Cornulin Is Induced in Psoriasis Lesions and Promotes Keratinocyte Proliferation via Phosphoinositide 3-Kinase/Akt Pathways

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Psoriasis is a chronic inflammatory skin disease characterized by abnormal proliferation of epidermal keratinocytes and infiltration of inflammatory cells. CRNN is a major component of the cornified cell envelope and implicated in several epithelial malignancies. Here, we show that CRNN expression was increased in the lesioned epidermis from the patients with psoriasis vulgaris and skin lesions from the imiquimod (IMQ)-treated mice. Expression of CRNN in cultured keratinocytes (HEKa and HaCaT) was also induced by M5, a mixture of five pro-inflammatory cytokines (i.e., IL-17A, IL-22, IL-1α, oncostatin M, and TNF-α). Lentiviral expression of CRNN increased cell proliferation by inducing cyclin D1. Conversely, knockdown of CRNN by small interfering RNA suppressed G1/S transition and attenuated the M5-induced proliferation. In addition, CRNN overexpression increased the phosphorylation and activation of phosphoinositide 3-kinase and Akt. Inactivation of the phosphoinositide 3-kinase and Akt pathways using small interfering RNA or selective inhibitors (LY294002 and MK2206) reduced the proliferative effects of CRNN. Furthermore, topical use of anti-psoriatic calcipotriol effectively decreased expression of CRNN, inhibited the Akt activation and improved the IMQ-stimulated psoriasis-like pathologies. Taken together, these results suggest that induced expression of CRNN may contribute to the pathogenesis of psoriasis.


INTRODUCTION

Psoriasis, a common inflammatory skin disease, is characterized as white silvery scales covered with erythematous plaques. It is a lifelong morbidity affecting about 2–3% of the population worldwide (Nestle et al., 2009). Psoriasis can severely attenuate the quality of life for those affected (Schön and Boehncke, 2005). While many treatment options exist for psoriasis, no cure is available. Abnormally rapid multiplication of keratinocytes is known to contribute to psoriasis pathogenesis (Zhang et al., 2015); however, the mechanism for keratinocyte hyperproliferation in psoriasis remains unclear.

CRNN, also called c1orf10 or SEP53, is a cornified squamous epithelial protein (Contzler et al., 2005). CRNN was initially discovered in esophageal cells (Xu et al., 2000). Subsequent studies have shown that CRNN is also expressed in other squamous cells and epithelial cells (Arnow et al., 2009; Ciregia et al., 2016; Contzler et al., 2005; Imai et al., 2005; Lieden et al., 2009; Salahshourifar et al., 2015; Schröder et al., 2016; Xiao et al., 2015). It is located primarily in the upper layers of differentiated squamous tissues and plays an important role in epidermal differentiation (Contzler et al., 2005). CRNN was considered as a marker of late keratinocyte differentiation.

A distinctive trait of psoriasis is the increased mitotic rate of the basal keratinocytes, which results in a hyperproliferative epidermis and incomplete cornification. However, the role of CRNN in psoriasis remained unknown. The purpose of this study, therefore, was to determine whether CRNN contributes to the pathogenesis of psoriasis and, if so, the signaling pathway responsible for it. We first investigated CRNN expression in psoriasis patient samples and imiquimod (IMQ)-induced psoriasis-like skin inflammation in mice, and then examined the biological function of CRNN by overexpression or small-interfering RNA (siRNA) approach in vitro. Our results showed that CRNN expression was positively correlated with psoriasis and CRNN may be implicated in the pathogenesis of psoriasis by promoting keratinocyte proliferation via activation of PI3K/Akt pathways.
Fixed paraffin-embedded tissue sections from psoriasis patients (Psoriasis) and healthy individuals (Normal) were examined by histopathologic assay. As shown in Figures 1a and 1b, in normal skin tissues, samples from patients with psoriasis by immunohistochemistry. We first investigated CRNN protein expression in tissue inflammation in mice. Up-regulation of CRNN protein expression in patients with psoriasis and in IMQ-induced psoriasis-like skin inflammation in mice. Formalin-fixed paraffin-embedded tissue sections from psoriasis patients (Psoriasis) and healthy individuals (Normal) were examined by histopathologic assay. 

