Original Article
hsa-miR-155 targeted NCSTN 3’UTR mutation promotes the pathogenesis and development of acne inversa

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Abstract: Objectives: To investigate molecular mechanisms of nicastrin (NCSTN) mutations inducing acne inversa (AI). Methods: New and old lesional and non-lesional skin samples were obtained from an AI patient. Healthy skin samples were obtained from the buttocks of 100 non-AI patients. Hematoxylin-eosin staining and immunohistochemistry of NCSTN protein were examined. All exon-intron and exon boundary sequences were polymerase chain reaction (PCR) -amplified and sequenced. Bioinformatic analyses of NCSTN 3’-untranslated regions (3’UTR) were conducted using RegRNA2.0. 3’UTR of NCSTN was cloned vector of psiCHECK-2 vector; the mutant 3’UTR NCSTN-psiCHECK-2 was constructed on a template of NCSTN 3’UTR. A dual-luciferase reporter gene assay, real-time reverse transcription (qRT)-PCR and Western blot analysis were conducted to evaluate functional changes associated with the mutation. Results: We identified a novel deletion mutation of the NCSTN gene in the NCSTN 3’UTR region (designated c.2584-2585del CA) at the binding site of human micro-RNA-155 (hsa-miR-155). Levels of NCSTN protein were potently lower in epidermis and hair follicles of AI patients with lesions than in healthy skin. The hsa-miR-155+mutant NCSTN significantly downregulated in dual luciferase assay, qRT-PCR, and Western blot. The novel deletion mutation was confirmed to be a pathological cause of AI. Conclusions: miR-155 downregulates the expression of NCSTN by binding NCSTN 3’UTR, providing a possible new mechanism of loss of NCSTN function in AI patients. hsa-miR-155 functions as a promoter in AI, and is a potential therapy target for AI.

Keywords: Acne inversa, NCSTN, mutation, hsa-miR-155, dual-luciferase reporter assay

Introduction
Acne inversa (AI), also known as hidradenitis suppurativa (HS), is a relapsing, inflammatory and disabling follicular occlusive disease, with chronic duration. It usually afflicts individuals after puberty but it may also affect children [1]. When AI coexists with acne conglobate, dissecting cellulitis, and/or pilonidal cysts, it is considered a follicular occlusion triad and/or tetrad [2]. Loo et al. termed HS, Dowling-Degos, and multiple epidermal cysts as a new follicular occlusion triad; because they have a common histopathology of abnormal follicular keratosis [3]. The primary lesions mostly appear in apocrine gland regions, for example, axillae, genitals, the groin, breast, buttocks, and the perianal area. Clinical manifestations of this condition include deep nodules, boils, pustule, and a foul discharge. The disease gradually progresses into an abscess, sinus tracts, fistula, and scars.

The average prevalence of AI is 1% in Europe [4], with a mean incidence of 6.0 per 100 000 person-years. In Africa, the female-male ratio of AI is nearly 3:1, and skin lesions in males tend to be more severe [5]. Furthermore, progression of AI is associated with comorbidities such as inflammatory bowel disease, particularly Crohn’s disease [6], pyoderma gangrenosum [7], and spondyloarthritis [8]. In severe and long-lasting cases, the condition may develop into non-melanoma cancers including squamous cell carcinoma (SCC) [5], perianal mucinous adenocarcinoma [9], liver cancer, and other diseases. The disease may cause severe pain, loss of ability to work, and significantly decrease the living quality of patients [10].

