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RESEARCH ARTICLE

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MiR-126 correlates with increased disease severity and promotes keratinocytes proliferation and inflammation while suppresses cells' apoptosis in psoriasis

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Background: This study aimed to investigate the miR-126 expression in lesional skin and its correlation with clinical features in psoriasis patients and to explore the effect of upregulated miR-126 on cells' proliferation, apoptosis, and inflammation in human keratinocytes.

Methods: A total of 102 psoriasis patients were consecutively enrolled in this study. MiR-126 expressions in lesional skin and paired nonlesional skin were detected by quantitative polymerase chain reaction (qPCR). Human keratinocytes (HaCaT cells) were transfected with miR-126 mimic plasmids and blank mimic plasmid. Cell Counting Kit-8 and annexin V/propidium iodide assays were performed to assess the cells' proliferation and apoptosis, and protein levels of apoptotic markers (cleaved caspase-3 [C-caspase-3] and B-cell lymphoma-2 [Bcl-2]) were detected by Western blot assay. Inflammatory cytokines mRNA and protein levels were detected by qPCR and Western blot assays, respectively.

Results: MiR-126 expression was upregulated in lesional skin tissue compared with paired nonlesional skin tissue, and its expression positively associated with Psoriasis Area and Severity Index score in psoriasis patients. MiR-126 expression was increased in miR-126 mimic group compared with negative control (NC) mimic group after plasmids transfection into HaCaT cells, and cells' proliferation was enhanced while cells' apoptosis rate was reduced in miR-126 mimic group than NC mimic group. Protein expressions of C-caspase and Bcl-2 also indicated miR-126 mimic decreased the cells' apoptosis. In addition, miR-126 mimic increased TNF-α, IFN-γ, IL-17A, and IL-22 expressions while decreased IL-10 expression.

Conclusion: In conclusion, miR-126 correlates with elevated risk and increased disease severity in psoriasis patients, and upregulation of miR-126 promotes cells' proliferation and inflammation while inhibits cells' apoptosis in keratinocytes.

KEYWORDS

apoptosis, inflammation, miR-126, proliferation, psoriasis

1 | INTRODUCTION

Psoriasis, a common chronic and systemic inflammatory skin disease, affects 2%-3% of the population worldwide.[1,2] In China, an epidemiological survey of psoriasis in 6 provinces shows that the prevalence of psoriasis is 0.47%.[3] Patient with psoriasis has a higher risk of developing multiple complications, including psoriatic arthritis, cardiovascular diseases, Crohn's disease, anxiety, and depression, all of them substantially contributing to increased morbidity

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and mortality.[4,5] Although the application of topical therapy, physiotherapy, systemic therapy, and novel biological agents (such as tumor necrosis factor alpha [TNF-α] inhibitor, interleukin [IL]-12/23 inhibitor and IL-17 inhibitor) have achieved a certain effect, the low response rate and high recurrence rate remain critical issues to clinicians.[1,6-8] Therefore, it is of great importance to investigate the underlying mechanism of psoriasis and explore novel treatment target.

MicroRNAs (miRNAs) are a class of small noncoding RNAs that play crucial roles in the regulation of biological processes in signal transduction, cells' proliferation, differentiation, and apoptosis.[9-12] Accumulating studies disclose that dysregulation of miRNAs including miR-31, miR-155, and miR-26b is involved in the pathological processes of a variety of immune diseases including psoriasis.[13-15] MiR-126, located on human chromosome 9q34.3, has been reported to participate in the development and progression of multiple immune diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and inflammatory bowel disease (IBD).[16-18] However, to the best of our knowledge, the role of miR-126 in the etiology of psoriasis has not been reported. Therefore, this study aimed to investigate the miR-126 expression of lesional skin and its correlation with clinical features in psoriasis patients and to explore the effect of upregulation of miR-126 on cells' proliferation, apoptosis, and inflammation in human keratinocytes.

