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Potential diagnostic role of salivary miR-let-7a-5p as a biomarker in oral squamous cell carcinoma and oral lichen planus patients: A research article

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Research Article

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Abstract

Background:

Oral squamous cell carcinoma is one of the most common oral lesions that rapid diagnosis is extremely beneficial for conventional treatment. Oral lichen planus is a pre-malignant condition with the transformation potential to oral squamous cell carcinoma. Recently, the microRNAs have been considered as the novel regulator biomarkers for gene expression and early diagnosis of malignant lesions as well as evaluation of the potential for **pre-malignant** changes in the lesions. So, we evaluated the miR-Let-7a-5p expression in the saliva of patients with oral squamous cell carcinoma, oral lichen planus, and control groups to achieve an early diagnostic marker.

Methods:

This cross-sectional study was conducted for 15 months at the Dental Research Center, Mashhad University of Medical Sciences, Mashhad, Iran. Fresh salvia was collected from oral lichen planus patients (*n* = 27), oral squamous cell carcinoma patients (*n* = 28), and healthy individuals (*n* = 25). The expression of miR-let-7a-5p was evaluated among the three groups by quantitative polymerase chain reaction.

Results:

In this study, three groups were quantitatively and qualitatively evaluated for miR-let-7a-5p expression in saliva. The results showed that there was a statistically significant correlation in the mean quantitative and qualitative expression of miR-let-7a-5p among the three groups.

Conclusions:

The levels of miR-let-7a-5p expression were significantly lower in patients with oral squamous cell carcinoma and oral lichen planus compared to healthy controls. The miR-let-7a-5p can be consideredas a biomarker in saliva that we propose to be potentiallyreliable in the diagnosis and prognosis of oral squamous cell carcinoma and also oral lichen planus transformation.

1. Background

The oral squamous cell carcinoma (OSCC) is one of the most common cancers in the world. The survival rate is approximately 80% in patients with early-stage and 20-40% in individuals with no recovery at the advanced-stages, so the reports indicated about 300,000 annual deaths of worldwide (1). Another oral disease, oral lichen planus (OLP), is a relatively common chronic skin disease that often affects the oral mucosa in adults. The prevalence of OLP is 0.5-2.2%. Although the word OLP implies an immune-mediated mucosal skin disease, but it is more closely linked to T-cells, cytokines, enzymes, and oxidative imbalance in the pathogenesis (2, 3). The OLP lesions can be identified with six patterns, namely reticular, papular, plaque-like, atrophic/erosive, ulcerative, and bullous types (4). Recent studies showed that OLP is a pre-malignant condition with the transformation potential to OSCC (5).

The high incidence rate of metastasis and recurrence, poor prognosis and late diagnosis in advanced-stages, with extended lesions potential in OSCC, it should be considered the estimating and evaluating of malignant transformation potential oflesions (6, 7).The novel prognostic approaches based on detection of molecular biomarkers can be usefull for prediction of OSCC and OLP patients outcomes (7, 8). Detection of biomarkers can lead to the development of preventative treatment methods to control the disease in early phases. Biopsies and histological examination with clinical oral examinationare the gold standard methods for identification of oral disease. The biopsy operation is quite invasive, and preparing the slides take few days. The differential diagnosis between pre-malignant from malignant lesions is restricted (1, 9). Saliva collection is non-invasive, low-cost, easy to use, and store method that can applicable for detection of molecular biomarkers in oral cancerprecess. Biomarkers, which reflected molecular pathways of normal physiology process or pathological condition are found on this body fluid. The saliva detection of DNA, RNA, miRNA, proteins (cytokines: IL-8, IL-1b, and TNF), hormones, and numerous metabolites can be applied for oral cancer diagnosis (9-13).

The miRNA is 19-24 nucleotide's non-coding RNA that have been considered a regulatory biomarker for gene expression since 1993 (14). The miRNA is involved in many biological pathways, including cell cycle regulation, cell growth, apoptosis, cell differentiation, response to stress, and essential role in carcinogenesis (15, 16). These regulators are present in all body fluids, including serum, saliva, urine, *etc*. Evaluation of miRNA expression in saliva can help us to identify the biomarkers for early diagnosis of oral cancer. Because of the miRNA pluripotent functions, alternation in their expression impact the oncogenesis network's interaction and can be consider as cancer diagnostic biomarker or therapeutic targets. For instance, a correlation has previously hasbeen found between head and neck cell carcinoma (HNSCC) and miR-211, miR-21, and miR-184 (10). From our initial study, a subset miR-let-7a-5p expressed has been selected to determine its clinical utility as a potential biomarker. Its functions include inhibiting cell migration and invasion as well as targeting several oncogenes. It was reported miR-let-7a-5p expression altered in human cancers such as prostate (15), colorectal carcinoma (17), breast (18), and lung (16). This study aimed to evaluate the expression of miR-Let-7a-5p in the saliva of patients with OSCC and OLP in compare to control group to achieve wether miR-Let-7a-5p can apply asan early diagnostic marker in these patients.

