miR-125b inhibits keratinocyte proliferation and promotes keratinocyte apoptosis in oral lichen planus by targeting MMP-2 expression through PI3 K/Akt/mTOR pathway

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1. Introduction

Lichen planus (LP) is a chronic inflammatory disease of the skin and mucosa that usually affects older and middle-aged people [1], and is characterized histologically by a subepithelial band-like lymphocytic infiltrate and epithelial basal cell destruction with the formation of apoptotic bodies [2]. Oral lichen planus (OLP) is restricted solely to the oral cavity, affecting approximately 2% of the population, mainly middle-aged women [3]. Once established, the lesions rarely undergo self-remission and, in some cases, have a malignant potency [4]. However, the etiopathogenesis of OLP remains unclear.

The pathological process of OLP is tightly linked to the degeneration of basal keratinocytes [5]. Keratinocytes are a major cell type in human skin and play a fundamental role in normal skin metabolism [6], with important regulatory roles in the exacerbation and perpetuation of OLP. HaCaT cells are immortalized human skin keratinocytes and suitable substitutes for oral keratinocytes because they can be easily grown and passaged indefinitely [7]. Gram-negative bacterial lipopolysaccharide (LPS) has long been known to induce inflammatory mediators [7]. Accordingly, we established an in vitro inflammatory model of OLP lesions to a certain extent by introducing LPS on cultured keratinocytes.

MicroRNAs (miRNAs) are 19–22 nucleotide-long single-stranded noncoding RNAs that can mediate post-transcriptional silencing by binding with partial complementarity to the 3'
untranslated region (UTR) of the target mRNA [8]. According to our current understanding of miRNA function, a unique miRNA can regulate the expression of multiple proteins, and the expression of a specific protein may be controlled by several miRNAs [9]. These miRNAs, which are mainly highly conservative, have been shown to participate in many fundamental life processes, such as development, differentiation, growth control, autophagy, and apoptosis, along with tumorigenesis. Hence, reduced miRNA expression has been shown to contribute to cancer, heart diseases, infectious diseases, inflammatory diseases, and other medical conditions, making them potential targets for medical diagnosis and therapy [10].

It is believed that matrix metalloproteinases (MMPs) are among the potential key mediators of cell proliferation, cancer invasion and angiogenesis [11]. In human skin, MMPs are up-regulated in response to cytokines, growth factors, cell-matrix interactions, and LPS [6,12]. Moreover, studies have indicated the role of MMP-2 and MMP-9 as inflammatory markers [13]. Currently, the effect of MMP-2 on inflammation has been recognized, but the regulatory mechanism linking MMP-2 with the progression of skin inflammation, especially OLP, remains poorly understood.

As skin inflammation may serve as a model for chronic inflammatory disorders, it is likely that miRNAs involved in skin inflammation will eventually be implicated in other metabolic disorders, such as keratinocyte proliferation and apoptosis, suggesting that some of these miRNAs may become disease markers and therapeutic targets in OLP. Decreased expression of miR-125b has previously been shown in OLP samples compared to normal oral mucosa [14]. The reduced expression of miR-125b may indicate a higher risk of poor outcomes in patients with oral squamous cell carcinoma (OSCC) [15]. In addition, OLP has been associated with a low but clinically relevant increased risk of OSCC [16]. The elevated expression of MMP-2 has been examined in OLP samples [17] and further, increased MMP-2 expression has been closely associated with OSCC [18]. Hence, in the present study, we mainly focused on the regulatory role of miR-125b in MMP-2 expression, as well as on keratinocyte proliferation and apoptosis in an LPS-induced OLP model.