2 | MATERIALS AND METHODS

2.1 | Participants

One hundred and two psoriasis patients were consecutively enrolled in this study between January 2016 and December 2016. The inclusion criteria consisted of: (i) Diagnosed as psoriasis. (ii) In moderate to severe disease condition which was defined as lesional body surface area (BSA; psoriasis-affected) ≥10% and Psoriasis Area and Severity Index (PASI) score ≥8 points. (iii) Age above 18 years. Patients with: (i) solid tumor, (ii) hematological malignancy, (iii) severe infection, (iv) hepatic or renal dysfunction were excluded from our study. This study was approved by the Ethics Committee of Fuling Centre Hospital of Chongqing, and all patients signed the informed consents.

2.2 | Samples and miR-126 detection

After enrollment, 3 mm punch biopsies were taken from the lesional skin of psoriasis patients. Meanwhile, paired nonlesional skin 5 cm away from the lesion was taken as controls. Total RNA was extracted using TRIzol (Invitrogen, USA), and miR-126 expression in tissue of lesional skin was detected by quantitative polymerase chain reaction (qPCR; Detailed methods were described in "qPCR assay" subsection).

2.3 | Data collection

Patients' characteristics including age, gender, body mass index (BMI), disease duration, lesional BSA, PASI score, and treatments were documented after the enrollment.

2.4 | Cells' culture

Human keratinocytes (HaCaT cells) were kindly given by Fudan University (Shanghai, China). After resuscitation, the HaCaT cells were subsequently cultured in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco) at 37°C with 5% (v/v) CO_2.

2.5 | miR-126 mimic plasmids transfection and followed assays

HaCaT cells were transfected with blank mimic plasmid and miR-126 mimic plasmid as negative control (NC) mimic group and miR-126 mimic group, respectively. MiR-126 expression was detected by qPCR assay at 24 hour after transfection, and cells' proliferation was determined by Cell Counting Kit (CCK)-8 assay at 0, 24, 48, and 72 hour after transfection. And cells' apoptosis rate was measured by annexin V/propidium iodide (AV/PI) assay at 72 hour after transfection. Meanwhile, the expressions of apoptotic markers (cleaved caspase-3 [C-caspase-3] and B-cell lymphoma-2 [Bcl-2]) were detected by Western blot assay at 72 hour after transfection; In addition, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-10, IL-17A, and IL-22 mRNA expressions were detected by qPCR assay and their protein levels were detected by Western blot assay at 24 hour after transfection.

2.6 | qPCR assay

Total RNA in lesional skin tissue, nonlesional skin tissue or HaCaT cell line was extracted using TRIzol (Invitrogen) according to manufacturer's instructions. And 1 ug total RNA was reversely transcribed into cDNA using ReverTra Ace® qPCR RT Kit (Toyobo, Japan), then the cDNA was used for qPCR amplification by SYBR® Green Quantitative RT-qPCR Kit (KAPA, Japan). U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal reference for miR-126 and mRNA, respectively. qPCR was performed as follows: heat activated at 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 5 seconds, annealing, and extension at 61°C for 30 seconds. And the PCR amplification was performed in triplicate. Subsequently, the qPCR results were calculated using $2^{-\Delta\Delta Ct}$ method. The primer sequences were exhibited in Table 1.

2.7 | Western blot assay

RIPA (Thermo fisher, MA, USA) was used to extract total protein from tissue or cells. The extracted total protein was then quantified using Pierce™ BCA Protein Assay Kit (Thermo fisher). 20 μg of total protein after denaturation at 95°C for 5 minutes was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the isolated protein was transferred into nitrocellulose membranes (Millipore, USA). After that, the nitrocellulose membranes were blocked in 5% nonfat powdered milk at room temperature for 2 hour and were incubated with primary antibodies

overnight at 4°C overnight, followed by incubation with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 hour. Afterward, the bands were visualized using an enhanced chemiluminescence (ECL) kit (Millipore, USA) under X-ray. Additionally, the antibodies information was shown in Table 2.

2.8 | CCK-8 assay

Cells' proliferation was assessed using the CCK-8 kit (Dojindo, Japan). After transfection, the cells were cultured with 100 μL RPMI-1640 medium (Gibco) in 96-well plates, and 10 μL CCK-8 dye solution (Dojindo, Japan) was added into each well at 0, 24, 48, and 72 hour after transfection. The cells were incubated for 2 hour in the dark, then using a Microplate reader (BioTek, USA) to read the fluorescence value of the plates.

