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REPORT

Diagnostic and prognostic relevance of salivary microRNA-21, -125a, -31 and -200a levels in patients with oral lichen planus - a short report

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Abstract

Background Oral lichen planus (OLP), a relatively common chronic inflammatory disease of the oral mucosa, is considered to be a premalignant disorder of the oral cavity. Previously, several biomarkers have been tested for their diagnostic potential. Here, we aimed to investigate the diagnostic potential of four miRNAs, miR-21, -125a, -31 and -200a, known to be involved in oral squamous cell carcinoma (OSCC) development, in the saliva of OLP patients as also their putative relation to OSCC development in these patients.

Materials and methods Saliva samples from 30 patients with OLP were collected, 15 of whom were diagnosed with dysplasia upon histopathologic examination. In addition, 15 saliva samples from patients with OSCC and 15 saliva samples from healthy donors were collected. After RNA extraction, the respective miRNA levels were assessed by quantitative RT-PCR.

Results We found that the miR-21 levels were significantly increased in saliva samples derived from patients with OLP, dysplastic OLP and OSCC, compared to those from healthy controls ($p = 0.012$, $p = 0.0017$ and $p < 0.0001$, respectively). Conversely, significant decreases in miR-125a levels were found in the OLP, dysplastic OLP and OSCC samples, compared to those from healthy controls ($p < 0.0014$, $p < 0.0001$ and $p < 0.0001$, respectively). In addition, significant increases in miR-31 levels were found in samples derived from dysplastic OLP and OSCC patients, but not in those from nondysplastic OLP patients, compared to those in healthy controls ($p = 0.01$ and $p = 0.004$, respectively). Finally, we found that the miR-200a levels were significantly decreased only in samples derived from OSCC patients ($p < 0.0001$).

Conclusions From our data we conclude that increased miR-21 levels in conjunction with decreased miR-125a levels in saliva of OLP patients may be indicative for a poor prognosis. Conversely, we conclude that lack of significant alterations in miR-31 and miR-200a levels in saliva of OLP patients may be indicative for absence of malignant transformation.

Keywords Oral lichen planus · microRNA · Saliva · Oral squamous cell carcinoma

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1 Introduction

Lichen planus is a chronic inflammatory disease of mucous membranes. This disease affects 1 to 2% of the general population. In the oral cavity, buccal mucosa, tongue and gingiva are commonly affected. Other parts may be affected as well [1]. The clinical presentations of the lesions include popular, reticular, plaque zed type, erosive, atrophic and bullous forms. Oral lichen planus (OLP) has recently been recognized as a potentially premalignant disorder. Because the annual incidence of oral cancer is 5 per 100,000, the annual risk of malignant transformation is hundredfold increased in OLP patients [2]. Cancer does not necessarily develop within the OLP lesion itself, but may occur in any part of the oral cavity [3]. Although in the past various OLP biomarkers have been considered, microRNAs (miRNAs) have so far been subject of only a limited number of studies [4–6]. In the recent past, miRNAs have been found to be secreted by different cancer cells and they can, as such, be employed to non-invasively detect and characterize different types of cancer, including oral squamous cell carcinoma (OSCC) [6–9].

MiR-21 has been widely studied in oral, head and neck and the other cancers [10, 11]. Experimental evidence indicates that this miRNA may inhibit tumor suppression and apoptosis in, among others, OSCC [4, 12]. A survey has shown that miR-21 may be related to inflammation and cancer and a meta-analysis has revealed that circulating miR-21 may serve as a biomarker for the occurrence of various carcinomas. The latter notion has highlighted its potential as biomarker for early cancer detection [13]. Others have shown that miR-21 may play a role in malignant transformation [14] and that increased miR-21 levels may be found in OLP tissues compared to normal mucosa [15]. It has also been found that members of the miR-125 family may play crucial roles in cancer-related processes. Specifically, it has been found that miR-125a may act as a tumor suppressor through the down-regulation of target oncogenes. As such, miR-125a may serve as a potent diagnostic/prognostic biomarker, also in OSCC [16]. As yet, miR-125a levels have not been determined in saliva samples of OLP patients. Also miR-31 ranks amongst the most frequently altered miRNAs in human cancers. It has been reported that its level may be increased in liver, head and neck and colorectal cancers, and decreased in breast, gastric and prostate cancers [17, 18]. In addition, miR-31 levels have been found to be significantly increased in OSCC patients and, as such, its use as biomarker for early OSCC detection has been recommended [5]. Although miR-31 has been detected in OLP tissue samples [19], its putative diagnostic/prognostic potential in OLP patient-derived saliva still remains to be established. The miR-200 family comprises 5 members, i.e., miR-200a, miR-200b, miR-200c, miR-141 and miR-429. It has been reported that miR-200a overexpression may affect epithelial-mesenchymal transition (EMT) and, thus, tumor development [20]. Lower miR-200a levels have been observed in OSCC patient-derived saliva samples compared to those derived from healthy subjects [4, 21].

