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Effects of silencing NLRP3 gene on proliferation of psoriasis-like HaCaT cells and expressions of cytokines

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Abstract

We aimed to explore the effects of silencing NOD-like receptor protein 3 (NLRP3) on proliferation of psoriasis-like HaCaT cells and expressions of cytokines. HaCaT cells were treated with human keratinocyte growth factor (KGF) and were divided into KGF group, negative control group, NLRP3-RNAi group and control group. Cells proliferation was detected by CCK8, cell clone formation rate was detected by clone formation assay, distribution of cells cycle was detected by flow cytometry, expressions of cyclin B1 (Cyclin B1), cyclin-dependent kinase 2 (CDK2), Ki67 and proliferating cell nuclear antigen (PCNA) proteins were detected by Western blot, and levels of interleukin (IL)-17, IL-23, IL-6 and tumor necrosis factor α (TNF-α) were detected by enzyme-linked immunosorbent assay. Compared with control group, expressions of NLRP3 mRNA and protein, proliferation rate and clonal formation rate were increased in KGF group, percentage of cells in G0/G1 phase was decreased, percentage of cells in S phase was increased, expressions of Cyclin B1, CDK2, Ki67 and PCNA proteins were increased, and levels of IL-17, IL-23, IL-6 and TNF-α were increased. Compared with negative control group, expressions of NLRP3 mRNA and protein, proliferation rate and clonal formation rate were decreased in NLRP3-RNAi group, percentage of cells in G0/G1 phase was increased, percentage of cells in S phase was decreased, expressions of Cyclin B1, CDK2, Ki67 and PCNA proteins were decreased, and levels of IL-17, IL-23, IL-6 and TNF-α were decreased. Silencing NLRP3 gene can inhibit the proliferation of psoriasis-like HaCaT cells, arrest cell cycle, inhibit the expressions of cell proliferation-related proteins and reduce levels of pro-inflammatory factors.

Keywords: Psoriasis, HaCaT cell, Nod-like receptor protein 3, Cell proliferation, Inflammatory response.

1. Introduction

Psoriasis, a persistently challenging and relapsing dermatological disorder, manifests as a chronic, scaly condition. It is intricately triggered by a confluence of environmental factors and is under the complex governance of polygenic inheritance, while also being intricately mediated by the multifaceted mechanisms of the immune system. This condition distinctively exhibits itself through the abnormal differentiation of keratinocytes and the extensive infiltration of various immune cells, underscoring its complexity and stubborn resistance to straightforward therapeutic interventions [1]. In China, the prevalence of psoriasis has increased from 0.123% in 1984 to 0.47% in 2008. Although this rate is lower compared to the 2% to 4% prevalence in European and American countries [2], psoriasis is associated with an elevated risk of cardiovascular diseases and metabolic syndrome. The condition also presents symptoms like skin itching, burning sensation, thick silvery scales on the scalp, and joint swelling or pain, significantly impacting the daily life and work of those affected [3]. Therefore, understanding the pathological mechanisms of psoriasis is essential for guiding its prevention and treatment.

The immune pathway mediated by helper T cell 17 (Th17) is widely recognized as a crucial mechanism in the development of psoriasis [4]. Research has shown that components of the NLRP3 inflammasome, such as Nod-like receptor protein 3 (NLRP3), are highly expressed in psoriatic lesions and exacerbate the severity of the disease by disrupting the Treg/Th17 balance [5]. RNA interference (RNAi) technology, which utilizes double-stranded RNA to trigger the specific degradation of mRNA, thereby reducing or silencing the expression of target genes, is known for its efficiency, specificity, and ease of use. It is commonly employed in gene function studies and as a tool for drug screening [6].

In this study, we leveraged RNAi technology to silence NLRP3 gene expression. Through in vitro cell modeling, we explored the impact of silencing the NLRP3 gene on the proliferation of psoriasis-like HaCaT cells and their cytokine profiles. This approach sheds light on the potential role of NLRP3 in psoriatic pathology and offers insights into new therapeutic targets for managing this complex condition. The study's emphasis on the NLRP3 component within the NLRP3 inflammasome highlights its significance in the immune dysregulation observed in psoriasis,

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particularly regarding the balance between regulatory and inflammatory T cells. By investigating the cellular and molecular changes following NLRP3 gene silencing, this research contributes to a deeper understanding of psoriasis pathogenesis and opens avenues for novel treatment strategies that could alleviate the burden of this disease on patients.