2. Methods And Material

2.1. Ethics statement

The experimental protocols of the present study were approved by the ethics committee of Mashhad University of Medical Sciences (IR.MUMS.DENTISTRY.REC.1399.166), and all subjects visiting as outpatients were enrolled in the Department of the Research Council of Mashhad University of Medical Sciences, Faculty of Dentistry, Mashhad,Iran. They were well informed about the purpose of this study and signed written informed consent.

2.2. Participantsand sample collections

This cross-sectional study was conducted in 15 months (from January 4, 2021, to March 21, 2022). Eighty subjects were recruited from the Dental Research Center, Mashhad University of Medical Sciences, Mashhad, Iran.

In a sterile RNase/DNase free falcon tube, 1.5 CC of fresh human saliva was collected between 6 am and 11 am and kept on ice. Next, thesamples stored at -80°C. The total sample size for the study was 80participants inthree groups: OLP (*n* = 27), OSCC (*n* = 28), and healthy control (*n* = 25).

2.3. Inclusion Criteria

Participants were randomely selected with definitive clinical diagnosisin OSCC and OLP. The healthy controls had no medical history of any malignancies or thesystemic diseases. The candidate patients had not undertaken therapy before including surgery, chemotherapy, and radiotherapy. Three groups were similar in age and sex.

2.4. Exclusion Criteria

Participants with a history of other malignancies and a reluctance to cooperate to continue the project, samples that did not have the required quality were excluded from the study.

2.5. Salivary RNA extraction

In this study, microRNA was extracted from saliva without the use of specific kits with high quality and purity, which was one of the important achievements of this study. The procedure was as follows:

The 300-500 µl saliva samples were centrifuged for 20 minutes at 12000 RPM and 4°C, then the supernatant was discarded. We applied RNXplus (Sina Clon, IRAN) reagent and changed the procedure instructions. To begin with, each pellet was given 800 µl RNXplus and vortexed for 15 seconds to homogenize it. Then we incubated them for 3-4 minutes at room temperature. After that, each tube received 200 µl of chloroform (Merck Company), which was vortexed for 15 seconds before being incubated at room temperature for 3-5 minutes. The materials were centrifuged for 20 minutes at 12000 RPM and 4°C. Each sample had about 600µl of the upper aqueous layer transferred into a fresh 1.5 ml DNase and RNase-free microtube. We repeated this step: 200 µl chloroform (Merck Company) was added to each tube and vortexed for 15 seconds, and incubated at room temperature for 3-5 minutes. Then centrifuged at 12000 RPM and 4°C for 20 minutes, and approximately 500 µl of the upper aqueous layer were carefully transferred to the new 1.5 ml DNase/ RNase-free microtubes. After that, for RNA precipitation, 500 µl of cold absolute ethanol (Merck Company) was added to each tube and the microtubes were turned upside down for a few seconds. The microtubes were incubated overnight at -20°C. Then, the samples were centrifuged for 20 minutes at 12000 RPM and 4°C. The supernatant was dispersed. The pellet was washed in 1 ml of cold 80% molecular-grade ethanol and centrifuged for 20 minutes at 12000 RPM and 4°C. This step was repeated two times. The pellet was air dried for 3–4 minutes at room temperature, and then it was resuspended in 20 µl of DEPC water and incubated for 5

minutes at room temperature. Finally, we collected material from the bottom of microtubes using a rapid spin.

The purity of the extracted RNA was determined by assessment of 260/280 nm absorbance ratio of wave length using nanodrop 1000 spectrophotometer (NanoDrop, Thermo Scientific 2000, USA). The RNAs with suitable quantification (100 ng/µl) and purity (1.5-2 ratio) are utilized for complementary DNA (cDNA) synthesis.

2.6. Reverse transcription

CDNA was synthesized by the Adscriptc DNA synthesis Kit (REF: 22701, Bio-Tech, Addbio, Korea). The cDNA synthesis was performed in 20 µl total volume according manufacture instructure including 10µl of 2X Reaction Buffer, 2µl of 10 mM dNTP mixture, 6µl of RNA, and 1 µl of RT primer (1 pM) for U6 or miR-Let-7a-5p and 1 µl of 20X enzymeby ABI thermocycler (One Step, USA). The temperature cycling protocol were preincubation in25 °C for 10 minutes, reverse transcription (RT) at 50 °C for 60 minutes, RT inactivation at 80 °C for 5 minutes, and holding at 12 °C.

2.7. QuantitativeReal-time PCR (qPCR)

The qPCR was performed to quantitate the relative expression of *miR-let7a-5p* and U6 using SYBR Green master mix (REF: 22701, Bio-Tech, addbio, Korea) with target primers (Table 1). All reactions were performed in duplicate in 20 µl total volumes in separate wells. Each reaction contained 0.5 µL of each primer (10 pM), 10 µl of SYBR Green master mix (XX), 7µl distilled sterile water, and 2 µlof cDNA. The qPCR temperature process was used by Light Cycler 96 (Roche, Germany) following preincubation step at 95 °C for 30 seconds, and 50 cycles at 95 °C for 5 seconds for denaturation and 60 °C for 30 seconds for annealing. Differential expression was analyzed by the ΔΔCT method. The U6 housekeeping miR was used as the reference gene (Figure 1). Moreover, we performed the analysis of the melting curve to confirm specific target amplification.

