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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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ABSTRACT

Oral lichen planus (OLP) is a chronic inflammatory mucosal disease that involves the degeneration of keratinocytes. However, the etiology and mechanisms of OLP pathogenesis have not been fully elucidated. In this study, we used keratinocytes HaCaT stimulated with lipopolysaccharide (LPS) to mimic a local OLP immune environment, and investigated the regulatory role of miR-125b in keratinocyte proliferation and apoptosis under OLP conditions. Immunohistochemical analysis and quantitative real-time PCR (qRT-PCR) assay showed that MMP-2 expression was up-regulated and miR-125b expression was down-regulated in both OLP mucosa tissues and LPS-incubated HaCaT cells. Western blot analysis indicated that miR-125b overexpression suppressed LPS-induced MMP-2 expression in HaCaT cells. Molecularly, our results confirmed that MMP-2 is a target gene of miR-125b in HaCaT cells. The effect of miR-125b on cell proliferation was revealed by CCK-8 assay, BrdU assay and cell cycle analysis, which illustrated that miR-125b overexpression impeded LPS-induced HaCaT cell proliferation. Flow cytometry analysis further demonstrated that miR-125b overexpression promoted HaCaT cell apoptosis. Moreover, these effects were involved in PI3 K/Akt/mTOR activation, as miR-125b overexpression inhibited LPS-enhanced expression of p-Akt and p-mTOR proteins. Taken together, these data confirm that miR-125b might inhibit keratinocyte proliferation and promote keratinocyte apoptosis in OLP pathogenesis by targeting MMP-2 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′

Abbreviations: OLP, oral lichen planus; LPS, lipopolysaccharide; miRNAs, microRNAs; qRT-PCR, quantitative real-time PCR; . UTR, un-translated region; MMPs, matrix metalloproteinases; OSCC, oral squamous cell carcinoma; FBS, fetal bovine serum; CCK-8, cell counting kit-8; FITC, fluorescein isothiocyanate; PI, propidium iodide; mTOR, mammalian target of rapamycin; ATCC, American Type Culture Collection; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; BrdU, bromodeoxyuridine; PI3 K, phosphatidylinositol 3 kinase; HNSCC, head and neck squamous cell carcinoma; FACS, fluorescence-activated cell sorting.

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un-translated 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 divided, 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% CO_2 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% H_2O_2 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 $2^{-\Delta\Delta Ct}$ method. The above experiments were performed in triplicate, and each assay included a negative control that lacked cDNA.

2.4. Cell transfection

Cultured cells (4×10^5) 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 subjected 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

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