TABLE 1 Primers used in this study

Genes	Sequence (5′→3′)
miR-126	F: GCTGTCAGTTTGTCAAATA
	R: GTGCAGGGTCCGAGGT
TNF-α	F: TTCCTCAGCCTCTTCTCCTTCCT
	R: ATCTCTCAGCTCCACGCCATTG
IFN-γ	F: TCGGTAACTGACTTGAATGTCCA
	R: TCGCTTCCCTGTTTTAGCTGC
IL-10	F: TGTTGCCTGGTCCTCCTGACT
	R: GCCTTGATGTCTGGGTCTTGGTT
GAPDH	F: TGACCACAGTCCATGCCATCAC
	R: GCCTGCTTCACCACCTTCTTGA
U6	F: CTCGCTTCGGCAGCACATATACTA
	R: ACGAATTTGCGTGTCATCCTTGC

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN-γ, interferon-γ; IL-10, interleukin 10; TNF-α, tumor necrosis factor α.

2.9 | AV/PI assay

Cells' apoptosis rate of HaCaT cells was quantified by flow cytometry (FCM; BD, USA) using the FITC AV apoptosis detection kit with PI (BD, USA) according to the manufacturer's protocol. Briefly, HaCaT cells were collected at 72 hour after transfection, and then were washed by PBS and resuspended in binding buffer. Afterward, AV solution was added and stand in the dark at room temperature for 15 minutes, and the PI solution was added before setting in the FCM. Then the results green (AV) and red (PI) fluorescence of each sample were quantitatively analyzed by FCM.

2.10 | Statistics

Statistical analysis was performed by SPSS software 22.0 (IBM, USA) and GraphPad Prism software 5.01 (GraphPad, USA). Data were mainly presented as mean ± standard deviation, median (1/4-3/4 quantiles) or count (%). Comparison between 2 groups was determined by *t* test, Wilcoxon signed rank sum test or Wilcoxon rank sum test. The association of miR-126 expression with continuous clinicopathological characteristics was evaluated by Spearman test. $P < .05$ was considered as significant.

3 | RESULTS

3.1 | Characteristics of psoriasis patients

As presented in Table 3, 102 psoriasis patients with mean of age 44.11 ± 10.92 years were enrolled in this study, among whom 61 patients were males and 41 patients were females. The mean BMI was 24.68 ± 2.63 kg/m^2, and the mean disease duration was 11.32 ± 6.83 years. The mean lesional BSA and PASI Score were 20.34% ± 8.38% and 12.52 ± 4.73 points, respectively. In addition, the numbers of cases treated by topical therapy, phototherapy, systemic nonbiologic treatment, and systemic biologic treatment were

TABLE 2 Antibody information

Antibody name	Company	Country	Dilution ratio
Primary antibody			
Rabbit monoclonal anticleaved caspase 3 antibody	Abcam	USA	1:2000
Rabbit polyclonal anticaspase 3 antibody	Abcam	USA	1:2000
Rabbit monoclonal anti-BCL-2 antibody	Abcam	USA	1:1000
Rabbit polyclonal anti-TNF-α antibody	Abcam	USA	1:2000
Rabbit polyclonal anti-IFN-γ antibody	Abcam	USA	1:1000
Rabbit polyclonal anti-IL-10 antibody	Abcam	USA	1:2000
Rabbit monoclonal anti-GAPDH antibody	Abcam	USA	1:10 000
Second antibody			
Goat anti-rabbit IgG H&L (HRP) antibody	Abcam	USA	1:2000

Bcl-2, B-cell lymphoma-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horse radish peroxidase; IFN-γ, interferon-γ; IgG, immunoglobulin G, IL-10, interleukin 10; TNF-α, tumor necrosis factor α.

TABLE 3 Characteristics of psoriasis patients

Parameters	Psoriasis patients (N = 102)
Age (y)	44.11 ± 10.92
Gender (male/female)	61/41
BMI (kg/m^2)	24.68 ± 2.63
Disease duration (y)	11.32 ± 6.83
Lesional BSA (%)	20.34 ± 8.38
PASI Score	12.52 ± 4.73
Treatment	
Topical therapy (n/%)	93 (91.2)
Phototherapy (n/%)	86 (84.3)
Systemic nonbiologic treatment (n/%)	70 (68.6)
Systemic biologic treatment (n/%)	11 (10.8)

BMI, body mass index; BSA, body surface area (affected by psoriasis); PASI, Psoriasis Area and Severity Index.
Data were presented as mean ± standard deviation or count (%).

