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Association between the ratio of aryl hydrocarbon receptor (AhR) in Th17 cells to AhR in Treg cells and SLE skin lesions

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ABSTRACT

Skin lesions are typical clinical manifestations of systemic lupus erythematosus (SLE) and the biomarker for predicting SLE skin injury is not clear. We conducted a hospital-based case-control study with aim to explore the predictive value of the ratio of aryl hydrocarbon receptor (AhR) in T helper 17 (Th17) cells to AhR in regulatory T (Treg) cells (AhR ratio) in SLE skin lesions. The clinical and laboratory data were obtained from their medical records, and the AhR relative expression levels were evaluated by reverse transcription-quantitative polymerase chain reaction. Flow cytometry was applied to determine the proportion of AhR-overexpressing cells in Th17 and Treg cells. Pearson's correlation and logistic regression analyses were used to evaluate the association between AhR ratio risk of skin lesions. Results showed that the expression level of *AhR* in peripheral blood mononuclear cells was increased > 3-fold in patients with SLE compared with that in healthy controls. Compared with control group, the percentage of AhR-overexpressing cells to Th17 cells was statistically higher in patients with SLE, whereas no significant difference was observed in the percentage of AhR-overexpressing cells to Treg cells between patients with SLE and control group. AhR ratio was also higher in SLE, and it was negatively correlated with complement 3 while positively correlated with erythrocyte sedimentation rate. In addition, compared with the low-AhR ratio group, more younger SLE patients with skin lesions, ultraviolet allergies and lower C3 levels were observed in the high-AhR ratio group, implicating that AhR ratio may be a potential biomarker for predicting SLE skin injury.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that can affect multiple organs, including the kidneys, skin, lungs and heart [1]. Skin lesions are typical clinical manifestations of SLE [2]. The pathogenesis of SLE skin injury is not entirely clear, and novel biomarkers should be explored to predict SLE with skin lesions. Previously, Ma et al. reported that the proportion of T helper 17 (Th17) cells to regulatory T (Treg) cells was increased in the skin of atopic dermatitis lesions [3]. Furthermore, Zhou et al. reported that numerous Th17-associated cytokines were expressed in the skin lesions of early systemic sclerosis [4]. Although abnormal levels of Th17 and Treg serve an important role in the pathogenesis of SLE [5,6], it is unclear the key molecule that regulates Th17 and Treg, and its role in predicting SLE, particularly in skin lesions.

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor [7]. Recently, AhR has gained increased attention in immune diseases. Talbot et al. reported that smoking could induce AhR

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activation in Th17 cells [8]. Accumulating data have demonstrated that AhR is essential for generating Th17 and Treg cells, and developing their immune function, particularly in autoimmune diseases [9–11]. However, to the best of our knowledge, less study has been conducted so far about the role of AhR in SLE.

In the present study, the expression level of *AhR* was detected in peripheral blood mononuclear cells (PBMCs) in patients with SLE. Then, the ratio of AhR in Th17 cells to that in Treg (AhR ratio) was calculated, and the correlation between AhR ratio and SLE activity was assessed. The results demonstrated that AhR had significantly higher expression in PBMCs and Th17 cells of patients with SLE, and AhR ratio was positively correlated with SLE activity. Furthermore, AhR ratio was significantly higher in SLE patients with skin lesions and ultraviolet allergy, and it was verified as an independent predictor for skin lesions in patients with SLE. Taken together, these results demonstrated that AhR ratio may be a potential predictive indicator for SLE, particularly for skin lesions, and AhR may be a key molecule for UV-induced skin injury particularly in SLE patients.