Saliva collection is rapidly emerging as a non-invasive, low cost, high speed and robust method for oral cancer assessment [22], including the occurrence of OLP [19, 23, 24]. As yet, however, no study has been reported in which miRNA levels have been compared in saliva samples from OLP and OSCC patients. Here, we set out to assess salivary miR-21, -125a, -31 and -200a levels in OLP patients with or without dysplasia and in OSCC patients in order to evaluate their diagnostic and prognostic potential.

2 Materials and methods

2.1 Patients and saliva sample collections

Patient information was collected using medical records, physical examinations, tissue biopsies and histopathologic evaluations. Through this survey, 30 OLP patients were selected based on clinical data and the presence of cellular changes in the biopsies [15, 25]. Of these 30 patients, 20 were female and 10 were male. The mean age of the patients was 49 (range: 18 to 76 years). According to histopathology, 10 patients did not exhibit dysplasia and 20 did. In addition, 15 OSCC patients (positive controls) were selected based on both clinical and histopathological criteria. Another 15 healthy subjects (negative controls) were carefully selected based on medical history and physical examination, as well as on age and gender (patient matched). Subjects with systemic diseases and/or oral lesions were excluded (Supplementary Figs. 1, 2).

Informed consent was obtained from all participants after conformation of lack of other malignant lesions, autoimmune diseases, immunodeficiencies, hepatitis infections, HIV, heart diseases, immunosuppressive therapy, using drugs inducing the lichenoid reaction, and adjacency of lesions to dental restorations, especially amalgam restorations [5, 26]. Patients with OLP were assessed by incisional biopsy from erosive and ulcerative, plaque-like and non-homogeneous areas with a higher possibility of malignant transformation in the presence or absence of dysplasia [27]. Unstimulated saliva samples were collected from patients with OLP and OSCC, as well as from healthy controls, before biopsy using the Navazesh method [28]. The participants were asked to avoid eating, drinking and using oral hygiene procedures at least one hour before sampling and to wash their mouth with water to reduce contamination and remove debris. The saliva samples (3 to 5 ml) were collected in 15 ml RNase-free Falcon tubes after which 1 ml RNase inhibitor/RNAlater RNA stabilization reagent (QIAGEN) was added. Next the samples were immediately frozen and stored at −80 °C until RNA extraction.

2.2 RNA extraction and qRT-PCR

MiRNA extractions from saliva supernatants (400 μl) were carried out using a mirVana PARIS kit (Ambion, USA), according to the manufacturer's instructions. Reverse transcription was carried out in a mixture (10 μl) containing 2 μg poly(A), 4 μl RNA, 1 μl RT miRNA primer, 0.5 μl RT enzyme, 2 μl RT buffer (Amplicon, UK) and ~2.5 μl nuclease-free water. The reactions were carried out at 42 °C for 60 min and stopped at 80 °C for 5 s. The resulting cDNA (1 μl) was mixed with 5 μl QuantiTect SYBR Green (Qiagen, Germany), 1 μl of each forward and backward miRNA primer and 2 μl nuclease-free water in a total volume of 10 μl. This reaction mixture was subsequently incubated at 95 °C for 2 min, followed by 45 cycles of 95 °C for 5 s, 63 °C for 20 s and 72 °C for 20 s. The melting curve was set at 60.0 to 95.0 °C at increments of 1 °C for 0.05 min. The miRNA levels were normalized to that of miRNA-U6 as internal control. The miRNA levels obtained were calculated using the $2^{-\Delta\Delta Ct}$ method [20]. Briefly: sampleΔCt target miR = sampleCt target miR - sampleCt miR-U6; test $\Delta\Delta$Ct target miR = test ΔCt target miR - Control ΔCt target miR as described previously [4].

2.3 Statistical analysis

The microRNA levels in patients compared to normal controls were assessed using Student's t-test. Other statistical analyses were performed using Graph pad prism software. A p value < 0.05 was considered to indicate statistical significance.