93 (91.2%) cases, 86 (84.3%) cases, 70 (68.6%) cases, and 11 (10.8%) cases, respectively.

3.2 | MiR-126 expression in lesional skin tissue and paired nonlesional skin tissue

Wilcoxon signed rank sum test was used to determine the difference in miR-126 expression between lesional skin tissue and paired nonlesional skin tissue, and the result showed that miR-126 expression in lesional skin tissue was higher compared with paired nonlesional skin tissue ($P < .001$, Figure 1).

3.3 | The association of miR-126 expression with clinical features

MiR-126 expression was positively associated with PASI score ($P = .003$, Figure 2F), however, no correlation of miR-126 expression with age ($P = .212$, Figure 2A), gender ($P = .195$, Figure 2B), BMI ($P = .629$, Figure 2C), disease duration ($P = .090$, Figure 2D) or lesional BSA ($P = .086$, Figure 2E) was found.

3.4 | The effect of upregulated miR-126 on HaCaT cells' proliferation

At 24 hour after transfection, miR-126 expression in miR-126 mimic group was increased compared with NC mimic group ($P < .001$, Figure 3A), which indicated that miR-126 mimic plasmid successfully increased the miR-126 expression. And at 72 hour after transfection, cells' proliferation by CCK8 assay was elevated in miR-126 mimic group compared to NC mimic group ($P < .05$, Figure 3B), which implied that miR-126 enhanced HaCaT cells' proliferation.

3.5 | The effect of upregulated miR-126 on HaCaT cells' apoptosis

At 72 hour after transfection, cells' apoptosis rate was detected by AV/PI assay (Figure 4A), and the results showed that cells' apoptosis rate was reduced in miR-126 mimic group compared with NC mimic group ($P < .01$, Figure 4B). In addition, apoptotic markers (C-caspase 3 and Bcl-2) were also detected by western blot assay, which displayed that C-caspase 3 expression was decreased while Bcl-2 expression was increased in miR-126 mimic group compared to NC mimic group (Figure 4C). These results suggested that miR-126 inhibited HaCaT cells' apoptosis.

3.6 | The effect of upregulated miR-126 on inflammatory cytokines expressions in HaCaT cells

To explore the influence of miR-126 on regulating inflammation in HaCaT cells, mRNA and protein expressions of TNF-α, IFN-γ, IL-10, IL-17A, and IL-22 were determined after transfection by qPCR assay and Western blot assay (Figure 5F) respectively, which disclosed that pro-inflammatory cytokines (TNF-α, IFN-γ, IL-17A, and IL-22) were increased (Figure 5A-B,D-E) while anti-inflammatory cytokine (IL-10) was decreased (Figure 5C) in miR-126 mimic group compared with NC mimic group. These results indicated that miR-126 promoted inflammation in HaCaT cells.

3.7 | The association of miR-126 expression with therapeutic methods

As listed in Table S1, the miR-126 expression was not associated with topical therapy ($P = .892$), phototherapy ($P = .451$), systemic nonbiological treatment ($P = .530$) or systemic biologic treatment ($P = .859$) in psoriasis patients.

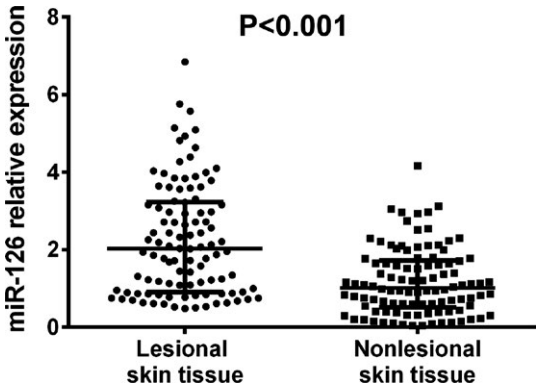

FIGURE 1 The miR-126 expression in lesional skin tissue and nonlesional skin tissue. The expression of miR-126 was increased in lesional skin tissue compared with nonlesional skin tissue. Comparison between 2 groups was determined by Wilcoxon signed rank sum test. $P < .05$ was considered significant