2. Materials and methods

2.1. Patients and controls

A total of 20 patients with SLE (3 males and 17 females; mean age, 38.90 ± 16.14 years) were enrolled in the present study. Patients using corticosteroids (≤1.0 mg per kg body weight per day of prednisone or equivalent) for ≥4 weeks of stable background treatment were accepted [12]. Between March and July 2017, these patients were diagnosed with SLE at the First Hospital of Lanzhou University according to the 1997 rised diagnostic criteria of the American College of Rheumatology [13]. The healthy control group was composed of 8 age- and gender-matched individuals (1 male and 7 females; mean age, 37.13 ± 16.91 years) without any risk factors or chronic diseases. The disease activity of patients with SLE was assessed on the base of SLE disease activity index 2000 (SLEDAI-2K) [14], and SLEDAI-2K > 4 was defined as SLE active stage [13]. Patients receiving a high dose of corticosteroids (≥1.5 mg/kg/day) in the previous month; those presenting with pregnancy, malignant diseases, end stage of renal diseases, heart failure, hematology disease, antiphospholipid syndrome, other autoimmune diseases such as rheumatoid arthritis and Sjogren Syndrome, liver disease (including hepatitis and liver cirrhosis); and those who had received blood transfusion during the previous 4 months, were excluded from the study. Considering tobacco smoking is an important risk factor for AhR, we also excluded tobacco smokers in both cases and controls. The study protocol was approved by the Research Ethics Committee of the First Hospital of Lanzhou University (approval no. LDYYLL201731), and informed consent was obtained from all the participants.

2.2. Data extraction

Demographic data clinical characteristics and laboratory test results of all enrolled subjects were extracted from their medical records. The following information was collected from each patient: age, gender, disease duration, C-reaction protein (CRP) levels, erythrocyte sedimentation rate (ESR), complement (C) 3 levels, C4 levels, 24-hour (24 h) urine protein, and serum immunoglobulin M (IgM), IgG and IgA levels. A total of 5 ml venous blood was sampled from each patient and healthy subject into heparin sodium-coated sterile tubes for various experiments.

2.3. Flow cytometry

For Treg staining ($CD4^+$ $CD25^+$ $CD127^{low}$), 100 μl blood samples ($1.6 * 10^3$–$4 * 10^4$ Treg per subject) were stained with fluorescein isothiocyanate (FITC)-cluster of differentiation (CD) 4 (25 μg/ml; catalog no. 11–0048; eBioscience), allophycocyanin (APC)-CD25 (7.5 μg/ml; catalog no. 17–0259; eBioscience) and phycoerythrin (PE)-CD127 (3 μg/ml; catalog no. 12–1278; eBioscience). The mixed samples were incubated in the dark for 30 min at 4 °C. Then, 300 μl red blood cell lysis buffer was used to lyse red blood cells. Upon adding 3 ml PBS and centrifuging at 300*g* for 5 min, the supernatant was removed. Upon fixation and permeabilization, peridinin chlorophyll protein complex (PerCP)-AhR (3 μg/ml; catalog no. 46-9854; eBioscience) was added and incubated in the dark for 30 min at 4 °C. The supernatant of the mixture was removed upon centrifugation at 300*g* for 5 min, and subjected to flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) following dilution in 500 μl PBS.

For Th17 staining ($CD3^+$ $CD8^-$ $IL\text{-}17A^+$), 250 μl blood samples ($0.5 * 10^3$–$4 * 10^4$ Th17 per subject) were mixed with 250 μl RPMI 1640 (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) in a tube, and 2 μl phorbol myristate acetate/ionomycin (catalog no. 70-CS1001; MultiSciences Biotech Co. Ltd.) alongside 2 μl brefeldin A/monensin mixture (catalog no. 70-CS1002; MultiSciences Biotech Co. Ltd.) was added to the tube. The samples were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 4–6 h. Then, 100 μl of the mixed samples was added into a new tube with 20 μl APC-CD3 (catalog no. 555335; BD Biosciences) and FITC-CD_8 (catalog no. 555366; BD Biosciences), and incubated in the dark at 4 °C for 30 min. Upon fixation and permeabilization, 20 μl PE-IL-17A (catalog no. 560436; BD Biosciences) and 5 μl PerCP-AhR (3 μg/ml; catalog no. 46-9854; eBioscience) were applied to the sample and incubated in the dark for 15 min at 4 °C. Finally, the cells were resuspended and evaluated by flow cytometry.