RESULTS
Up-regulation of CRNN protein expression in patients with psoriasis and in IMQ-induced psoriasis-like skin inflammation in mice
We first investigated CRNN protein expression in tissue samples from patients with psoriasis by immunohistochemistry. As shown in Figures 1a and 1b, in normal skin tissues, CRNN was weakly expressed in the basal layer of the skin in 49.12% (28 of 57) of samples. However, in psoriatic lesions, CRNN was strongly expressed in almost all layers of the epidermis, with a positive rate of 73.61% (53 of 72). In addition, we also examined the protein level of CRNN in psoriatic skin by using Western blotting. As shown in Figures 1c, the protein level of CRNN was higher in the skin of psoriatic patients than that in normal controls. As shown in Supplementary Table S1 (online), we summarized the relative information, including the lesion types and durations of the disease, Psoriasis Area Severity Index, age, and sex distribution. The correlations between the CRNN expression and key clinical features of the disease were analyzed. Weak but statistically significant correlations were observed between the overexpression and Psoriasis Area Severity Index score ($r = 0.239$, $P = 0.009$) and inversely with the guttate type of lesions ($r = -0.324$, $P = 0.021$). To assess whether CRNN was increased in the inflammatory skin lesions induced by topical IMQ application, we applied IMQ cream on the shaved back skin of BALB/c mice for 7 consecutive days. As shown in Figure 1d, mice treated daily with control cream did not show any sign of inflammation. However, 2 or 3 days after the start of IMQ application, the back skin of the mice started to display signs of erythema, scaling, and thickening. As shown in Figure 1e, analysis of immunohistochemistry-stained sections from the IMQ-treated skin showed increased CRNN protein level. We also assessed CRNN mRNA and protein levels in tissue samples from mice with or without IMQ treatment by using real-time quantitative PCR and Western blotting, respectively. As observed in Figure 1f–1g, IMQ induced a significant increase in CRNN at both mRNA and protein levels. 

Upregulation of CRNN protein expression in cell models of psoriasis
HaCaT cells are primary human epidermal keratinocytes isolated from adult skin. HaCaT cells are spontaneously transformed aneuploid immortal keratinocytes. The combination of IL-17A, IL-22, IL-1α, oncostatin M, and TNF-α (M5) has been shown to induce psoriasis-like changes in cultured keratinocytes (Rabeony et al., 2014). To determine the effects
of M5 on CRNN expression in keratinocytes, HEKa or HaCaT cells were treated with various concentrations of M5 (0, 2.5, 5 ng/ml) for up to 72 hours. As shown in Figure 2, M5 significantly increased CRNN expression in HEKa and HaCaT cells at both mRNA (Figure 2a–2d) and protein levels (Figure 2e–2h).

CRNN positively regulated keratinocyte proliferation
Psoriasis is characterized by dysregulated proliferation and differentiation of keratinocytes (Nestle et al., 2009). To determine the effect of CRNN on keratinocyte proliferation, we knocked down CRNN expression by using siRNAs in both HEKa and HaCaT cells and examined their impacts on cell proliferation. As shown in Figure 3a, knockdown of CRNN expression decreased cell proliferation in both HEKa and HaCaT cells. Moreover, cell cycle analysis indicated that CRNN knock-down led to growth arrest in the G1/S transition (Figure 3b) and, concomitantly, decreased expression levels of cell cycle regulator cyclin D1 (Figure 3c). Conversely, lentiviral overexpression of CRNN promoted cell proliferation (Figure 3d), stimulated G1/S transition (Figure 3e), and induced expression of cyclin D1 (Figure 3f) in keratinocytes.

M5 has been shown to induce keratinocyte proliferation in vitro (Rabeony et al., 2014). To study the potential role of CRNN in M5-induced keratinocyte proliferation, CRNN expression in HEKa and HaCaT cells was knocked down by using siRNA. As shown in Supplementary Figure S1a (online), M5-induced cell proliferation in HEKa and HaCaT cells was inhibited by knockdown of CRNN. CRNN silencing also abrogated the effect of M5 on cells G1/S transition (Supplementary Figure S1b). Furthermore, inhibition of CRNN abrogated the M5-induced expression of CRNN and cyclin D1 in both HEKa and HaCaT cells (Supplementary Figure S1c–S1d).

