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DNA demethylation of the perforin promoter in CD4$^+$ T cells from patients with subacute cutaneous lupus erythematosus

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ABSTRACT

Background: Recent evidence indicates that human lupus is an epigenetic disease characterized by impaired T cell DNA methylation. Perforin, a cytotoxic effector molecule, is overexpressed due to hypomethylation of its promoter regulatory elements in CD4$^+$ T cells from patients with systemic erythematosus lupus (SLE). However, it is unknown whether aberrant expression and methylation of perforin occur in CD4$^+$ T cells from patients with subacute cutaneous lupus erythematosus (SCLE).
Objective: We aimed to compare the perforin expression level and the methylation status of the perforin promoter region in CD4$^+$ T cells from SCLE patients and healthy controls.
Methods: We used real-time RT-PCR to compare the perforin mRNA levels, and Western-blot to compare perforin protein levels in CD4$^+$ and CD8$^+$ T cells from SCLE patients and healthy controls. Bisulfite sequencing was used to determine the methylation status of the perforin promoter region.
Results: Perforin is overexpressed in SCLE CD4$^+$ T cells. Demethylation of the perforin promoter region was seen in CD4$^+$ T cells from patients with SCLE.
Conclusions: DNA demethylation at the perforin locus contributes to perforin overexpression in SCLE CD4$^+$ T cells.

1. Introduction

The mechanisms initiating human lupus remain unknown. Aberrant epigenetics plays essential roles in the pathogenesis of systemic lupus erythematosus (SLE) [1,2]. Human and murine CD4$^+$ T cell become autoreactive in vitro when treating with the DNA methylation inhibitors including 5-azacytidine (5-azaC), procainamide and hydralazine [3–7]. These treated cells can induce a lupus-like autoimmune disease if transferred into host mice [8]. Our previous studies demonstrated that gene-specific hypomethylation, particularly of several methylation-sensitive autoreactivity-related genes including CD11a (*ITGAL*), perforin (*PRF1*), CD70 (*TNFSF7*) and CD40LG (*TNFSF5*) are all overexpressed in CD4$^+$ T cells from patients with SLE [5–7,9].

Subacute cutaneous lupus erythematosus (SCLE) is a disease midway between SLE and discoid lupus erythematosus (DLE), but as well, it can coexist with both SLE and DLE. Most patients with SCLE have a chronic or relapsing but benign condition, with few of the serious manifestations associated with SLE [10,11]. However, some patients with SCLE may develop into SLE, causing progressive systemic damage over a period of years [12]. The genetic and epigenetic factors that trigger the transition from SCLE to SLE remain unknown. We recently reported that global genomic DNA of SCLE CD4$^+$ T cells is hypomethylated, and SCLE CD4$^+$ T cells overexpressed CD11a [13] and CD70 (unpublished data). These indicated the importance of hypomethylation in the development of SCLE. Perforin, a pore-forming molecule encoded by the *PRF1* gene [14], is overexpressed in CD4$^+$ T cells from SLE patients, and perforin promoter sequence is hypomethylated in SLE CD4$^+$ T cells [6,15]. We hypothesized that the perforin promoter sequences is similarly overexpressed and hypomethylated in CD4$^+$ T cells from SCLE patients.

To test this hypothesis, we compared perforin expression in CD4$^+$ T cells from SCLE patients and healthy controls. We also compared the methylation status of the perforin promoter region in these groups. Our results provide further support a role for DNA hypomethylation in the development of SCLE.

2. Materials and methods

2.1. Subjects

Nine healthy subjects (aged 30.4 ± 5.8 years) were recruited from medical staff at the Second Xiangya Hospital. Twelve patients with SCLE (aged 32.8 ± 8.3 years) were recruited from the outpatient

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Table 1
Patient profiles.

Patient	Age/gender	ANA titer	Medications
1	32/F	1:40	Pred. 10 mg/day
2	22/F	1:40	Pred. 5 mg/day
3	28/F	1:80	Pred. 20 mg/day, HCQ 0.2 g/day
4	21/F	1:40	Pred. 20 mg/day
5	42/F	Negative	Thalidomide 200 mg/day
6	37/F	Negative	Pred. 10 mg/day, HCQ 0.2 g/day
7	30/F	1:20	HCQ 0.2 g/day, TG 30 mg/day
8	23/F	1:20	HCQ 0.2 g/day, TG 30 mg/day
9	41/F	Negative	HCQ 0.2 g/day, TG 30 mg/day
10	40/M	1:40	None
11	33/F	1:40	None
12	45/F	1:80	None

Pred., prednisone; HCQ, hydroxychloroquine; TG, tripterygium glycosides.

dermatology clinic and in-patient ward at the Second Xiangya Hospital of Central South University. Diagnosis of SCLE was based on the presence of characteristic clinical features, including photosensitivity, annular polycyclic erythema of the trunk, upper limbs and face as well as testing positive for the anti-Ro antibody [16–18]. All patients (3 ANA-negative, 9 ANA-positive) exhibited fewer than four of the American College of Rheumatology criteria for SLE, had no visceral involvement and tested negative for anti-double-stranded DNA, anti-U1RNP and anti-Sm antibodies. Relevant clinical information of the SCLE patients included in the study is listed in Table 1. Patients and controls were age and sex matched in all experiments. The protocols were reviewed and approved by the human ethics committee of the Central South University Xiangya Medical College, and written informed consent was obtained from all subjects.