Treatments should be developed with reference to clinical manifestations, body site, severity, and course of disease. Main treatment options include topical therapy, systemic therapy, analgesics, zinc gluconate, intramuscular gamma-
**Table 1.** Review of γ-secretase gene mutation and clinical findings in AI families and sporadic cases

<table>
<thead>
<tr>
<th>Gene</th>
<th>No.</th>
<th>Location</th>
<th>Mutation</th>
<th>Family History</th>
<th>Patients (M/F)</th>
<th>Sex/Age of proband (years)</th>
<th>Age at onset of proband (years)</th>
<th>Distribution</th>
<th>Obese/smoking</th>
<th>Ethnicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCSTN</td>
<td>1</td>
<td>Exon 11</td>
<td>c.1258 C&gt;T (n)</td>
<td>Yes</td>
<td>25 (13/12)</td>
<td>M (?)</td>
<td>?</td>
<td>Buttocks, nape of the neck, armpit</td>
<td>Nonobese</td>
<td>Chinese</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Exon 11</td>
<td>c.1300 C&gt;T (n)</td>
<td>Yes</td>
<td>10 (5/5)</td>
<td>?</td>
<td>Since puberty</td>
<td>Axillary, inguinal, and perianal folds</td>
<td>Nonobese</td>
<td>French</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Exon 15</td>
<td>c.1695 T&gt;G (n)</td>
<td>Yes</td>
<td>4 (1/3)</td>
<td>M (47)</td>
<td>26</td>
<td>Back of the neck, axillae, buttocks, and groin</td>
<td>?</td>
<td>Chinese</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Exon 15</td>
<td>c.1768 A&gt;G (m)</td>
<td>Yes</td>
<td>8 (3/5)</td>
<td>?</td>
<td>Since puberty</td>
<td>Buttocks (severe)</td>
<td>Nonobese</td>
<td>Chinese</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Exon 3</td>
<td>c.223 G&gt;A (m)</td>
<td>Yes</td>
<td>3 (3/0)</td>
<td>M (20)</td>
<td>16</td>
<td>Neck, mons pubis, and penis</td>
<td>?</td>
<td>Chinese</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Exon 5</td>
<td>c.553 G&gt;A (m)</td>
<td>No</td>
<td>-</td>
<td>F (45)</td>
<td>13</td>
<td>Axillae, chest, groin, buttocks</td>
<td>BMI=38 diabetes (2) smoker</td>
<td>Caucasian</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Exon 6</td>
<td>c.632 C&gt;G (m)</td>
<td>No</td>
<td>1 (1/0)</td>
<td>M (48)</td>
<td>38</td>
<td>Back of the neck, axillae, buttocks, and groin</td>
<td>?</td>
<td>Chinese</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Exon 6</td>
<td>c.647 A&gt;C (m)</td>
<td>Yes</td>
<td>6 (5/1)</td>
<td>M (46)</td>
<td>18</td>
<td>Back of neck, axillae, buttocks, and groin</td>
<td>?</td>
<td>Chinese</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Exon 9</td>
<td>c.1101+1 G&gt;A (m)</td>
<td>Yes</td>
<td>4 (4/0)</td>
<td>M (?)</td>
<td>16</td>
<td>Axillae, suprapubic area, groin, buttocks, thighs, and neck</td>
<td>BMI=30 15pack/year</td>
<td>British</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Intron 8</td>
<td>c.996+7 G&gt;A (m)</td>
<td>No</td>
<td>-</td>
<td>F (38)</td>
<td>35</td>
<td>Axillae, groin buttocks</td>
<td>BMI=37 smoker diabetes (2)</td>
<td>Caucasian</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Exon 11</td>
<td>c.1101+10 A&gt;G (m)</td>
<td>No</td>
<td>-</td>
<td>M (24)</td>
<td>16</td>
<td>Axillae, groin, buttocks, genitalia</td>
<td>BMI=24 smoker</td>
<td>Afro-Caribbean</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Exon 13</td>
<td>c.1551+1 G&gt;A</td>
<td>Yes</td>
<td>13 (6/7)</td>
<td>M (?)</td>
<td>?</td>
<td>Back, buttocks, chest, face, forehead, nape, and waist</td>
<td>?</td>
<td>Chinese</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>Exon 3</td>
<td>c.210_211delAG</td>
<td>Yes</td>
<td>7 (5/2)</td>
<td>F</td>
<td>?</td>
<td>Belly, back, buttocks</td>
<td>?</td>
<td>Chinese</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>Exon 5</td>
<td>c.487delC 5</td>
<td>Yes</td>
<td>5 (3/2)</td>
<td>?</td>
<td>Since puberty</td>
<td>Groin</td>
<td>Nonobese</td>
<td>French</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PSEN1</td>
<td>1</td>
<td>Exon 5</td>
<td>c.725delC</td>
<td>Yes</td>
<td>4 (3/1)</td>
<td>M</td>
<td>?</td>
<td>Armpits, groin, buttocks (severe); back, face, nape, and waist</td>
<td>?</td>
<td>Chinese</td>
</tr>
<tr>
<td>PSEN1</td>
<td>2</td>
<td>Exon 3</td>
<td>c.66_67insG</td>
<td>Yes</td>
<td>2 (0/2)</td>
<td>F (?)</td>
<td>15</td>
<td>Axillae, under breasts, groin</td>
<td>BMI=23 no smoke</td>
<td>British</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Exon 3</td>
<td>c.279delC</td>
<td>Yes</td>
<td>19 (9/10)</td>
<td>F</td>
<td>?</td>
<td>Back, buttocks, chest, face, forehead, nape, and waist</td>
<td>?</td>
<td>Chinese</td>
</tr>
</tbody>
</table>
globulin [11]. Recently, new therapies involve the use of tumor necrosis factor α (TNF-α) inhibitors, such as infliximab, adalimumab, etanercept, efalizumab, ustekinumab, and photodynamic therapy [12]. Although there are several alternative treatments, their efficacy is temporary in the early stages of treatment. Radical surgeries (including a procedure on the apocrine sweat gland) have a definite effect but cannot prevent relapse in patients with long-term AI. However, combination therapy is commonly and effectively used. The diagnosis is confirmed according to von der Werth criteria [13].

