Different Expression of MicroRNA-146a in Peripheral Blood CD4+ T Cells and Lesions of Oral Lichen Planus

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Abstract—Oral lichen planus (OLP) is a common T cell-mediated chronic inflammatory disease of unknown etiology. Recent increasing evidence indicates that microRNA-146a (miR-146a) plays a vital role in inflammatory diseases and T cell regulation. This study aimed to investigate the expression of miRNA-146a in peripheral blood CD4+ T cells and local OLP lesions and to evaluate its relationship with clinical forms of OLP. Sixteen patients with OLP were divided into two groups: erosive OLP and nonerosive OLP. The expression of miR-146a was examined by quantitative real-time polymerase chain reaction. The expression of miR-146a in peripheral blood CD4+ T cells showed no significant difference between OLP group and control group (P > 0.05), and among erosive OLP, nonerosive OLP, and control groups (P > 0.05 for all). The expression in local lesions of the OLP group was significantly higher than that of the control group (P = 0.003), and it was significantly higher in the erosive OLP group than in the non-erosive OLP (P = 0.010) and control groups (P = 0.007). However, miR-146a expression in the nonerosive OLP group did not significantly differ from that in the control group (P > 0.05). These data indicate that miR-146a might be more involved in the local immune disorder of OLP. MiR-146a might be utilized as a candidate biomarker to estimate the severity of OLP.

KEY WORDS: oral lichen planus; microRNA-146a; CD4+ T cells; lesion.

INTRODUCTION

Oral lichen planus (OLP) is a common T cell-mediated chronic inflammatory disease with a prevalence of 0.1–4%, and it has been classified as a precancerous condition by the World Health Organization (WHO) [1–3]. The disease affects the oral mucosa with different clinical presentations, mainly including erosive and nonerosive forms [2]. The typical pathological characteristics of OLP are the degeneration of basal cells and infiltration of dense lymphocytes, especially CD4+ T cells, into the lamina propria [4]. Considering the distinct characteristics of their spatial and temporal distribution, CD4+ T cells were believed to be greatly involved in the pathogenesis of OLP, via secreting various cytokines and activating CD8+ cytotoxic T cells [4, 5]. Our previous report revealed that CCL5/CCR5 might participate in the long-term immune disorder of OLP through the recruitment of CD4+ T cells [6]. Whether any other factors contribute to this activated CD4+ T cells immune response in OLP is still unclear.

MicroRNAs (miRNAs) play an important role in regulating immune response as well as in immune cell development [7–10]. MiRNAs are a class of 21–23 nucleotide long, endogenous small, single-stranded, and non-coding RNAs involved in regulating several important physiological and pathological processes [11, 12]. Numerous reports have focused on the role of miR-146a in CD4+
not received any medical immune treatment within 6 months. The group of OLP patients consisted of nine females and seven males with a mean age of 46.8 years, an age range from 36 to 62 years, and a duration range from 1 to 24 months. According to the clinical features, the patients were divided into two subgroups, including six erosive cases and ten nonerosive cases. Nine healthy volunteers were recruited as the controls, consisting of five females and four males with a mean age of 42.0 years and an age range from 21 to 57 years. There was no significant difference of age, gender, and disease duration between any two groups (P > 0.05). Normal tissue samples were from gingival tissues which adhered to the extracted teeth. The clinical characteristics of the subjects are listed in Table 1.

CD4+ T Cell Isolation and Tissue RNA Sample Preparation

Peripheral blood samples were drawn from each subject, which were added in heparin for lymphocytes isolation. After diluted with equivalent PBS, the blood with heparin was transferred to the centrifuge tubes with lymphocyte separation medium (Tianjin Haoyang Biological Manufacture Co. Ltd, Tianjin, China). Isolation of PBMCs was performed by Ficoll-Paque density gradient centrifugation. CD4+ T cells were negatively selected from PBMCs by using Human CD4 T Lymphocyte Enrichment Set-DM (BD Biosciences, Franklin Lakes, NJ, USA).

