Clin Oral Invest (2018) 22:1463–1467
https://doi.org/10.1007/s00784-017-2252-6

ORIGINAL ARTICLE

Correlation between tissue expression of microRNA-137 and CD8 in oral lichen planus

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Received: 20 June 2017 / Accepted: 10 October 2017 / Published online: 15 October 2017

Abstract
Objectives Oral lichen planus (OLP) is a chronic, inflammatory condition, classified by the World Health Organization as a premalignant lesion. We performed this study to evaluate the correlation between microRNA-137 (miR-137) and CD8 oral tissue expression in OLP patients.
Materials and methods Twenty OLP patients [classified into three groups: (a) papular, reticular, or plaque; (b) atrophic; and (c) erosive] and 20 healthy controls were subjected to biopsy of the oral mucosa. To evaluate CD8 tissue expression, we performed immunohistochemical examination, followed by immunostaining and computerized quantification. The expression of miR-137 was evaluated using real-time quantitative PCR. We used SPSS software (version 15 for windows) to perform the statistical analysis.
Results Our analysis showed an increased tissue expression of CD8 ($p < 0.01$) and reduced expression of miR-137 ($p < 0.001$) in OLP patients, compared to the control group. Moreover, there was a statistically significant difference ($p = 0.001$) between OLP subgroups in terms of CD8 tissue expression [highest in erosive OLP and lowest in papular/reticular/plaque OLP]. However, these subgroups showed no significant difference ($p = 0.168$) in terms of miR-137 expression. A negative correlation ($p < 0.05$) between tissue expression of miR-137 and CD8 was noted with a varying correlation coefficient in different OLP subgroups (-0.250 in erosive OLP, -0.491 in atrophic OLP and -0.616 in papular/reticular/plaque OLP).
Conclusions Our findings indicate reduced expression of miR-137 and a reverse correlation between tissue expression of miR-137 and CD8 in the oral mucosa of OLP patients.
Clinical relevance Future studies should investigate the therapeutic potential of miR-137 overexpression in OLP patients.

Keywords CD8 · miR-137 · Malignant transformation · Oral lichen planus

Introduction

Oral lichen planus (OLP) is a chronic, inflammatory, and mucocutaneous disease that affects 1 to 2% of the adult population with a female predilection [1]. Clinically, OLP has six forms that may coexist with each other, including white (papular, reticular, and plaque) and red forms (atrophic, erosive, and bullous) [2]. It is classified by the World Health Organization (WHO) as a premalignant lesion [3] with a malignant transformation frequency that ranges between 0.07% and 5.8% in individual studies [4–7]. A recent meta-analysis of 57 studies (20,095 patients) showed that 1.1% of OLP patients develop oral squamous cell carcinoma (OSCC) [8].

Although the etiology and pathogenesis of OLP are still unclear, numerous studies suggested the involvement of immune mechanisms due to the presence of basal cell degeneration and subepithelial T-cell (CD4 and CD8) band-like infiltrations in OLP lesions [9, 10]. The activation of T-lymphocytes is probably mediated by antigen presentation (by basal keratinocytes), mast cell degranulation, and

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activation of matrix metalloproteinases (MPPs). Later, cytotoxic (CD8) T-cells induce basal cell rupture and keratinocyte apoptosis, which play a role in determining the clinical form of OLP, especially the atrophic and erosive forms [11].

MicroRNA (miRNA) is a short (22 nucleotides), noncoding RNA that regulates gene expression at posttranscriptional levels by binding to specific messenger RNAs and promoting their degradation and/or translational inhibition [12]. Therefore, it plays a critical role in the control of cell division, proliferation, differentiation, and death. Alterations in miRNA expression are involved in several pathological processes, including chronic inflammatory and autoimmune diseases [13, 14], as well as cancer [15].

Few studies have indicated significant miRNA alterations in OLP patients [16–18]. In sera from 30 patients with multifocal OLP, Nylander et al. identified 15 miRNAs with significant differential expression compared to controls, including miR-21, miR-223, and miR-143 (connected to epithelial cancer) and miR-21 and miR-181a (connected to autoimmune diseases) [18]. MicroRNA-137 (miR-137) is a tumor-suppressor miRNA, located on chromosome 1p22 [19]. Different studies have referred the tumor suppressor effect of miR-137 to inhibiting the expression of SP-1 [20], p-16 [21], and bromodomain-4 oncogenes [22] in OSCC tissues.