2.4. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

According to the manufacturer's protocol, PBMCs were harvested with Ficoll® (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) from 4 ml blood samples ($0.32 * 10^7$–$1.6 * 10^7$ PBMCs per subject), and total RNA was extracted with TRIzol reagent. Complementary DNA synthesis was performed using the FastKing RT Kit (Tiangen Biotech Co., Ltd., Beijing, China). RT-qPCR was performed with a LightCycler® 480 System (Roche Diagnostics, Basel, Switzerland). Analysis of *AhR* and *GAPDH* gene expression levels was performed using SYBR Green Master Mix. The gene-specific primers that were used in the experiment are listed in Table 1. The PCR reaction was initiated with an incubation step of 15 min at 95 °C to activate the AmpliTaq Gold DNA Polymerase. This was followed by 40 cycles of a denaturation step for 10 s at 95 °C and annealing for 30 s at 64 °C for *AhR* and *GAPDH*. Each primer was designed using melting curve analysis to confirm that primer-dimers were not formed in the reaction. The levels of *AhR* transcript were normalized to those of *GAPDH* and reported as a fold change. The comparative CT method was used in this study.

2.5. Statistical analysis

The quantitative demographic data are presented as mean ± standard deviation and interquartile range for continuous variables or as counts (percentages) for categorical variables. Comparison of the

Table 1
Primers used in reverse-transcription quantitative polymerase chain reaction.

Gene	Sequences
AhR	
Forward primer	5′-CAAATCCTTCCAAGCGGCATA-3′
Reverse primer	5′-CGCTGAGCCTAAGAACTGAAAG-3′
GAPDH	
Forward primer	5′-CATGAGAAGTATGACAACAGCCT-3′
Reverse primer	5′-AGTCCTCCACGATACCAAAGT-3′

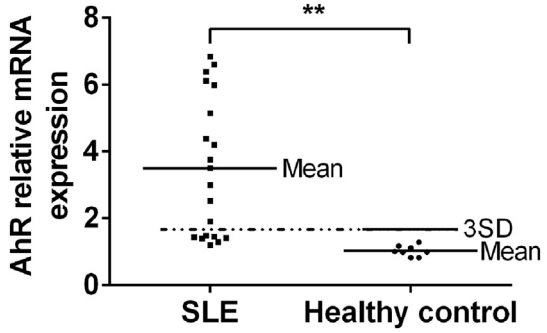

Fig. 1. The relative mRNA expression level of *AhR* was measured by real-time quantitative PCR in the PBMCs of healthy control and SLE patients. The 3SD indicated the value of mean plus 3 standard deviation of the ratio of AhR in PBMCs in healthy control. $^{**}P < 0.005$.

clinical characteristics was performed using the Student's *t*-test for two-group comparisons, the χ^2 test for categorical variables and the Mann-Whitney *U* test for continuous variables. Pearson's correlation analysis was used to examine the strength and direction of a linear relationship between AhR ratio and SLE activity-related patterns. The logistic regression analysis was also performed to estimate the odds ratio (OR) and confidence intervals (CIs) of skin lesions and AhR ration. All data were analyzed with SPSS 19.0 statistical software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Upregulation of AhR in PBMCs of patients with SLE

The expression levels of *AhR* were measured in the PBMCs of patients with SLE and healthy controls by RT-qPCR. Compared with healthy controls, significantly higher expression levels of *AhR* were observed in patients with SLE ($p < 0.01$), and it was > 3-fold higher in patients with SLE than healthy control (Fig. 1). The cut-off value (mean plus 3 standard deviations) of *AhR* from the healthy control group was 1.5, and 65% SLE patients have a higher *AhR* expression in PBMCs (Fig. 1).

