Increased HERV-E clone 4–1 expression contributes to DNA hypomethylation and IL-17 release from CD4$^+$ T cells via miR-302d/MBD2 in systemic lupus erythematosus

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Abstract

Background: Increased human endogenous retroviruses E clone 4–1 (HERV-E clone 4–1) mRNA expression is observed in systemic lupus erythematosus (SLE) patients and associates with the disease activity. In this study, we want to further investigate the mechanism of HERV-E clone 4–1 mRNA upregulation and its roles in SLE progression.

Methods: CD4$^+$ T cells were isolated from venous blood of SLE patients or healthy controls and qRT-PCR was used to detect HERV-E clone 4–1 mRNA expression. We then investigated the regulation of Nuclear factor of activated T cells 1 (NFAT1) and Estrogen receptor-α (ER-α) on HERV-E clone 4–1 transcription and the functions of HERV-E clone 4–1 3′ long terminal repeat (LTR) on DNA hypomethylation and IL-17 release.

Results: We found HERV-E clone 4–1 mRNA expression was upregulated in CD4$^+$ T cells from SLE patients and positively correlated with SLE disease activity. This is associated with the activation of Ca$^{2+}$/calcineurin (CaN)/NFAT1 and E2/ER-α signaling pathway and DNA hypomethylation of HERV-E clone 4–1 5′LTR. HERV-E clone 4–1 also takes part in disease pathogenesis of SLE through miR-302d/Methyl-CpG binding domain protein 2 (MBD2)/DNA hypomethylation and IL-17 signaling via its 3′LTR.

Conclusions: HERV-E clone 4–1 mRNA upregulation is due to the abnormal inflammation/immune/methylation status of SLE and it could act as a potential biomarker for diagnosis of SLE. HERV-E clone 4–1 also takes part in disease pathogenesis of SLE via its 3′LTR and the signaling pathways it involved in may be potential therapeutic targets of SLE.

Keywords: HERV-E clone 4–1, Systemic lupus erythematosus, Transcription factors, DNA hypomethylation, miR-302d, MBD2
Enhanced expression of mRNA from HERV-E clone 4–1 was reported in SLE than healthy controls (HCs) [5, 6], and our former study demonstrated that HERV-E clone 4–1 mRNA expression was increased in SLE patients, and the expression level of HERV-E clone 4–1 was associated with SLE disease activity index (SLEDAI) [7]. HERV-E clone 4–1 5’LTR/LTR2C was hypomethylated in CD4+ T cells from SLE patients [7–9] which might have close relationship with its expression.

In this study, we sought to further investigate the mechanism of HERV-E clone 4–1 mRNA upregulation and its roles in SLE progression, and to estimate the potential value of HERV-E clone 4–1 in acting as a biomarker and therapeutic target for SLE.

Methods

Ethics and selection of patients

This research was approved by the Institutional Research Ethics Committee of Shanghai General Hospital and abided by the ethical guidelines of the Declaration of Helsinki. All the patients involved in this study were adult and written informed consents were obtained from all the patients. All patients with SLE were diagnosed in accordance with the 1997 ACR revised criteria for classification of SLE. Disease activity was assessed using the SLE disease activity index (SLEDAI), and active disease was defined as an SLEDAI score ≥ 5. Age- and sex-matched healthy controls were recruited from the medical staff at Shanghai General Hospital.

Isolation, culture and treatment of CD4+ T cells

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood of SLE patients or healthy controls using Ficoll-paque density gradient centrifugation. Purified CD4+ T cells were negatively isolated from PBMCs by CD4+ T-cell isolation kits (STEMCELL Technologies, Vancouver, Canada) according to the manufacturer’s protocol. CD4+ T cell purity was routinely > 90% as verified through flow cytometry. The cells were then cultured in Xvivo 15 medium (Lonza, Walkersville, MD, USA) supplemented with 10% human AB serum (Valley Biomedical, Winchester, VA, USA) at 37 °C with 5% CO2. The treatments of the cells were: TNF-α (HY-P7058, MedChemExpress, NJ, USA), 10 ng/ml, 24 h; IL-6 (HY-P7044, MedChemExpress), 10 ng/ml, 24 h; 17β-estradiol (estradiol/E2) (HY-B0141, MedChemExpress), 100 nmol/L, 24 h; Lipopolysaccharides (LPS) (L8880, Solarbio, Beijing, China), 100 ng/ml, 24 h; ultraviolet B (UVB), 50 ml/cm² [10]; hydroxylchloroquine sulfate (HCQ sulfate) (HY-B1370, MedChemExpress), 6 μg/ml, 24 h; 5-Azacytidine (5-aza C) (HY-10586, MedChemExpress), 1 mM, 24 h; prednisolone (HY-17463, MedChemExpress), 10 ng/ml, 24 h; AZD9496 (HY-12870, MedChemExpress), 5 mM, 24 h.

