Regulation of Notch1 and Foxp1 by MiR-34a in Psoriasis Vulgaris

Huiqin Wang; Xiaoling Zhang; Juan Bu & Weidong Wu


SUMMARY: This study is to investigate the regulation of Notch1 and Foxp1 by miR-34a in the development of psoriasis vulgaris. RT-PCR was used to compare the levels of miR-34a in the skin lesions of 20 patients with psoriasis vulgaris and 20 normal skin tissues. Immunohistochemistry was used to detect the expression of Notch1 and Foxp1 in 51 patients with psoriasis vulgaris, which were further compared with that in 29 normal control tissues. In addition, in HaCaT cells, we used miR-34a mimics and inhibitors to overexpress and inhibit miR-34a, respectively, and detected the mRNA and protein levels of miR-34a, Notch1, and Foxp1. The level of miR-34a in the skin lesions of patients with psoriasis vulgaris was significantly higher than that in normal skin tissues (t=2.192, P<0.05). The positive rate of Notch1 in the skin lesions of patients with psoriasis vulgaris was 76.47 %, which was significantly higher than that in normal skin tissues (13.79 %) (t=29.215, P<0.01). The positive rate of FOXP1 in the psoriasis vulgaris group was 92.16 %, which was also significantly higher than that in the normal skin group (65.52 %) (t=9.087, P<0.01). In addition, overexpression of miR-34a significantly promoted the expression of Notch1 and Foxp1. However, inhibition of miR-34a significantly reduced Notch1 and Foxp1 levels. miR-34a is highly expressed in the skin tissues of patients with psoriasis vulgaris, and may participate in the development of psoriasis vulgaris by regulating Notch1 and Foxp1.

KEY WORDS: Psoriasis vulgaris; miR-34a; Notch1; Foxp1.

INTRODUCTION

Psoriasis is a chronic inflammatory skin disease, which is mostly characterized by inflammatory cell infiltration, excessive proliferation of epidermal cells, dermal vascular proliferation and local immunological abnormalities (Di Meglio et al., 2014). Notch1 and Forkhead box P1 (Foxp1) are cell cycle regulators, which can regulate cell proliferation and differentiation. Studies have shown that Notch1 and Foxp1 play an important role in the pathogenesis of psoriasis (Ma et al., 2018; Yuan et al., 2020). However, the pathogenesis of psoriasis is still not well clarified.

MicroRNAs (miRNAs) are endogenous non-coding RNA molecules composed of approximately 22 nucleotides. By promoting target mRNA degradation and/or translation inhibition, they negatively regulate gene expression at the post-transcriptional level (Wu et al., 2018). The miRNAs are expressed in keratinocytes and immune cells, and play an important role in regulating their development and function (Shen et al., 2017). MiR-34a acts as a tumor suppressor (Chou et al., 2016), which participates in cell proliferation, apoptosis, survival, migration, invasion and angiogenesis by regulating target genes. It has been shown that miR-34a is correlated with tumor cell proliferation regulator Foxp1 and Notch1 (Slabáková et al., 2017).

Here, in this study, we aim to investigate the expression of miR-34a in skin tissues of patients with psoriasis vulgaris and its regulation on Notch1 and Foxp1. The levels of miR-34a in the skin tissues of patients with psoriasis vulgaris and normal skin tissues were analyzed with RT-PCR. The expressions of Notch1 and Foxp1 were detected by immunohistochemical staining. Further through overexpression or inhibition of miR-34a expression, we explored the regulation of Notch1 and Foxp1 by miR-34a in clinical practice.
HaCaT cells, and clarified how miR-34a may affect the pathogenesis of psoriasis vulgaris through Notch1 and Foxp1.

