Role of the long non-coding RNA HOTAIR/miR-126 axis in an *in vitro* psoriasis model

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Abstract. Psoriasis is a T-cell-mediated inflammatory skin disease that is characterized by excessive keratinocyte proliferation and persistent skin inflammation. Accumulating evidence suggests that long non-coding RNAs (lncRNAs) are dysregulated in a number of inflammatory conditions. In the present study, an *in vitro* psoriasis cell model was established. Human HaCaT keratinocytes were activated using the inflammatory factor IL-22. Briefly, HaCaT cells were starved in serum-free DMEM for 24 h and then stimulated with 100 ng/ml IL-22 in serum-free DMEM for 24 h. Previous research indicated that HOX transcript antisense RNA (HOTAIR) may participate in the development of psoriasis. First, reverse transcription-quantitative PCR (RT-qPCR) analysis was performed to detect HOTAIR expression. The results indicated that HOTAIR expression was reduced in IL-22-stimulated HaCaT cells. Subsequently, a dual-luciferase reporter assay was performed to verify the binding site between HOTAIR and microRNA (miR)-126. The RT-qPCR results indicated that miR-126 expression was increased in IL-22-stimulated HaCaT cells. Moreover, the effects of HOTAIR and miR-126 on IL-22-stimulated HaCaT cell proliferation and apoptosis were assessed. HaCaT cells were transfected with control-plasmid, HOTAIR-plasmid, HOTAIR-plasmid + mimic control or HOTAIR-plasmid + miR-126 mimic for 24 h. At 24 h post-transfection, the cells were stimulated with 100 ng/ml IL-22 for 24 h and experiments were conducted. IL-22 induced cell proliferation and suppressed apoptosis. However, HOTAIR-plasmid inhibited cell viability and induced apoptosis in IL-22-stimulated HaCaT cells. In addition, the western blotting results indicated that HOTAIR-plasmid increased cleaved caspase-3 expression and the cleaved caspase-3/caspase-3 ratio, whereas the HOTAIR-plasmid-mediated effects were reversed by miR-126 mimic. Collectively, the results of the present study demonstrated that the lncRNA-HOTAIR/miR-126 axis may be implicated in the regulation of psoriasis progression and may serve as a potential therapeutic target for psoriasis.

Introduction

Psoriasis is a relatively common chronic inflammatory skin disease that adversely affects the lives of the patients (1). Psoriasis affects 2-3% of the population worldwide (2,3). Previous research indicates that psoriasis is associated with a number of complications, including psoriatic arthritis, cardio-vascular diseases, Crohn's disease, anxiety and depression, with the occurrence of these diseases increasing the mortality risk (4,5). It has been reported that certain therapeutic strategies may be used to treat psoriasis with good results, including local therapy, physical therapy, systemic therapy and new biological agents (such as TNF-α, IL-12/23 and IL-17 inhibitors), but the recurrence rate of psoriasis remains high (6-8). Therefore, identifying a novel therapeutic strategy for psoriasis would be beneficial. It was previously demonstrated that IL-22 regulates human keratinocyte proliferation and increases IL-22 expression in the serum and skin lesions of patients with psoriasis (9). Therefore, IL-22 may play a key role in the development of psoriasis (10). In addition, IL-22-induced skin inflammation was reported in a mouse model, further suggesting that IL-22 is associated with psoriasis (10).

Integrated genomic and transcriptome sequencing results have demonstrated that >90% of DNA sequences are actively transcribed, 98% of which are transcribed into non-coding RNA (ncRNA), including microRNAs (miRNAs/miRs) and long ncRNAs (lncRNAs) (11,12). lncRNAs are transcripts that are >200 nucleotides in length with limited potential for protein coding (13). lncRNAs regulate gene expression at different levels and participate in a variety of biological processes (14,15). Previous research has indicated that lncRNA may be abnormally expressed in mammalian and plant cells (16,17). An increasing number of studies have demonstrated that lncRNAs may be used as biomarkers for diagnosing and predicting different types of cancer (18-23).