Quantitative reverse transcription-PCR (qRT-PCR)

Total RNAs of cells were extracted using Trizol (Invitrogen) according to the instructions provided by the manufacturer. Reverse transcription was performed using the Primescript RT Master Mix (Takara, Otsu, Japan), and cDNA was amplified using SYBR-Green Premix (Takara). The expression of HERV-E clone 4–1 gag was normalized to the expressions of GAPDH. The data were analyzed by delta Ct method. Primers of HERV-E clone 4–1 gag used in this study were imported from other published articles [5–7] and the primers were, F: 5’-CACATGGTGAGAGTCGTTGTTT-3’ and R: 5’-GCTTGGCGTTCCTTAGTAG-G-3’; GAPDH, F: 5’-GGAGTCCTGCGGTCTTCTTC-3’ and R: 5’-GCTGATGATCTGGAGCTGTGTTG-3’. Primers for HERV-E clone 4–1 3’LTR were, F: 5’-TCGCCATTCTCCTGGTGTC-3’ and R: 5’-TATTCCGCGGAGATCATTTTG-3’.

Oligonucleotide, plasmids and transfection

SiRNA, miR-302d mimics and corresponding negative controls were transfected by Hiperfect transfection reagent (Qiagen, Valencia, CA, USA) and plasmids were transfected by Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) into cells. Nuclear factor of activated T cells 1 (NFAT1) siRNA and Estrogen receptor-α (ER-α) siRNA were obtained from Santa Cruz Biotechnology (sc-36, 055, sc-29,305, Santa Cruz, CA, USA). The 3’LTR of HERV-E clone 4–1 were cloned into pcDNA 3.1 plasmid and the recombinant plasmid was transfected into cells to obtain the 3’LTR mRNA overexpression.

Western blot analysis

Cells were lysed using radioimmunoprecipitation (RIPA) lysis buffer (Beyotime, Shanghai, China). Protein concentrations were detected using bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). Total proteins were separated by sodium dodecy1 sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). Antibodies used in the assays were NFAT1 antibody (ab2722, Abcam, Cambridge, UK), ER-α antibody (#8644, Cell Signaling Technology) and GAPDH antibody (#5174, Cell Signaling Technology). IRF9 antibody (#76684, Cell Signaling Technology), Methyl-CpG binding domain protein 2 (MBD2) antibody (ab38646, Abcam) and IL-17 antibody (ab77171, Abcam).

Luciferase assay

An NFAT luciferase reporter plasmid (pNFAT-Luc) containing NFAT1 binding promoter elements was used to detect the NFAT1 transcriptional activity. CD4+ T cells were co-transfected with a mixture of 300 ng pNFAT-Luc reporter and 5 ng pRL-TK Renilla luciferase reporter. After different treatment, the luciferase
activities were measured using the Dual Luciferase Reporter assay (Promega, Madison, WI, USA). pRL-TK Renilla luciferase reporter was used to normalize the transfection efficiency.

Full-length sequences of HERV-E clone 4–1 5’ LTR containing wild-type of NFAT1 or ER-α predicted binding site was inserted into pGL3-Basic luciferase reporter vector (Promega). Mutant reporter plasmids were prepared using Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Cells were co-transfected with a mixture of 300 ng firefly luciferase reporter, 5 ng pRL-TK Renilla luciferase reporter, and NFAT1 or ER-α plasmids. After 48 h of incubation, the luciferase activities were quantified using the Dual Luciferase Reporter assay (Promega, Madison, WI, USA). The sequences of 3’LTR of HERV-E clone 4–1 mRNA or MBD2 3’UTR containing potential wild-type or mutant binding sites of miR-302d were constructed into pmirGLO vectors (Promega). The luciferase vectors and miR-302d mimics were transfected into CD4+ cells along with pRL-TK vector. The dual-luciferase Reporter assay system (Promega) was used to detect luciferase activity. pRL-TK Renilla luciferase reporter was used to normalize the transfection efficiency.