CRNN activated Akt and extracellular signal–regulated kinase in keratinocytes
To elucidate the molecular mechanism of the effect of CRNN on keratinocytes proliferation, we analyzed the effect of CRNN on the Akt and ERK 1/2 signaling pathways, two serine/threonine-specific protein kinases that positively regulate cell proliferation (Adams et al., 2005). As shown in Figure 4a, overexpression of CRNN by lentivirus significantly increased the phosphorylation of Akt and ERK in both HEKa and HaCaT cells. Conversely, knockdown of CRNN decreased the phosphorylation of Akt and ERK (Figure 4b). In Supplementary Figure S2 (online), we showed that M5 treatment increased the levels of p-Akt and p-ERK in control
Figure 3. CRNN positively regulated keratinocyte proliferation. (a) HEKa (left) and HaCaT (right) cells were seeded in 96-cell plates and transfected with CRNN (#1 and #2) or Control (Ctrl) siRNA for 24, 48, and 72 hours before measuring keratinocyte proliferation by MTT assay (n = 3). (b) HEKa (left) and HaCaT (right) cells were seeded in six-cell plates and transfected with CRNN (#1 and #2) or Control (Ctrl) siRNA for 48 hours before measuring cell cycle profiles by flow cytometry (n = 3). (c) HEKa (left) and HaCaT (right) cells were seeded in six-cell plates and transfected with CRNN overexpressing lentivirus (CRNN) or Control (VEC) for 48 hours before measuring keratinocyte proliferation by MTT assay (n = 3). HEKa (left) and HaCaT (right) cells were seeded in 96-cell plates and transfected with CRNN overexpressing lentivirus (CRNN) or Control (VEC) for 48 hours before measuring cell cycle profiles by flow cytometry or CRNN and cyclin D1 protein levels by Western blot (n = 3). *P < 0.05, **P < 0.01 versus Control group (VEC or Ctrl siRNA), which indicates a statistically significant difference. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NS, not significant; siRNA, small interfering RNA.
Figure 3. Continued
**Figure 4. CRNN activated Akt and ERK in keratinocytes.** (a) HEKa (left) and HaCaT (right) cells were seeded in six-cell plates and transected with CRNN overexpressing lentivirus (CRNN) or Control (VEC) for 48 hours. The protein levels of Akt, phospho-Akt, ERK1/2, phospho-ERK1/2, and CRNN were analyzed by Western blot analysis (n = 3). (b) HEKa (left) and HaCaT (right) cells were seeded in six-cell plates and transfected with CRNN (#1 and #2) or Control (Ctrl) siRNA for 48 hours before measuring Akt, phospho-Akt, ERK1/2, phospho-ERK1/2, and CRNN protein levels by Western blot (n = 3). *P < 0.05, **P < 0.01 versus Control group (VEC or Ctrl siRNA), which indicates a statistically significant difference. ERK, extracellular signal–regulated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NS, not significant; siRNA, small interfering RNA.

**Inhibition of Akt, but not ERK, abrogated the effects of M5 on keratinocyte proliferation**

To further confirm the functional roles of Akt and ERK in the keratinocyte proliferations, we pretreated the HEKa and HaCaT cells with highly selective Akt inhibitor MK2206 and ERK inhibitor PD89059 before exposure to M5. As shown in Figure 5a and Supplementary Figure S3a (online), MK2206 or Akt siRNA abolished the M5-induced cell proliferation as assessed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and FACS assays. However, PD89059 had few effects (Supplementary Figure S3b). Further, either MK2206 or Akt siRNA effectively attenuated the M5-induced cyclin D1 upregulation (Figure 5b and Supplementary Figure S3c).

Phosphatidylinositol 3,4,5-trisphosphate phosphatase to negate the PI3K/Akt pathway (Georgescu, 2010). To study whether CRNN activated Akt via the downregulation of PTEN, we overexpressed CRNN in HEKa and HaCaT cells for 48 hours, but found no effect on PTEN expression level (data not shown).

**M5 stimulated the NF-κB signaling pathway in keratinocytes through CRNN**

NF-κB is a key transcription factor for the expression of genes involved in immunity, inflammation, proliferation, and survival. NF-κB is critically implicated in the pathogenesis of psoriasis (Goldminz et al., 2013). By using immunofluorescence, we found that knockdown of CRNN significantly decreased the protein level of NF-κB/p65 (Supplementary Figure S4a online). In both HEKa and HaCaT cells, CRNN knockdown decreased the M5-induced translocation of p65 (Supplementary Figure S4b), indicating that CRNN also played a role in the M5 activation of the NF-κB signaling pathway.