2. Materials and methods

2.1. Tissue samples and cell culture

The study population consisted of 33 cases (9 healthy individuals, and 24 cases of OLP) from the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China). The patients had been clinically and histologically diagnosed with OLP according to the modified diagnostic criteria from the WHO [19], without previous treatment for OLP or antibiotic use, within the last month. After local anesthesia of the lesion site in the OLP group, 8-mm punch biopsies were collected; in the control group, biopsy specimens were obtained from the buccal mucosa using the same procedure. This study was approved by the Ethics Committee of Zhengzhou University and conducted according to the Declaration of Helsinki Principles. Prior to biopsy, all participants were informed about the procedure and informed consent was obtained. All biopsies obtained from OLP lesions of representative areas were immediately embedded in Tissue Tek OCT (Miles Inc., Elkhart, IN, USA), snap frozen in liquid nitrogen, and then stored at −80°C.

The human embryonic kidney cell line HEK293T and the human immortalized skin keratinocyte cell line HaCaT were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HaCaT cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Rockville, MD, USA), and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin; Life Technologies, Rockville, MD, USA) at 37°C in a humidified 5% CO2 incubator. HEK293T cells were grown in DMEM medium supplemented with high glucose, L-glutamine, sodium pyruvate (Life Technologies, Rockville, MD, USA), and 10% FBS.

2.2. Immunohistochemistry

A standard immunohistochemical protocol was used in this assay. Briefly, ~5 μm serial sections were cut and mounted on 3-aminopropyltriethoxysilane-coated slides, and then deparaffinized, and antigen retrieval was performed using a commercially available solution (DAKO, Carpinteria, CA, USA) at pH 6 for 1 h. Endogenous peroxidase was blocked using methanol and 3% H2O2 for 20 min, and incubation with 0.1% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) was performed for 15 min to block non-specific immunoreactions. The primary antibody used was anti-MMP-2 antibody (Oncogene Research Products, Cambridge, MA) at a dilution of 1:200 for 30 min. Biotinylated secondary antibody and streptavidin peroxidase (Universal Dako LSAB Kit; Dako, Sydney, NSW, Australia) were incubated for 15 min each at room temperature. The immune response was visualized using diaminobenzidine (DAB; Vector, Burlingame, CA, USA), with a brown reaction product indicating positive labeling. After counterstaining with hematoxylin, immunopositive cells were examined for the presence of MMP-2 immunoreactivity under a microscope (BX50; Olympus, Japan).

2.3. Quantitative real-time PCR (qRT-PCR) assay

Total RNA in the tissue samples or cultured cells, including miRNAs, was extracted using a miRNA Isolation Kit (Ambion, USA) according to the manufacturer's instructions. The purity and concentration of the RNA samples were determined using a dual-beam ultraviolet spectrophotometer (Eppendorf, Hamburg, Germany). The expression levels of mature miR-125b were analyzed using Taqman miRNA assay (Applied Biosystems, Foster City, CA, USA) and normalized to RNU48 expression. The relative miR-125b level was quantified using the 2−ΔΔCt method. The above experiments were performed in triplicate, and each assay included a negative control that lacked cDNA.

2.4. Cell transfection

Cultured cells (4 × 105) were seeded in 6-well plates (Corning Inc., Corning, NY, USA) for 24 h and transfected with miR-125b mimics, anti-miR-125b mimics or control mimics (GenePharma, Shanghai, China) at a final concentration of 30 nM using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transfection efficiency was evaluated by qRT-PCR assay. The cells were subject to further analysis after 72 h post-transfection as presented in the results section.

2.5. Luciferase reporter assay

The possible site of binding between MMP-2 and miR-125b was searched in a microRNA database (http://www.microrna.org). The 3′-UTR of human MMP-2 containing the miR-125b targeting sequence was inserted into the pMIR-REPORT™ miRNA Expression Reporter Vector System (Ambion, USA). The reporter vector plasmid containing either MMP-2-wt 3′-UTR or MMP-2-mut 3′-UTR sequence was subsequently co-transfected with corresponding miRNAs into HEK293T cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Cultured cells were harvested for luciferase assays 48 h after transfection. The
2.5. Cell counting kit-8 (CCK-8) assay