Chromatin immunoprecipitation (ChIP)
ChIP assay was conducted using EZ ChIP kit (Millipore, Billerica, MA, USA) and NFAT1 antibody (ab2722, Abcam) or ER-α antibody (#8644, Cell Signaling Technology) according to the instruction of the manufacturer. The primers specific to HERV-E clone 4–1 5’ LTR were: 5’-CTCCCCAACCTCCCCCTTTTC-3’ and 5’-TGAGAAACATGACTGGGGGC-3’. Normal rabbit IgG (A7016, Beyotime, Shanghai, China) was used to control the non-specific immunoprecipitation.

DNA extraction and global methylation analysis
Assays of DNA extraction and global methylation analysis was described in our previous study [11].

Enzyme-linked immunosorbent assay
The concentration of IL-17 in culture supernatants were measured by Human IL-17 ELISA Kit (ab119535, Abcam) according to the manufacturer’s instructions. Optical density values were read at 450 nm using ELx800 Absorbance Microplate Reader (BioTek, VT, USA).

Statistical analysis
Statistical analysis was performed using the SPSS program (version 18.0; SPSS, Chicago, IL, USA). The statistical significance of differences between two groups was tested using Student’s t test. Spearman’s analysis was used to test correlation. P < 0.05 was considered as statistically significant.

Results
HERV-E clone 4–1 mRNA expression was upregulated in CD4+ T cells from SLE patients
In our former study, we found that HERV-E clone 4–1 mRNA expression was higher in lupus CD4+ T cells than in cells from healthy controls and the HERV-E clone 4–1 mRNA expression level was positively correlated with SLE disease activity [7]. To continue our study, first, we used new samples (Additional file 1: Table S1) to prove HERV-E clone 4–1 mRNA expression was higher in SLE CD4+ T cells than in cells from healthy controls using Quantitative reverse transcription-PCR (qRT-PCR) (Fig. 1a). We also found that HERV-E clone 4–1 mRNA expression level was higher in active patients than that of inactive patients (Fig. 1b) and positively correlated with SLE disease activity (Fig. 1c). We also followed-up some patients who got oral prednisolone and hydroxychloroquine treatment and the activity of SLE changed from active to inactive. We found that the HERV-E clone 4–1 mRNA expressions decreased as the SLEDAI decreased (Fig. 1d and e). But the HERV-E clone 4–1 mRNA expressions of the inactive patients were also higher than that of HCs (Fig. 1f). The Area Under Curve (AUC) was 0.760, the 95% confidence interval (95% CI) was 0.622 to 0.897, and the best Youden’s index is 0.5. This indicated that HERV-E clone 4–1 mRNA might have good diagnostic value for SLE and could act as a potential diagnostic biomarker for SLE.