2. Materials and Methods

2.1. Experimental materials

The immortalized human keratinocyte cell line HaCaT was purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Human keratinocyte growth factor (KGF), RNA extraction kit, antibodies for Cyclin B1, Cyclin-Dependent Kinase 2 (CDK2), Ki67, and Proliferating Cell Nuclear Antigen (PCNA) were obtained from Sigma (St. Louis, MO, USA). NLRP3-RNAi lentivirus and the corresponding control lentivirus were procured from Shanghai Jiema Pharmatech Co., Ltd. (Shanghai, China). The 2× SYBR Green qPCR Master Mix was sourced from Beijing Bairuiji Biotechnology Co., Ltd. (Beijing, China), and the CCK-8 cell proliferation kit was from Anhui Biojing Medical Technology Co., Ltd. (Hefei, China). The cell cycle kit was acquired from Shanghai Yiji Industrial Co., Ltd. (Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) kits for interleukin (IL)-17, IL-23, IL-6, and tumor necrosis factor-alpha (TNF-α) were from Hangzhou Lianke Biotech Co., Ltd. (Hangzhou, China). Equipment included a microplate reader (VarioskanLUX, Thermo Scientific, Waltham, MA, USA), a quantitative PCR instrument (LightCycler 96, Roche, Basel, Switzerland), a cell culture incubator (CellXpert® C170i, Eppendorf, Germany), and a flow cytometer (Cyto FLEX, Beckman Coulter, Franklin Lakes, NJ, USA).

2.2. Cell culture

HaCaT cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO2 incubator. Cells in the logarithmic phase were seeded at a density of 1×10^5 cells/well. When cell confluence reached 30%, NLRP3-RNAi lentivirus and the control lentivirus were used to transfect the HaCaT cells, forming the RNAi-NLRP3 and negative control groups, respectively. After 36 hours of transfection, cells were collected for qRT-PCR and Western blot analysis to measure NLRP3 mRNA and protein expression.

2.3. qRT-PCR

To establish a psoriasis cell model, HaCaT cells were treated with KGF (40 ng/mL) and divided into KGF, negative control, and NLRP3-RNAi groups, with a normal growth cell group as the control. Cells were collected 24 hours post-KGF treatment for subsequent experiments. Total RNA was extracted using the RNA extraction kit and reverse transcribed to cDNA for qRT-PCR amplification (95°C for 2 min, followed by 38 cycles of 95°C for 5s and 56°C for 10s). Primer sequences for NLRP3 were 5'-AAAGCCAAGAATCCACAGTGTAAC-3' (forward) and 5'-TTGCCTCGCAGGTAAAGG-3' (reverse), and for actin were 5'-GGCTACAGCTTCACCACCAC-3' (forward) and 5'-TGCGCTCAGGAGGAGC-3' (reverse). NLRP3 mRNA levels were calculated using the 2-ΔΔCt method.

2.4. Western blot

Cell lysates were prepared to extract total protein, whose concentration was measured, and 70 μg of total protein was subjected to SDS-PAGE, followed by electroblotting, blocking with 5% non-fat milk for 2 hours, and overnight incubation at 4°C with primary antibodies against human NLRP3, CDK2, CCNB1, Ki67, PCNA, and β-actin (1:1000). This was followed by 1-hour incubation with secondary antibody (1:1000), washing, chemiluminescence detection, and protein band analysis using an imager.

2.5. CCK-8

Cell proliferation was assessed by adding 10 μL of CCK-8 solution to the cells and incubating for 2 hours before measuring the absorbance at 490 nm. Proliferation rate was calculated as ((experimental group absorbance - control group absorbance) / control group absorbance) × 100%.

2.6. Cell clonal formation

For clonal formation, cells were seeded in culture dishes (300 cells/dish) and treated with KGF (40 ng/mL) for KGF, negative control, and NLRP3-RNAi groups, while the control group was left to grow normally. After 14 days of culture, cells were fixed with formaldehyde, and stained with crystal violet, and the number of clones formed was counted. Clonal formation rate was calculated as (number of clones formed / number of seeded cells).