MATERIAL AND METHOD

Subjects. We recruited 20 patients (10 males and 10 females) with psoriasis vulgaris who were treated in the Dermatology Department of the People's Hospital of Xinjiang Uygur Autonomous Region from November 2018 to May 2019. The age of the patients ranged from 8 to 73 years, with an average of (42.91±18.36) years old. Skin lesion biopsy tissues were collected. In addition, 20 healthy volunteers (8 males and 12 females; age range 22 to 60 years old; average age 35.41±14.19 years old) who received plastic surgery during the same period were enrolled as controls. Normal human skin tissues were collected during plastic surgery. The clinical data of the two groups were not statistically significant (P<0.05) (data not shown). Inclusion criteria: All patients with psoriasis had typical skin lesions and were diagnosed by histopathology; the patients had not used tretinoin, glucocorticoids, and immunomodulators for systemic treatment within 3 months prior to sample collection; the patients had stopped phototherapy and drug treatment for at least 1 month; the patients did not have other immune diseases or tumor. Exclusion criteria: Patients with other systemic diseases such as lung, liver and kidney diseases; patients with other autoimmune diseases; patients with malignant tumors; breastfeeding, menstrual and pregnancy women. In the normal control group, we excluded subjects with autoimmune disease, genetic diseases, and family history of psoriasis. This study was approved by the Ethics Committee of People's Hospital of Xinjiang Uygur Autonomous Region, and was conducted in accordance with the principles expressed in the Declaration of Helsinki. All patients signed the informed consent.

Immunohistochemistry. Skin tissue sample sections were collected from 51 patients with psoriasis vulgaris and 29 normal control subjects. The sample sections were routinely deparaffinized, hydrated and treated with 3 % H2O2 at room temperature for 10 minutes. After that, the sample was microwaved in citrate buffer (pH=6.0) for 10 min (92~98°C) for antigen retrieval. After cooling, 5 % calf serum was added for blockage at room temperature for 30 min. Then, primary antibodies including anti-Notch1 (ab51627) (1:150) were added respectively and incubated at room temperature for 10 minutes. After that, the sample was microwaved in citrate buffer (pH=6.0) for 10 min (92~98°C) for antigen retrieval. After cooling, 5 % calf serum was added for blockage at room temperature for 30 min. Then, primary antibodies including anti-Foxp1 (ab134055) (1:1000) and anti-Notch1 (ab51627) (1:150) were added respectively and incubated overnight at 4°C. Then, the goat anti-rabbit IgG H&L (HRP) (ab6721) (1:1000) was added and incubated at room temperature for 30 min. Finally, color development with DAB and hematoxylin counterstaining were performed. After mounting, the sample was observed and photographed under a light microscope.

Transfection. HaCaT cells (1 × 10^4) were seeded in a 6-well plate (200 mL/well). After the confluence of cell growth reached 70 %, cell transfection was performed with lipofectamine2000 (Invitrogen, USA). The miR-34a mimics, mimics negative control (NC), miR-34a inhibitor, and inhibitor NC were respectively transfected into HaCat cells an incubated for 6 h.

MTT assay. The cells were seeded in 96-well plates and cultured overnight at 37°C. Cells were transfected with miR-34a mimics or inhibitors. At 24 h, 48 h, and 72 h after transfection, respectively, the cells were added with 10mL MTT and incubated at 37°C for 4h. After adding 150 mL DMSO, the cells were incubated for 10min at 37°C. The OD 568 of each well was measured by a microplate reader (Molecular Devices, Flexstation® 3, USA) to calculate cell viability.

RT-qPCR. Total RNA was extracted from patient tissues and Haca cells. Tissue RNA was used to detect miRNA, and cellular RNA was used to detect miRNA, Notch1, and Foxp1. Reverse transcription was performed with TIANScript RT Kit (Tiangen, Beijing, China). The reaction system for reverse transcription included 2 µL 5×PrimeScript Buffer, 0.5 µL PrimeScript RT Enzyme Mix I, 0.5mL Oligo(dt)15 (15 µM), 0.5 µL Random 6 mers (100 µM), total RNA 500 ng, and RNase free dH2O to 10µL. SuperReal PreMix Plus (SYBR Green) (Tiangen, Beijing, China) was used for RT-qPCR. The PCR primers were synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). U6: Forward: CTCGCTTCGGCAGCACA, Reverse:
were: 95 °C, 1 min, 40 cycles of 95 °C, 10 s and 60 °C 34 s, 94 °C 1 min 30 s, 60 °C 1 min, 60 °C →94 °C, 1.1 °C/s heating. The gene expression level was calculated by the ΔΔCt method.

Western blot. At 48h after transfection, cells were collected and lysed with RIPA buffer (including PMSF). After centrifugation at 12000 rpm at 4 °C for 5 min, the supernatant was collected and the protein concentration was determined by the BCA method (Solarbio, Beijing, China). Western blot was used to detect protein expression. Briefly, after the protein samples were electrophoresed and transferred to the membrane, the membrane was blocked with 5 % BSA at room temperature for 1 h. After that, primary antibody of Notch1 and Foxp1 (1:1000, Abcam, USA) was added and incubated overnight at 4 °C. Then, the secondary antibody of HPR-rabbit-anti-human IgG H&L (1:10000) was added and incubated at room temperature for 1 h. After color development, the protein levels were analyzed with Quantity-one image software (Bio-Rad Gel imaging system, USA). The optical density value was scanned, and the density ratio of the target protein to the GAPDH (%) was calculated.