The etiology of AI is not completely understood. However, most studies identify AI as an autosomal dominant disease. The transmembrane enzymatic complex γ-secretase consists of four subunits: catalytic unit presenilin and three other cofactors, namely presenilin Enhancer 2, Nicastrin, and Anterior Pharynx Defective-1. The γ-secretase complex catalyzes the splitting of protein substrates important for developmental signaling pathways [14].

In all, 22 mutations of PSEN1, PSENEN, and NCSTN have been reported to be responsible for AI, observed as 1 mutation, 3 mutations, and 18 mutations, respectively (Table 1) [5, 15-22], or as 4 nonsense mutations (18.2%), 5 missense mutations (22.7%), 5 splice mutations (22.7%), 1 insert mutation (4.5%), and 7 delete mutations (31.8%), which are the most common mutations. Gao et al. reported that the AI gene located at chromosome 1p21.1-1q25.3 [23]. Wang et al. first identified six Chinese HS families had six different mutations in γ-secretase [5].

MicroRNAs (miRNAs) are a group of small non-coding sequences that regulate expression of different genes in eukaryotic cells at the post-transcription stage. By binding with the target 3'-untranslated regions (3'UTRs) of miRNAs, they can induce direct mRNA degradation or translation inhibition [24]. It has been reported that human miRNA-155 (hsa-miR-155) is associated with oral SCC25. However, no reports have associated it with the incidence and progression of AI.

In this study, we observed a novel mutation at 3’UTR of the NCSTN gene in a Chinese AI patient whose relatives did not suffer from this disease; the gene was designated c.2584-2585del CA. Then it was identified based on its interaction with hsa-miR-155. This study was undertaken to investigate molecular mechanisms of NCSTN mutations inducing AI.

Materials and methods

Subjects

A 26-year-old Chinese male was admitted to the Department of Dermatology and Venerology of Nanfang Hospital, Guangdong Province, China. The patient developed recurrent acne on his face and abscesses on his body, progressing to scars and sinus tracts starting in his twentieth year. All of his relatives were healthy. The patient denied having knowledge of any responsible disease. He was identified and carefully examined by at least two dermatologists. The diagnosis was confirmed by histological examination of skin biopsy specimens. Blood and skin biopsies were collected from the patient as well as randomly from 100 unrelated, unaffected, and healthy controls. All samples, including those of blood and tissue, were taken with the informed consent of patients and volunteers. This study complies with the principles of the Helsinki Declaration and was approved by the Ethics Committee of Southern Medical University, Guangdong province, China.