**Calcipotriol downregulated CRNN and inhibited Akt phosphorylation in IMQ-induced skin lesions**

Calcipotriol, a synthetic derivative of vitamin D, has been shown to reduce the redness, thickness, and scaling in psoriasis (Kragballe and Iversen, 1993). To determine the effect of calcipotriol on CRNN expression in psoriasis, IMQ-treated mice were topicaly applied with calcipotriol for 7 days. As shown in Supplementary Figure S5a (online), calcipotriol treatment significantly decreased erythema in IMQ-treated mice. The efficacy was associated with reduced levels of CRNN, cyclin D1, and phosphorylated Akt (Supplementary Figure S5b). Pathologically, calcipotriol clearly ameliorated
the histological changes in IMQ-treated mice (Supplementary Figure S5c).

**DISCUSSION**

Human CRNN gene is encoded on chromosome 1q21 where the epidermal differentiation complex localized (Sławczyk-Macieja et al., 2015). CRNN is a 53-KDa protein of 495 amino acids with a calcium-binding motif of about 90 residues at its N-terminus and a conserved consecutive repeat sequence of 60 amino acids. Its structural characteristics are similar to those of “fused gene” family members of the epidermal differentiation complex. In recent years, increasing evidence has implicated the role of CRNN in a wide range of human squamous-cell epithelium. To the best of our knowledge, however, its contribution to the development of psoriasis has not been reported. In this study, we found that CRNN expression was upregulated in skin samples from patients with psoriasis as well as mice with IMQ-induced psoriasis-like skin inflammation. CRNN expression was also increased after stimulation with M5 in two human keratinocytes cell lines. We provided evidence that PI3K/Akt is an important signaling pathway mediating the effects of CRNN. Furthermore, the expression of CRNN was decreased after topical application of calcipotriol in IMQ-treated mice. Taken together, these in vitro and in vivo results pointed toward a potential role of this molecule in the pathogenesis of psoriasis.

In psoriasis, the mitotic rate of the basal keratinocytes is increased, which leads to an abnormally rapid multiplication of the cells of the epidermal layer of the skin. We found that overexpression of CRNN induced cyclin D1 and promoted keratinocytes proliferation. Conversely, knockdown of CRNN resulted in growth arrest in the G1/S stage transition and inhibited cell proliferation. The combination treatment of IL-17A, IL-22, IL-1a, oncostatin M, and TNFα (M5) has been shown to induce psoriasis-like changes, including increased cell proliferation in cultured keratinocytes (Rabeony et al., 2014). In this study, we also found that CRNN knockdown abrogated M5-induced increases in cell proliferation in cultured keratinocytes, suggesting an important role of CRNN in regulating keratinocyte proliferation.

In human oral squamous cell carcinoma, Imai et al. (2005) showed that upregulation of CRNN expression resulted in an arrest in the G1 phase of the cell cycle and a downregulation...
of cyclin D1 expression. In esophageal squamous cell carcinoma, CRNN played a role in cell cycle arrest at G1/S checkpoint by upregulating the expressions of P21WAF1/CIP1 and Rb (Chen et al., 2013). In the current study, we demonstrated that CRNN promoted keratinocyte proliferation by regulating the G1/S transition. CRNN depletion attenuated the expression of multiple G1/S regulator cyclin D1. Because psoriasis is also a hyperproliferative disease, the proliferative effect of CRNN described here is seemingly not in agreement with a tumor suppressor’s function. Several proteomic studies have suggested that loss of CRNN may be mainly associated with malignant transformation (Schaaaij-Visser et al., 2009; Xiao et al., 2015). In this context, distinct pathologic differences between psoriasis as a benign process and squamous cell carcinomas as neoplastic transformations may be one of the explanations.

How CRNN modulates the cell cycle proteins is largely unknown. Here, we revealed that PI3K/Akt pathway is a key signaling mechanism. Overexpression of CRNN activated Akt, induced cyclin D1 and promoted keratinocytes proliferation, while the inactivation of PI3K and Akt with specific siRNA or highly selective inhibitors attenuated the CRNN- and pro-inflammatory cytokines (M5)-elicited proliferation in keratinocytes. Certainly, questions as to how CRNN couples complex stimuli to PI3K/Akt yet remain unsolved. In light of a central function of PI3K in integrating diverse membrane and extracellular signals, it is plausible that dysregulated CRNN may affect the relay of signals from the receptors for growth factors, innate immunity and metabolites to PI3K/Akt and other pathways. Considering that CRNN is a member of epidermal differentiation complex gene family including well-characterized epidermal differentiation genes, such as involucrin, loricrin, and psoriasin, potential effects of CRNN on epidermal differentiation also warrant further investigation (Henry et al., 2012; Kypriotou et al., 2012; Marenholz et al., 1996). NF-kB is a transcription factor regulating the expression of a large number of pro-inflammatory genes. Many of the triggering factors for psoriasis initiate inflammation by activation of NF-kB (Moorchung et al., 2014). Indeed, silencing CRNN gene reduced NF-kB in keratinocytes. Taken together, given the established roles of the PI3K/Akt and NF-kB signaling pathways in the key pathogenesis of psoriasis, including immune-inflammation and epidermal proliferation, triggering these pathways may account for a potentially pathogenic mechanism of the dysregulated CRNN expression.