Statistical analysis. All data were processed by SPSS 13.0 software and expressed as mean ± standard deviation (SD). One-way analysis of variance was performed on the measurement data, and the LDS method was used for pairwise comparison. The measurement data was compared with t test, which was of normal distribution and represented by mean ± SD. The count data was analyzed by χ² test. A P value <0.05 is considered as significant difference.

RESULTS

The miR-34a is highly expressed in skin tissues of psoriasis vulgaris. RT-qPCR was conducted to analyze miR-34a expression in skin tissues. The results showed that miR-34a in the skin lesions of patients with psoriasis vulgaris was significantly higher than normal skin tissues (Table I, t=2.192, P<0.05).

Table I: miR-34a levels in normal skin tissue and psoriasis vulgaris lesion tissue.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>miR-34a</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal tissue</td>
<td>20</td>
<td>0.01±0.014</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lesion tissue</td>
<td>20</td>
<td>0.026±0.021*</td>
<td>2.192</td>
<td>0.016</td>
</tr>
</tbody>
</table>

Note: * P<0.05 compared with normal tissue.

Notch1 and Foxp1 are highly expressed in skin tissues of psoriasis vulgaris. Notch1 and Foxp1 expression in skin tissues of psoriasis vulgaris were detected with immunohistochemistry. As shown in Figure 1, Notch1 was mainly expressed in the cytoplasm of keratinocytes, and occasionally in the nucleus or cell membrane. Notch1 was expressed in the entire epidermis in the skin lesions of patients with psoriasis vulgaris. Statistical analysis showed that a positive rate of Notch1 was 76.47 % (39/51) in patients with psoriasis vulgaris. However, in normal skin tissues, Notch1 was mainly expressed in the basal layer of the epidermis. In addition, the spinous layer and granular layer cells showed weak or no expression. The positive rate of Notch1 in normal tissues was 13.79 % (4/29), which was significantly lower than that of patients with psoriasis vulgaris (χ²=29.215, P<0.01) (Table II).

Table II: Notch1 expression in psoriasis vulgaris lesions and normal skin tissues.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
<th>Positive (%)</th>
<th>χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psoriasis vulgaris</td>
<td>39</td>
<td>12</td>
<td>51</td>
<td>76.47</td>
<td>29.215</td>
<td>0.000</td>
</tr>
<tr>
<td>Normal control</td>
<td>4</td>
<td>25</td>
<td>29</td>
<td>13.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>37</td>
<td>80</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

In addition, Foxp1 in the psoriasis vulgaris group was mainly expressed in the entire epidermis, while in normal skin tissues, it was mainly distributed in the basal layer and spinous layer of the epidermis (Fig. 1). The positive rate of Foxp1 in the psoriasis vulgaris group was 92.16 % (47/51), significantly higher than that in normal skin (65.52 % (19/29)) (χ²=9.087, P<0.01) (Table III).

Verification of miR-34a transfection efficiency. Through RT-qPCR, we first confirmed the expression of miR-34a after transfection of miR-34a mimics and miR-34a inhibitor. After transfection of miR-34a mimics, miR-34a was significantly higher than that of NC control group (Fig. 2A). On the contrary, after miR-34a inhibitor treatment, the level of miR-34a in the cells was significantly lower than that of NC control group (Fig. 2B).
The miR-34a promotes cell proliferation. The effects of miR-34a mimics and miR-34a inhibitor on the proliferation of HaCat cells were detected by MTT (Fig. 3). The results showed that there was no significant difference between the groups after treatment for 24h and 48h. However, after 72h, miR-34a mimics obviously increased proliferation of HaCaT. In addition, miR-34a inhibitor significantly reduced the proliferation of HaCaT cells.

The miR-34a upregulates the levels of Notch1, and Foxp1 mRNA. After transfection of miR-34a mimics and miR-34a inhibitor into HaCat cells, the levels of miR-34a, Notch1, and Foxp1 mRNA were detected by RT-qPCR (Fig. 4A-4C). Among them, miR-34a, Notch1, and Foxp1 mRNA were significantly higher in the miR-34a mimics group than NC control group. However, miR-34a, Notch1, and Foxp1 mRNA in the miR-34a inhibitor group were significantly lower than NC control group.