Skin samples

New and old lesional and non-lesional skin samples were obtained from the patient. Healthy skin samples were obtained from the buttocks of non-AI patients undergoing surgery. Skin samples were fixed for section.

Hematoxylin and eosin staining

Skin samples were formalin fixed, alcohol dehydrated, and paraffin embedded. Then the blade cut the paraffin blocks into about 3 microns thick to histopathologic observation and stained with hematoxylin and eosin.

Polymerase chain reaction

The exons and exon-intron boundaries of the NCSTN Genomic DNA were sequenced, using DNA isolated from peripheral blood using the Blood DNA Mini Kit (Simgen, Hangzhou, China, Cat. No. 3001050) and amplified by
NCSTN mutation causes AI

Table 2. NCSTN’ primers for PCR

<table>
<thead>
<tr>
<th>NO.</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TGGAAACACGAACTCCCGGT</td>
<td>GGCAAAGGGAAGCCTGGT</td>
<td>368</td>
</tr>
<tr>
<td>E2</td>
<td>TACCCCAACGCACTCCGGGT</td>
<td>TGCCCCATGACAGGCTGGT</td>
<td>341</td>
</tr>
<tr>
<td>E3</td>
<td>GGATAGGGATGAGAAGCAGATCTCTCTCTCT</td>
<td>CTGGTCTCCCATCTCAGGCTGGT</td>
<td>667</td>
</tr>
<tr>
<td>E4</td>
<td>CCCCTTATCAGTGCTCCT</td>
<td>TGGCCCATAGGAATGGGAGT</td>
<td>1049</td>
</tr>
<tr>
<td>E5</td>
<td>TGGCTGTGCTGCGAGCTGCCTCT</td>
<td>GGTTGACGCTGAAGGCTGGT</td>
<td>1782</td>
</tr>
<tr>
<td>E6</td>
<td>TTATGGTCCCATCTTCCCTTCTTCTCT</td>
<td>TTGTATTTATGCCAGTTAGCAGTCTGT</td>
<td>1565</td>
</tr>
<tr>
<td>E7&amp;8</td>
<td>AGGATCCACAGAAGCACCTCT</td>
<td>TTCAACAACAGTGGAGCTCT</td>
<td>946</td>
</tr>
<tr>
<td>E9&amp;10</td>
<td>TTAGGGTACGGATGATGCTG</td>
<td>AAGCTGTCGGTCTCAGGAGGAGT</td>
<td>619</td>
</tr>
<tr>
<td>E11</td>
<td>AGGAAATCAGAGAGCCTGGT</td>
<td>CTAAGACGTGGAGGAGGAGT</td>
<td>457</td>
</tr>
<tr>
<td>E12&amp;13</td>
<td>ATTGTCTCTACCCCTTCTTCTCTCT</td>
<td>AGACTGGCCAGATAGAGCTCAGT</td>
<td>640</td>
</tr>
<tr>
<td>E14-16</td>
<td>CCCATGACTGGTGTCCTCAGT</td>
<td>GCCCTCTAAGGGAACAGTCT</td>
<td>1238</td>
</tr>
<tr>
<td>E17</td>
<td>AGTGCCTAGGCTGGAGGAGT</td>
<td>CTAGGCTGCTGACAGCT</td>
<td>1013</td>
</tr>
</tbody>
</table>

polymerase chain reaction (PCR), followed by direct automated sequencing using ABI PRISM 3130 genetic analyzers (Applied Biosystems, Foster City, CA, USA).

All coding exons of the NCSTN gene together with boundary exon-intron sequences were amplified (Table 2) [5]. The PCR program was as follows: HotStarsTaq activation at 94°C for 5 min, followed by 28 cycles, each with denaturation at 94°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 45 s, except that in the first 8 cycles the annealing temperature was decreased from 62°C to 58°C by 0.5°C per cycle, and the final extension was at 72°C for 5 min. The new variants were then analyzed in 200 normal chromosomes for the possibility of polymorphisms.