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Additional studies have reported that the abnormal expression of lncRNAs is closely associated with the occurrence and development of psoriasis (24,25). Jia *et al* (26) demonstrated that the lncRNA MEG3 affects the proliferation and apoptosis of psoriatic epidermal cells via targeting miR-21/caspase-8. Gao *et al* (27) reported that the lncRNA MIR31HG may be a potential diagnostic biomarker and therapeutic target for psoriasis. Qiao *et al* (28) reported that the lncRNA msh homeobox 2 pseudogene 1 may participate in the occurrence of psoriasis. Previous research also indicated that the stress-induced lncRNA psoriasis-susceptibility-related RNA gene may play a key role in psoriasis (29). Tang *et al* (30) demonstrated that the lncRNA homeobox transcript antisense RNA (HOTAIR) acts as an oncogene in human cancer. Moreover, Yang *et al* (31) indicated that HOTAIR enhanced liver cancer cell proliferation via promotion of epithelial-to-mesenchymal transition. A recent study indicated the presence of an association between a genomic variant within HOTAIR and the risk of psoriasis (32). However, the specific role and related mechanisms of HOTAIR in psoriasis remain to be further elucidated.

Numerous studies have reported that the expression of multiple miRNAs, such as miR-125b, miR-155 and miR-26b, is associated with the progression of psoriasis (33-35). It has also been demonstrated that miR-126 is highly expressed in the tissues of patients with psoriasis, but with lower plasma expression levels. Furthermore, miR-126 expression in the plasma of patients was found to be inversely correlated with the risk and severity of psoriasis. In addition, a mutual binding site between lncRNA HOTAIR and miR-126 has been identified (36,37). Therefore, it may be hypothesized that the lncRNA HOTAIR is involved in the occurrence and development of psoriasis through regulation of miR-126 expression.

The aim of the present study was to explore whether HOTAIR was involved in psoriasis through modulation of miR-126 expression, so as to provide more strategies and a theoretical basis for the treatment of psoriasis.

Materials and methods

Cell culture and transfection. Human HaCaT keratinocytes (CLS Cell Lines Service GmbH) were cultured in DMEM (Hyclone; Cytiva) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin (Beyotime Institute of Biotechnology) at 37°C in a 5% CO_2 incubator. For overexpression of HOTAIR, full-length HOTAIR was amplified by PCR (primers: 5'-GACTCGCCTGTGCTCTGGAGCT-3' and 5'-TTGAA AATGCATCCAGATTTTT-3') and then cloned into the multiple cloning site of the pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.) to construct the HOTAIR-plasmid. The empty pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.) was used as the control-plasmid. Subsequently, HaCaT cells (5x10^4 cells per well; 6-well plate) were transfected with 100 ng control-plasmid, 100 ng HOTAIR-plasmid, 100 nM mimic control (sense: 5'-UUCUCCGAACGUGU CACGUTT-3'; anti-sense: 5'-ACGUGACACGUUCGGAGA ATT-3'; GenePharma), 100 nM miR-126 mimic (sense: 5'-UCG UACCGUGAGUAAUAAUGCG-3'; antisense: 5'-CAUUA UUACUCACGGUACGAUU-3'; GenePharma), 100 ng HOTAIR-plasmid + 100 nM mimic control or 100 ng HOTAIR-plasmid + 100 nM miR-126 mimic using Polyplus transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). At 24 h post-transfection, the cells were used for subsequent experiments.

Psoriasis model establishment. HaCaT cells (5x10^4 cells per well) were seeded into a 6-well plate. After reaching 80-90% confluence, the medium was replaced with serum-free DMEM at 37°C for 24 h. HaCaT cells were then treated with 100 ng/ml IL-22 and serum-starved at 37°C for an additional 24 h (38).