Cultured cells (5 × 10⁵) were seeded in 96-well plates (Corning Inc.) after transfection or not and incubated in the absence or presence of 10 μg/mL LPS. Cell proliferation was assessed at different time points (0 h, 24 h, 48 h, and 72 h), using the CCK-8 assay (Dojindo, Tokyo, Japan) according to the manufacturer’s protocol. The absorbance value at a wave length of 450 nm, which shows a positive relationship with cellular proliferation, was measured using a microplate reader (Model 450; Bio-Rad Laboratories, Hercules, CA, USA).

2.7. Bromodeoxyuridine (BrdU) assay

Cultured cells (5 × 10⁵) were seeded in 96-well plates (Corning Inc.) after transfection or not and incubated in the absence or presence of 10 μg/mL LPS for 24 h. BrdU incorporation during DNA synthesis was analyzed using a cell proliferation enzyme-linked immuno sorbent assay (ELISA; BrdU kit; Beyotime, Shanghai, China) according to the manufacturer’s protocol. All experiments were performed in triplicate, and the absorbance value of each well was determined at 450 nm using a microplate reader.

2.8. Cell cycle analysis

Cultured cells (4 × 10⁵) were seeded in 6-well plates after transfection or not and incubated in the absence or presence of 10 μg/mL LPS for 24 h. Following collection and fixation in 70% ethanol overnight at 4°C, cells were stained with propidium iodide (PI, 50 μg/mL; Sigma, St. Louis, MO, USA) and DNase-free RNase A (100 μg/mL; Sigma, St. Louis, MO, USA), and then incubated at 37°C for 30 min in the dark before analysis. Quantitative analysis of the DNA content was performed on a fluorescence-activated cell sorting (FACS) Calibur flow cytometer (Becton-Dickinson Immunocytochemistry Systems, San Jose, CA, USA), and the fraction of the cell population in each phase of the cell cycle was determined by flow cytometry analysis.

2.9. Apoptosis assay

Cell apoptosis was measured by flow cytometry analysis following the instructions for the Annexin V-fluorescein isothiocyanate (FITC)/PI Apoptosis Detection Kit (R&D systems, Abingdon, UK). Cultured cells were washed in PBS twice, suspended with 500 μL binding buffer, and then labeled with 5 μL Annexin V–FITC/PI for 15 min in the dark at room temperature. Thereafter, flow cytometry measurement was performed on a FACSAria flow cytometer (BD Biosciences; San Jose, CA, USA). Data were analyzed using BD FACSDiva software (BD Biosciences), and presented as dot plots showing the fluorescence intensity of Annexin-V FITC and PI.

2.10. Western blot analysis

Cell lysates were collected and protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein were processed for western blot analysis following the standard protocol. The primary antibodies used were anti-Akt, anti-phospho-Akt (Ser473), anti-mTOR, and anti-phospho-mTOR (Ser2448) antibodies (Cell Signaling Technology, Beverly, MA, USA); anti-MMP-2 antibody (Oncogene Research Products, Cambridge, MA); and anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The protein bands resulting after incubation with proper secondary antibody were visualized by ECL.

Fig. 1. The expression of MMP-2 and miR-125b in clinical specimens and LPS-treated or non-treated HaCaT cells. (A) MMP-2 expression in normal oral mucosa tissue samples (n = 9) and OLP tissue samples (n = 24) was detected by immunohistochemical analysis. Magnification ×200. (B) MMP-2 expression in LPS-treated or non-treated HaCaT cells was determined by western blotting and representative blots are shown. (C) miR-125b expression levels in clinical specimens were determined by qRT-PCR assay. (D) miR-125b expression levels in LPS-treated or non-treated HaCaT cells were determined by qRT-PCR assay. All experiments were repeated at least three times. *P < 0.05 vs. healthy samples or non-treated HaCaT cells.
compared Spring, 3.2.11. LPS-incubated was revealed control HaCaT (Beyotime, China). The absorbance values of the target proteins and related quantification analysis were determined using Gel-Pro Analyzer version 4.0 software (Media Cybernetics, Silver Spring, MD, USA), and data were normalized to β-actin.