Immunohistochemistry

Immunohistochemistry was performed to measure the levels of NCSTN protein in old and new skin lesions of the AI patient and healthy controls. Paraffin sections were incubated overnight at 4°C with anti-NCSTN antibody (1:100, Bioss, Beijing, China. Cat. No. bs-6058R). Then, slides were incubated with a biotin-conjugated secondary antibody for 30 min at room temperature, and treated with diaminobenzidine tetrahydrochloride to ensure the visualization of the antigen-antibody complex. Finally, the sections were lightly counterstained with hematoxylin.

Cell culture

Human embryonic kidney 293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO®, Shanghai, China, Cat. No. C11995500BT) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Shanghai, China, Cat. No. 10099-141). Cell culture was maintained at 37°C in a humidified atmosphere with 5% CO₂ (Eppendorf, USA, Galaxy 179R/S). One day before transfection, the cells were inoculated into 24-well plates (Corning, Shanghai, China, Cat. No. 3524) containing 500 µL DMEM. On the day of transfection, they were transferred to 300 µL OPTI-MEM medium (Invitrogen, Shanghai, China, Cat. No. 31985062).

Dual luciferase reporter assay

Reg RNA2.0 (http://regrna2.mbc.nctu.edu.tw/) was used to predict miRNA targets for putative mRNAs. The psiCHECK-2 luciferase vector (Promega, Shanghai, China, Cat. No. C8021) was used for dual luciferase assays. The 286 bp sequence of the NCSTN 3’UTR was inserted into the vector at the 3’ end of the hRluc (Renilla luciferase) reporter gene. NCSTN 3’UTR was inserted using the XhoI/NotI sites (NEBcutter V2.0 http://nc2.neb.com/NEBcutter2/). PCR products and psiCHECK-2 were digested using XhoI (TaKaRa, Dalian, China, Cat. No. D1094A) and NotI (TaKaRa, Dalian, China, Cat. No. 1166BH) to construct wild type recombinant plasmid. The PCR products were cloned downstream of the Renilla luciferase gene containing psiCHECK-2 vector, generating a psiCHECK-2-3’UTR-NCSNT (termed wt-NCSTN), using T4 DNA Ligase (TaKaRa, Dalian, China, Cat. No. D2011A). On the following day, a single clone was randomly selected and a reconstructed plasmid was extracted (AXYGEN, California, USA, Cat. No. AP-MN-P-50) for digestion, PCR, and identification of sequences.

PsiCHECK-mt-NCSTN plasmid, containing a mutated binding site at the position 513-519 of NCSTN 3’UTR, was constructed using the following primers: 5’ACTGTCCTTTCTCCAGGCCCTTGCGATGCTGGGTGCTGCGGGTGGGTAT3’ (forward) and 5’ATACCCACCCGAGC-
ACGCCACCCCTAATGCCATCTGAGGGCCTGGAG-AAAGGACAGT3’ (reverse). In this case, wt-NCSTN was used as the template. PCR products were preliminarily analyzed by 1% agarose gel electrophoresis and digested with DpnI (Promega, Shanghai, China, Cat. No. R6231) for 4 h at 37°C. Then 5 µL product was transferred to 50 µL competent cells (DH5α) for overnight culture at 37°C, with water replacing plasmid as a NC. The following day, another clone was randomly selected and a reconstructed plasmid was extracted for cleave, PCR, and sequence identification.

Luciferase assays were performed with a dual-luciferase assay kit (Promega, Shanghai, China, Cat. No. E1910). HEK 293-T cells were co-transfected with the indicated psiCHECK2 construction (0.5 µg/well) in 24-well plates, hsa-miR-155, NC (50 nM) or miRNA inhibitor, and NC inhibitor (100 nM) (RIBOBIO, Guangzhou, China) using Lipofectamine 2000 (Invitrogen, Shanghai, China, Cat. No. 11668-019). After 48 h, cells were harvested and luciferase activity was determined using a GloMax™ 20/20 luminometer (Promega, Madison, Wisconsin, USA). Luciferase data were expressed as a ratio of Renilla luciferase (RL) to firefly luciferase (FL) to normalize transfection variability between samples and experiments were repeated in triplicate using independent samples.