2.8. Statistical analyses

All data analysis was performed using SPSS software (Version 25). The Data normalized by threshold cycle number ΔCt, and the relative miRNA expression level was calculated as 2^-(ΔCt) which is commonly used in miRNA expression. The p-value less than 0.05 was considered significant.

3. Results

3.1. Characteristics of study subjects

In this study, miR-let-7a-5p expression were evaluated in saliva of 28 OSCC, 27 OLP patients, and 25 healthy controls. A total of 80 individuals were studied including 44 women (55%) with a mean age of 50.57±13.18 and age range of 28 to 73 years, and 36 men (45%) with a mean age of 56.19±13.73 years and age range of 25 to 78 years. First, the normality of quantitative variables was investigated using

Shapiro-Wilke, and the variables of age and expression of miR-let-7a-5p did not have a normal distribution (Table 2). The Correlation between salivary miR-let-7a-5p expression and clinicopathological indices of OSCC patients are demonsterated in Table 3. The results of non-parametric Chi-Square and Kruskal-Wallis tests showed there was no significant relationship between age and sex in study groups. There was also no significant assosiation between gene expression, age and sex in different groups.

3.2. The quantitative expression of the saliva let-7a-5p levels among the three groups

The expression of miR-let-7a-5p expression in the three studied groups was quantitatively assessment by the Kruskal-Wallis test. The mean quantitative expression of miR-let-7a-5p in the three groups including OSCC,OLP, and control were 87.0± 13.1, 40.2±3.2, and 77.3± 3.2, respectively. The results showed that there was a statistically significant difference the mean inexpression of miR-let-7a-5p between the three groups (p-value <0.05).

3.3. The qualitative expression of the saliva let-7a-5p levels among the three groups

According to the expression of miR-let-7a-5p in the study groups and according to the previous studies, the results of qRT-PCR were also evaluated qualitatively. The samples were classified into three levels: no expression, low expression, and high expression. The samples were placed according to the amount of fold change in the no expression group (fold change≤1), in the low expression group (1≤ fold change≤2), and in the high expression group (fold change≥2). The Chi-square test showed statistically significant difference in the three levels of miR-let-7a-5p expression among different groups (p-value <0.000) (Table 4).

3.4. Comparison of quantitative and qualitative expression ofmiR-let-7a-5p in OSCC group according to the grades

The statistical analysis by Fisher's Exact Test did not show difference between grades I, II, III in OSCC patients (p = 0.554). Although the expression of miR-let-7a-5p in grade III was higher than grades I and II (Table 5).

3.5. Comparison of quantitative and qualitative expression of miR-let-7a-5p in OSCC group according to the stage

Our results demonsterated no significant difference between the OSCC stages (p = 0.554). The mean quantitative expression of miR-let-7a-5p in early-stage was 1.097 ± 1.287, and in the advanced-stage were 0.464 ± 0.650. The mean expression of miR-let-7a-5p in early-stage patients was higher than advanced-stage (Figure 2).

3.6. Multiple comparisons of miR-let-7a-5p expression in study groups

The least significant difference (LSD), post-test, was performed to determine which specific group rejected the null hypothesis. According to the LSD test, all three groups showed statistically significant difference

from each othe r(p-value < 0.05)(Table 6).

