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Alteration of interspersed repetitive sequence methylation in T lymphocytes of people living with HIV: A preliminary study

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ABSTRACT: Interspersed repetitive sequence (IRS) consists of various classes. IRS methylation possesses different levels in cell type, cell condition, and pathogenesis of the disease. The current study aimed to evaluate IRS methylation in HIV-1 infected T lymphocytes in patients living with human immunodeficiency virus (HIV) (PLWH). We examined HERV-E LTR2C, HERV-K LTR5Hs, LINE-1, and Alu methylation status in CD4+ T cells and non-CD4+ T cells such as CD8+ T cells of people living with HIV (PLWH) in comparison with healthy donors. Later, we infected peripheral blood mononuclear cells (PBMCs) from healthy donors with HIV-1 and observed methylation level changes of HERV-K and HERV-E for up to 14 days. We found that the methylation of LINE-1 and Alu did not change significantly in either CD4+ or CD8+ T cells. Interestingly, we observed a significant increase in HERV-K methylation and a significant reduction in HERV-E methylation in both cell types. In the HIV-1 infected T lymphocytes experiment, after 4 days, we noticed a reduction in HERV-K methylation and an induction of HERV-E methylation. Nevertheless, there are trends of increased HERV-K methylation and decreased HERV-E methylation on days 7, 10, and 14. The IRS methylation changes were not associated with the HIV-1 quantity. In summary, IRS methylation level was cell type specific. HERV elements methylation had a different pattern in PLWH. The present study provided fundamental knowledge of IRS methylation in T cells of PLWH. The mechanisms and consequences of these virus-associated epigenetic changes should be further investigated.

KEYWORDS: HIV-1, interspersed repetitive sequence, IRS, HERV, methylation, T cells

INTRODUCTION

HIV infection is a major disease worldwide, including Thailand. According to the high mutation rates of the HIV genome, 9 subtypes and more than 30 circulating recombinant forms (CRF) were classified based on genetic diversity [1]. HIV-1 CRF01_AE, or mosaic virus, is a major subtype found in Thailand, with a minor (5–10%) proportion of subtype B [2]. According to the World Health Organization, up to 38.5 million individuals could be living with HIV worldwide as of 2021 [3]. However, no fully effective HIV vaccine has yet been created. A considerable problem with HIV-1 is its high rate of mutation. Moreover, there are not any strategies that can eradicate the latently infected CD4 T cells, including antiretroviral therapy (ART) [4]. Recent research demonstrated that host epigenetic modifications caused by the virus are essential to HIV pathogenesis [5]. Cytosine-guanine dinucleotide (CpG) methylation, an epigenetic modification, is one of the heritable epigenetic modifications. It involves adding a methyl group to cytosine, especially within CpG (cytosine/guanine) pairs [6,7]. Moreover, CpG methylation is involved in a dynamic process that can precipitate both transient and stable changes in gene expression [8].

The CpG is also present in the interspersed repetitive sequences (IRSs) of transposable elements (TEs) in the human genome, as well as the gene promoter region. The TE was found at approximately 45% of the whole genome and can be classified into two main groups: DNA transposons (2.8%) and retrotransposons (42.2%). The subdivided retrotransposons belong to long terminal repeat as LTR and non-LTR retrotransposons. Non-LTR retrotransposons comprise about 34% of the human genome [9]. The non-LTR