**RNA isolation and real-time reverse transcription (qRT)**

Total RNA was extracted using RNAiso Plus. Briefly, 1 mg RNA was used to perform reverse transcription using PrimeScript RT reagent Kit with gDNAErase (TaKaRa, Tokyo, Japan). RT-PCR amplification of the transcribed cDNA was performed with the SYBR Premix Ex TaqTM II purchased from (TaKaRa, Tokyo, Japan). The
sequences of the primer sets were listed as follows: NCSTN-F1: 5’GCAGTGCCAGGATCCAAGTA; NCSTN-R1: 5’CAGTTCAAAGGCAGGAGACA; 18s-rRNA-F: 5’CCTGGATACCGCAGCTAGGA; 18s-rRNA-R: 5’GCGGCGCAATACGAATGCCGC. Quantification of gene expression was determined by comparative quantity, using 18s-rRNA expression as inner control.

**Western blot analysis**

Cells were treated with RIPA lysis buffer supplemented with protease inhibitor cocktail and protein phosphatase inhibitor. The concentration of the protein was quantified by a DC protein kit (Bio-Rad Laboratories, Hercules, CA, USA). Samples were separated and transferred to PVDF membranes. Then, they were incubated with their primary antibodies overnight at 4°C. Primary antibodies used were against NCSTN and GAPHD. In the following day, the membranes were incubated with secondary antibody for 2 h at room temperature. Expression levels of the respective proteins were determined by enhanced chemiluminescence reagent.

**Statistical analysis**

Statistical analysis was performed using SPSS 16.0 software (SPSS Inc., IL, USA). Binding ability = (R/F) samples/(R/F) controls (firefly luciferase: F; Renilla luciferase: R). Data are expressed as mean ± standard deviation (SD) from at least three independent experiments. The differences among groups were analyzed using one-way ANOVA. Multiple comparisons were made using the method of least significant difference to ascertain the condition of equal variance for 3’UTR mut-NCSTN. For the condition of heterogeneity of variance, Welch and Dunnett T3 methods were used for 3’UTR NCSTN. One-way ANOVA was used for detecting psiCHECK-2. The difference in 3’UTR NCSTN and psiCHECK-2 groups was not statistically significant, and there was no need for multiple comparisons. A two-tailed P value less than 0.05 was considered statistically significant.

**Results**

**Clinical and histopathologic findings**

Recurrent acne on the face and abscesses on the body of the AI patient was investigated. Red or skin-colored, depressed or hypertrophic scars caused by recurrent inflammatory pustules, nodules, abscesses, and cysts were seen on the forehead, nose, underjaw, cheek (Figure 1A), backside (Figure 1B), and buttock (Figure 1C). Hyperplasia and hypertrophy manifested on the nasal tip and nasal alae, while the face developed mild leontiasis (Figure 1A). New cysts on the buttock became fluctuant with a few purulent secretions (Figure 1C). In new abscess lesions, HE staining showed a slight hyperkeratosis of the epidermis, hyperplasia of stratum spinosum, and extension of epidermal skin (Figure 1D). Large hair follicles within the dermis were full of horny substances (Figure 1E). Flake inflammatory cells, including neutrophils, lymphocytes, histocytes, and multinucleated giant cells had infiltrated around dermal blood vessels and cutaneous appendages,
NCSTN mutation causes Al

causing hyperplasia, expansion, and congestion of blood capillary (Figure 1F). In old scar lesions, histological features included slight hyperkeratosis of the epidermis and hyperplasia of the stratum spinosum (Figure 1G), and extensive collagen-fiber hyperplasia in the dermis (Figure 1G, 1H). There was atrophy of hair follicles and sebaceous glands (Figure 1H), multiple lanugo hairs, and the absence of a valid hair follicle structure (Figure 1I). These results proved that his Al condition was very pronounced.