4. Discussion

The microRNAs, particularly the miR-let-7 family and their regulatory proteins, are out of control in a wide range of cancers and have a complex impact on cancer cells maintenance, metabolism, tumorigenesis, and metastasis (19, 20). The miR-Let-7 family has a major impact on the many cellular pathways linked to neoplastic alterations. The miR-Let-7 family is known as a tumor suppressorin most malignancies that either underexpressed or not expressed. Based on the findings of previou sresearch expression of miR-Let-7 was evaluated in patients and Let-7a-based alternative medicines have been proposed as the potential target in cancer therapeutic approaches. Then, novel therapeutic platforms that target cancer cells can help patients to overcome resistance to therapy. These treatment can be employed on their own or in conjunction with existing chemotherapy (21-24). A microRNA can regulate the expression of a wide number of target genes and affect genes other than the ones intended. On the other hand, a single gene may be regulated by a large number of microRNAs, in which case changing the expression of a single microRNA will not be sufficient to influence the target gene for therapeutic purposes. It has been reported by strong evidence that miR-let-7a targeted genes in several types of cancer, for instance, cysteine-aspartic acid protease-8 (CASP8), **Bcl-2 Associated X-protein** (BAX), **B-cell lymphoma 2** (BCL2), cyclin D2 (CCND2), **EWS RNA-binding protein 1** (EWSR1), Forkhead Box A1 (FOXA1), ribonuclease type III (DICER1), **high mobility group A1** (HMGA1), High-mobility group AT-hook 2 (HMGA2), KRAS, MYC, HRAS, myeloproliferative leukemia protein (MPL), Neurofibromatosis type II (NF2), PR domain zinc finger protein 1 (PRDM1) and NRAS (25, 26). Several studies have reported that let-7a could inhibit the growth of cells in lung cancer and lymphoma, and Signal transducer and activator of transcription 3 (STAT3) signalling was influenced by miR-let-7a in cervical carcinogenesis (10, 27, 28). It seems using candidate microRNAs such as miR-let-7a-5p in target cancer trerapy has less of the issues described above. Therefore, it was confirmed that the expression of this microRNA is reduced in patients with various malignancies (19, 29). Because the let-7 family directly inhibits cell development by targeting numerous oncogenes, enough knowledge about its expression is critical for treating patients effectively but there was restrict investigations related to the saliva expression of miR-let-7a-5p. The miR- let-7a-5p has been reported as a biomarker in other cancers such as prostate, breast, and colorectal (15, 18, 30, 31), but It has not been studied extensively in oral cancer. STAT3 signallingand HMGA2 gene was influenced by miR-let-7a-5p that lead to increase cell adhesion and also it enhances BAX, BCL2 genes which ultimately lead to reduced apoptosis and OSCC development. Down regulate of miR-let-7a-5p promotes PI3K/Akt/mTOR pathway that lead to cell growth (Figure 3)(16). So, we investigated the expression of a member oflet-7 family (miR-let-7a-5p) in three groups OSCC and OLP patients as well as healthy individuals. For the first time, we compared saliva miR-let-7a-5p expression in the patients with pre-malignant and malignant lesions with healthy controls. This is also the first research of miR-let-7a-5p expression in OLP patients. The results of our study showed that 71.4% of OSCC patients not expressed miR- let-7a-5p, and 21.4% and 7.1% of them demonsterated low expression, and high expression, respectively. While, the control group exhibited 84% high expression. We report significant down-regulation of miR-let-7a-5p in the saliva samples of

OSCC patients. Also, thelevels of miR-let-7a-5p expression in saliva revealed significant sensitivity and specificity to differentiate between OSCC, OLP patients, and healthy controls. Although the decrease in salivary expression of miR-let-7a-5p of OSCC patients was not significantly related to stage and grade, but could be due to the our small study sample size. It was so significant in OSCC patients incompared to the OLP and healthy control groups. However, when comparing the OLP patients to healthy controls, there was a reduction in the expression of miR-let-7a-5p, which might be related to the function of this microRNA in inflammation or the possibility of malignancy in this lesion. The OSCC group demonstrated lower expression of miR-let-7a-5p than the healthy control group, which makes sense given the significance of this microRNA in tumor development and progression. Fadhil, *et al* .(10) showed that expression of miR- let-7a-5p can be suitable biomarker in the prognosis and diagnosis of head and neck cancer. Lee *et al.* (32) evaluated the expression of miR-let-7a-5p in saliva, in healthy individuals and patients with invasive periodontitis. Their results also showed that miR-let-7a-5p expression is a non-invasive screening method for the development of salivary diagnosis in patients with invasive periodontitis. Alwhaibi *et al.* (15) demonstrated that reducing in miR-let-7a-5p levels was effective in the treatment, screening, and inhibition and migration of cancer cells in patients with prostate cancer. Previous studies suggested that miR-let-7a-5p can apply as a potential new therapeutic target for the treatment of cervical (33) and liver cancers (34). It has also been shown that in patients with pulmonary adenocarcinoma, concomitant reduction of let-7a-5p exosomal is useful as a predictive biomarker for survival (16). The miR-let-7a-5p was also assessed in breast cancer and showed that Let-7a-5p could be used as a biomarker in breast cancer (18, 30). Furthermore, our experiment was consistent with previous findings. In this study, we also examined the expression of let-7a-5p in OLP patients, which showed low expression and high expression in 74.1% and 29.9% of them, respectively and also lower expression than healthy controls. Based on our searches in different databases, there are no studies for evaluation of miR-let-7a-5p as a biomarker during precancerous changes of OLP. Therefore, We are reporting novel biomarkers (miR- let-7a-5p) in saliva that we had identified to find lesions with malignant transformation potential. According to the results of present study, it is recommended that this microRNA be used in the treatment of OSCC patients, after clinical trial phases. However, according to the role of this microRNA in reduction of inflammation, it is suggested to evaluate the expression of this microRNA in the serum and saliva of patients in future studies.

5. Conclusion

Our results concluded that the salivaryexpression of the miR-let-7a-5p were significantly decreased in OSCC and OLP patients in compared to the healthy controls. We are reporting miR- let-7a-5p as saliva that can be suitable for diagnosis and prognosis of OSCC and for detection of precancerous changes and inflammation in OLP during carcinogenesis process of oral cancer. According to our study from reliable sources, this is the first study that compares OSCC as the most common oral malignancy and OLP as a lesion with the potential of malignant changes in terms of miR- let-7a-5p biomarkers. Detection of salivary biomarker is valuable because sample collection is a non-invasive method for screening the OLP lesions

with susceptibility to malignancy transformation OSCC as malignancy. The miR-let-7a-5p in equilibrium are involved in the maturation and differentiation of oral squamous cells.