Therefore, studying different miRNA expression profiles might lead to the discovery of a novel biomarker for OLP diagnosis and possible malignant transformation. We performed this study to evaluate the correlation between tissue expression of miR-137 and CD8 in OLP patients.

Materials and methods

Subjects and tissue specimens

Twenty OLP patients [diagnosed according to the WHO modified diagnostic criteria [3]] and 20 OLP-free controls were recruited from the outpatient clinic (oral medicine, periodontology and oral surgery department, Faculty of Oral and Dental Medicine, Cairo University) during the period between January 2013 and December 2016. Later, OLP patients were divided into three subgroups according to the clinical type (a. papular, reticular, or plaque [white types]; b. atrophic; and c. erosive [red types]). All OLP patients were nonsmokers, nonalcoholics, free from other oral (including OLP malignant transformation) and systemic diseases, and did not receive any OLP treatment for at least 3 months before the biopsy. The study protocol was approved by the Local Committee of Research Ethics at Cairo University. Because the majority of enrolled participants were illiterate, each of them gave an oral consent after a thorough explanation of the study objective and procedures.

Clinical data were collected during the first visit, including age, sex, as well as follow-up duration and OLP clinical type. The oral mucosa biopsy from the control group was obtained from the non-inflamed operculum during surgical third molar removal in the oral surgery department or non-inflamed gingiva during aesthetic procedures in the periodontology department. Each biopsy specimen was divided into two parts: one part was fixed in formalin, and then embedded in paraffin for histopathological examination, while the other part was stored at − 80 °C until assayed for miR-137 expression.

Histopathological examination (CD8 detection)

The tissue blocks were cut into sections, each of 4 μm thickness. Hematoxylin and eosin examination was performed to confirm OLP diagnosis and detect any dysplastic features. We later performed immunohistochemical examination to compare CD8 tissue expression in OLP and control patients. The demonstration of CD8 expression in tissue specimens of different OLP subtypes was done by immunostaining. This involved a two-step process in which the unlabeled primary monoclonal antibody (CD8 anti T-cell) was added, followed by a labeled secondary antibody. For detection and visualization of the bound antibody, a detection kit (BIOGENEX, Fremont, USA) was used. Later, we employed a quantitative computerized method to evaluate the staining distribution of CD8.

MicroRNA tissue extraction

miRNA was extracted from oral biopsy samples by miRNeasy extraction kit (Qiagen, Valencia, California, USA), using 750 μL of QIAzol lysis reagent. This was followed by repeated centrifugation at 8000 to 12,000×*g* and addition of biochemical buffers [RWT (700 μL) and RPE (500 μL)]. The extracted miRNA was later stored at − 80 °C until use.

Reverse transcription and real-time quantitative PCR

Reverse transcription (RT) was carried out on miRNA in a final volume of 20 μL RT reactions (incubated for 60 min at 37 °C, followed by 5 min at 95 °C) using the miScript-II-RT kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Real-time quantitative PCR (qPCR) was performed using a Green (MiScript SYBR) PCR kit and miScript primer assays for miR-137 (Qiagen, Valencia, CA, USA). Twenty nanograms of cDNA were used as a template in a total volume of 20 μL reaction with the following conditions: denaturation at 95 °C for 15 min followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s, in which fluorescence was acquired and detected by Rotor-Gene Q Real-time PCR system (Qiagen, USA). After the PCR cycles,

melting curve analyses were performed to validate the specific generation of the expected PCR product. We used SNORD as an endogenous control.

The expression of miR-137 in OLP and control groups was compared using the $\Delta\Delta Ct$ method. The cycle threshold (Ct) value is the number of qPCR cycles required for the fluorescent signal to cross a specified threshold. Therefore, ΔCt was calculated by subtracting the Ct values of SNORD from those of target microRNAs, while $\Delta\Delta Ct$ was calculated by subtracting the ΔCt of the control samples from the ΔCt of OLP samples. The fold change in miR-137 expression was calculated by the equation ($2^{-\Delta\Delta Ct}$).