Oral tissue samples were collected from the lesion sites of OLP patients and the gingival tissues adhering to the extracted teeth of control subjects, respectively. The tissues were cracked and homogenized with QIAzol lysis buffer. The homogenate was transferred into a new RNA enzyme EP tube, and chloroform was added. After centrifugation, the supernatant was taken and added ethanol to use.

RNA Extraction and Reverse Transcription

Total RNA was extracted using miRNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). Concentration and purity were then determined by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Dubuque, IA, USA). Using miScript Reverse Transcription Kit (QIAGEN GmbH, Hilden, Germany), the total RNA sample was incubated at 37 °C for 60 min and then change to 95 °C for 5 min to convert total RNA back into cDNA.

MATERIALS AND METHODS

Subjects

All OLP patients were from the Department of Oral Medicine, School and Hospital of Stomatology, Wuhan University. All participating subjects gave their informed consents. The experiments followed the principles outlined in the Declaration of Helsinki in the use of human samples and were approved by Ethical Committee Board of School and Hospital of Stomatology, Wuhan University. OLP patients enrolled in the present study were clinically diagnosed and pathologically confirmed as OLP according to the definition of OLP made by the WHO [18]. The participating subjects had neither any systemic disorders (such as cardiovascular disease and diabetes mellitus) nor any soft tissue lesions in the oral mucosa. All subjects were not smokers and severe alcoholics. And, patients of OLP had not received any medical immune treatment within 6 months.

The group of OLP patients consisted of nine females and seven males with a mean age of 46.8 years, an age range from 36 to 62 years, and a duration range from 1 to 24 months. According to the clinical features, the patients were divided into two subgroups, including six erosive cases and ten nonerosive cases. Nine healthy volunteers were recruited as the controls, consisting of five females and four males with a mean age of 42.0 years and an age range from 21 to 57 years. There was no significant difference of age, gender, and disease duration between any two groups (P > 0.05). Normal tissue samples were from gingival tissues which adhesion to the extracted teeth. The clinical characteristics of the subjects are listed in Table 1.

Given that OLP is a T cell-mediated precancerous inflammatory autoimmune condition, we hypothesized that miR-146a might be involved in the pathogenesis of OLP. However, very few studies have reported the role of miR-146a in OLP, and the expression of miR-146a in peripheral blood CD4+ T cells is still unknown. Therefore, in the present study, we detected the expression of miR-146a in the peripheral blood CD4+ T cells and lesion tissues and analyzed its association with the different clinical forms of OLP.

CD4+ T Cell Isolation and Tissue RNA Sample Preparation

Peripheral blood samples were drawn from each subject, which were added in heparin for lymphocytes isolation. After diluted with equivalent PBS, the blood with heparin was transferred to the centrifuge tubes with lymphocyte separation medium (Tianjin Haoyang Biological Manufacture Co. Ltd, Tianjin, China). Isolation of PBMCs was performed by Ficoll-Paque density gradient centrifugation. CD4+ T cells were negatively selected from PBMCs by using Human CD4 T Lymphocyte Enrichment Set-DM (BD Biosciences, Franklin Lakes, NJ, USA).

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Quantitative Real-Time Polymerase Chain Reaction (Real-Time PCR)

The analyses of miR-146a expression was done with miScript SYBR Green PCR Kit (QIAGEN GmbH, Hilden, Germany) using ABI 7500 Real-Time PCR system (Applied Bio-Systems Inc., Foster City, CA, USA) in a one-step real-time PCR 96-well optical plate. The PCR initial activation step was at 95 °C for 15 min, followed by 15 s at 94 °C for denaturation, 30 s at 55 °C for annealing, and 34 s at 70 °C for extension. The cycle number was set as 35 cycles. MiR-146a expression was normalized to endogenous snRNA-U6 as internal control. The forward primer and reverse primer of U6 were synthesized as C T C G C T C C G C A G C A C A G G A C C T C and A A C C G C T T C A C C G A T T T G G T, respectively. The forward primer sequence of miR-146a was designed as T G A G A A C T G A T T C A T T C A T (Invitrogen™ Life Technologies, Shanghai, China), and a miscript universal primer had already been offered in the PCR kit. The threshold cycle (Ct) of three replicates for miR-146a examining and two replicates for internal control per sample was used to calculate $2^{-\Delta\Delta CT}$.