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was extracted using TRIzol™ reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA extraction was considered successful when three bands were observed in the nucleic acid gel. When RNA extraction was successful, total RNA was reverse-transcribed into cDNA using a reverse transcription kit (Vazyme Biotech Co., Ltd.) according to the manufacturer's instructions. Subsequently, qPCR was performed using SYBR Green PCR kit (Vazyme Biotech Co., Ltd.). The thermocycling conditions were as follows: Initial denaturation for 5 min at 95°C; followed by 38 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. mRNA and miRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (39) and normalized to the internal reference genes GAPDH and U6, respectively. All samples were assessed in triplicate and all experiments were repeated three times. The primer sequences used were as follows: GAPDH, forward 5'-ATTCCATGGCA CCGTCAAGGCTGA-3' and reverse 5'-TTCTCCATGGTG GTGAAGACGCCA-3'; U6, forward 5'-GCTTCGGCA GCACATATACTAAAAT-3' and reverse 5'-CGCTTCACGAA TTTGCGTGTCAT-3'; HOTAIR, forward 5'-CAGTGGGGGA ACTCTGACTCG-3' and reverse 5'-GTGCCTGGTGCTGTC TTACC-3'; and miR-126 forward 5'-GCTGTCAGTTTGTCAA ATA-3' and reverse 5'-GTGCAGGGTCCGAGGT-3'.

Western blotting. Total protein was extracted from cells using RIPA buffer (Beyotime Institute of Biotechnology) and quantified using a bicinchoninic acid assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of proteins (40 μg per lane) were separated via 12% SDS-PAGE for 40 min and transferred to PVDF membranes (EMD Millipore). The membranes were blocked for 1.5 h at room temperature with 5% non-fat milk. Subsequently, the membranes were incubated at 4°C overnight with the following primary antibodies: Anti-cleaved caspase-3 (cat no. ab32042; 1:1,000; Abcam), anti-pro-caspase-3 (cat no. ab32499; 1:1,000; Abcam) and GAPDH (cat no. ab9485; 1:1,000; Abcam). Following primary incubation, the membranes were incubated with an anti-rabbit horseradish peroxidase conjugated IgG secondary antibody (cat no. 7074; 1:2,000; Cell Signaling Technology, Inc.) for 2 h. Protein bands were visualized using the enhanced chemiluminescence method (Cytiva). GAPDH was used as the loading control.

Flow cytometry. Cell apoptosis was assessed using the Annexin V/propidium iodide (PI) Apoptosis Detection

kit (Beyotime Institute of Biotechnology). Briefly, cells (5x10^4 cells per well) were plated in 6-well plates overnight. On the following day, HaCaT cells were transfected with plasmid or mimic. Subsequently, cells were directly collected, centrifuged at 1,000 x g at 4°C for 5 min, and resuspended in 100 μl FITC-binding buffer. Subsequently, cells were incubated with 5 μl ready-to-use Annexin V-FITC (BD Biosciences) and 5 μl PI in the dark at 4°C for 30 min. Cell apoptosis was assessed using a BD FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using CellQuest™ software, version 5.1 (BD Biosciences).

Dual-luciferase reporter assay. The wild-type (WT) or mutant (MUT) 3' untranslated region (UTR) of HOTAIR was cloned into the pmiRGLO vector (Promega Corporation). Recombinant plasmids were acquired using the EndoFree Plasmid Maxi kit (Vazyme Biotech Co., Ltd.). HaCaT cells (5x10^4 cells per well) were co-transfected with 100 nM miR-126 mimic or 100 nM mimic control and 1 ng MUT-3'UTR-HOTAIR or 1 ng WT-3'UTR-HOTAIR at 37°C for 48 h. *Renilla* luciferase pRL-TK vector (Promega Corporation) was used as the control. At 48 h post-transfection, luciferase activity was measured using a dual-luciferase reporter assay system (Promega Corporation). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

MTT assay. Cell viability was assessed by performing an MTT assay. Transfected HaCaT cells were treated with 100 ng/ml IL-22 at 37°C for 24 h. HaCaT cells were plated into a 96-well plate and incubated at 37°C for 24, 48 or 72 h. Subsequently, 20 μl MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA) was added into each well at 37°C for 4 h. Absorbance was measured using a multifunctional plate reader (BD Biosciences) at a wavelength of 570 nm. Data are presented as the mean ± standard deviations of three separate experiments.

Caspase-3 activity detection. Caspase-3 activity was detected using a caspase-3 activity detection kit (Beyotime Institute of Biotechnology). Following transfection, HaCaT cells were treated with 100 ng/ml IL-22 at 37°C for 24 h. Subsequently, cells were collected by centrifugation (600 x g for 5 min at 4°C) and caspase-3 enzyme activity in cells was measured at a wavelength of 405 nm using a multifunctional plate reader (BD Biosciences).