Statistical analysis

Data were expressed in terms of mean ± standard deviation (SD) or frequencies and percentages when appropriate. We used the Student's t test to compare numerical variables between the two main groups and the Wilcoxon signed rank test for within-group comparisons. For comparing categorical data, the chi-square (χ^2) test was performed. The Fisher's exact test was used instead when the expected frequency was less than 5. The correlation between various variables was performed using the Spearman rank correlation equation. A p value of less than 0.05 was considered statistically significant. All statistical calculations were performed using the Statistical Package for Social Sciences (SPSS, Chicago, IL, USA) software (Version 15 for Windows).

Results

We enrolled 20 patients with OLP [including 14 females (70%) and 6 males (30%)] and 20 healthy control subjects [including 7 females (35%) and 13 males (65%)]. The mean age values were 48.85 (±8.9) and 32.04 (±10.02) years for OLP patients and control subjects, respectively. The follow-up period of OLP patients ranged from 1 to 48 months. Table 1 shows the baseline characteristics of enrolled OLP patients in our study.

CD8 tissue expression

Data analysis showed a statistically significant difference ($p < 0.01$) in CD8 tissue expression between OLP (4.7 ± 2.43) and control (0.70 ± 2.43) groups. Moreover, CD8 tissue expression differed significantly ($p = 0.001$) between different OLP subgroups [highest in erosive OLP (7.20 ± 1.13), followed by atrophic OLP (4.10 ± 1.74) and lowest in papular/reticular/plaque OLP (2.09 ± 0.91)].

Table 1 The baseline characteristics of enrolled oral lichen planus (OLP) patients in the current study

Patient no.	Sex	Age (years)	Follow-up duration (months)	Clinical OLP type
1	Female	60	6	Atrophic
2	Male	48	3	Erosive
3	Female	40	–	Papular
4	Male	60	4	Atrophic
5	Male	47	More than 12	Plaque
6	Female	58	More than 3	Atrophic
7	Female	59	9	Atrophic
8	Female	26	1	Atrophic
9	Male	45	48	Erosive
10	Female	43	2	Erosive
11	Female	55	5	Bullous erosive
12	Female	50	6	Atrophic
13	Female	34	1	Erosive
14	Female	49	–	Plaque
15	Male	47	–	Atrophic
16	Female	49	5	Erosive
17	Female	42	2	Papular
18	Female	53	1	Erosive
19	Male	55	–	Reticular
20	Female	57	4	Atrophic

microRNA137 gene expression

There was a statistically significant difference ($p < 0.001$) in miR-137 expression in the oral tissue mucosa between OLP (2.02 ± 1.17) and control (6.47 ± 1.53) groups. However, no statistically significant difference in miR-137 expression was detected between different OLP subgroups ($p = 0.168$). Table 2 summarizes the results of evaluated outcomes.

Correlation of miR-137 gene expression with CD8 tissue levels in OLP subgroups

Our analysis showed a negative correlation ($p < 0.05$) between the tissue expression of miR-137 and CD8 with a varying correlation coefficient in different OLP subgroups [-0.250 in erosive OLP, -0.491 in atrophic OLP, and -0.616 in papular/reticular/plaque OLP].

Correlation between tissue expression and disease-associated risk factors

Using the Student's t test, no statistically significant association was found between the age of OLP patients and lesional expression of CD8 or miR-137 ($p > 0.05$). Similarly, the

Table 2 Summary of CD-8 and miRNA-137 tissue expression results

	Oral lichen planus patients ($N = 20$)			Control group ($N = 20$)	p value
CD8 tissue expression	4.7 ± 2.43			0.70 ± 0.23	< 0.01*
	Papular/reticular/plaque	Atrophic	Erosive		
	2.09 ± 0.91	4.10 ± 1.74	7.20 ± 1.13		0.001#
miRNA-137 tissue expression	2.02 ± 1.17			6.47 ± 1.53	< 0.001*
	Papular/reticular/plaque	Atrophic	Erosive		
	2.74 ± 0.55	1.96 ± 1.33	1.58 ± 1.18		0.168#

Data are mean ± standard deviation. The p value was considered significant if ≤ 0.05

*Comparing OLP and control groups, #Comparing different OLP groups

Fisher's exact test revealed no significant association ($p > 0.05$) between the gender of OLP patients and the tissue expression of CD8 and miR-137 in OLP lesions.