6. Abbreviations

OLP, Oral lichen planus; OSCC, Oral squamous cell carcinoma; HNSCC, Head and eck squamous cell carcinoma; EGFR, Epidermal growth factor receptor inhibitors; IL-8, Interleukin 8; IL-1b, Interleukin 1 beta; TNF, **Tumour Necrosis Factor;** CASP8, Cysteine-aspartic acid protease-8; BAX, **Bcl-2 Associated X-protein**; BCL2, **B-cell lymphoma 2**; CCND2, Cyclin D2; EWSR1, **EWS RNA-binding protein 1**; FOXA1, Forkhead Box A1; DICER1, Ribonuclease type III; HMGA1, **High mobility group A1**; HMGA2, High-mobility group AT-hook 2; MPL, Myeloproliferative leukemia protein; NF2, Neurofibromatosis type II; PRDM1, PR domain zinc finger protein 1; cDNA, Complementary DNA; qPCR, Quantitative Real-time PCR; LSD, Least significant difference.

7. Declarations

7.1. Ethics approval and consent to participate:

The study were approved by the ethics committee of Mashhad University of Medical Sciences (IR.MUMS. DENTISTRY. REC. 1399.166).

7.2. Consent for publication:

Not applicable.

7.3. Availability of data and materials:

The data that support the findings of this study are available from [Dr. Farnaz Mohajertehran] but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of [Dr. Farnaz Mohajertehran].

7.4. Competing interests:

The authors declare that they have no competing interests

7.5. Funding:

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7.6. Authors' contributions:

FM made the design of the work, supervised and supported this study and quality controlled of data and algorithms. FM made substantial contributions to the conception. AF, SJO , AS, NM, , KKh, SHAB carried out the research, collected the data for this study and performed the data extraction, the management and interpretation of the results as well as the draft of this paper and contributed in writing the manuscript. PMM helped in the interpretation and article editing. All authors read and approved the final manuscript.

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References

1. Al Rawi N, Elmabrouk N, Kou RA, Mkadmi S, Rizvi Z, Hamdoon Z. The role of differentially expressed salivary microRNA in oral squamous cell carcinoma. A systematic review. Arch. Oral Biol. 2021;125:105108.
2. Ghazi N, Aali N, Shahrokhi V-R, Mohajertehran F, Saghravanian N. Relative expression of SOX2 and OCT4 in oral squamous cell carcinoma and oral epithelial dysplasia. Rep. Biochem. Mol. Biol. 2020; 9:171.
3. Glick M. Burket's oral medicine. 12th ed. PMPH USA; 2015.
4. Jana A, Thomas J, Ghosh P. Erosive oral lichen planus inflicts higher cellular stress than reticular type. JOMFP. 2021; 25:279.
5. Mohtasham N, Ayatollahi H, Saghravanian N, Zare R, Shakeri M-T, Sahebkar A, *et al.* Evaluation of tissue and serum expression levels of lactate dehydrogenase isoenzymes in patients with head and neck squamous cell carcinoma. Anticancer. Agents. Med. Chem. 2019; 19:2072–2078.
6. Salazar C, Nagadia R, Pandit P, Cooper-White J, Banerjee N, Dimitrova N, *et al.* A novel saliva-based microRNA biomarker panel to detect head and neck cancers. Cel. Oncol. 2014; 37:331–338.
7. Baran K, Kordiak J, Jabłoński S, Antczak A, Brzeziańska-Lasota E. The Significance of the Alter miR let-7a and miR-335 Expression Level Regulating the CCR7/CCL19 Axis as Potential Biomarkers of Tumor Progression in NSCLC. J. Clin. Med. 2022; 11:655.
8. Pfaffe T, Cooper-White J, Beyerlein P, Kostner K, Punyadeera C. Diagnostic potential of saliva: current state and future applications. Clin. Chem. 2011; 57:675–687.
9. Farah CS, McIntosh L, Georgiou A, McCullough MJ. Efficacy of tissue autofluorescence imaging (VELScope) in the visualization of oral mucosal lesions. Head Neck. 2012; 34:856–862.
10. Fadhil RS, Wei MQ, Nikolarakos D, Good D, Nair RG. Salivary microRNA miR-let-7a-5p and miR-3928 could be used as potential diagnostic bio-markers for head and neck squamous cell carcinoma. PloS One. 2020; 15:e0221779.
11. Korostoff A, Reder L, Masood R, Sinha UK. The role of salivary cytokine biomarkers in tongue cancer invasion and mortality. Oral Oncol. 2011; 47:282–287.