Statistical analysis. Statistical analyses were performed using GraphPad Prism software (version 6; GraphPad Software, Inc.). Comparisons between two groups were analyzed using the unpaired Student's t-test. Comparisons among multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data are presented as the mean ± SD from at least three independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

HOTAIR is downregulated in IL-22-stimulated HaCaT cells. To explore HOTAIR expression in psoriasis, an *in vitro* cell model of psoriasis was established. Cells were starved in serum-free DMEM for 24 h and then stimulated with 100 ng/ml
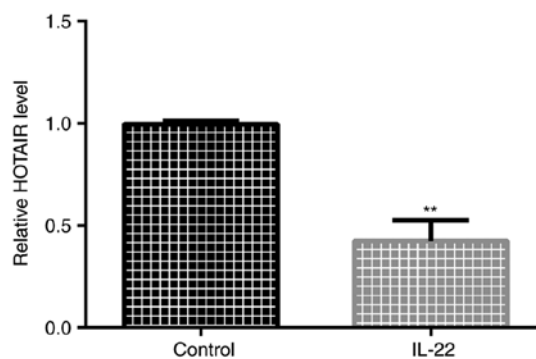

Figure 1. HOTAIR expression in IL-22-stimulated HaCaT cells. Reverse transcription-quantitative PCR analysis was performed to detect the relative expression of HOTAIR in IL-22-treated HaCaT cells. Data are presented as the mean ± SD. **P<0.01 vs. control group. HOTAIR, HOX transcript antisense RNA; IL, interleukin.

IL-22 in serum-free DMEM for 24 h. The RT-qPCR results indicated that HOTAIR was downregulated in IL-22-treated HaCaT cells (Fig. 1).

HOTAIR binds to miR-126. Previous studies (36,37) reported the existence of a mutual binding site between HOTAIR and miR-126 (Fig. 2A). A luciferase assay was performed to investigate the interaction between HOTAIR and miR-126. HaCaT cells were co-transfected with a luciferase plasmid containing the lncRNA HOTAIR sequence (HOTAIR-WT and HOTAIR-MUT) and miRNA-126 mimic. The results indicated that miR-126 suppressed the luciferase activity of HOTAIR-WT but did not alter the luciferase activity of HOTAIR-MUT (Fig. 2B). RT-qPCR analysis was performed to detect miR-126 expression in HaCaT cells that were treated with IL-22 and untreated cells. The results indicated that miR-126 was upregulated in IL-22-stimulated HaCaT cells (Fig. 2C).

Transfection efficiency of HOTAIR and miR-126 in HaCaT cells. HaCaT cells were transfected with control-plasmid or HOTAIR-plasmid. The RT-qPCR results indicated that, compared with the control-plasmid group, HOTAIR-plasmid increased HOTAIR expression in HaCaT cells (Fig. 3A). HaCaT cells were transfected with mimic control or miR-126 mimic. The RT-qPCR results indicated that, compared with the mimic control group, miR-126 mimic increased miR-126 expression in HaCaT cells (Fig. 3B). HaCaT cells were also transfected with HOTAIR-plasmid + mimic control or HOTAIR-plasmid + miR-126 mimic for 24 h. The results suggested that HOTAIR-plasmid significantly reduced miR-126 expression in HaCaT cells, which was reversed by miR-126 mimic (Fig. 3C).