3.2. Increased percentage of AhR-overexpressing cells in Th17 cells of patients with SLE

In order to identify the expression level of AhR in subsets of PBMCs, the percentages of AhR-positive cells in Th17 and Treg cells, which are important components of PBMCs, were investigated in patients with SLE and healthy controls by flow cytometry. Compared with healthy controls, the percentage of AhR-overexpressing Th17 cells to total Th17 cells in patients with SLE was remarkably higher ($p < 0.01$) (Fig. 2A–C). There was no significant difference in the percentage of AhR-overexpressing Treg cells to total Treg cells between patients with SLE and healthy controls (Fig. 2D–G). Furthermore, the comparison with the frequencies of Th17 and Treg with AhR-overexpression was analyzed. The results showed that the frequency of Th17 with AhR-overexpression was significantly higher than that of Treg (Supplementary Fig. 1). Besides, the correlation between AhR ratio and AhR expression for total lymphocytes was analyzed, and the results showed that AhR ratio was positive correlation with AhR expression in total lymphocytes (Supplementary Fig. 2).

3.3. The relationship between AhR ratio and SLE activity

We also calculated correlation coefficient to measure the strength and direction of a linear relationship between AhR ratio and SLE activity. Compared with healthy controls, AhR ratio, which was calculated by flow cytometry, was statistically significantly higher in patients with SLE ($p < 0.05$) (Fig. 3A). A strong positive correlation was observed between AhR ratio and ESR ($r = 0.578$; $p = 0.012$) (Fig. 3B). Furthermore, AhR ratio was negatively correlated with C3 levels ($r = -0.61$; $p = 0.003$) (Fig. 3C). Besides, the relationship between AhR ratio and SLEDAI-2K was analyzed, and the results showed that