retrotransposons affect the human genome in different ways, including insertion mutations, genomic instability, and gene expression changes. Among non-LTR retrotransposons, long interspersed nuclear elements-1 (LINE-1) (16.9%) and short interspersed nuclear elements (Alu) (10.6%) have the highest population of IRSs [9]. Human endogenous retroviruses (HERVs) are the majority of LTR retrotransposons (8.2%). HERVs have been classified into three classes. Class I consists of gamma-like retroviruses known as type C. This class is subdivided into 6 groups, including HERV-H, HERV-I, HERV-W, and HERV-E. Class II contains beta-like retroviruses known as types A, B, and D, which are subdivided into 10 groups, including HERV-K. Finally, Class III comprises foamy virus-related HERVs, members of which include HERV-L [10]. The methylation levels of various IRS elements, including LINE-1, Alu, and some types of HERV sequences, have been studied. The hypomethylation of LINE-1, Alu, HERV-E, and HERV-K has been found in various conditions such as cancers, embryogenesis, aging, congenital malformations, exposure to certain environments, malnutrition, diabetes, and autoimmune diseases [11,12,13,14,15]. Interestingly, aging cells have hypomethylation of the Alu element and HERV-K but not LINE-1 [16]. Thus, the loss of genome-wide methylation in IRSs is cell type-specific in each condition. Methylation changes in IRSs can result in several consequences. First, hypomethylation increases IRS RNA and protein, causing a variety of consequences based on RNA and protein functions and their variations [17]. Additionally, it was revealed that LINE-1 and HERV both have gene regulatory functions mediated by DNA methylation [18,19,20].

There is currently no information of IRSs methylation in HIV-1 infected normal human cells. Thus, in the current study, we investigated the IRS methylation profile of LINE-1, Alu, HERV-E, and HERV-K in CD4+ and CD8+ T cells in PLWH (People Living with HIV) and healthy donors. Furthermore, we determined the kinetics of HERV-K and HERV-E methylation levels in HIV-1 infected peripheral blood mononuclear cells (PBMCs) from healthy donors.

MATERIALS AND METHODS

Ethics statement

The current study was approved by the Institutional Review Board for the Faculty of Medicine at Chulalongkorn University (IRB No.358/46), and all study subjects provided written informed consent.

Study participants

The volunteers were recruited at the King Chulalongkorn Memorial Hospital, the Anonymous Clinic, and the Thai Red Cross AIDS Research Center in Bangkok, Thailand. Ten Thai PLWH, and ten Thai healthy donors (HIV-1 negative) with age- and sex-matched were enrolled. CD4 and viral loads were collected from each individual. All subjects were naive about antiretroviral treatment. CD4+ T cell counts ranged from 104 to 1,036 cells/mm^3 (median 281 cells/mm^3), CD8+ T cell counts ranged from 492 to 3,101 cells/mm^3 (median 1,007 cells/mm^3), and plasma HIV-RNA levels ranged from <40 to 132,609 copies/ml (mean 55,544 copies/ml), as determined by the Chiron b-DNA Monitor test (Quantiplex v3.0, Bayer Diagnostics, Walpole, MA, USA). Clinical data are summarized in Table 1. Moreover, ten healthy donors were included to study *ex vivo* with HIV-1 infected cells.

PBMCs preparation and cell isolation

PBMCs were isolated by density gradient separation with Isoprep (Robbins Scientific Corporation, Sunnyvale, CA, USA) and cryopreserved for further immunological assays. Briefly, after removing plasma, ACD-treated whole blood was diluted 1:1 with RPMI1640 medium containing 2 mM L-Glutamine (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and layered over Isoprep. Following a 30 min centrifugation of the samples at 1,500 rpm, the PBMC layer was collected and washed twice with RPMI 1640. Harvested PBMCs were then resuspended in R10 medium (RPMI1640 supplemented with 100 U/ml of penicillin, 100 U/ml of streptomycin, and 10% heat-inactivated fetal bovine serum (FBS, Bio Whittaker, MD, USA)). For cell isolation, the CD4+ T cell subpopulation was purified through the use of anti-CD4 antibody-coated immunomagnetic beads (Dynal, Great Neck, NY, USA). The non-CD4+ T cell fractions were referred to as CD8+ T cells. The purity of CD4+ T cells and CD8+ T cells was more than 98% measured by flow cytometry.