3 Results and discussion

After qRT-PCR analysis of the miR-21 level in the saliva samples from the different patients and healthy controls included, we found that this level was higher in patients with OSCC compared to that in patients with OLP, both with and without dysplasia ($p < 0.0001$, $p = 0.0017$ and $p = 0.012$, respectively). The lowest miR-21 levels were observed in normal healthy control samples (Figs. 1, 2a). Previously, miR-21 levels have been measured in OSCC tissue samples, and significant increases have been reported [8, 11, 29]. Up till now, however, its levels have not been measured in saliva samples of OSCC patients. Our current findings are in support of those derived from the OSCC tissue samples. In addition, Cervigne and Brito observed increased miR-21 levels in precancerous progressive leukoplakia lesions [14, 30], and Madkour and colleagues observed significantly increased miR-21 levels in tissue samples derived from patients with reticular, erosive and atrophic types of OLP, suggesting a role of this miRNA in OLP malignant transformation [31]. The results of these previous studies on OLP tissue lesions are in conformity with our current observations in OLP salivary samples. These similarities may be related to a role of miR-21 in inflammation, the regulation of inflammatory cytokines and immune responses associated with T-cells, as has been proposed for patients with OLP [31]. We found that the miR-21 levels were significantly increased in the dysplastic OLP saliva samples compared to those without dysplasia. This increase may be related to the process of malignant transformation and/or the inhibition of tumor suppression [15].

Next, we found that the miR-125a saliva levels were highest in healthy subjects and, surprisingly, significantly decreased in patients with OLP ($p < 0.001$), dysplastic OLP and OSCC compared to the healthy control group ($p < 0.0001$). The OSCC miR-125a levels were not significantly different from those with dysplastic OLP, but were significantly lower than those in OLP patients without dysplasia ($p = 0.004$). The miR-125a levels were found to be significantly lower in saliva from patients with dysplastic OLP compared to those from OLP patients without dysplasia ($p = 0.002$, Figs. 1, 2b). Previously, miR-125a levels have been evaluated in both tissue and salivary samples from OSCC patients, as well as in leukoplakia tissue samples, and the noticeable reductions that were observed in the levels of this miRNA [18, 32] are again in conformity with our current results. Although up till now no studies have been published on salivary OLP miR-125a levels, it has been reported that in sera from OLP patients significantly decreased levels of this miRNA compared to healthy controls may be found. This reduction was found to be related to the severity and extent of lesions [33]. Here, we observed reductions in miR-125a levels in all cases, although more significantly in dysplastic OLP and OSCC patients. These similar results in OLP patients may be due to the effect of this miRNA on inflammatory chemokines that induce the activity and infiltration of T-cells in OLP [34]. Considering the additional decreased miR-125a levels observed in the dysplastic OLP and OSCC samples, this miRNA may serve as a biomarker for the detection of malignant transformation in OLP patients, which is novel.

Subsequently, we found that the miR-31 levels were higher in OSCC patients compared to those in the control group ($p = 0.004$), whereas its levels were lower in dysplastic OLP patients compared to those in the healthy control group ($p = 0.01$). The miR-31 levels in patients with OLP without dysplasia were not significantly different from those in the healthy control group, whereas its levels in patients with dysplastic OLP were significantly higher compared to those in the nondysplastic cases ($p = 0.01$, Figs. 1, 2c). Previously, altered miR-31 levels have been reported in OLP tissue samples and, although malignant transformation was not discussed, the authors noted a relationship between miR-31 levels and OLP as a premalignant condition [18, 19]. Others have reported similar results in OSCC patients [35, 36]. In a recent study,

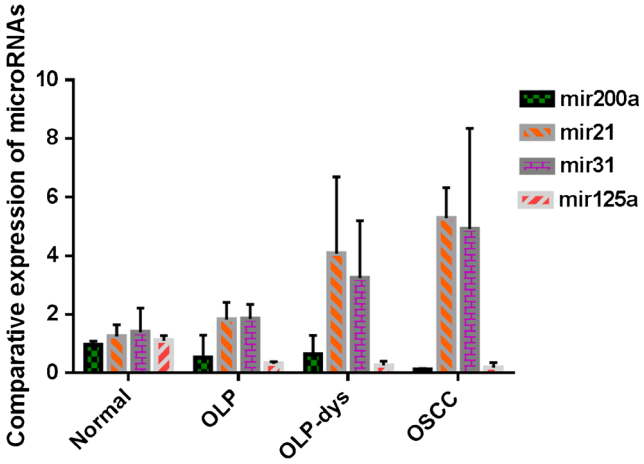

Fig. 1 Comparative salivary miR-200a, miR-21, miR-31 and miR-125a levels. Histograms showing the respective miRNA levels in the different patient groups and the normal control group as determined by qRT-PCR and normalized to miR-U6 Triplicate assays were performed for each RNA sample. OLP (Oral Lichen Planus), OLP-dys (Oral Lichen Planus + dysplasia), OSCC (Oral Squamous Cell Carcinoma)

miR-31 overexpression was noted only in OSCC tumor cells, possibly as a late event in neoplastic evolution after formation of an invasive lesion [36]. These observations appear to be in conformity with our current finding of increased miR-31 levels in saliva of patients with dysplastic OLP and OSCC, but not in those of patients with OLP without dysplasia [36]. Others also observed overexpression of miR-31 in OSCC tissue samples and dysplastic leukoplakia, but not significantly in OLP patients without dysplasia [37], again in conformity with our results. These effects may be attributed to an oncogenic role of miR-31 in early OSCC stages [35]. Accordingly, also this miRNA may serve as a novel biomarker for the detection of malignant transformation in OLP patients.