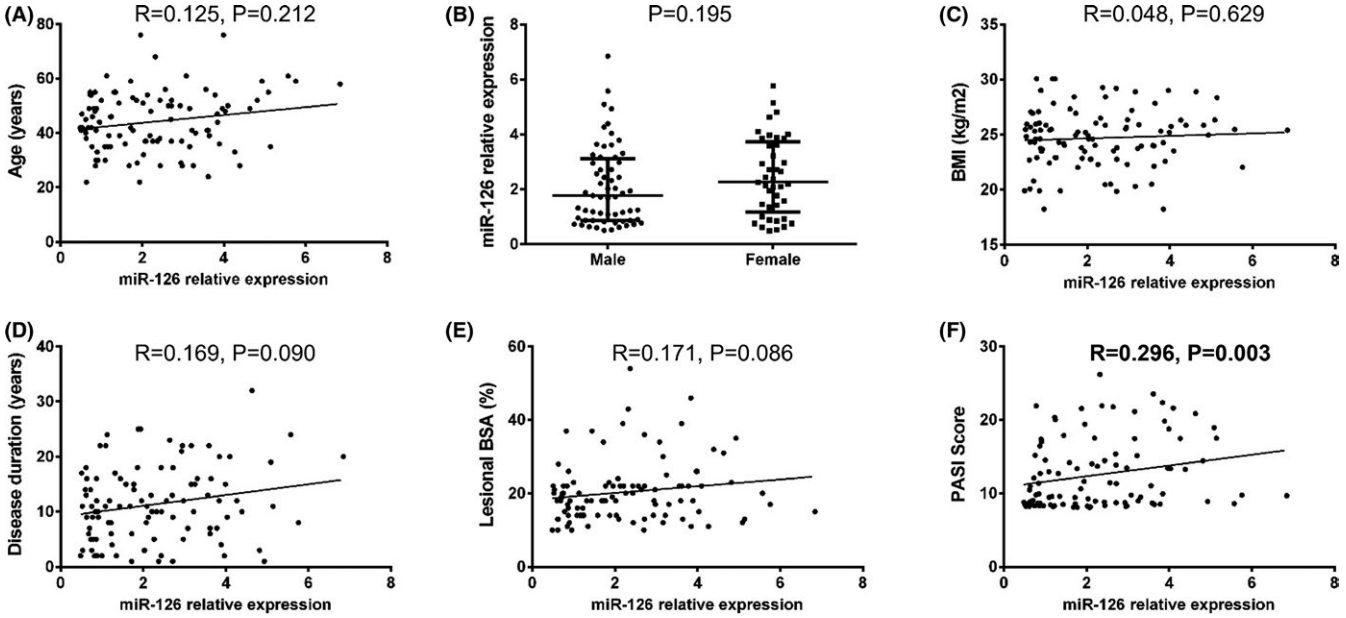

FIGURE 2 The association of miR-126 expression with pathological features in psoriasis patients. MiR-126 level in lesional skin tissue positively correlated with PASI Score (F) but did not associate with age (A), gender (B), BMI (C), disease duration (D), or lesional BSA (E). The correlation of miR-126 expression in lesional skin tissue with age, BMI, disease duration, and lesional BSA was determined by Spearman test. Comparison between 2 groups was determined by Wilcoxon signed rank sum test. $P < .05$ was considered significant. BMI, body mass index; BSA, body surface area; PASI Score, Psoriasis Area and Severity Index Score