Discussion

Our study showed that OLP patients have higher mucosal tissue levels of CD8 and reduced-miR-137 expression, compared to individuals with healthy oral mucosa. For the first time, it provides an evidence of a negative correlation between miR-137 and CD8 expression in the oral mucosal tissue of OLP patients. It also showed that among different clinical types of OLP, the erosive and atrophic forms have significantly higher levels of CD8 tissue expression than papular and reticular types.

A former study by Dang and colleagues compared miR-137 and p16 methylation frequency between normal individuals, OLP patients, and those with OSCC. The authors found that OLP tissue has a higher frequency of miR-137 methylation than the normal tissue; however, that was significantly lower than OSCC tissues [21]. Therefore, the lower expression of miR-137 in our study can be explained by its increased methylation during OLP pathogenesis. Being a tumor suppressor RNA, reduced expression of miR-137 is possibly linked to the malignant transformation of OLP. The same finding was previously reported in other cancer types, including colorectal cancer [23] and cancers of the head and neck [19]. Interestingly, Dang et al. also showed that miR-137 methylation was restricted to the epithelium only, suggesting that OLP malignant transformation starts in the epithelium then spreads to the underlying connective tissue. Their findings were consistent with previous reports that showed different methylation patterns of miR-137 in normal and malignant epithelial tissues in the oral cavity [24, 25].

Other miRNA alterations have been reported in OLP patients before. Zhang et al. showed that in comparison to normal controls, 46 miRNAs were differentially expressed in OLP patients, including 38 downregulated and 8 upregulated miRNAs [17]. Another study by Arao et al. demonstrated the upregulation of miR-146a and miR-155 in OLP patients [16]. Moreover, Danielsson et al. detected a non-significant trend towards reduced expression of miR-21 in patients who had OLP for ≥ 5 years [26].

Due to the unclear pathogenesis of OLP, the role of miR-137 in this process remains unconfirmed. Several studies have indicated an increased proliferation rate of the basal epithelial cells in OLP lesions [27–29]. The reduced expression of miR-137, detected in our study, may help explain this finding. Other studies suggested that infiltrating inflammatory cells induce DNA mutations through pro-inflammatory cytokines and carcinogenic metabolites [10]. The negative correlation between miR-137 and CD8 tissue expression suggests anti-inflammatory or immunomodulatory functions for miR-137.

Based on our results and those of other studies [20, 22], miR-137 may have an antiproliferative function, which may be of use in the management of OLP and OSCC. Sun and colleagues showed that miR-137 overexpression suppressed the cellular proliferation in tongue SCC, as well as the expression of SP-1 oncogene and the epithelial-mesenchymal transition (a pathological criteria in oral carcinomas) [20]. Similarly, Tianpeng et al. showed that miR-137 overexpression inhibited cellular proliferation, migration, and invasion in OSCC tissues [22].

Few risk factors, such as smoking, alcohol consumption, and use of immunotherapeutic drugs are suggested to be involved in the pathogenesis of OLP or its malignant transformation [8, 30]. A recent meta-analysis showed an increased risk of OLP malignant transformation in smokers [8]. Established as a carcinogen, the debate in the literature is about whether smoking is an independent risk factor for OSCC or just increases the malignant potential of OLP. Other risk factors, such as the use of immunotherapeutics for OLP treatment or HCV infection may weaken the immune surveillance, increasing the malignant potential of OLP [8, 30]. However, further evidence is needed to establish the association between these risk factors and OLP malignant transformation.

Our analysis failed to document a correlation between CD8 and miR-137 tissue expression and the age or gender of

enrolled patients. Considering the small sample size in our analysis, larger studies are needed to confirm the lack of association and to test this correlation with other disease-associated factors, such as smoking and the use of immunotherapeutic agents.

Overall, our findings indicate a negative correlation between tissue expression of miR-137 (reduced) and CD8 (elevated) in the oral mucosa of OLP patients. Future studies should investigate the therapeutic potential of miR-137 overexpression in OLP and OSCC. Moreover, the effect of smoking, alcohol consumption, and immunotherapeutic drugs on the mucosal tissue expression of miR-137 should be further investigated.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical approval Approval was obtained from the Local Committee of Research Ethics at Cairo University, Egypt.

Informed consent All participants gave an oral consent to undergo biopsy from the oral mucosa.

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