The miR-34a upregulates the protein levels of Notch1 and Foxp1. Western blot was used to detect the effects of miR-34a mimics and miR-34a inhibitor on the expression of Notch1 and Foxp1. The results showed that miR-34a mimics could significantly promote the expression of Notch1 (Fig. 5). However, miR-34a inhibitor significantly inhibited Notch1 expression. In addition, after miR-34a transfected HaCaT cells, miR-34a mimics significantly promoted the expression of Foxp1. However, miR-34a inhibitor caused a significant decrease in Foxp1 expression (Fig. 5).
Figure 3: Cell proliferation after transfection of miR-34a mimics and miR-34a inhibitor. MTT was used to detect cell proliferation. *P<0.05

Figure 4: RT-PCR detection of miR-34a, Notch1, and Foxp1 mRNA expression after transfection of miR-34a mimics and miR-34a inhibitor. Relative expression of (A) miR-34a, (B) Notch1, and (C) Foxp1 mRNA were shown. Compared with NC group, *P<0.05.

Figure 5: Comparison of the protein expression levels of Notch1 and Foxp1 after transfection of miR34a mimics and miR-34a inhibitor. Representative and quantitative Western blot results were shown. Compared with NC group, *P<0.05.
DISCUSSION

Psoriasis is a chronic inflammatory disease regulated by multiple factors. It is a skin disease characterized by abnormal proliferation of epidermal keratinocytes and hypokeratosis. Studies have shown that miRNAs are involved in skin physiology, biochemistry, immunity and many other processes (Wang et al., 2017a,b). The miR-34a is located on the human 1p36 chromosome and has a total of 110 nucleotide sequences (Wang et al., 2016). Transfection of tumor cells with miR-34 can cause cell cycle arrest in G1 phase (Wang et al., 2013). Meanwhile, miR-34 reactivation in cancer cells can induce caspase-regulated apoptotic pathways. In addition, miR-34 may also play a synergistic effect with other miRNAs.

miR-34a participates in biological processes such as cell proliferation, apoptosis, survival, migration, invasion and angiogenesis through the regulation of target genes (Wang et al., 2013). miR-34a is directly regulated by the transcription factor p53, and is abnormally expressed in a variety of diseases (Lou et al., 2010). Study has also found that miRNA-34a is not only directly regulated by p53, but can also activate the p53 signaling pathway by inhibiting the expression of SIRT1 or E2F3, thereby forming a p53-miRNA-34a positive feedback loop (He et al., 2007). Psoriasis is a type of chronic inflammatory skin disease with characteristic cell hyper-proliferation. Effectors such as p14ARF, MDM2, p21WAF1, Bcl-2/Bax, and c-myc in the p53 pathway are involved in the occurrence of psoriasis. It is speculated that miRNA-34a may play a role through the p53 pathway in the occurrence of psoriasis. Therefore, in this study, we explored the expression of miR-34a in the skin lesions of patients with psoriasis vulgaris. In addition, through in vitro experiments, we found that miR-34a significantly promoted the expression of its target genes Notch1 and Foxp1 in psoriasis vulgaris.

First, RT-qPCR results indicate that miR-34a was highly expressed in psoriasis vulgaris lesions. This result is consistent with previous studies (Yang & Tang, 2012). Secondly, Notch signaling plays a key role in cell differentiation and proliferation. Blanpain et al. (2006) found that Notch receptors released Notch intracellular domain (NICD) after binding to ligands on the cell surface. NICD then entered the nucleus and combined with CSL (CBF1/Suppressor of Hairless/Lag-1) to form transcription activation complex, thereby promoting the expression of downstream target gene (Blanpain et al., 2006). The activation of Notch1 could activate CCND1 and CDK2 through the CSL pathway, prompting cells to enter the S phase (Blanpain et al., 2006). In most cases, Notch1 is used as an oncogene to promote tumor growth. The cell cycle of keratinocytes in psoriasis vulgaris lesions is shorter than that of normal cells, which is similar to the occurrence of tumors (Ma et al., 2017). We detected the level of Notch1, the downstream target protein of miR-34a, by immunohistochemistry, and the results showed that Notch1 was mainly expressed in the entire epidermis in psoriasis vulgaris tissues, and the positive rate was significantly higher than that of normal skin tissues. These results are consistent with the results of Abdou et al. (2012).