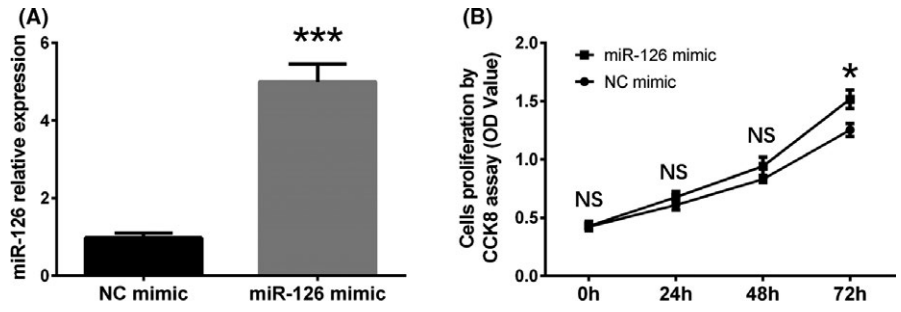

FIGURE 3 The effect of miR-126 upregulation on proliferation in HaCaT cells. MiR-126 expression in HaCaT cells was increased in miR-126 mimic group compared with NC mimic group at 24 h after transfection (A). And the cells' proliferation was increased in miR-126 mimic group compared with NC mimic group at 72 h after transfection (B). Comparison between 2 groups was determined by *t* test. $P < .05$ was considered significant. $^{*}P < .01$, $^{***}P < .001$. HaCaT, human keratinocytes; NC, negative control

3.8 | The effect of downregulation of miR-126 on HaCaT cells' proliferation and apoptosis

MiR-126 expression in miR-126 inhibitor group was lower than that in NC inhibitor group ($P < .001$) (Figure S1A). And there was no difference in cells' proliferation ($P < .05$) (Figure S1B) or cells' apoptosis ($P < .05$) (Figure S1C,D) between miR-126 inhibitor group and NC inhibitor group.

4 | DISCUSSION

The results of our study presented that: (i) MiR-126 expression was elevated in lesional skin tissue compared with paired nonlesional skin tissue in psoriasis patients, and it was positively associated with PASI score. (ii) Upregulated miR-126 promoted cells' proliferation and inflammation while reduced cells' apoptosis in HaCaT cells.

Although the etiology of psoriasis is still obscure, it is generally accepted that psoriasis is a T cell-mediated inflammatory dermatosis, and hyperproliferation of keratinocytes and sustained inflammation in skin tissue are the major pathological processes of psoriasis.[19,20] Inflammatory myeloid dendritic cells release IL-12 and IL-23 to activate IL-17-producing T cells, Th1 cells, and Th22 cells to secrete multiple inflammatory cytokines related to psoriasis consisting of IL-17, IFN-γ, TNF, and IL-22, which mediate keratinocytes to amplify psoriatic inflammation.[19] However, the molecular mechanisms behind these processes are still not fully understood. As regulatory