12. Ogawa Y, Taketomi Y, Murakami M, Tsujimoto M, Yanoshita R. Small RNA transcriptomes of two types of exosomes in human whole saliva determined by next generation sequencing. Biol. Pharm. Bull. 2013; 36:66–75.
13. Shahabinejad M, Zare R, Mohajertehran F, Mahdiraji AA. Cytokeratins (CK7 and CK20) Genes Expression Association with Clinicopathological Indices in Oral Squamous Cell Carcinoma and Dysplastic Oral Epithelium. Rep. Biochem. Mol. Biol. 2021; 10:126.
14. Farshbaf A, Mohtasham N, Zare R, Mohajertehran F, Rezaee SA. Potential therapeutic approaches of microRNAs for COVID-19: Challenges and opportunities. J. Oral Biol. Craniofac. Res. 2021; 11:132–137.
15. Alwhaibi A, Parvathagiri V, Verma A, Artham S, Adil MS, Somanath PR. Regulation of Let-7a-5p and miR-199a-5p Expression by Akt1 Modulates Prostate Cancer Epithelial-to-Mesenchymal Transition via the Transforming Growth Factor-β Pathway. Cancers. 2022; 14:1625.
16. Zhang L, Hao C, Zhai R, Wang D, Zhang J, Bao L, *et al.* Downregulation of exosomal let-7a-5p in dust exposed-workers contributes to lung cancer development. Respir. 2018; 19:1–8.
17. Liu T-P, Huang C-C, Yeh K-T, Ke T-W, Wei P-L, Yang J-R, *et al.* Down-regulation of let-7a-5p predicts lymph node metastasis and prognosis in colorectal cancer: Implications for chemotherapy. Surg. Oncol. 2016; 25:429–434.
18. Fuso P, Di Salvatore M, Santonocito C, Guarino D, Autilio C, Mulè A, *et al.* Let-7a-5p, miR-100-5p, miR-101-3p, and miR-199a-3p hyperexpression as potential predictive biomarkers in early breast cancer patients. J. Pers. Med. 2021; 11:816.
19. Ma Y, Shen N, Wicha MS, Luo M. The Roles of the Let-7 Family of MicroRNAs in the Regulation of Cancer Stemness. Cells. 2021; 10:2415.
20. Alles J, Fehlmann T, Fischer U, Backes C, Galata V, Minet M, *et al.* An estimate of the total number of true human miRNAs. Nucleic Acids Res. 2019; 47:3353–3364.
21. Roush S, Slack FJ. The let-7 family of microRNAs. Trends Cell Biol. 2008; 18:505–516.
22. Balzeau J, Menezes MR, Cao S, Hagan JP. The LIN28/let-7 pathway in cancer. Front. Genet. 2017; 8:31.
23. Mohtasham N, Mahdavi-Shahri N, Salehinejad J, Ejtehadi H, Torabi-Parizi M, Ghazi N. Detection of nucleoproteins in squamous cell carcinoma, and dysplastic and normal mucosa in the oral cavity by methyl green-pyronin staining. J. Oral Sci. 2010; 52:239–243.
24. Sharifi-Sistani N, Zartab H, Babakoohi S, Saghravanian N, Jamshidi S, Esmaili H, *et al.* Immunohistochemical comparison of the expression of p53 and MDM2 proteins in ameloblastomas and keratocystic odontogenic tumors. J. Craniofac. Surg. 2011; 22:1652–1656.
25. Hsu S-D, Tseng Y-T, Shrestha S, Lin Y-L, Khaleel A, Chou C-H, *et al.* miRTarBase update 2014: an information resource for experimentally validated miRNA-target interactions. Nucleic Acids Res. 2014; 42:D78-D85.
26. Forbes SA, Beare D, Gunasekaran P, Leung K, Bindal N, Boutselakis H, *et al.* COSMIC: exploring the world's knowledge of somatic mutations in human cancer. Nucleic Acids Res. 2015; 43:D805-D811.

27. Shishodia G, Shukla S, Srivastava Y, Masaldan S, Mehta S, Bhambhani S, *et al.* Alterations in microRNAs miR-21 and let-7a correlate with aberrant STAT3 signaling and downstream effects during cervical carcinogenesis. Mol. Cancer. 2015; 14:1–13.
28. Mohajertehran F, Sahebkar A, Zare R, Mohtasham N. The promise of stem cell markers in the diagnosis and therapy of epithelial dysplasia and oral squamous cell carcinoma. J. Cel. Physiol. 2018; 233:8499–8507.
29. Liu C, Chen Z, Fang M, Qiao Y. MicroRNA let-7a inhibits proliferation of breast cancer cell by downregulating USP32 expression. Transl. Cancer Res. 2019; 8:1763.
30. Chen C, Liu X, Chen C, Chen Q, Dong Y, Hou B. Clinical significance of let-7a-5p and miR-21-5p in patients with breast cancer. Ann. Clin. Lab. Sci. 2019; 49:302–308.
31. Jahanbin A, Hasanzadeh N, Abdolhoseinpour F, Sadr-Nabavi A, Raisolsadat M-A, Shamsian K, *et al.* Analysis of MTHFR gene C. 677C > T and C. 1298A > C polymorphisms in Iranian patients with non-syndromic cleft lip and palate. Iran. J. Public Health. 2014; 43:821.
32. Lee N-H, Lee E, Kim Y-S, Kim W-K, Lee Y-K, Kim S-H. Differential expression of microRNAs in the saliva of patients with aggressive periodontitis: a pilot study of potential biomarkers for aggressive periodontitis. J. Periodontal Implant Sci. 2020; 50:281.
33. Gao J, Ding C, Zhou J, Wu G, Han Z, Li J, *et al.* Propofol suppresses lung cancer tumorigenesis by modulating the circ-ERBB2/miR-7-5p/FOXM1 axis. Thorac. Cancer. 2021; 12:824–834.
34. Liu L, Zhao J, Peng Y, Yang M, Zhang L, Jin X. miR-let-7a-5p Inhibits Invasion and Migration of Hepatoma Cells by Regulating BZW2 Expression. OncoTargets Ther. 2020; 13:12269.