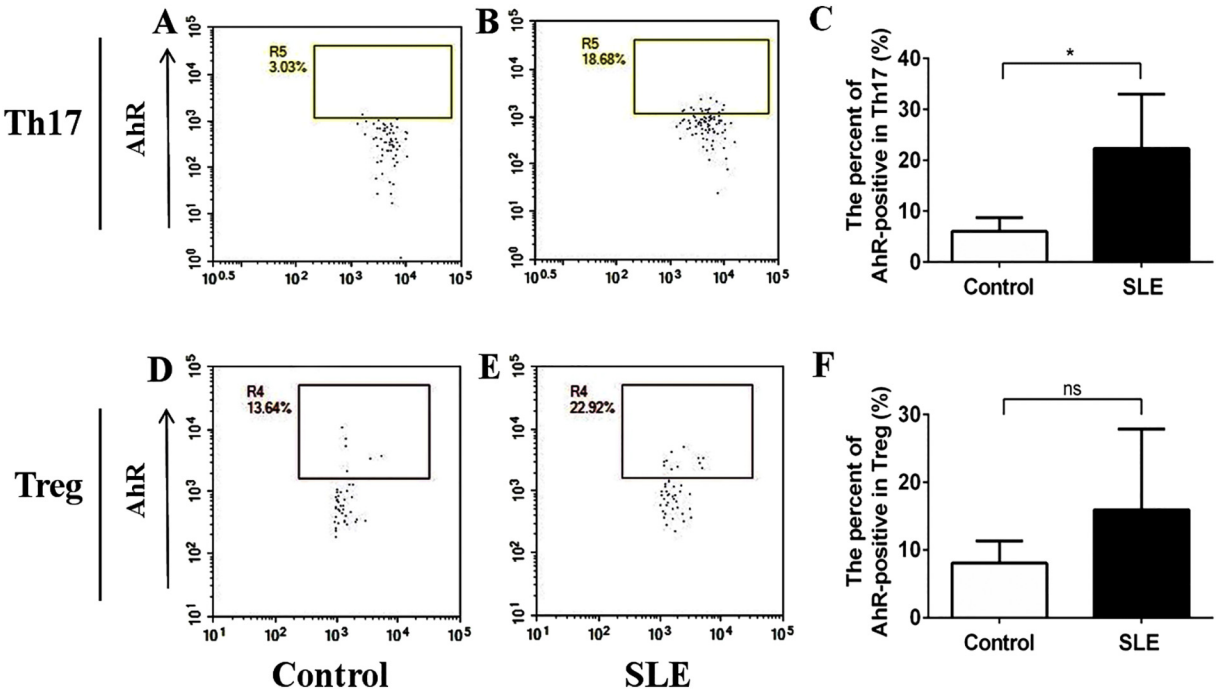

Fig. 2. The percentages of AhR-positive cells in Th17 cells of healthy controls (A) and patients with SLE (B) as detected by flow cytometry, and the two groups were compared (C). Percentages of AhR-positive cells in Treg cells of healthy controls (D) and patients with SLE (E) were detected by flow cytometry, and the two groups were compared (F). $^{*}P < 0.05$; NS, not significant.

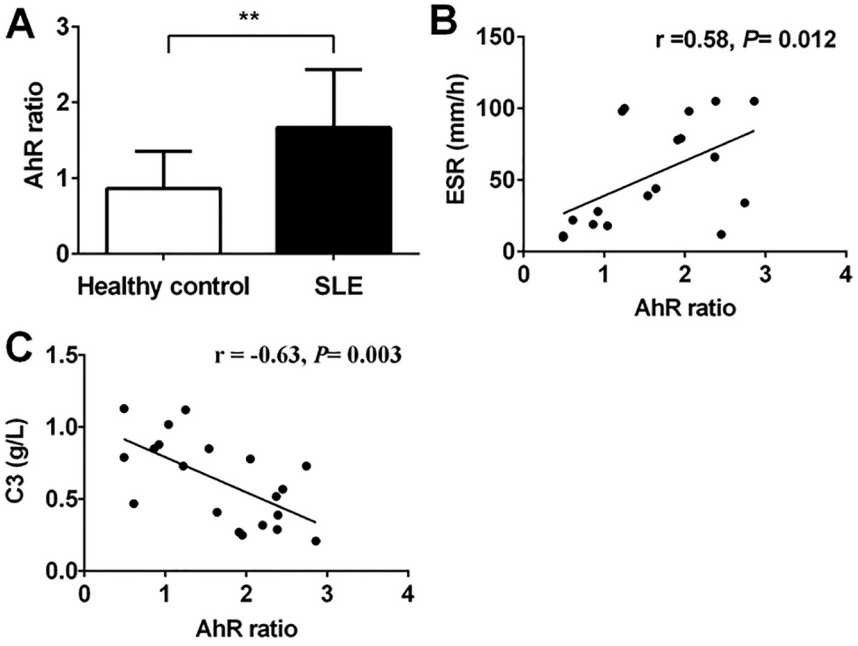

Fig. 3. AhR ratio in healthy controls and patients with SLE (A). Correlation between AhR ratio and ESR (B) and C4 levels (C). *P < 0.05, **P < 0.01. AhR ratio, the ratio of AhR in Th17 cells to that in Treg cells.

AhR ratio was not significantly correlated with SLEDAI-2K ($p = 0.091$) (Supplementary Fig. 3). Unfortunately, no significant correlation was observed between AhR ratio and SLEDAI-2K score, CRP or C4 levels (data not shown).

3.4. Laboratory data and clinical manifestation in low- and high-AhR ratio in patients with SLE

The normal reference range of AhR ratio was determined according to the 95% confidence interval (CI) in the control group (0.46–1.27). Then, patients with SLE were divided into two groups (a low-AhR ratio group and a high-AhR ratio group) according to the normal upper limits. AhR ratio above the normal upper limits was defined as the high-AhR ratio group, while AhR ratio below the normal upper limits was identified as the low-AhR ratio group.