2.11. Statistical analysis

Data are expressed as mean ± SD of results derived from three independent experiments performed in triplicate. Statistical analysis was performed using Student’s t-test and ANOVA. *P < 0.05 and **P < 0.01 were considered statistically significant compared to the respective control.

3. Results

3.1. miR-125b expression was down-regulated and MMP-2 expression was up-regulated in both OLP mucosa tissues and LPS-incubated HaCaT cells

Immunohistochemical analysis indicated that MMP-2 expression was significantly up-regulated in OLP tissue samples compared to normal oral mucosa (Fig. 1A). qRT-PCR assay showed that the levels of miR-125b were markedly reduced in OLP samples compared to healthy mucosa samples (Fig. 1C). In addition, LPS incubation lowered the miR-125b level, but augmented MMP-2 expression in HaCaT cells (Fig. 1B and D). The results revealed that miR-125b expression was down-regulated and MMP-2 expression was up-regulated in both OLP mucosa tissues and LPS-incubated HaCaT cells.

3.2. miR-125b overexpression suppressed LPS-induced MMP-2 expression in HaCaT cells

Western blot analysis indicated that MMP-2 expression was up-regulated by LPS incubation, whereas miR-125b transfection abolished the enhanced expression of MMP-2 protein induced by LPS incubation in HaCaT cells (Fig. 2). These data implied that miR-125b might be a promising candidate for directly targeting MMP-2 in HaCaT cells.

3.3. miR-125b directly targeted MMP-2

Bioinformatic prediction showed that there was one putative binding site between miR-125b and the 3’-UTR of MMP-2 (Fig. 3A). To confirm this, a luciferase reporter assay was performed by evaluating the luciferase activity of HEK293T cells transfected with the pMIR-MMP-2 3’-UTR plasmids and comparing this activity with that of cells transfected with control plasmids. The results showed that miR-125b significantly suppressed the luciferase expression of MMP-2-wt, whereas MMP-2-mut had no suppressive effect (Fig. 3B). In addition, qRT-PCR analysis confirmed that miR-125b transfection led to an increase in mature miR-125b in HaCaT cells (Fig. 3C). The protein level of MMP-2 was restrained following miR-125b transfection in HaCaT cells (Fig. 3D). In summary, these results indicate that miR-125b directly targets MMP-2 in HaCaT cells.

3.4. miR-125b overexpression impeded HaCaT cell proliferation in LPS-induced OLP model

To investigate the effects of LPS and miR-125b on the proliferation of HaCaT cells, CCK-8 assay, BrdU assay, and FACs cell cycle analysis were performed. The OD value in the CCK-8 assay revealed that miR-125b overexpression inhibited LPS-promoted cell proliferation in a time-dependent manner compared with the blank control group (Fig. 4A). The miR-125b-induced inhibition of DNA synthesis was further analyzed by measuring BrdU incorporation: a 64% decrease in BrdU incorporation was detected in response to miR-125b transfection compared to blank control cells (Fig. 4B). In addition, we carried out FACs analysis of DNA content, the results of which revealed that LPS led to a 12.1% reduction in the number of cells in the G0/G1 phase of the cell cycle as compared to the blank control group; conversely, miR-125b overexpression promoted an distinct accumulation of cells at the G0/G1 phase, resulting in cell cycle arrest (Fig. 4C). Thus, these findings illustrated that miR-125b overexpression impeded HaCaT cell proliferation in an LPS-induced OLP model.