As a safe and efficacious treatment for psoriasis, topical calcipotriol inhibits epidermal proliferation and promotes epidermal differentiation. We found that calcipotriol improved psoriasis-like lesions and decreased the epidermal CRNN level in IMQ-treated mice.

In summary, CRNN expression was upregulated in patients with psoriasis as well as in vivo and in vitro models of psoriasis. CRNN promoted keratinocyte proliferation, which was associated with activation of Akt via PI3K. Furthermore, CRNN promoted cell inflammation in keratinocyte via NF-kB pathway. Taken together, our results suggested that CRNN may be implicated in the pathogenesis of psoriasis.

MATERIALS AND METHODS

Reagents
DMSO, MIT, propidium iodide, DAPI, RNaseA, and LY294002 were purchased from Sigma-Aldrich (St. Louis, MO). Puromycin and NF-kB p65 antibody (ab32536) were purchased from Abcam (Cambridge, UK). CRNN antibody (11799-1-AP) was purchased from Proteintech (Chicago, IL). The antibodies against Akt (pan) (#4691), phospho-Akt (#2965), ERK 1/2 (#9102), phospho-ERK 1/2 (#4376), and anti-rabbit DyLight 488-conjugated secondary antibodies (#4412) were purchased from Cell Signaling Technology (Danvers, MA). Mouse monoclonal antibodies to cyclin D1 (sc-246), β-actin (sc-47774), and horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG polyclonal antibodies were procured from Santa Cruz Biotechnology (Santa Cruz, CA). DMEM and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). MK2206 and PD98059 were purchased from MedChemExpress (Monmouth Junction, NJ).

Patient samples
Patient samples were obtained from the tissue bank of the Department of Dermatology, The Second Affiliated Hospital of Xi’an Jiaotong University. There were a total of 129 samples collected, including 72 psoriasis vulgaris (from 43 males and 29 females, aged 7–88 years old), and 57 normal skin tissues (from 25 males and 32 females, aged 22–59 years old) from cosmetic surgery. The average duration of psoriasis was 3 years (range 10 days to 40 years). There were 46 cases of plaque psoriasis and 26 cases of guttate psoriasis. The average Psoriasis Area Severity Index score of psoriasis patients was 8.85. The staining results of all samples were identified by two experienced pathologists. Written informed consent for tissue procurement was obtained from all patients before study initiation, and ethics approval was obtained from the Institutional Ethics Committee of Xi’an Jiaotong University.

IMQ-induced mouse psoriasis model
Male BALB/c (6–8 weeks; 20–25 g), free of specific pathogens, were used in the experiments. The animals were obtained from the Animal Center of Xi’an Jiaotong University. The animals were kept under standard laboratory conditions of 12-hour light to dark cycle and 24–26°C ambient temperature. All experimental animals used in this study were under a protocol approved by the Institutional Ethics Committee of Xi’an Jiaotong University. Mice received a daily topical dose of 62.5 mg of commercially available IMQ cream (5%; Sichuan Mingxin Pharmaceutical Co., Ltd, Chendu, China) on the shaved back for 7 consecutive days, as described previously (van der Fits et al., 2009). Control mice were topically applied a control vehicle cream (Nanchang Baiyun Pharmaceutical Co., Ltd, Nanchang, China). In therapeutic mode, mice were treated with calcipotriol (C, 40 mg/cm², twice daily; Leo Pharmaceutical Co. Ltd, Ballerup, Denmark) 2 days after starting IMQ application. The mice were sacrificed and the skin lesions were excised for real-time quantitative PCR, Western blot, and immunohistochemistry analysis.

Cell culture
The HEKa and the HaCaT cells (human keratinocyte cell line) were cultured in DMEM containing 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere with 5% CO₂.