2.2. $CD4^+$ and $CD8^+$ T cells isolation

Using MACS® magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), we isolated $CD4^+$ and $CD8^+$ T cells from patients with SCLE and healthy controls as previously described [13]. The purity of enriched $CD4^+$ and $CD8^+$ T cell isolates was evaluated by flow cytometry and was generally higher than 95%.

2.3. Perforin mRNA quantitation

Perforin transcripts were quantitated by real-time RT-PCR using a Rotor-Gene™ 3000 (Corbett Research, Mortlake, NSW, Australia) and previously published primers and protocols [13,15]. Perforin mRNA levels are presented relative to β-actin transcripts as described previously [13,15].

2.4. Immunoblotting analysis

To determine whether perforin expression is altered at the protein level, we generated Western-blots using mouse anti-human perforin monoclonal antibody (Abcam, British), along with HRP-conjugated secondary antibody and Pierce® ECL substrate (Pierce, Rockford, IL); and quantified band intensities with Quantity One® 1-D analysis software (Bio-Rad Laboratories, Hercules, CA).

2.5. Bisulfite genomic sequencing

Isolation of genomic DNA and bisulfite conversion was performed as described previously [13,15]. The 454 bp (−439 to +15) *PRF1* promoter fragment was amplified by nested PCR. The fragments were cloned into pGEM T vector (Promega, American), and five independent clones were sequenced for each of the amplified fragments. The following primers were used: round I: forward primer (−492 to −452): 5′-ATTGAATTCTAATTTT-TAGGGTTTATATGATTTATAATTTT-3′; reverse primer (58 to 17): 5-TCCTCTAGAAATAACATCAACCCCCCAAACAACCCAC TATAA-3; round II: forward primer (−439 to −398) 5′-AAGGAATTCAG GTATAGTGAGGTTGAAGAATTTTATTAGTTT-3′; reverse primer (15 to −25) 5′-TCCTCTAGACAACCACCACTCACATCACTTCTACTTCCTA-3′.

2.6. Statistical analysis

The Student's *t*-test for equality of means was used to compare values. *P*-values of less than 0.05 were considered significant. All analyses were performed with SPSS Version 12.0 software.

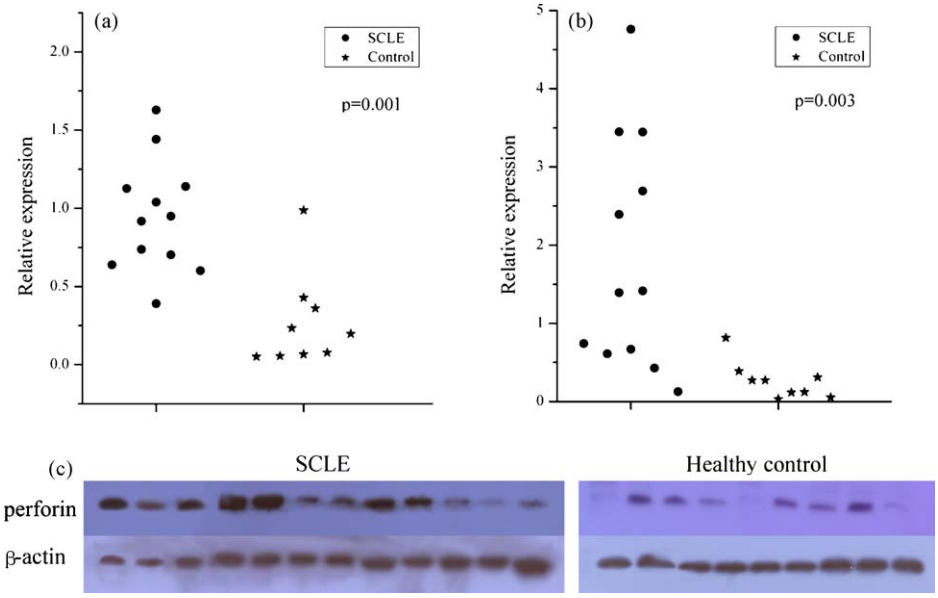

Fig. 1. Expression of perforin in $CD4^+$ T cells from patients with SCLE. (a) Relative expression level of perforin mRNA in $CD4^+$ T cells measured by quantitative real-time PCR. Results are presented as expression levels normalized to β-actin. ($p = 0.001$). (b) Relative expression level of perforin protein in $CD4^+$ T cells measured by Western-blot. Results are presented as expression levels normalized to β-actin ($p = 0.003$). (c) Western-blot analysis of perforin and β-actin expression in $CD4^+$ T cells from SCLE patients and healthy controls.