HOTAIR suppresses cell proliferation and induces apoptosis in IL-22-stimulated HaCaT cells by regulating miR-126 expression. Subsequently, MTT assays and flow cytometry were performed to detect cell proliferation and apoptosis, respectively. The MTT assay suggested that, compared with the control group, HaCaT cell proliferation was increased in the IL-22 group. In addition, compared with the IL-22 + control-plasmid group, HOTAIR-plasmid suppressed
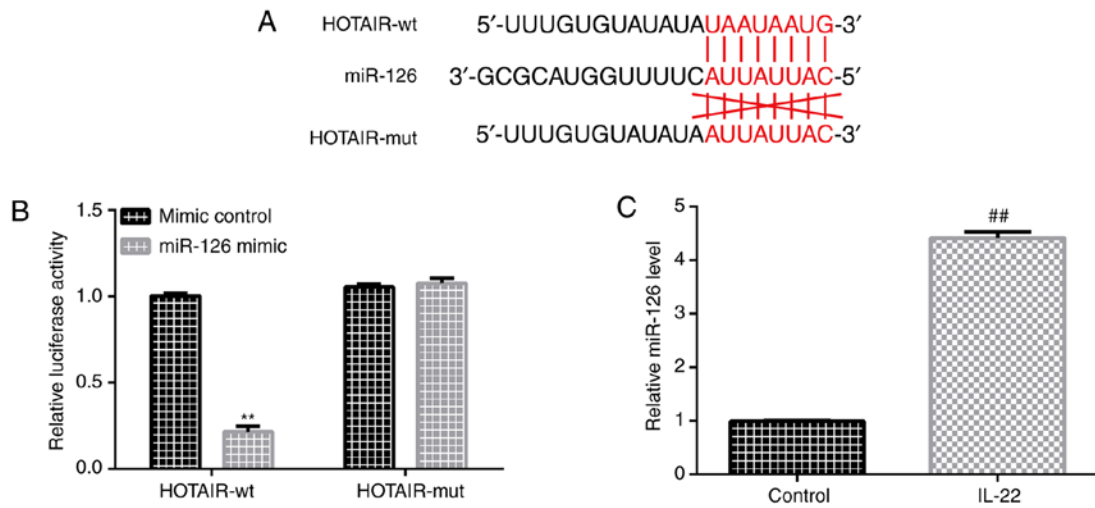
Figure 2. Binding sites between HOTAIR and miR-126. (A) Potential binding sites between HOTAIR and miR-126. (B) The dual-luciferase reporter gene assay was performed to confirm the binding sites between HOTAIR and miR-126. (C) Reverse transcription-quantitative PCR analysis was performed to detect miR-126 expression in IL-22-treated HaCaT cells. Data are presented as the mean ± SD. **P<0.01 vs. mimic control group; ##P<0.01 vs. control group. HOTAIR, HOX transcript antisense RNA; miR, microRNA; wt, wild-type; mut, mutant; IL, interleukin.
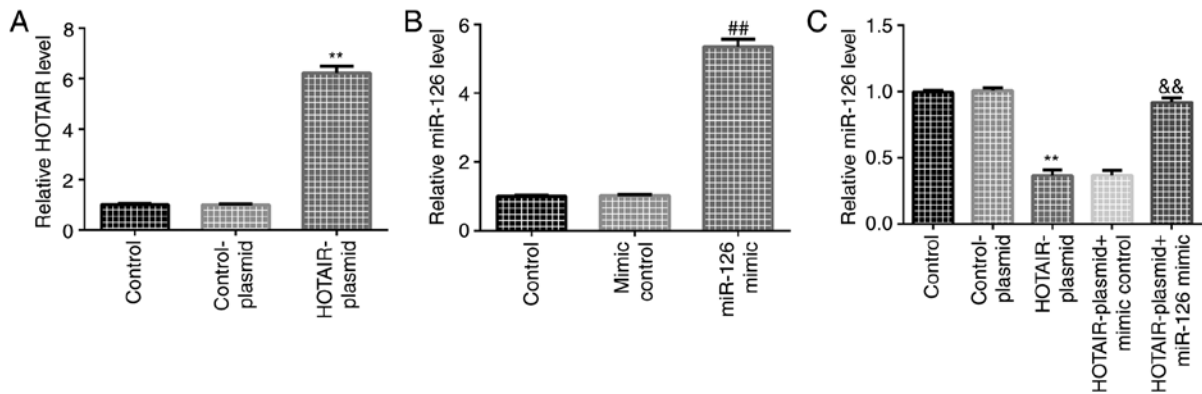

Figure 3. Transfection efficiency of HOTAIR and miR-126 in HaCaT cells. (A) RT-qPCR analysis was performed to detect HOTAIR expression in the control-plasmid and HOTAIR-plasmid groups. (B) RT-qPCR analysis was performed to detect miR-126 expression in the mimic control and miR-126 mimic groups. (C) RT-qPCR analysis was performed to detect miR-126 expression in the HOTAIR-plasmid + mimic control and HOTAIR-plasmid + miR-126 mimic groups. Data are presented as the mean ± SD. **P<0.01 vs. control-plasmid group; ##P<0.01 vs. mimic control group; &&P<0.01 vs. HOTAIR-plasmid + mimic control group. HOTAIR, HOX transcript antisense RNA; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; IL, interleukin.