Statistical Analyses

Data were presented as mean±standard deviation (SD) or median (min–max), and statistical significance was defined as $P$ value <0.05. The test of data normality (Shapiro–Wilk), Mann–Whitney $U$, one-way ANOVA, and Kruskal–Wallis tests were analyzed with SPSS 13.0 for windows software (SPSS, Inc., Chicago, IL, USA).

RESULTS

Expression of miR-146a in Peripheral Blood CD4+ T Cells and Different Clinical Forms of OLP

The expression level of miR-146a in peripheral blood CD4+ T cells of OLP group (2.66, 0.09–14.84) was about 1.8 times of the control group (2.17, 0.003–5.16), but no significant difference was found ($P$>0.05) (Fig. 1a).

When OLP subjects were divided into erosive and non-erosive forms according to their clinical manifestations, we found that there was no statistical difference between the two forms of OLP subjects in the expression of miR-146a ($P$>0.05). Furthermore, neither erosive (6.69, 0.09–14.84) nor nonerosive OLP (2.66, 0.17–4.58) group showed statistical difference in the expression of miR-146a, compared with control group (2.17, 0.003–5.16) ($P$>0.05) (Fig. 1b).

Expression of miR-146a in Local Tissues and Different Clinical Forms of OLP

The expression of miR-146a of OLP group (2.61, 0.40–16.98) was significantly higher than that of the control group (1.27, 0.23–2.05) ($P=0.003$) (Fig. 2a).

The expression level of miR-146a in erosive OLP group (6.49 ± 5.97) was significantly higher than that in the control group (1.07 ± 0.60) ($P=0.007$), the former was about 6.1 times that of the latter. The expression of miR-146a of the erosive group (6.49 ± 5.97) was significantly higher than that of the nonerosive group (2.21 ± 1.01) ($P=0.010$), the former was about 2.9 times that of the latter. The expression level of miR-146a in nonerosive OLP group (2.21 ± 1.01) was about 2.1 times of the control group (1.07 ± 0.60), but there was no statistical difference ($P$>0.05) (Fig. 2b).

DISCUSSION

MiRNAs play a vital role in the regulation of inflammatory and autoimmune diseases, and their overexpression or underexpression may result in several pathogenic disorders via affecting the human immune response [9, 19]. The present study showed that miR-146a expression in peripheral blood CD4+ T cells in patients with OLP was not significantly different from that in the control group. However, Arão et al. [20] found that most of the samples...
of OLP (67.7%) showed increased expression of miRNA-146a in peripheral blood mononuclear cells (PBMCs). We speculated that this difference could be attributed to several factors. Firstly, Arão et al.’s study provided a descriptive explanation of data rather than quantitative statistical analysis. Secondly, PBMCs contain monocytes and all kinds of lymphocytes [21]. Therefore, increased expression of miR-146a in PBMCs may be associated with other cells rather than CD4+ T cells.