Cell proliferation assay
Cell proliferation was assessed by using MTT assay. The HEKa and the HaCaT cells were plated at 5 × 10⁴ of cell density in 96-well
plates and transfected with control siRNA (si-Ctrl) or CRNN siRNA (si-CRNN-1 and si-CRNN-2) followed by M5 or not. Twenty microliters of MTT solution (5 mg/ml) was added to each well. After 4 hours of incubation at 37°C, the supernatants were replaced by 150 μl DMSO, and the absorbance of each well at 490 nm was determined. Mean readings from three independent experiments were plotted for each time point. Five technical replicates were performed for each data point.

**Cell cycle analysis**

Cell cycle analysis was performed by counting the cell with DNA stained with propidium iodide (Tang et al., 2008). Cells were harvested, fixed with 70% ethanol, and stored overnight at 4°C. After fixation, propidium iodide staining solution (50 μg/ml propidium iodide and 100 mg/ml ribonuclease A) was added to the cells, which were then incubated for 30 minutes in the dark at 37°C. DNA content was analyzed by using flow cytometry (FACS Calibur from BD Biosciences, San Jose, CA). Modifit, version 3.3 software (Verity Software House, Topsham, ME) was used to analyze the results. At least three independent experiments were performed.

**RNA extraction and real-time quantitative PCR**

Total RNA was extracted using Trizol reagent (Invitrogen). The cDNA was reverse transcribed from total RNA. The PCR primers were synthesized by Sangon Biotech (Shanghai, China). Primer sequences are listed in Supplementary Table S2 (online). Reactions were performed using SYBR premix EX Taq I (Takara, Japan) and analyzed by the 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA). Relative mRNA levels were calculated using the comparative Ct (ΔΔCt) method. Experiments were repeated three times and three technical replicates were included for each data point.

**Protein extraction and Western blot analysis**

Total protein was extracted from cells with lysis buffer (50 mM Tris-HCl, pH 7.5, 15 mM EGTA, 100 mM NaCl, 0.1% Triton X-100) supplemented with the protease inhibitor cocktail (Roche Diagnostics, Risch-Rotkreuz, Switzerland). The BCA protein assay reagents (Pierce, Rockford, IL) were used to assess the protein concentration. Protein samples were separated on SDS-PAGE and transferred onto polyvinylidene fluoride membrane. The blots were incubated with primary antibodies and horseradish peroxidase–conjugated secondary antibodies, and then visualized by using an enhanced chemiluminescence system. The intensity of the bands was quantified by using Image Pro Plus software (Media Cybernetics, Rockville, MD).

**Immunohistochemistry**

Immunohistochemical staining was executed using a standard immunoperoxidase staining procedure (Pinheiro et al., 2008). The staining results were appraised under microscope by two independent pathologists and quantitated according to the following scoring system. A positive rate score was first designated based on the percentage of positive cells (≤5%, scored 0; 6%–25%, scored 1; 26%–50% scored 2; 51%–75% scored 3; >75%, scored 4). A second score of staining intensity was then designated. The overall score for each microscopic field was computed by the product of the two scores. The average score of five fields was received as the final score of CRNN expression for each slide.

**Transient transfection of siRNA**

The control (Ctrl), Akt, PI3K p110, and CRNN siRNA oligonucleotides were synthesized by Shanghai Gene-Pharma (Shanghai, China) and their sequences are shown in Supplementary Table S3 (online).

Cells were transfected with siRNAs according to the recommended procedures of Lipofectamine 2000 Transfection Reagent (Invitrogen).

**Lentivirus transduction**

Lentiviruses of control vector and CRNN were from GenePharma (Shanghai, China). Transduction was carried out by adding 10 μl of virus suspension (titer 1 × 10⁸ TU/ml) to cells cultured in 1 ml complete culture medium containing 5 μg/ml polybrene. Forty-eight hours after transfection, cells were selected with puromycin (5 μg/ml) containing medium for 2 weeks.

**Statistical analysis**

Data are presented as mean ± standard deviation from at least three independent experiments. Data analysis was performed using GraphPad Prism (version 6; GraphPad Software, La Jolla, CA). Student t test (comparisons between two groups) or one-way ANOVA followed by Newman–Keuls post-hoc test (more than two groups) were used to analyze the statistical significance. P < 0.05 was considered statistically significant.

**Data availability**

Genbank accession numbers for qRT-PCR primers used are provided in Supplementary Table S2.

**CONFLICT OF INTEREST**

The authors state no conflict of interest.

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**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.06.184.

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