Finally, we found that the miR-200a levels were relatively highest in saliva samples from the healthy control group. No significant differences in miR-200a levels were found in the saliva samples from the OLP patients, both with and without dysplasia, whereas its levels were found to be significantly reduced in saliva samples from the OSCC patients compared to the healthy control group ($p < 0.0001$). (Figs. 1, 2d). Previously, it was found in a microarray-based study that the miR-200a levels were reduced in three OLP tissue samples [18]. This seemingly contradictory result may be explained by the (limited) sample sizes, the nature of the various specimens included and the different detection methods used. Others observed decreased miR-200a levels in saliva samples from OSCC patients [4, 20, 21], which is consistent with the results obtained in the present study. Based on these data, we conclude that miR-200a may not be considered as a suitable biomarker for the detection of dysplastic OLP. It may, however, be used for the detection of dysplastic lesions in transformation to OSCC.

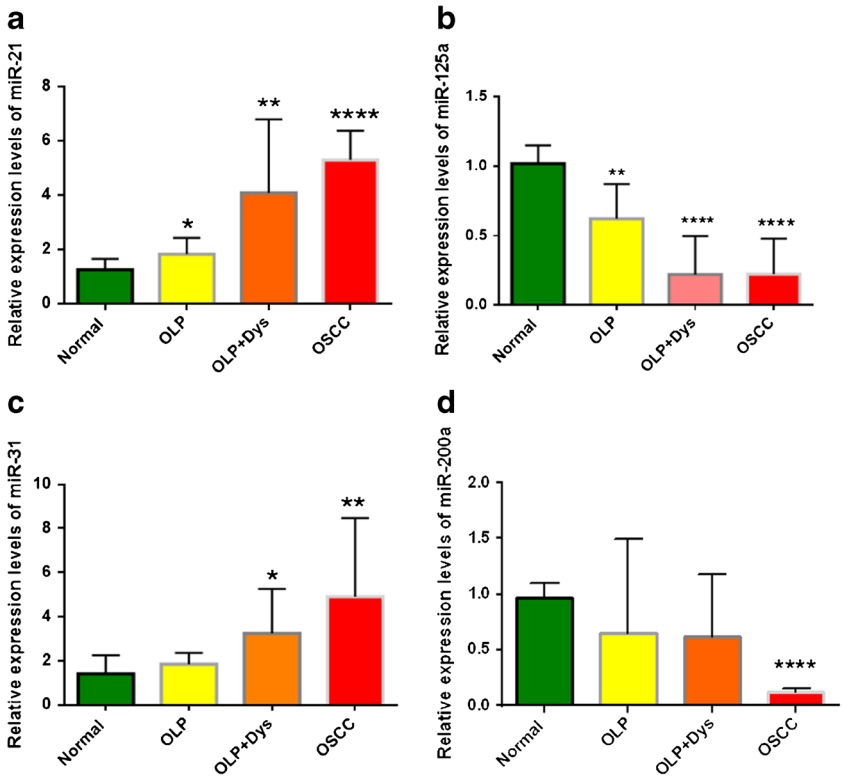

Fig. 2 Histograms showing relative miRNA levels. Relative salivary miRNA-21 (**a**), -125a (**b**), -31 (**c**) and -200a (**d**) levels among different patient-control groups as determined by qRT-PCR. OLP (Oral Lichen Planus), OLP-Dys (Oral Lichen Planus + dysplasia), OSCC (Oral Squamous Cell Carcinoma). (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$)

Taken together, we found that the salivary miR-21 and miR-125a levels in OLP patients were significantly altered, whereas those of miR-31 and miR-200a were not significantly altered in these patients (Fig. 2). Our present data indicate that these four salivary miRNAs may serve as a useful biomarker panel to monitor OLP lesions. Decreased salivary miR-125a levels in conjunction with increased miR-21 levels may be indicative of a poor prognosis in OLP patients. As such, these miRNAs may be used for the detection of malignant transformation in OLP patients. Conversely, lack of significant changes in miR-200a and miR-31 levels may specify absence of malignant transformation in oral mucosal lesions in OLP patients. These novel findings may be instrumental for rapid, convenient and noninvasive OLP monitoring. The analysis of larger cohorts is recommended to validate our current results and to establish exact cut-off values for these miRNAs in relation to each stage of the disease.

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