NFAT1 activity was increased in SLE and associated with increased HERV-E clone 4–1 mRNA
To explain why HERV-E clone 4–1 mRNA was upregulated in CD4+ T cells from SLE patients, we wondered if some transcription factors could promote the transcription of HERV-E clone 4–1 mRNA. Since the 5’ LTR contained the transcription factor binding sites [12], this region was used to predict the potential transcription factors. Using TransFac and JASPAR database, we found some transcription factors that might regulate the expression of HERV-E clone 4–1 mRNA. NFAT1, which was proved to play critical roles in SLE [13] caught our attention. First, full length fragment of the human Human endogenous retroviral DNA (4–1) 5’LTR with wild type (wt) or mutant (mut) predicted NFAT1 binding site was inserted into the luciferase reporter plasmid (Fig. 2a). Then, we use NFAT1 overexpression plasmids to overexpress NFAT1 and NFAT1 siRNA to knockdown NFAT1 (Fig. 2b-e). Luciferase reporter analysis showed that overexpression of NFAT1 led to an increase in
luciferase activity of the wt HERV-E clone 4–1 5’LTR plasmid in CD4⁺ T cells, while mut NFAT1 binding site attenuated the increase of luciferase activity (Fig. 2f). In addition, Chromatin immunoprecipitation (ChIP) assay clearly showed that the predicted NFAT1-binding site in HERV-E clone 4–1 5’ LTR presented the ability to bind to NFAT1 protein (Fig. 2g). Moreover, qRT-PCR analysis showed that overexpression of NFAT1 could increase the expression of HERV-E clone 4–1 mRNA and knockdown of NFAT1 with siRNA could decrease the expression of HERV-E clone 4–1 mRNA (Fig. 2h and i). Then, we collected CD4⁺ T cells of SLE patients and HCs to detected NFAT1 activity using NFAT luciferase reporter assay and HERV-E clone 4–1 mRNA expression. We found NFAT1 activity was upregulated in CD4⁺ T cells from SLE patients (N = 27) and higher in active patients than that of inactive patients (Fig. 1k). What’s more, the relative NFAT1 activity had strong correlation with HERV-E clone 4–1 mRNA expression (Fig. 2l).
So, these results all together suggested that NFAT1 could induce HERV-E clone 4–1 mRNA expression via binding to its 5′LTR. We also detected the influence of some factors on NFAT1 activity and HERV-E clone 4–1 mRNA expression (Fig. 2m and n).

E2 could upregulate HERV-E clone 4–1 mRNA expression via ER-α in CD4+ T cells from SLE patients

When selecting the potential transcript factors that might regulate the expression of HERV-E clone 4–1 mRNA, ER-α, which was the receptor of E2 drew our
attention. This is because SLE has a predilection for females of child-bearing age who have relatively high estrogen level and estrogen is also a risk factor for SLE [14] and HERV-E was upregulated in breast cancer and ovarian cancer [15, 16]. Then, we further explored the role of E2 and ER-α in SLE. Accordingly, full length fragment of the human endogenous retroviral DNA (4–1 5' LTR with wild type (wt) or mutant (mut) predicted ER-α binding site was inserted into the luciferase reporter plasmid (Fig. 3a). Then, we use ER-α overexpression plasmids to overexpress ER-α and ER-α siRNA to knockdown ER-α (Fig. 3b-e). Luciferase reporter analysis showed that overexpression of ER-α led to an increased luciferase activity of the wt HERV-E clone 4–1 5'T plasmid in CD4+ T cells, while mut ER-α binding site attenuated the increase of luciferase activity (Fig. 3f). In addition, ChIP assay clearly showed that the predicted ER-α binding site in HERV-E clone 4–1 5' LTR presented the ability to bind to ER-α protein (Fig. 3g). Moreover, qRT-PCR analysis showed that ER-α plasmids could increase the expression of HERV-E clone 4–1 mRNA while ER-α antagonist AZD9496 maleate and ER-α siRNA could decrease the expression of HERV-E clone 4–1 mRNA (Fig. 3h-i). In addition, AZD9496 and ER-α siRNA could reverse the upregulated HERV-E clone 4–1 mRNA expression induced by E2 (Fig. 3k and l). So, these results all together suggested that E2 could also upregulate HERV-E clone 4–1 mRNA expression via ER-α in CD4+ T cells from SLE patients.

DNA hypomethylation of HERV-E clone 4–1 5’LTR contributed to the increase of HERV-E clone 4–1 mRNA

In our former study, we found the HERV-E clone 4–1 5’LTR was hypomethylated in CD4+ T cells from SLE patients and its methylation could be inhibited by 5-aza C [7]. Here, we investigated whether this DNA hypomethylation was involved in the NFAT1 or ER-α induced HERV-E clone 4–1 mRNA upregulation. We found that HERV-E clone 4–1 mRNA expressions were upregulated when NFAT1 or ER-α was overexpressed or 5-aza C was used in CD4+ T cells from SLE patients and HC (Fig. 4a-d). In CD4+ T cells from SLE patients and HCs, HERV-E clone 4–1 mRNA expressions were higher when both NFAT1 was overexpressed and 5-aza C was used than that when NFAT1 was overexpressed or 5-aza C was used (Fig. 4a and b); accordingly, HERV-E clone 4–1 mRNA expressions were higher when both ER-α was overexpressed and 5-aza C was used than that when ER-α was overexpressed or 5-aza C was used (Fig. 4c and d). Besides, the times of HERV-E clone 4–1 mRNA upregulation were higher in CD4+ T cells from SLE patients than that of HCs when NFAT1 or ER-α was overexpressed (Fig. 4e and f), and the times of HERV-E clone 4–1 mRNA upregulation were higher in CD4+ T cells from HCs than that of SLE patients when 5-aza C was used (Fig. 4g). These results together suggested that DNA hypomethylation contributed to the upregulation of HERV-E clone 4–1 mRNA induced by NFAT1 and ER-α.