Foxp1 is an important target gene of miR-34a. It is located on chromosome 3p14.1 and has a wide range of expression in human tissues to varying degrees (Jiang et al., 2010; Benayoun et al., 2011). It belongs to the P subfamily of FOX (Forkhead Box) family. The FOX family is a large, versatile and evolutionarily conserved transcription factor family, which is characterized by a forkhead/winged helix (Jiang et al., 2010; Benayoun et al., 2011). FOX family proteins are involved in processes such as embryonic development, cell cycle regulation, carbohydrate and lipid metabolism, and immune regulation (Feng et al., 2010; Jiang et al., 2010; Benayoun et al., 2011). Its mutation and abnormal expression can lead to developmental malformations, metabolic disorders, and tumor occurrence (Feng et al., 2010; Jiang et al., 2010; Benayoun et al., 2011). Foxp1 is regulated by miR-34a and participates in a variety of biological processes, and is closely related to the physiology and immunity of skin diseases. It is shown that the positive rate of Foxp1 protein in skin basal cell carcinoma was significantly higher than that of adjacent tissues (Lu M, Huang C, Li B and Xu Y). Here, we found that the positive rate of Foxp1 in normal skin tissue was significantly lower than that in psoriasis vulgaris. Similarly, after transfecting HaCaT cells with miR-34a mimic and miR-34a inhibitor, Western Blot results showed that miR-34a overexpression promoted the expression of Foxp1. However, miR-34a inhibitors significantly inhibited the expression of Foxp1. Studies have found that miRNA-34a can be used as a specific marker for diffuse large B-cell lymphoma and non-small cell lung cancer (Gallardo et al., 2009; Fang et al., 2012). At present, the diagnosis of psoriasis vulgaris is mainly based on clinical symptoms and physical signs. No sensitive, specific, simple, and easy-to-detect psoriasis vulgaris markers have been identified. Therefore, the results of this study may provide potential markers for the early diagnosis of psoriasis vulgaris and potential therapeutic target for the treatment of psoriasis vulgaris.

In summary, we concluded that miR-34a, which was highly expressed in the pathogenesis of psoriasis vulgaris, may be involved in the abnormal proliferation of epidermal cells by regulating its target genes Notch1 and Foxp1. Therefore, we believe that miR-34a and its target genes Notch1 and Foxp1 may be closely related to the pathogenesis of psoriasis vulgaris.
RESUMEN: El objetivo de este estudio fue investigar la regulación de Notch1 y Foxp1 por miR-34a en el desarrollo de la psoriasis vulgar. Se utilizó RT-PCR con el fin de comparar los niveles de miR-34a en las lesiones cutáneas de 20 pacientes con psoriasis vulgar y 20 tejidos de piel normales. Se utilizó inmunohistoquímica para detectar la expresión de Notch1 y Foxp1 en 51 pacientes con psoriasis vulgar, que se compararon además con la de 29 tejidos normales control. Además, en las células HaCaT, usamos miméticos e inhibidores de miR-34a para sobreexpresar e inhibir miR-34a, respectivamente, y detectamos los niveles de ARNm y proteína de miR-34a, Notch1 y Foxp1. El nivel de miR-34a en las lesiones cutáneas de pacientes con psoriasis vulgar fue significativamente mayor que en los tejidos normales de la piel (t=2,192, P<0,05). La tasa de positividad de Notch1 en las lesiones cutáneas de pacientes con psoriasis vulgar fue del 76,47 %, que fue significativamente mayor que la de los tejidos normales de la piel (13,79 %) (t=29,215, P<0,01). La tasa positiva de FOXP1 en el grupo de psoriasis vulgar fue del 92,16 %, que también fue significativamente mayor que la de los tejidos normales de la piel (13,79 %) (t=9,087, P<0,01). Además, la sobreexpresión de miR-34a promovió significativamente la expresión de Notch1 y Foxp1. Sin embargo, la inhibición de miR-34a redujo de manera importante los niveles de Notch1 y Foxp1. miR-34a se expresa en gran medida en los tejidos de la piel en pacientes con psoriasis vulgar y puede participar en el desarrollo de la psoriasis vulgar mediante la regulación de Notch1 y Foxp1.

PALABRAS CLAVE: Psoriasis vulgar; miR-34a; Mucosal; Foxp1.

REFERENCES