3.5. miR-125b overexpression promoted HaCaT cell apoptosis in LPS-induced OLP model

To further assess the effect of miR-125b on HaCaT cells in an LPS-induced OLP model, the percentage of apoptotic cells in miR-125b-transfected HaCaT cells was evaluated by flow cytometry. The ratio of apoptotic cells in the group treated with LPS and miR-125b transfection was markedly increased compared to the group treated with LPS alone (Fig. 5). This result suggested that miR-125b overexpression could accelerate cell apoptosis in LPS-incubated HaCaT cells.

3.6. miR-125b overexpression inhibited LPS-induced PI3 K/Akt/mTOR activation in HaCaT cells

To explore the potential molecular mechanism of miR-125b in the LPS-induced OLP model, we evaluated the phosphorylation of Akt and mTOR in LPS-incubated HaCaT cells. Western blot analysis demonstrated that the phosphorylation of Akt and mTOR proteins was enhanced under LPS pretreatment, which was robustly suppressed by miR-125b transfection (Fig. 6). Accordingly, the results implied that miR-125b may act as a suppressor in the pathological process of OLP via the PI3 K/Akt/mTOR signaling pathway.
Pathologically, OLP is an inflammatory process involving either oral and/or skin mucosal epithelial surfaces [20], the biological assessment of which frequently involves keratinocytes [21]. Previous studies have indicated that miRNAs are short, single-stranded, noncoding RNAs that play important roles in the regulation of gene expression and bioinformatic predictions indicate that miRNAs regulate more than 30–60% of protein-coding genes in the human genome [22]. Therefore, in this study, HaCaT keratinocytes, a spontaneously transformed human epithelial cell line, were used to help investigate the functional role of miR-125b in OLP and elucidate the related molecular mechanism.

Expression profiling in healthy human organs/tissues has indicated that miR-125b expression is found in most tissues, and in normal skin, it is mainly expressed in resident cells such as fibroblasts, keratinocytes, and melanocytes [23]. Studies have shown that OLP is a chronic inflammatory process accompanied by the degeneration of basal keratinocytes. In inflammatory skin conditions, including psoriasis [23], atopic eczema [24], head and neck squamous cell carcinoma (HNSCC) [25], and OSCC [26],

Fig. 3. MMP-2 is a direct target gene of miR-125b. (A) MicroRNA database search revealed one possible binding site in the MMP-2 3’-UTR for miR-125b. (B) Luciferase reporter assay to measure the reporter activity of MMP-2-wt 3’-UTR or MMP-2-mut 3’-UTR sequence was performed in HEK293T cells. *P < 0.05 vs. control mimics group. (C) miR-125b expression levels in HaCaT cells were determined by qRT-PCR assay 72 h after transfection with the corresponding miRNAs. (D) MMP-2 protein levels in HaCaT cells were determined by western blotting 72 h after transfection with the corresponding miRNAs. All experiments were repeated at least three times. *P < 0.05 vs. Blank control group.