HERV-E clone 4–1 3’LTR induced DNA hypomethylation and IL-17 release via miR-302d/MBD2

Since 3’UTRs of mRNAs were reported to act as natural miRNA sponges and could serve as competitive endogenous RNAs (ceRNAs) of other genes through sharing the common miRNAs [17–20]. We want to explore whether the 3’LTR of HERV-E clone 4–1 mRNA could act as a miRNA sponge and act ceRNAs of other genes. Through programs based on microRNA.org and Targetscan, we found that there was a potential binding site of miR-302d in the 3’LTR of HERV-E clone 4–1 mRNA (Fig. 5a). Then, we performed luciferase reporter assays to determine this interaction. Luciferase assay showed that miR-302d mimics could decrease the luciferase activity of reporter containing wt 3’LTR of HERV-E clone 4–1 while mut binding site attenuated the increase of luciferase activity (Fig. 5b). This suggested that 3’LTR of HERV-E clone 4–1 could bind to miR-302d and act as a sponge for miR-302d. We also found MBD2 was another potential target of miR-302d (Fig. 5a) and verified the interaction between MBD2 3’UTR and miR-302d using luciferase assay (Fig. 5c). Then, we found that overexpression of 3’LTR of HERV-E clone 4–1 (Fig. 5d) increased the protein levels of MBD2 and miR-302d mimics could rescue the increase of MBD2 protein by the 3’LTR (Fig. 5e). These results suggested that HERV-E clone 4–1 acts as a ceRNA of MBD2 to positively regulate MBD2 expression in 3’LTR and miR-302d dependent manners. We also detected the expression of IRF-9 which was a proved target of miR-302d in SLE [21] and found that overexpression of 3’LTR of HERV-E clone 4–1 increased the protein levels of IRF9 and miR-302d mimics could rescue the increase of IRF9 protein by the 3’LTR (Fig. 5e).

The mRNA levels of MBD2 in was increased in CD4+ T cells of SLE patients and inversely correlated with global DNA methylation and positively correlated with and SLE-DAI score [22, 23]. What’s more, MBD2 was found to stimulate Th17 cell differentiation and IL-17 release in other autoimmune diseases [24–26] and IL-17 play critical functions in the pathophysiology of SLE [27, 28] So, MBD2 might play important roles in SLE progression. Then, we intended to further study the role of HERV-E clone 4–1, miR-302d and MBD2 in global DNA methylation and IL-17 expression in CD4+ T cells of SLE patients. CD4+ T cells were transfected with HERV-E clone 4–1 3’LTR expression plasmids, miR-302d mimics or MBD2 expression plasmids. Global DNA methylation levels, intracellular IL-17 level and IL-17 level in culture supernatants were subsequently measured. The results showed that global DNA methylation level decreased when CD4+ T cells of SLE were transfected with 3’LTR expression plasmids or MBD2 expression plasmids.
and increased when transfected with \textit{miR-302d} mimics (Fig. 5d). Intracellular IL-17 level and IL-17 level in culture supernatants increased when CD4\textsuperscript{+} T cells of SLE were transfected with 3'LTR expression plasmids or \textit{MBD2} expression plasmids and decreased when transfected with \textit{miR-302d} mimics (Fig. 5g-j). All together, these results suggested that \textit{HERV-E clone 4–1} 3'LTR induce DNA hypomethylation and IL-17 release via \textit{miR-302d}/\textit{MBD2} in CD4\textsuperscript{+} T cells of SLE.