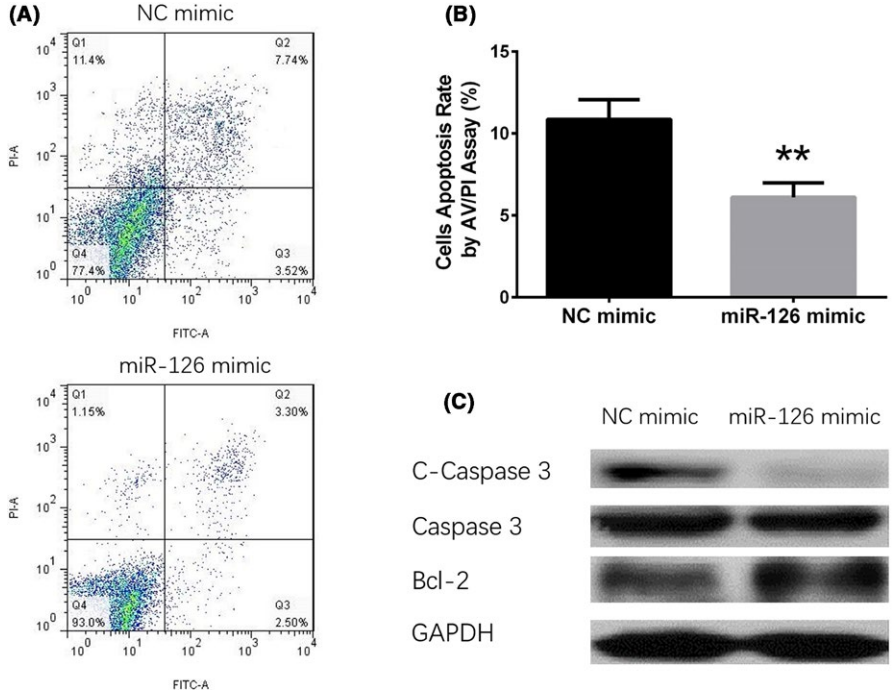

FIGURE 4 The effect of miR-126 upregulation on apoptosis in HaCaT cells. At 72 h after transfection, the cells' apoptosis was detected by AV/PI (A), and compared with NC mimic group, the cells' apoptosis rate in miR-126 mimic group was decreased (B). The levels of C-caspase 3 and caspase 3 in miR-126 mimic group were higher than that in NC mimic group, while Bcl-2 expression was decreased in miR-126 mimic group compared with NC mimic group (C). Comparison between 2 groups was determined by *t* test. $P < .05$ was considered significant. $^{**}P < .01$. AV/PI, annexin V/propidium iodide; Bcl-2, B-cell lymphoma-2; C-caspase 3, cleaved caspase 3; HaCaT, human keratinocytes; NC, negative control