cell proliferation, which was reversed by miR-126 mimic (Fig. 4A). The flow cytometry results indicated that, compared with the control group, cell apoptosis was decreased in IL-22-induced HaCaT cells (Fig. 4B and C). Furthermore, the caspase-3 activity assay results indicated that compared with the control group, caspase-3 activity was significantly decreased in the IL-22 group (Fig. 4D). The western blotting results indicated that compared with the control group, cleaved caspase-3 protein expression and the cleaved caspase-3/caspase-3 ratio was decreased in IL-22-induced HaCaT cells (Fig. 4E and F). Compared with the IL-22 + control-plasmid group, HOTAIR-plasmid increased cell apoptosis (Fig. 4B and C), promoted caspase-3 activity (Fig. 4D), increased cleaved caspase-3 protein expression and enhanced the cleaved caspase-3/caspase-3 ratio (Fig. 4E and F), whereas miR-126 mimic reversed these effects.

HOTAIR-plasmid reduces miR-126 expression. Finally, RT-qPCR analysis was performed to detect miR-126

expression. Compared with the control group, miR-126 expression was significantly increased in the IL-22 group. Compared with the IL-22 + control-plasmid group, HOTAIR-plasmid reduced miR-126 expression, which was reversed by miR-126 mimic (Fig. 5).