In the present study, the normal upper limit of the AhR ratio was 1.27, and 8 out of 20 patients with SLE (40.00%) were included in the low-AhR ratio group, while 12 out of 20 patients with SLE (60.00%) were classified into the high-AhR ratio group. Then, demographic characteristics, clinical manifestation and laboratory indexes were analyzed between the two groups. Compared with the low-AhR ratio group, the patients with SLE were younger ($P < 0.05$); a higher number of patients suffered skin injury and ultraviolet allergy (12.50 vs. 83.33%; $P = 0.005$ and 12.50 vs. 75.00%; $P = 0.020$, respectively); and C3 levels were significantly lower ($P < 0.05$) in the high-AhR ratio group (Table 2). Interestingly, no significant differences were observed in terms of gender, disease duration, the SLE patients with arthritis, hematologic disorder, neurological symptoms, peritonitis or pleural effusion, renal damage, and the levels of CRP, ESR, C4, 24-h urine protein, serum IgM, IgG and IgA between the two groups (Table 2).

3.5. AhR ratio may be an independent risk factor for skin lesions in SLE

Table 3 showed the OR of SLE skin lesions with AhR ratio by multiple logistic regression analysis. After adjusted potential risk factors, such as age, CRP, ESR, C3 levels and C4 levels, the OR (95% CI) of SLE skin lesions with high AhR ratio was 6.33 (1.21–33.11), compared with low AhR ratio.

Table 2
Baseline characteristics of patients with SLE with low- and high-AhR ratio (the ratio of AhR in T helper 17 to AhR in regulatory T cells).

	Low-AhR ratio ($n = 8$)	High-AhR ratio ($n = 12$)	p-Value
Demographic characteristics			
Age (years)	49.25 ± 14.30	32.00 ± 13.79	0.015*
Gender (M/F)	2/6	1/11	0.537
Disease duration (month)	60.00 (7.50, 168.50)	47.00 (4.50, 7200)	0.301
Clinical manifestation			
Arthritis, n (%)	7 (87.50)	5 (41.67)	0.070
Hematologic disorder, n (%)	5 (62.50)	4 (33.33)	0.362
Neurological symptoms, n (%)	2 (25.00)	2 (16.67)	1.000
Peritonitis or pleural effusion, n (%)	3 (37.50)	2 (16.67)	0.347
Renal damage, n (%)	4 (50.00)	4 (33.33)	0.648
Skin injury, n (%)	1 (12.50)	10 (83.33)	0.005**
Ultraviolet allergy, n (%)	1 (12.50)	9 (75.00)	0.020*
SLEDAI-2K score	13.29 ± 8.92	16.25 ± 6.16	0.962
Laboratory indexes			
CRP (mg/L)	3.38 (3.38, 37.60)	3.38 (3.38, 9,36)	0.698
ESR (mm/h)	22.00(14.50, 63.00)	44.00(12.00, 79.00)	0.630
C3 (g/L)	0.88 ± 0.24	0.51 ± 0.22	0.012*
C4 (g/L)	0.24 ± 0.19	0.09 ± 0.07	0.162
24 hour urine protein (g/24 h)	0.23 (0.98, 0.50)	0.16 (0.10, 0.37)	0.748
Serum IgM (g/L)	1.27 ± 0.89	0.90 ± 0.39	0.271
Serum IgG (g/L)	12.88 ± 5.60	13.52 ± 4.09	0.743

Data are presented as mean ± standard deviation or median and interquartile range for continuous variables or as counts (percentages) for categorical variables. P-values were determined by independent Student's t-test for continuous variables, including age, systemic lupus erythematosus disease activity index 2000 score, C3 and C4 levels. The difference in disease duration, C-reactive protein levels and erythrocyte sedimentation rate between the two groups was determined by Mann-Whitney U test. χ^2 test or Fisher exact test was used to determine the difference in clinical manifestations. C, complement; Ig, immunoglobulin; AhR, aryl hydrocarbon receptor. *P < 0.05, **P < 0.01.

Table 3
Logistic regression association between risk of skin injury in SLE and AhR ratio.