Tables

Table 1: Specific primers for expression of miR-let7a-5p

Primers		sequences
microRNA Let-7a-5p	Forward	5’-GGTGATGAGGTAGTAGGTTGT-3’
	Reverse	5´-GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACAACTAT-3’
U6	Forward	5'-AAGGATGACACGCAAATTC-3'
	Reverse	5'-GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACAAAATATGG-3'

Table 2: Normal age distribution and miR-Let-7a-5p expressionby Shapiro-Wilk test

Tests of Normality					
	group	Shapiro-Wilk			
		Test statistic	Degrees of Freedom	P value	
Age	scc	.986	28	.958	Normal
	lp	.917	27	.033	Abnormal
	c	.934	25	.109	Normal
miR-let-7a expression	scc	.711	28	.000	Abnormal
	Lp	.538	27	.000	Abnormal
	C	.822	25	.001	Abnormal

Table 3 : Correlation between salivary miR-let-7a expression and clinicopathologicindices of OSCC patients

Clinicopathologic feature	Number (%)	miR-let-7a expression			P value
		No	low	High	
Age, y					
0≥6	11 (39.3%)	8 (72.7%)	2 (18.2%)	1 (9.1%)	0.999
<60	17 (60.7%)	12 (70.6%)	4 (23.5%)	1 (5.9%)	
Gender					
Male	16 (57.1%)	13 (65.0%)	3 (65.0%)	0 (0.0%)	0.194
Female	12 (42.9%)	7 (58.3%)	3 (35.0%)	2 (100.0%)	
Clinical stage					
Early	18 (64.3%)	12 (60.0%)	4 (66.7%)	2 (100.0%)	0.823
Advanced	10 (35.7%)	)40.0%8 (	)33.3%2 (	0 (0.0%)	
Grade					
I	15 (53.5%)	10 (50.0%)	4 (66.7%)	1 (50.0%)	0.554
II	9 (32.2%)	)35.0%7 (	)33.3%2 (	0 (0.0%)	
III	4 (14.3%)	)15.0%3 (	0 (0.0%)	1 (50.0%)	

Table 4: The comparison of qualitative expression of the saliva let-7a-5p levels among the three groups

			group			Total	Chi-Square Tests
			SCC	LP	C		
Group Expression	no expression	Frequency (%)	20 (71.4)	0 (0.0)	0 (0.0)	20 (25.0)	Chi-Square= 74.045
	high expression	Frequency (%)	2 (7.1)	7 (25.9)	21 (84.0)	30 (37.5)	Df=4
	Low expression	Frequency (%)	6 (21.4)	20 (74.1)	4 (16.0)	30 (37.5)	p-value=0.000
Total		Frequency (%)	28 (100.0)	27 (100.0)	25 (100.0)	80 (100.0)	

Table 5: Qualitative expression of miR-let-7a-5p in OSCC group (by grades)

Group	Grade		miR-let-7a-5pQualitative expression of			Total	Fisher's Exact test
			no expression	high expression	Low expression		p-value
OSCC	I	Frequency (%)	10 (50.0)	1(50.0)	4 (66.7)	15 (53.6)	0.554
	II	Frequency (%)	7 (35.0)	0 (0.00)	2 (33.3)	9 (32.1)	
	III	Frequency(%)	3 (15.0)	1 (50.0)	0 (0.00)	4 (14.3)	
	Total	Frequency	20	2	6	28	

Table 6: Multiple comparison of miR-let-7a-5p expression in study groups

Multiple comparison						
miR-let-7a-5p expression						
LSD test						
Group (I)	Group (J)	Mean difference (I-J)	Std. deviation	Sig.	Confidence Interval95%	
					Lower bound	upper bound
OSCC	OLP	-1.53*	.53	.005	-2.597030676	-.471014038
	Healthy Control	-2.90*	.54	.000	-3.988532621	-1.819654605
OLP	Healthy Control	-1.37*	.55	.015	-2.463941986	-.276200525