**Discussion**

Some studies had proved that \textit{HERV-E clone 4–1} mRNA expression was increased in SLE patients, and the expression level of \textit{HERV-E clone 4–1} was associated with SLE disease activity [5–7], however, they didn't thoroughly investigate the function and mechanism of \textit{HERV-E clone 4–1} in SLE. In this study, we investigated the mechanism of \textit{HERV-E clone 4–1} mRNA upregulation in CD4\textsuperscript{+} T cells...
from SLE patients and its roles in SLE progression. First, we found NFAT1 could induce HERV-E clone 4–1 mRNA expression by binding to its 5′ LTR. NFAT1, which is a key factor of Ca2+/calcineurin (CaN)/NFAT signaling pathways, was verified to be activated in SLE [13]. We also demonstrated that NFAT1 activity was upregulated in SLE and positively correlated with HERV-E clone 4–1 mRNA expression. NFAT1 are phosphorylated and reside in the cytoplasm in resting cells; upon stimulation, they are dephosphorylated by calcineurin, translocate to the nucleus, and become transcriptionally active [29–31]. Then the activated NFAT1 can regulate transcription of some inflammatory cytokines such as IL-6, IL-8, TNF-α and interferon-γ (IFN-γ) [32–35]. Furthermore, we found TNF-α, IL-6, E2, LPS, UVB could upregulate NFAT1 activity and HERV-E clone 4–1 mRNA expression and these factors play critical roles in SLE [14, 36–38]. These results together may explain the roles of NFAT1 in HERV-E clone 4–1 mRNA expression in SLE.

Adreno cortico hormones are an important class of anti-inflammatory/immunosuppressive drugs. They can inhibit the expression of TNF-α and IL-6 and decrease the activity of SLE [39]. Ca2+/CaN/NFAT signaling is an important pathway in the T-cell activation of SLE and some calcineurin inhibitors such as cyclosporine A and tacroliimus have been used in the clinical treatment of SLE [40]. Hydroxychloroquine, which could block Ca2+/CaN/NFAT signaling pathway through inhibiting the sustained Ca2+ storage release from the endoplasmic reticulum [41], was found to repress NFAT1 activity and HERV-E clone 4–1 expression. Prednisolone and hydroxychloroquine are first-line drugs in the treatment of SLE and all the patients followed-up got oral prednisolone and hydroxychloroquine treatment. These reasons interpret it well why HERV-E clone 4–1 mRNA expressions decreased after prednisolone and hydroxychloroquine treatment. So, we hold that the upregulation of HERV-E clone 4–1 mRNA is mainly due to the abnormal inflammation / immune status of SLE which involving many inflammatory cytokines and other risk factors. We also found that E2 could upregulate HERV-E clone 4–1 mRNA expression via ER-α. ER-α is one of the estrogen receptors which can be activated by estrogen and regulate gene transcription in nucleus [42]. Interestingly, HERV-E was upregulated in breast cancer and ovarian cancer [15, 16] and this probably also has close relationship with E2 and ER-α. ER-α antagonist is also a good Fig. 4 DNA hypomethylation of HERV-E clone 4–1 5′LTR contributed to upregulation of HERV-E clone 4–1 mRNA induced by NFAT1 and ER-α. CD4+ T cells from SLE patient or HC were treated with NFAT1 plasmids, ER-α plasmids, 5-aza C alone or in combination with 5-aza C in vitro. Relative HERV-E clone 4–1 mRNA expression (a–d) were detected. e and f Times of HERV-E clone 4–1 mRNA upregulation were compared by the Student’s t test in CD4+ T cells from SLE patient and HC when NFAT1 or ER-α was overexpressed. g Times of HERV-E clone 4–1 mRNA upregulation were compared by the Student’s t test in CD4+ T cells from SLE patient and HC when 5-aza C was used. Data were represented as mean ± SD, N = 3. *P < 0.05, **P < 0.01, ***P < 0.001
approach to restrain the expression of HERV-E clone 4–1. Taken together, we think these signaling pathways are good therapeutic targets for HERV-E clone 4–1.