Mutation screening for NCSTN genomic sequence

In all, 17 exons of the NCSTN gene were amplified and analyzed via agarose gel electrophore-
sis [1]. Direct sequencing of all coding and exon-intron boundary sequences identified a deletion mutation at the 3'UTR, designated c.2584-2585del CA (red arrow in Figure 2A), which was not a SNP (Genome Browser and Blat, http://genome.ucsc.edu; dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/) and had not been reported yet (The Human Gene Mutation Database, http://www.hgmd.org/). Unrelated controls did not show this change (Figure 2B).

**Immunohistochemistry**

Using anti-NCSTN antibody, immunohistochemical staining was positive in the epidermis (Figure 3A-E), the root sheath of hair follicles (Figure 3B, 3C, 3F), and the sebaceous gland (Figure 3C and 3F). However, compared to positive controls (Figure 3G-I), expression of NCSTN protein in AI lesions (Figure 3A-F) decreased, while old scar lesions were more obvious (Figure 3A-C). In addition, NCSTN protein was highly expressed in unrelated healthy skin (Figure 3G-I). The NC is shown in Figure 3J-L, indicating that NCSTN protein is potently lower in epidermis and hair follicles of AI patients with lesions than in healthy skin.

**Bioinformatic analysis for the identification of miRNAs that regulate NCSTN 3’UTR**

RegRNA 2.05 was used to generate potential miRNAs with a high probability of binding to the mutNCSTN 3’UTR (Figure 4A). Hsa-miR-155 showed a complementary sequence of mutNCSTN 3’UTR. The binding sites were located in the former 2 bp and after 5 bp of c.2584-2585del CA.

**Dual luciferase reporter assay**

Two digested partners were ligated together to generate the wild-type construction (wt-NCSTN). Site-directed mutagenesis was used to generate the mutant construct (mt-NCSTN). The wt-NCSTN fragment and mt-NCSTN were fused to luciferase and transiently transfected into 293T cells with approximately 50-60% confluence 10. The binding ability of the predicted NCSTN-targeting miRNAs was depicted in Figure 4B. The binding ability of mut-NCSTN 3’UTR (0.817±0.208) co-transfected with hsa-miR-155 had a stronger combining force than the psiCHECK-2 alone (0.955±0.021), 3’UTR NCSTN (1.019±0.010), blank (1), and NC (1.010±0.050) (Figure 4B). Compared to the blank (P=0.001, *P<0.01), inhibitor (P=0.000, *P<0.01), NC (P=0.001, *P<0.01), and NC inhibitor (P=0.001, *P<0.01), hsa-miR-155 (Figure 5B) and 3’UTR mutNCSTN significantly reduced luciferase activity (Figure 5B). In the group of cells transfected with wt-NCSTN (Figure 5A, 3’UTR NCSTN) and luciferase vector (Figure 5C, psiCHECK-2), the binding ability of miRNAs was similar to NC and blank.

**Protein expression**

Compared to the cell, empty, and wt-NCSTN groups, the gene expression of mut-NCSTN at the mRNA level was decreased in Figure 6A. Under the same conditions as Figure 6A-C showed mRNA and protein levels of cell, mock, and wt-NCSTN groups (**P<0.05). Thus, the mRNA and protein level of mut-NCSTN was remarkably decreased compared with the cell and wt-NCSTN groups.
NCSTN mutation causes AI

Discussion

We examined NCSTN for mutations in one AI patient and identified a deletion mutation in NCSTN 3’UTR, designated c.2584-2585delCA, which was not observed in 100 ethnically matched controls. Using computational algorithms of bioinformatic analysis, we first identified that hsa-miR-155 had a high probability of binding to the mutNCSTN 3’UTR. Hsa-miR-155 plays a vital role in the progression of oral SCC [25] and other diseases. However, it is not yet known whether miR-155 is involved in the pathogenesis of AI. Thus, we cloned a reporter plasmid containing wide-type 3’UTR of NCSTN at the 3’ position of the firefly luciferase reporter gene. In parallel, we constructed reporter plasmids in which the conserved target sequence at positions 515-516 of NCSTN 3’UTR were mutated, and transfected HEK 293T cells with these constructs with miR-155 mimics, miR-155 inhibitor, NC, or NC inhibitor. Luciferase activity was significantly decreased in groups transfected with miR-155 mimics and mutant 3’UTR (3’UTR mutNCSTN) reporter plasmids, compared to cells transfected with miR-155 mimics and 3’UTR (3’UTR NCSTN) reporter plasmids.