2.7. Flow cytometry

Cell cycle distribution was analyzed using flow cytometry. Cells were collected, centrifuged at 1000 rpm for 5 minutes with a centrifugal radius of 10 cm, washed with phosphate-buffered saline, fixed in 75% ethanol for 24 hours, treated with 0.4 μl of PI in the dark for 30 minutes, and analyzed using the flow cytometer's built-in software to determine the percentage of cells in different cell cycle stages.

2.8. ELISA

For inflammatory factor level measurement, cells were collected and centrifuged at 3000 rpm for 5 minutes with a centrifugal radius of 10 cm. The supernatant was used to measure IL-17, IL-23, IL-6, and TNF-α levels using ELISA, strictly following the instructions of the kit.

2.9. Statistical analysis

To analyze the data, Statistic Package for Social Science (SPSS) software version 25.0 (IBM, Armonk, NY, USA) was utilized. The data are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for the comparison of means among different groups. For post-hoc pairwise comparisons, the Least Significant Difference test (LSD-t test) was applied. The level of significance was set at $\alpha = 0.05$.

3. Results

In the study of various cell groups, it was observed that in the KGF group, the NLRP3 mRNA levels and protein expression in HaCaT cells were significantly higher than those in the control group ($P<0.05$). In contrast, in the NLRP3-RNAi group, the NLRP3 mRNA levels and protein expression in HaCaT cells were markedly lower than those in the negative control group ($P<0.05$). Detailed data

can be found in Table 1 and Figure 1.

The cell proliferation rate and clone formation rate were compared among different groups. In the KGF group, both the cell proliferation rate and clone formation rate of HaCaT cells were significantly higher than those in the control group (P<0.05). In contrast, in the NLRP3-RNAi group, both the cell proliferation rate and clone formation rate of HaCaT cells were significantly lower than those in the negative control group (P<0.05). Please refer to Table 2 and Figure 2 for detailed data.

The protein expression of cell cycle-related proteins, including Cyclin B1, CDK2, Ki67, and PCNA, was compared among different groups. In the KGF group, the protein expression of Cyclin B1, CDK2, Ki67, and PCNA in HaCaT cells was significantly higher than that in the control group (P<0.05). Conversely, in the NLRP3-RNAi group, the protein expression of Cyclin B1, CDK2, Ki67, and PCNA in HaCaT cells was significantly lower than that in the negative control group (P<0.05). Please refer to Table 3 and Figure 3 for detailed data.

The levels of inflammatory factors in different cell

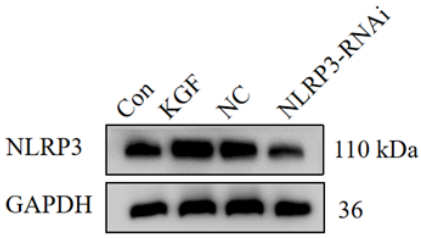

Fig. 1. NLRP3 protein expression in HaCaT Cells. Con=Control Group, KGF=KGF Group, NC=Negative Control Group NLRP3-RNAi=NLRP3-RNAi Group.

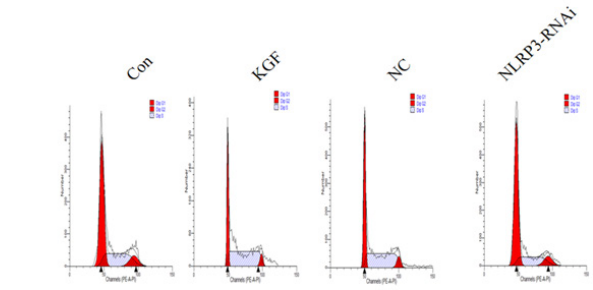

Fig. 2. Clone formation experiment in HaCaT cells.

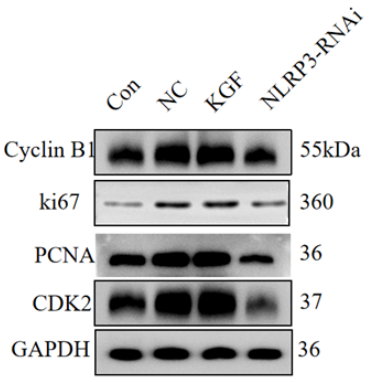

Fig. 3. Protein expression of Cyclin B1, CDK2, Ki67, and PCNA in different cell groups.