Discussion

lncRNAs have been found to be closely associated with the occurrence and development of psoriasis (24,25). The focus of the present study was the lncRNA HOTAIR, which has been extensively investigated (40). Accumulating evidence suggests that abnormal expression of HOTAIR is implicated in cancer development, including lung and gastric cancer, as well as hepatocellular carcinoma (41-43). Zhang *et al* (41) reported that HOTAIR promoted cell viability, migration and invasion. Gao *et al* (43) demonstrated that reduced HOTAIR expression decreased hepatocellular carcinoma growth. Liu *et al* (42) indicated that HOTAIR overexpression increased
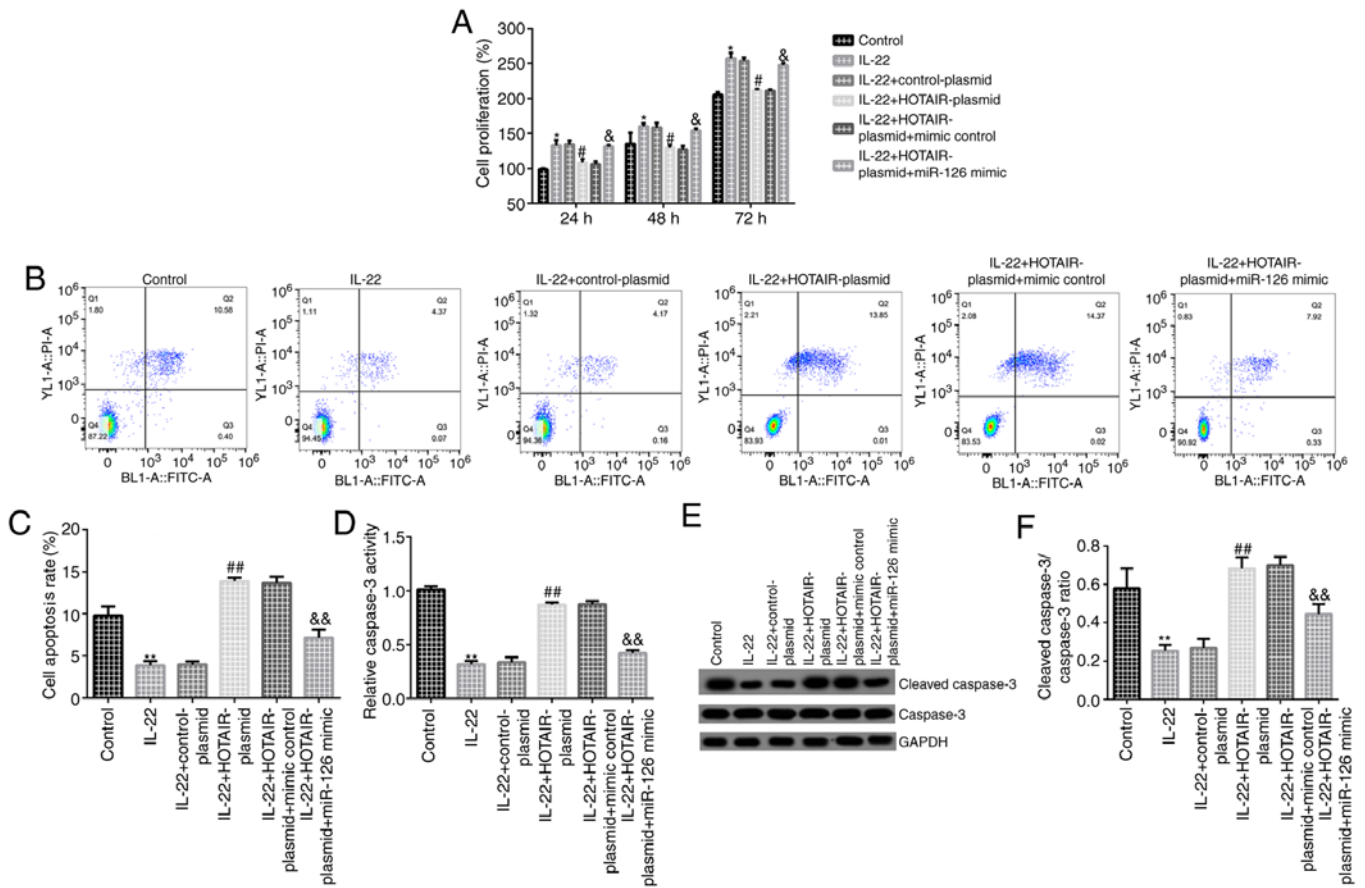
Figure 4. Effects of HOTAIR on IL-22-stimulated HaCaT cell proliferation and apoptosis. HaCaT cells were transfected with control-plasmid, HOTAIR-plasmid, HOTAIR-plasmid + mimic control or HOTAIR-plasmid + miR-126 mimic for 24 h. After 24 h, cells were stimulated with 100 ng/ml IL-22 for a further 24 h. (A) The MTT assay was conducted to detect cell proliferation at 24, 48 and 72 h. (B) Flow cytometry was performed to detect cell apoptosis. (C) Cell apoptosis rate. (D) Caspase-3 activity. (E) Western blotting was performed to measure cleaved caspase-3 and caspase-3 protein expression levels in different groups. (F) Cleaved caspase-3/caspase-3 ratio. Data are presented as the mean ± SD. $^*P<0.05$, $^{**}P<0.01$ vs. control group; $^#P<0.05$, $^{##}P<0.01$ vs. IL-22 + control-plasmid group; $^&P<0.05$, $^{&&}P<0.01$ vs. IL-22 + HOTAIR-plasmid + mimic control group. HOTAIR, HOX transcript antisense RNA; miR, microRNA; IL, interleukin.
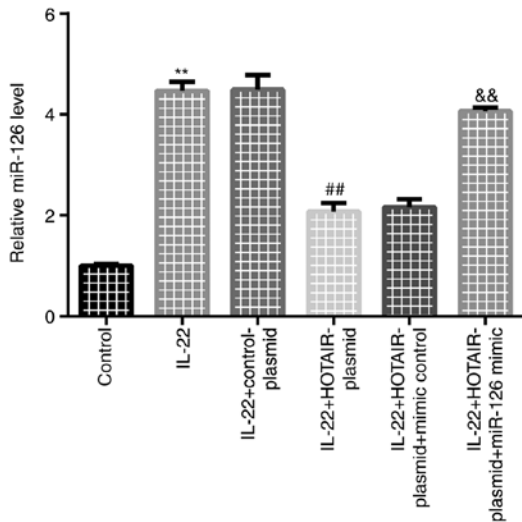