Nicastrin, encoded by NCSTN and located in 1q22-q23, is an essential subunit of the γ-secretase complex, which is important to the intramembranous cleavage of Notch [26]. Mice with defective γ-secreted enzyme showed hair-follicle hyperkeratosis and the formation of epidermal cysts, similar to human abnormalities such as AI. While mice lack apocrine sweat...
glands, it has been demonstrated that hair follicle embolism may be an initial abnormality and a major pathogenic factor associated with AI [27]. In the skin, the expression of Notch is associated with the development or differentiation of epidermis and hair follicles. Notably, disruption of the Notch signaling pathway causes epidermal and follicular hyperkeratosis as well as the formation of epidermal cysts. Therefore, a decrease in Notch signaling occurs due to a loss of functional mutation in γ-secretase genes, which play an important role in the pathogenesis of AI via aberrant trichilemmal keratinization.

In recent years, it has been found that miRNAs play an important role in the etiology of several diseases. Hundreds of miRNAs have been shown to be critical regulators of vascular diseases, inflammation, arterial remodeling, angiogenesis, smooth muscle cell regeneration, hypertension, apoptosis, neointimal hyperplasia, and signal transduction. Elevated expression of miR-155 is associated with many disorders, such as B cell lymphoma, stomach cancer, lung cancer, and colorectal cancer [28]. Furthermore, miR-155 is also a central modulator of the T-cell response, which is associated with inflammation and autoimmunity. It regulates the production of cytokines in CD4+ T cells and CD8+ T cells [29]. Elevated levels of TNF-α may also be associated with miR-155 and involved in cytokine production (e.g., IL-6, IL-17, and IL-22). Additionally, negative germiculture is common in patients with AI, and anti-inflammatory drugs are not effective. Therefore, we hypothesize that the disease may be associated with abnormalities of the immune system. The high levels of TNF in serum, expression of TLR abnormalities in keratinocytes of AI, and increased TLR2 of macrophages and dendritic cells in AI lesions all suggest that the disease is associated with immunity.

miR-155 plays a critical role in promoting the differentiation of Th17 cells in atopic dermatitis. It promotes the accumulation of follicular T helper cells, causing chronic, low-grade inflammation [30]. The differential expression of miR-155 has been studied in several inflammatory diseases; the results indicate that it can be used as a potential biomarker or as a therapeutic element in the treatment of autoimmune diseases. These data suggest that, besides directly targeting NCSTN, miR-155 plays an important role in the pathogenesis of AI in the immune system.

Thus, we hypothesized that by binding mutNCSTN 3′UTR, miR-155 decreased the expression and function of the NCSTN protein, which may then have influenced Notch signaling. This is consistent with current opinion that decreased Notch signaling due to loss of function mutation in γ-secretase genes plays a key role in the pathogenesis of AI.

However, there are some limitations to this study. First, only one patient with the mutation has been detected. However, this is a novel discovery, and future studies will now be able to look for the same mutation elsewhere. Another limitation is that we used immunohistochemistry and the dual luciferase assay to verify how the novel mutation is able to result in AI. While these findings are still important, future studies must also be conducted to determine the expression of NCSTN protein and mRNA by Western blot and quantitative PCR in different cell lines transfected with miR-155, miR-155 inhibitor, NC, and NCI.

In conclusion, our findings detect that miR-155 inhibits the expression of NCSTN, provide new insight into the mechanism of loss of NCSTN function in AI patients. These results suggest that miR-155 functions as a promotion in AI and a potential target for AI therapy.

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Disclosure of conflict of interest

None.

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NCSTN mutation causes AI


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