Table 1. Comparison of NLRP3 mRNA levels and protein expression in Different cell groups

Group	*n*	NLRP3 mRNA Levels	NLRP3 Protein Expression
Control Group	8	1.02±0.08	0.61±0.09
KGF Group	8	3.44±0.24*	1.19±0.06*
Negative Control	8	3.38±0.31	1.22±0.07
NLRP3-RNAi Group	8	0.41±0.06#	0.37±0.05#
F		485.498	301.836
P		<0.001	<0.001

*P<0.05 vs. control group; #P<0.05 vs. negative control group.

Table 2. Comparison of cell proliferation rate and clone formation rate in different groups.

Group	*n*	Cell Proliferation Rate (%)	Clone Formation Rate (%)
Control Group	8	—	32.08±5.41
KGF Group	8	62.33±4.45*	84.63±6.14*
Negative Control	8	63.14±4.39	83.87±5.49
NLRP3-RNAi Group	8	28.36±5.46#	47.46±4.08#
F			196.637
P		<0.001	<0.001

*P<0.05 vs. control group; #P<0.05 vs. negative control group.

Table 3. Protein expression of Cyclin B1, CDK2, Ki67, and PCNA in different cell groups.

Group	*n*	Cyclin B1	CDK2	Ki67	PCNA
Control Group	8	0.34±0.04	0.24±0.06	0.42±0.05	0.53±0.06
KGF Group	8	0.84±0.06*	0.73±0.05*	0.93±0.08*	0.88±0.08*
Negative Control	8	0.82±0.07	0.70±0.08	0.91±0.07	0.85±0.09
NLRP3-RNAi Group	8	0.43±0.04#	0.31±0.05#	0.53±0.05#	0.46±0.05#
F		184.410	139.733	133.677	72.388
P		<0.001	<0.001	<0.001	<0.001

*P<0.05 vs. control group; #P<0.05 vs. negative control group.

Table 4. Levels of inflammatory factors in different cell groups.

Group	*n*	IL-17(pg/mL)	IL-23(pg/mL)	IL-6(pg/mL)	TNF-α (pg/mL)
Control Group	8	62.45±4.88	54.12±5.33	87.49±6.21	196.43±10.18
KGF Group	8	211.69±13.69*	198.48±8.84*	304.09±18.98*	698.72±56.99*
Negative Control	8	207.82±15.08	194.87±9.13	298.46±20.18	703.48±52.14
NLRP3-RNAi Group	8	159.32±12.00#	102.36±10.08#	155.09±17.49#	412.66±46.40#
F		264.996	556.057	332.828	234.332
P		<0.001	<0.001	<0.001	<0.001

*P<0.05 vs. control group; #P<0.05 vs. negative control group.

groups were compared. In the KGF group, the levels of IL-17, IL-23, IL-6, and TNF-α in HaCaT cells were all significantly higher than those in the control group (P<0.05). Conversely, in the NLRP3-RNAi group, the levels of IL-17, IL-23, IL-6, and TNF-α in HaCaT cells were all significantly lower than those in the negative control group (P<0.05). Please refer to Table 4 for detailed data.

4. Discussion

Psoriasis is an immune- or inflammation-mediated disease, and its associated excessive proliferation, differentiation of keratinocytes, and immune dysregulation contribute to a vicious cycle [8]. NLRP3, a member of the Nod-like receptor protein family, plays a role in coordinating the immune system and cellular stress responses. Research [9] has shown that ginsenoside Rg1 can improve psoriasis-like skin lesions by inhibiting NLRP3 and proliferation of keratinocytes. Another study [10] has indicated a significant increase in the expression of NLRP3 inflammasomes, IL-18, and IL-1β in psoriasis epidermis compared to normal skin.

KGF, also known as Keratinocyte Growth Factor, is a growth factor secreted by subcutaneous tissue cells and plays a crucial role in regulating cell regeneration, differentiation, and migration [11]. In this study, a psoriasis cell model was established by stimulating HaCaT cells with KGF, and the results showed that KGF stimulation increased NLRP3 mRNA and protein expression in HaCaT cells. Furthermore, this study successfully constructed psoriasis-like HaCaT cells with NLRP3 gene silencing, confirmed by comparing NLRP3 mRNA levels and protein expression.