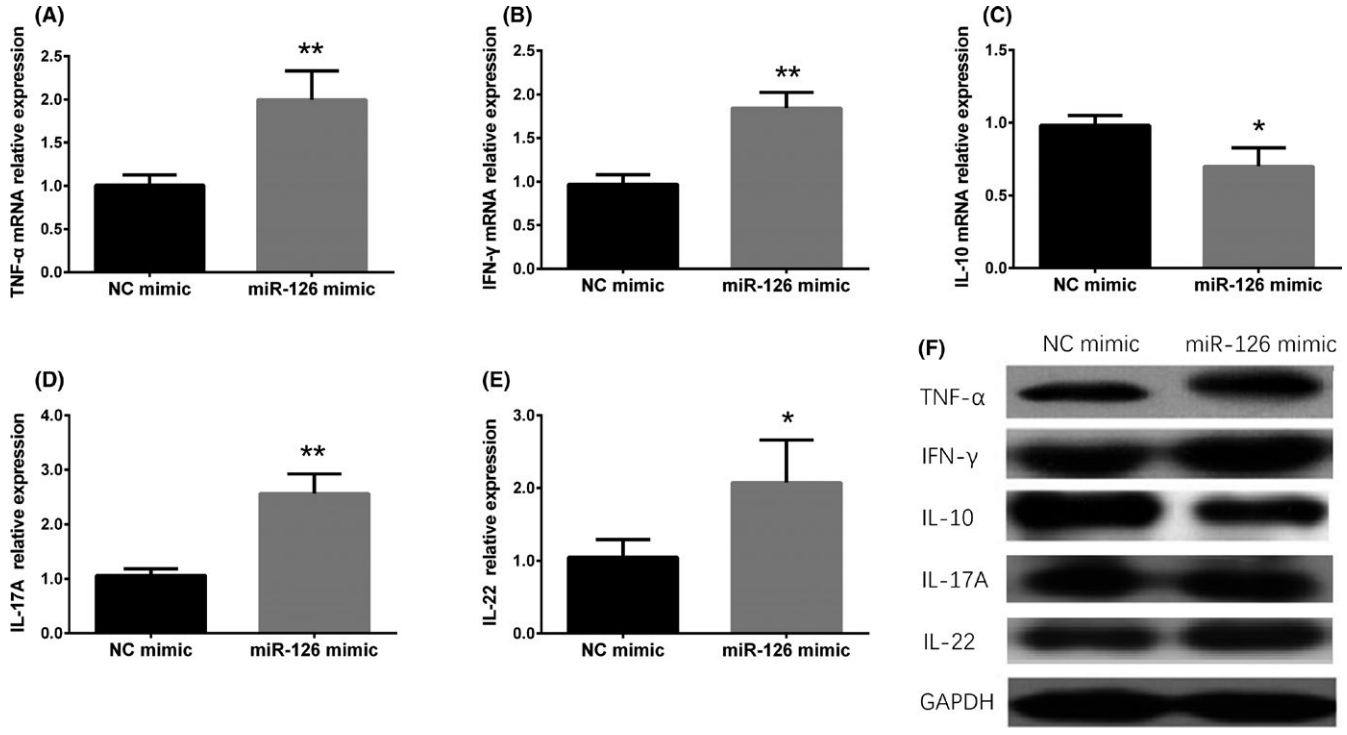

FIGURE 5 Correlation of miR-126 expression with the mRNA and protein levels of inflammatory cytokines. Compared with NC mimic group, TNF-α (A), IFN-γ (B), IL-17A (D), and IL-22 (E) mRNA levels were increased while IL-10 (C) level was reduced in miR-126 mimic group. Besides, compared with NC mimic group, TNF-α, IFN-γ, IL-17A, and IL-22 proteins were increased but IL-10 protein was decreased in miR-126 mimic group (F). Comparison between 2 groups was determined by *t* test. $P < .05$ was considered significant. $^{*}P < .01$, $^{**}P < .01$. IFN-γ, interferon-γ; IL-10, interleukin 10; IL-17A, interleukin 17a; IL-22, interleukin 22; NC, negative control; TNF-α, tumor necrosis factor α

elements, miRNAs play critical roles in immune diseases including psoriasis via participating in the development of immune cells, maintaining immune homeostasis, regulating innate and adaptive immunity, as well as regulating inflammatory factors.[21-24]

MiR-126 is a member of miRNAs that are reported to be involved in the development and progression of several autoimmune diseases such as RA and SLE.[16,25] A study elucidates that miR-126 expression is increased in the CD4+ T cells of SLE

patients and promotes the progression of SLE by enhancing T-cell autoreactivity through directly targeting DNA methyltransferase 1 (Dnmt1).[25] And another study illustrates that upregulation of miR-126 enhances cells proliferation and diminishes apoptosis of RA synovial fibroblasts by targeting phosphoinositide-3-kinase regulatory subunit 2 (PIK3R2) and regulating phosphoinositide-3-kinase regulatory subunit 1 (PI3K)—serine/threonine kinase 1 (AKT) signal pathway.[16] Feng et al exhibit that miR-126 level in colonic tissue is increased in active ulcerative colitis (UC) patients compared to inactive UC patients, irritable bowel syndrome patients and healthy controls, and their in vitro experiments reveal that elevated miR-126 expression decreases the level of NF-κB inhibitor alpha (IκBα), which is an inhibitor of the pro-inflammatory factor nuclear transcription factor kappa B (NF-κB), in colonic cancer cells, thus promoting the inflammatory responses in UC patients.[26] These previous studies indicate that miR-126 plays an important role in the development of autoimmune diseases. However, to the best of our knowledge, no study has disclosed the function of miR-126 in modulating disease risk and severity of psoriasis. In this study, we found that the expression of miR-126 in the lesional skin tissue was upregulated than that in paired non-lesional skin tissue, and its expression positively correlated with the PASI score in psoriasis. The possible reason might be that up-regulation of miR-126 enhanced the inflammation and promoted proliferation of keratinocytes in psoriasis, which was validated in our subsequent experiments.

Abnormal cells' proliferation of human keratinocytes is a predominant feature of psoriasis, and exaggerated keratinocytes proliferation is closely related to the occurrence of this disease. Keratinocyte act as a crucial layer in the pathology of psoriasis by initiating, sustaining, and amplifying the inflammatory responses via expressing molecules involved in T-cell recruitment, retention, and activation.[27,28] To further illustrate the effect of miR-126 on keratinocytes proliferation, apoptosis, and inflammatory cytokines expressions in psoriasis, we upregulated the expression of miR-126 by transfecting miR-126 mimic in HaCaT cells, and the results disclosed that the cells' proliferation was increased while cells' apoptosis was decreased in HaCaT cells' in miR-126 mimic group compared with NC mimic group. In addition, TNF-α, IFN-γ, IL-17A and IL-22 levels were elevated while IL-10 level was lowered in miR-126 mimic group compared with NC mimic group. Here are several possible explanations for our results: (i) according to previous studies, the overexpression of miR-126 could promote cells' proliferation and inhibits cells' apoptosis by regulating multiple pathways[16]; (ii) and miR-126 also upregulates the expression of pro-inflammatory cytokines by modulating immune-related factors such as Dnmt1 and NF-κB.[25,26] These results in our study indicated that miR-126 could be served as a potential treatment target in psoriasis patients.

In conclusion, miR-126 correlates with elevated risk and increased disease severity in psoriasis patients, and upregulated miR-126 promotes cells' proliferation and inflammation while inhibits cells' apoptosis in keratinocytes.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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