Index	ORs (95% CIs)[2]	p-Value
AhR ratio (High vs. Low)	6.33 (1.21–33.11)	0.029*
Age	1.11 (1.00–1.24)	0.050
CRP	1.48 (0.78–2.77)	0.228
ESR	1.34 (0.99–1.79)	0.052
C3	0.00 (0.00–19.35)	0.088
C4	0.01 (0.00–8.67)	0.175

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; C3, complement 3; C4, complement 4; AhR ratio, the ratio of AhR in T helper 17 to AhR in regulatory T cells. *$P < 0.05$.

4. Discussion

Multiple organ damage, including the kidneys, skin, lungs and heart, is the main feature of SLE. Skin is the second most commonly affected organ, and 72–85% of patients develop skin lesions over the course of SLE [15,16]. However, the biomarker for predicting SLE skin injury is not clear. The present study revealed that the mRNA level of *AhR* in PBMCs was significantly higher in SLE patients than healthy controls. Th17 and Treg cells are the cell subsets of PBMCs and serve a key role in SLE development [6,17]. To the best of our knowledge, the present results firstly demonstrated that a statistically significant higher frequency of AhR-overexpressing cells in Th17 cells of patients with SLE compared with healthy controls, while no significant difference was observed in the percentage of AhR-overexpressing cells in Treg between patients with SLE and healthy subjects. These results indicated that AhR may serve as an important role in mediating Th17/Treg imbalance of SLE pathogenesis, and increased AhR levels in Th17 cells may play a key role in over-production Th17 cells and result in organ damage of SLE. Similarly, Castañeda et al. reported that AhR in dendritic cells can enhance Th17 polarization [18]. Besides, the AhR-overexpressing may be also founded in Treg- and Th17-negative T cells, the frequencies of them as well as correlation between AhR ratio and AhR expression for each T cell type will be analyzed in future.

Furthermore, the present study demonstrated that AhR ratio was also significantly higher in patients with SLE compared with healthy controls, which suggested that AhR may polarize Th17 cells in SLE. According to the 95% CI value of healthy controls [19], patients with SLE were divided into two groups (a low-AhR ratio group and a high-AhR ratio group) based on the upper normal limits. No significant differences were observed in SLE with arthritis, hematologic disorder, neurological symptoms, peritonitis or pleural effusion, renal damage between the two groups, but SLE patients who suffered skin injury and ultraviolet allergy revealed significantly high-AhR ratio. Ultraviolet light, as one of the AhR ligands [20], is the best-known inducer of SLE-related deterioration, particularly in SLE skin lesions. Our results may indirectly confirm that ultraviolet light can induce SLE skin lesions through AhR activity, which is consistent with previous studies reporting that ultraviolet light can deteriorate SLE via strongly activate AhR [21]. In addition, the youngest patients in the high-AhR ratio group may have higher exposure to outdoor physical activity and ultraviolet radiation.

The serum levels of C4 and in particular C3 are reduced in the active phase of SLE [22]. In our study, the serum levels of C3 were significantly lower in the high-AhR ratio group compared with the low-AhR ratio group. Reich et al. reported that the most commonly immune reactants involve C3, IgM, IgG and IgA along the dermal-epidermal junction in patients with SLE patients and skin lesions [23]. We speculated that the decreased serum C3 levels in the active phase of SLE may be due to two reasons. On one hand, the circulating immune complex in the blood of patients with SLE could consume complement. On the other, C3 may be deposited in the skin and/or basement membrane of patients with SLE. C3, ESR, SLEDAI-2K, CRP and C4 could reflect the activity of SLE, and they are not synchronized in SLE disease. Our study indicated that AhR ratio was strongly negatively correlated with C3 and positively correlated with ESR, but it was not correlated with SLEDAI-2K, CRP or C4. This suggested that AhR ratio was associated with SLE activity. In addition, AhR ratio was an independent index in patients with SLE and skin lesions.

In conclusion, AhR ratio may be a potential biomarker for predicting SLE, particularly in skin lesions of patients with SLE.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.01.039.

Conflicts of interest

The authors declare that there is no conflict of interest associated with this work.

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