The thickening of psoriasis lesions is associated with abnormal proliferation of skin tissue. Research [12] has shown that inhibiting the toll-like receptor 4/nuclear factor-kappa B pathway regulates NLRP3 inflammasome and can suppress the proliferation of keratinocytes. The results of this study indicate that cell proliferation and clone formation rates were significantly increased in cells induced by KGF compared to normal growing cells, while NLRP3-RNAi cells showed significantly reduced rates compared to their negative control cells. This suggests that NLRP3 is involved in the proliferation capacity of HaCaT cells induced by KGF, and inhibiting NLRP3 gene expression can suppress cell proliferation.

Cell proliferation involves the increase in cell numbers and population expansion, where cells need to complete DNA replication in a specific order to prepare for the next round of cell proliferation [13]. The smooth operation of the cell cycle is crucial for cell proliferation. The cell cycle consists of several phases, with each phase playing a key role in cell proliferation. G1, S, and G2 phases are involved in DNA synthesis, collectively known as interphase, while the M phase is the cell division phase. G0 is the phase where cells temporarily stop dividing and perform other biological functions, known as the resting phase [14]. Inhibiting DNA synthesis during interphase can directly block the cell cycle, and the results of this study showed that KGF treatment promoted the transition of HaCaT cells from the G0/G1 phase to the S phase, while inhibiting NLRP3 gene expression caused G0/G1 phase arrest in HaCaT cells, affecting cell cycle progression.

Cell proliferation and the smooth operation of the cell cycle are regulated by cell cycle-related genes and proteins. Cyclin B1 forms a complex with its corresponding cyclin-dependent kinase (CDK) to promote cells' entry into mitosis [15]. CDK2 can form complexes with various cell cycle proteins and affect the transition of cells from G1 to the S phase, and it can also bind to PCNA to promote cell proliferation. Ki67 is expressed in G1, S, and G2 phases and reflects the level of cell proliferation activity [16]. The results of this study showed that the protein expression of Cyclin B1, CDK2, Ki67, and PCNA in HaCaT cells induced by KGF was higher compared to normally growing cells. In contrast, NLRP3-RNAi cells exhibited significantly reduced protein expression of Cyclin B1, CDK2, Ki67, and PCNA compared to their negative control cells. This suggests that inhibiting NLRP3 gene expression may be associated with reduced expression of these cell cycle-related proteins, contributing to cell cycle arrest.

In normal physiological conditions, immune cells recognize and attack pathogens or abnormal cells. In psoriasis patients, the immune system is dysregulated, leading to immune cell attacks on normal keratinocytes, resulting in excessive proliferation and an inflammatory response. IL-17 is produced by activated T cells and promotes the production of interleukins and cell adhesion molecules by stimulating epithelial cells, leading to an inflammatory response. Research has suggested that IL-17A can promote the recruitment of IL-1β-producing myeloid cells to initiate autoimmune reactions [17]. IL-23, produced by activated dendritic cells and macrophages, is a pro-inflammatory cytokine primarily mediated by Th17 cells and natural killer cells [18]. IL-6 and TNF-α are both pro-inflammatory cytokines involved in various inflammatory reactions. The results of this study showed that IL-17, IL-23, IL-6, and TNF-α levels were higher in HaCaT cells induced by KGF compared to normally growing cells, while these markers were lower in NLRP3-RNAi cells compared to their negative control cells. This suggests that inhibiting NLRP3 gene expression can alleviate the inflammatory response in HaCaT cells.

In summary, silencing the NLRP3 gene has an inhibitory effect on the proliferation capacity of psoriasis-like HaCaT cells. This may be associated with reduced cell cycle protein expression and the cell cycle being blocked. It also

lowers the levels of pro-inflammatory cytokines. This study provides evidence for the involvement of NLRP3 in the pathogenesis of psoriasis in vitro. However, the mechanism of psoriasis is complex, and further research will be needed using in vivo animal models to explore it in depth.

Conflict of interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
No human or animals were used in the present research.

Informed consent
The authors declare not used any patients in this research.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request

Authors' contributions
Yuepeng An and Qing Zhang designed the study and performed the experiments, Rui Yuan and Shanshan Wang collected the data, Rui Yuan, Shanshan Wang and Suqing Yang analyzed the data, Yuepeng An and Qing Zhang prepared the manuscript. All authors read and approved the final manuscript.

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