Figures

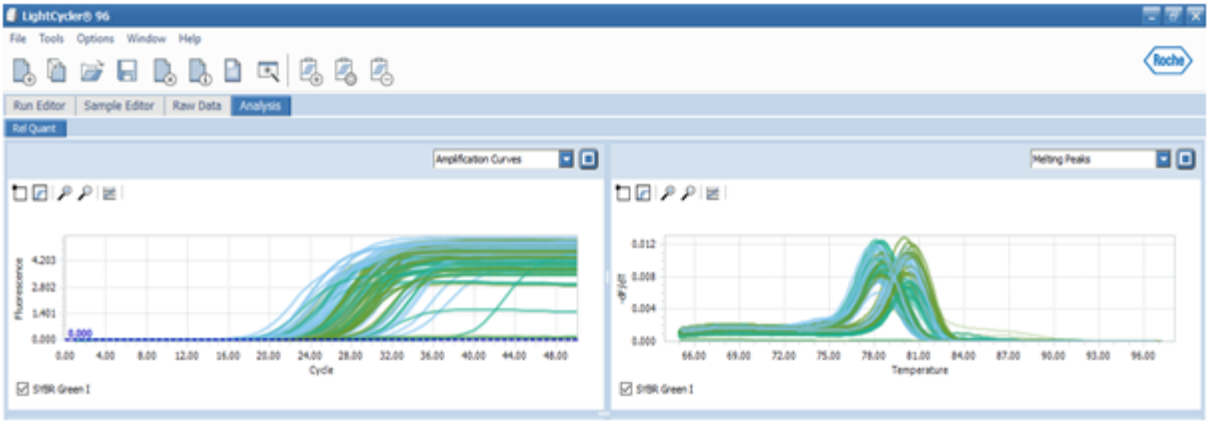

Figure 1

The melting curve (right) and amplification plot (left) of let-7a-5p and U6 expressions in real-time PCR

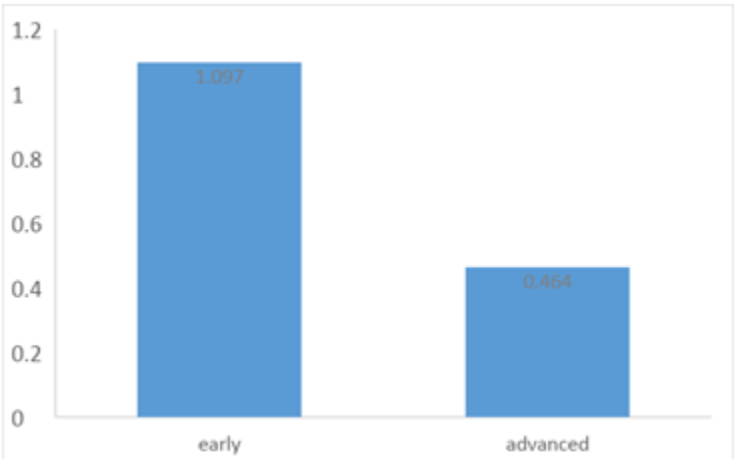

Figure 2

Comparison of quantitative expression of miR-let-7a-5p in OSCC group (by stage)

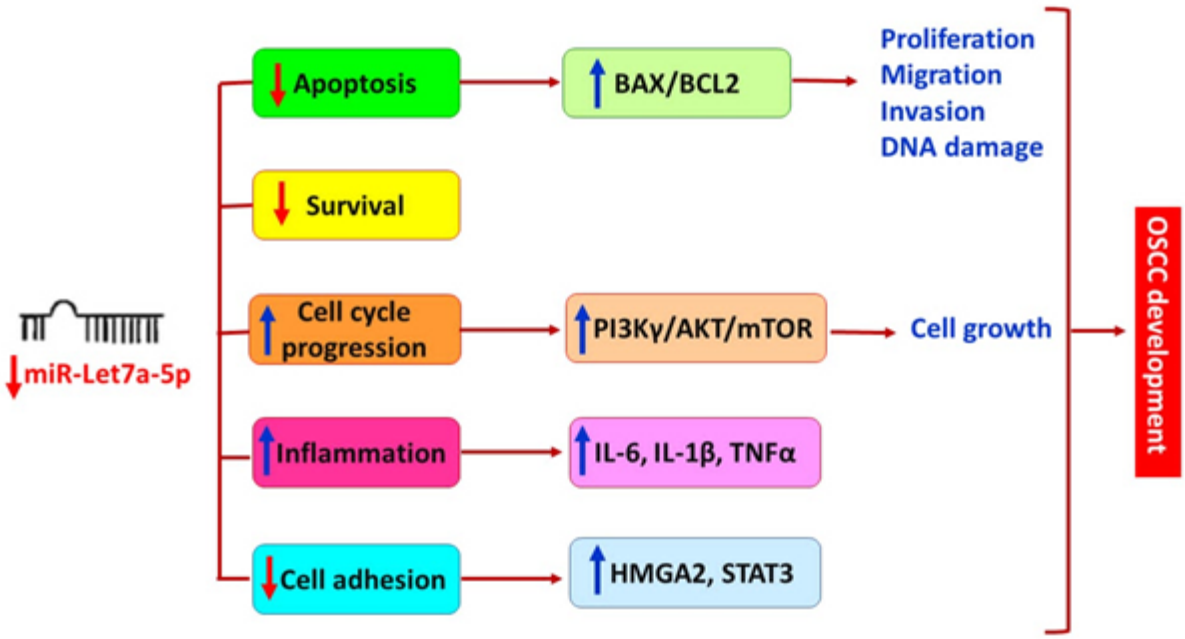

Figure 3

The impact of reduction in miR-let7a-5p expression on OSCC development