Fig. 4. Effects of LPS and miR-125b on cell proliferation in HaCaT cells. Cultured HaCaT cells were transfected with 30 nM miR-125b mimics or not in 96-well plates or 6-well plates, incubated in the absence or presence of 10 μg/mL LPS for 24 h, 48 h, or 72 h as indicated, and then processed for respective detection. (A) Cell growth was determined by CCK-8 assay. (B) Cell proliferation assay was performed using an ELISA BrdU kit. (C) Representative cytometric histogram of cell distribution in the G0/G1, S, and G2/M phases was determined by flow cytometry analysis. The individual DNA content was determined by the fluorescence intensity of PI. All experiments were repeated at least three times. *P < 0.05 vs blank control group. #P < 0.05 vs. LPS group.
Fig. 5. Effects of LPS and miR-125b on cell apoptosis in HaCaT cells. Cultured HaCaT cells were transfected with 30 nM miR-125b mimics or not in 6-well plates, and then incubated in the absence or presence of 10 μg/mL LPS for 24 h. The cell apoptosis was detected by flow cytometry analysis. All experiments were repeated at least three times. *P < 0.05 vs. Blank control group. #P < 0.05 vs. LPS group.
miR-125b is down-regulated compared to healthy skin. In addition, as an inflammatory marker, the up-regulation of MMP-2 has been illustrated during the carcinogenesis of cutaneous squamous cell carcinoma [13]. The effects of miR-125b on the invasion of glioblastoma CD133-positive cells are associated with the alteration of MMP-2 expression [27], and modulation of miR-125b can distort MMP-2 expression in hepatocellular cancer cells [28]. Clinically, both reduced miR-125b levels and enhanced MMP-2 expression have been implicated in the pathogenesis of OLP compared to normal oral mucosa [14,17]. The HEK293T is an immortalized cell line and easy to cultivate. Both the levels of miR-125 expression and MMP-2 protein were low in HEK293T cells, which may not interfere the results of luciferase reporter assay. So the HEK293T cells were used in luciferase reporter assay as indicated. Thus, in our study, decreased miR-125b levels and increased MMP-2 expression were observed in both OLP mucosa tissues and LPS-incubated HaCaT cells, and further, MMP-2 protein was demonstrated to be a target gene of miR-125b in HaCaT cells. miR-125b is a multifunctional miRNA that plays important roles in many physiological and pathological processes, and can function as a tumor suppressor in humans. In particular, miR-125b down-regulation has been implicated in the progression of OLP and OSCC [14,15]. Overexpression of miR-125b in primary human keratinocytes suppresses proliferation and induces the expression of several known differentiation markers in human psoriasis [29], and miR-125b down-regulation has been associated with cell proliferation and radioresistance mechanisms in OSCC [30]. Restored expression of miR-125b can inhibit cell proliferation, migration and invasion, arrest cell cycle progression, and induce cell apoptosis in Ewing’s sarcoma cell line A673 [31]. Likewise, our current findings revealed that miR-125b overexpression impeded HaCaT cell proliferation and promoted cell apoptosis in an LPS-induced OLP model.

The mammalian target of rapamycin (mTOR) has emerged as a major effector of fundamental cell processes, such as cell survival, proliferation, protein synthesis, and angiogenesis [32]. The critical regulator of mTOR is AKT, a serine/threonine protein kinase, which is activated by nutrients and growth factors in a phosphatidylinositol 3 kinase (PI3K)—dependent manner [33]. It is frequently activated in human cancers and precancerous lesions, and is considered a key regulator of normal and cancerous cell growth and fate decisions [32]. Furthermore, dysregulation in upstream and downstream molecules of mTOR signaling appears to occur in 90–100% of HNSCC, suggesting that the markers and targets in the Akt/mTOR pathway may be of particular clinical relevance [8].

Aberrations in the Akt/mTOR signaling pathway have been identified as one of the most commonly implicated pathways in various types of human cancer, including OSCC [34]. Overexpression of miR-125b decreases cell proliferation, and induces apoptosis through the up-regulation of phospho-Akt and phospho-mTOR proteins in HeLa cervical cancer cells [35]. Interestingly, it is has been suggested that Akt/mTOR activation occurs in the context of OLP patients, which possibly contributes to the premalignant potential of individual cases [36]. In this study, we also demonstrated that miR-125b lowered the enhanced phosphorylation of Akt protein and its downstream target, mTOR protein, in LPS-incubated HaCaT cells, highlighting the potential contribution of PI3 K/Akt/mTOR signaling pathway aberrations in OLP malignant potential.

In conclusion, in our experiments, we proved that miR-125b directly targets MMP-2 expression in HaCaT cells, and further, inhibits keratinocyte proliferation and promotes keratinocyte apoptosis through the PI3 K/Akt/mTOR signaling pathway in an LPS-induced OLP model. This study suggests that miR-125b is a potential therapeutic target for OLP treatment.

Conflicts of interest

The authors declare that they have no conflict of interest.

References