Figure 5. HOTAIR-plasmid reduces miR-126 expression in IL-22-induced HaCaT cells. HaCaT cells were transfected with control-plasmid, HOTAIR-plasmid, HOTAIR-plasmid + mimic control or HOTAIR-plasmid + miR-126 mimic for 24 h. After 24 h, cells were stimulated with 100 ng/ml IL-22 for a further 24 h. Reverse transcription-quantitative PCR analysis was performed to measure miR-126 expression. Data are presented as the mean ± SD. $^{**}P<0.01$ vs. control group; $^{##}P<0.01$ vs. IL-22 + control-plasmid group; $^{&&}P<0.01$ vs. IL-22 + HOTAIR-plasmid + mimic control group. HOTAIR, HOX transcript antisense RNA; miR, microRNA; IL, interleukin.

cell viability via miR-331-3p in gastric cancer. Furthermore, a previous study indicated that HOTAIR participated in psoriasis development (32). However, the expression and role of HOTAIR in psoriasis is not completely understood. Psoriasis is a T-cell-mediated chronic skin condition manifesting clinically as erythema and scaly skin. The primary pathological process of psoriasis is excessive keratinocyte proliferation and continuous skin inflammation (44,45), but the pathogenesis of the condition has yet to be fully elucidated. Therefore, the aim of the present study was to investigate the underlying mechanism in order to identify novel therapeutic targets for psoriasis.

First, the expression of HOTAIR was determined in an *in vitro* cell model of psoriasis, which was established by stimulating HaCaT cells with 100 ng/ml IL-22 for 24 h in serum-free DMEM. The findings indicated that HOTAIR was downregulated in IL-22-stimulated HaCaT cells. In addition, Yan *et al* (36) demonstrated that HOTAIR had a binding site with miR-126 in gastric cancer (36). Therefore, a dual-luciferase reporter assay was performed in the present study to investigate the interaction between HOTAIR and miR-126. The results also indicated that there was a mutual binding site between HOTAIR and miR-126.

miRNAs have been demonstrated to play key roles in psoriasis (33-35). Furthermore, miRNAs participate in the

development of immune cells, maintain immune homeostasis and regulate certain immune regulatory factors (46). miR-126 has been found to participate in the occurrence and development of multiple immune diseases, such as rheumatoid arthritis, systemic lupus erythematosus (SLE), inflammatory bowel disease and psoriasis (47-50). Qu *et al* (47) reported that miR-126 promoted cell proliferation via the PI3K/AKT signaling pathway. miR-126 was upregulated in the CD4$^+$ T cells of patients with SLE and enhanced T-cell autoreactivity to promote SLE progression. Feng *et al* (50) demonstrated that miR-126 was upregulated in patients with psoriasis. Consistent with previous results, the present study demonstrated that miR-126 was upregulated in IL-22-stimulated HaCaT cells, and that miR-126 expression was negatively correlated with HOTAIR expression. Subsequently, the effects of HOTAIR on IL-22-stimulated HaCaT cell proliferation and apoptosis were investigated. The results indicated that HOTAIR-plasmid suppressed cell viability and increased cell apoptosis, and these effects were reversed by miR-126 mimic. Therefore, the findings of the present study suggested that HOTAIR/miR-126 may represent promising novel targets for the treatment of psoriasis.

In conclusion, HOTAIR inhibited IL-22-induced HaCaT cell proliferation and increased apoptosis by regulating miR-126 expression, indicating that HOTAIR may play a protective role in psoriasis. However, this was an *in vitro* basic study of HOTAIR in psoriasis, and there were certain limitations: Experiments *in vivo* were not conducted; the target genes of miR-126 were not analyzed in depth; in addition, the correlation between the expression of HOTAIR/miR-126 and clinicopathological parameters in patients with psoriasis was not analyzed. Despite these limitations, however, the present study provides new potential targets for the clinical treatment of psoriasis and a theoretical basis for the development of psoriasis treatment strategies. In view of the aforementioned limitations, the role of HOTAIR/miR-126 in psoriasis will be further investigated in future studies.

Acknowledgements

Funding

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WZ contributed to study design, data collection, statistical analysis, data interpretation and manuscript preparation. BG, SC, JL and YS contributed to data collection and statistical analysis. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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