MiR-146a expression in local lesions in the OLP group was significantly higher than that in the control group. In our previous study, we observed activation of NF-κB-p65 and overexpression of TNF-α in local lesions of OLP; this positive regulatory loop may amplify and perpetuate inflammation [22]. Other researchers have reported that increased miR-146a expression in T cells is positively correlated with elevated TNF-α level, and that miR-146a could negatively regulate NF-κB pathway by repressing the target genes TRAF6 and IRAK1 [10, 23–25]. Therefore, we speculated that the increased expression of miR-146a in OLP lesions may be due to upregulated NF-κB-associated inflammatory cytokine TNF-α. Based on the upregulated expression of miR-146a in lesions, the present study revealed that miR-146a might participate in local immune disorder and the persistent inflammation in OLP. In addition, Rodríguez-Núñez et al. [26] found that the mean serum levels of Ig and the complement in patients with OLP were all within the corresponding normal ranges.

Fig. 1. Expression of miR-146a in peripheral blood CD4+ T cells. 2^\text{-ΔΔCT} was used to calculate the relative expression of miR-146a, and results were represented as box plots. The boxes stretch from the 25th to the 75th percentile; the lines across the boxes indicate the median values; the lines stretching from the boxes indicate extreme values. Mann–Whitney test and Kruskal–Wallis t test were used respectively. P value is shown in the blank.

Fig. 2. Expression of miR-146a in local lesions. Results were represented as box plots. The boxes stretch from the 25th to the 75th percentile; the lines across the boxes indicate the median values; the lines stretching from the boxes indicate extreme values. Mann–Whitney test and one-way ANOVA test were used. *P < 0.05, **P < 0.01.
Based on the above, miR-146a might be more involved in local immune disorder of OLP, compared to systemic immune response.

Several studies have demonstrated that the clinical manifestations, pathological features, treatment, and the risk of malignant transformations differ considerably between erosive and nonerosive OLP [26–31]. Rodriguez-Núñez et al. found that the mean proportions of CD4⁺CD45RO⁺ and DR⁺ lymphocytes in the peripheral blood were significantly higher in atrophic-erosive OLP than in reticular OLP, whereas the mean proportion of CD8⁺CD45RA⁺ lymphocytes was significantly lower in atrophic-erosive OLP [26]. Mazzarella et al. found increased expression of mRNA for matrix metalloproteinase in biopsy samples from lesions in erosive OLP compared to reticular OLP, while some other investigators discovered that salivary levels of a series of cytokines, such as IL-8, TNF-α, and IL-4, in the atrophic/ulcerative form of OLP were significantly higher than in the reticular form [28, 29, 31, 32]. Further, our previous study showed a negative correlation between miR-125a content and reticular, atrophic, and erosive lesion (RAE) scores used to assess the severity of OLP in different clinical forms, suggesting that miRNAs might have varied expression in different clinical forms of OLP [6]. In the present study, we observed a significant increase of miR-146a expression in local lesions of patients with erosive OLP (about 2.9 times) compared with nonerosive OLP. Together with the findings from previous studies, our results have confirmed that different clinical forms of OLP may have a distinct immune modulatory background.

Several studies [12, 33, 34] have shown that abnormal expression of miRNAs plays an important role in cancer development through regulating the expression of carcinogenic genes and/or tumor suppressor genes. Abnormal expression of miR-146a was found in a variety of human malignancies, such as prostate [35], breast [36], cervical [37], pancreatic [38], and gastric cancers [39, 40]. OLP is a chronic inflammatory disease, which was listed as a precancerous condition by WHO [41]. Hung et al. [42] found upregulation of miR-146 in oral squamous cell carcinoma tissues and speculated that miR-146a expression could contribute to oral carcinogenesis by targeting the IRAK1, TRAF6, and NUMB genes. Erosive OLP shows more severe clinical symptoms than nonerosive OLP and has a higher chance of recurrence and malignancy. The results of this study showed that the expression of miR-146a in erosive OLP was significantly higher than in nonerosive OLP; thus, we concluded that miR-146a may be involved in the malignancy associated with erosive OLP. However, further studies are warranted to confirm this speculation.

In conclusion, the present results demonstrate that miR-146a might play a role in local immune response in OLP and miR-146a could be used as a potential biological marker to evaluate the severity of OLP.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no competing interests.

REFERENCES