Some studies found the HERV-E clone 4–1 5’LTR was hypomethylated in CD4+ T cells from SLE patients [7–9]. We found that DNA hypomethylation contributed to upregulation of HERV-E clone 4–1 mRNA induced by NFAT1 and ER-α. We think DNA hypomethylation of HERV-E clone 4–1 5’LTR is an indispensable factor that account for the upregulation of HERV-E clone 4–1 mRNA for that upregulation of HERV-E clone 4–1 mRNA mainly exists in SLE while not in some other diseases that involving NFAT1 and ER-α activation.

In this study, we found that HERV-E clone 4–1 3’LTR could act as natural miRNA sponges for miR-302d to restrain miR-302d activity. MiR-302d was proved to be downregulated in SLE patient monocytes and could inhibit the type I IFN pathway which was a major contributor to SLE pathogenesis via its target IRF-9 [21]. HERV-E clone 4–1 3’LTR could positively regulate MBD2 expression by acting as a ceRNA of MBD2 via miR-302d and HERV-E clone 4–1 3’LTR could induce DNA hypomethylation and IL-17 release in CD4+ T cells of SLE. DNA hypomethylation of immune cells in SLE is associated with immune dysfunction and play important roles in the initiation
and development of SLE [43, 44]. IL-17 is a proinflammatory cytokine produced by activated T cells and plays a crucial role in disease pathogenesis and represent an attractive therapeutic target for SLE [27, 28]. Thus, we hold that HERV-E clone 4–1 takes part in disease pathogenesis of SLE through miR-302d/MBD2/DNA hypomethylation and IL-17 signaling via its 3’LTR. So, HERV-E clone 4–1 3’LTR may be a potential therapeutic target of SLE. Taken together, we draw a network diagram hypothesis showing relationship between HERV-E clone 4–1 and SLE which shows the important roles of HERV-E clone 4–1 in SLE pathogenesis (Fig. 6).

However, we should admit that we didn’t further investigate the role of HERV-E clone 4–1 proteins and this is a shortcoming of this study. This mainly because there is no specific antibody for these proteins.

**Conclusions**

In conclusion, we found that HERV-E clone 4–1 mRNA expression was upregulated in CD4+ T cells from SLE patients and could act as a good biomarker for diagnosis of SLE. This is associated with the activation of Ca2+/CaN/NFAT1 and E2/ER-α signaling pathway and DNA hypomethylation of HERV-E clone 4–1 5’LTR. HERV-E clone 4–1 also takes part in disease pathogenesis of SLE through miR-302d/MBD2/DNA hypomethylation and IL-17 signaling via its 3’LTR. These signaling pathways may be potential therapeutic targets of SLE.

**Additional file**

**Additional file 1:** Figure S1. The structure of HERV-E clone 4–1. Table S1. Clinical characteristics of SLE patients and healthy controls. (DOCX 26 kb)

**Abbreviations**

5-aza C: 5-Azacytidine; AUC: Area Under Curve; CaN: Calcineurin; ChIP: Chromatin immunoprecipitation; E2: 17β-estradiol/estradiol; ER-α: Estrogen receptor-α; HCQ: Hydroxychloroquine; HERV: Human endogenous retroviruses; IFN-γ: Interferon-γ; LPS: Lipopolysaccharides; LTRs: Long terminal repeats; MBD2: Methyl-CpG binding domain protein 2; NFAT: Nuclear factor of activated T cells; ORFs: Open Reading Frames; PBMC: Peripheral blood mononuclear cells; qRT-PCR: Quantitative reverse transcription-PCR; ROC: Operating Characteristic; SLE: Systemic lupus erythematosus; SLEDAI: SLE disease activity index; TNF-α: Tumor necrosis factor-α; UVB: Ultraviolet B

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**Authors’ contributions**

XW, CZ (Chaoshuai Zhao) and CZ (Chengzhong Zhang) designed and performed the experiments; ZW, XM, JS, YS and WS analyzed and interpreted the data; WS wrote the manuscript. ZW critically revised the manuscript. All authors read and approved the manuscript.

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**Availability of data and materials**

Not applicable.

**Ethics approval and consent to participate**

This research was approved by the Institutional Research Ethics Committee of Shanghai General Hospital and abided by the ethical guidelines of the
Declarations of Helsinki. Informed consents were obtained from all the patients involved in this study.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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