Ex vivo infection assays

For viral infection, the low dosage of 0.01 multiplicity of infection (MOI) HIV-1 infection was chosen to be 100TCID50 in 200 µl of culture medium. The 100TCID50 is the usual viral infection dosage or the quantity of virus necessary to infect 50% of tissue culture cells [20,21]. Individual PBMCs (200,000 per well) was pretreated with phytohemagglutinin (PHA) for 3 days before receiving 100TCID50 of the specified HIV-1 isolates in 200 µl per well in triplicate in 96-well round-bottomed culture plates. Cells were collected on days 0, 4, 7, 10, and 14. The p24 Ag intracellular staining assay was performed on the cells, and the DNA was prepared for methylation assay.

p24 Antigen by intracellular staining assay

Cells on days 0, 4, 7, 10, and 14 were transferred to V-bottomed plates and washed once in wash buffer (0.1% bovine serum albumin (BSA) and 0.5% Na_2O_3 in phosphate buffered saline (PBS)). Cells were

Table 1 Clinical characteristics of participants ($n = 30$).

		Participant			*Ex vivo* study
	Total	Healthy donor	PLWH	p-value	Healthy donor
Number of participants	30	10	10		10
Female	12 (40%)	5 (50%)	5 (50%)	1.000	2(20%)
Male	18 (60%)	5 (50%)	5 (50%)	1.000	8(80%)
Median age (Range)	35 (20–59)	37 (28–46)	34 (20–59)	0.646	39.5 (21–58)
Median CD4+ T cells (cells/mm^3)	695 (104–1399)	827 (670–1399)	281 (104–1036)	0.001	917 (484–1837)
Median CD8+ T cells (cells/mm^3)	795 (492–3101)	696 (554–1282)	1,007 (492–3101)	0.041	760 (430–1058)
Median plasma HIV-1 RNA (copies/ml)	55,544	–	55,544		–
Median plasma HIV-1 RNA (log10)	4.74	–	4.74		–

PLWH, People living with HIV.

stained with surface marker antibodies (CD8-PE/CD3-PerCP/CD4-APC) for 20 min at 4 °C. After incubation, cells were washed and fixed with 4% paraformaldehyde (PFA) for 10 min at 37 °C. Then, cells were permeabilized by 1X FACSPerm for 10 min at dark room temperature. Finally, cells were stained with p24 Ag antibody (KC57-FITC; Beckman Coulter Inc., Indianapolis, IN, USA) for 30 min at 4 °C. Non-stained cells were utilized as negative controls. Cells were analyzed using FACSCalibur™ flow cytometry (Becton Dickinson, Frankin Lases, NJ, USA).

DNA extraction

DNA was isolated from PBMCs and HIV-1 infected lymphocytes through the use of a standard phenol-chloroform method. In brief, for PBMCs, six ml of blood from each PLWH and control were collected and kept at 4 °C until processing within 24 h. All samples were centrifuged at 1600*g* for 10 min, and plasma was separated from each sample. The lysis buffer was added and centrifuged at 1000*g* for 8 min to eliminate the red blood cells. For HIV-1 infected lymphocytes, after incubation with HIV-1 on days 0, 4, 7, 10, and 14, the lymphocytes were harvested and centrifuged at 1600*g* for 10 min. Then, the pellet was incubated with 10% SDS/proteinase K (20 mg/ml) at 50 °C for 72 h, and the digested cells in all samples were subjected to phenol-chloroform extraction and ethanol precipitation. The precipitated DNA was suspended in 50 µl of sterile distilled water.

DNA conversion by sodium bisulfite

Bisulfite conversion of DNA was carried out using the EZ DNA methylation Kit™ (Zymo Research, Orange, CA, USA), according to the manufacturer's protocol. In brief, 20 µl of 500 ng of DNA was dissolved in 130 µl of the reagent mixture of bisulfite solution and then was performed in thermal cycler under the following conditions: 98 °C for 10 min, 64 °C for 2.5 h, and 4 °C storage up to 20 h. Then, Zymo-Spin™IC Column was utilized for isolating the bisulfite-treated DNA. The DNA was eluted with 10 µl of M-Elution Buffer. The bisulfite-treated DNA samples were stored at −20 °C until further uses.

IRS-quantitative combined bisulfite restriction analysis (qCOBRA)

The qCOBRA technique was utilized for methylation detection in LINE-1, Alu, HERV-E, and HERV-K (qCOBRA LINE-1, qCOBRA Alu, qCOBRA HERV-E, and qCOBRA HERV-K). It has been validated previously with pyrosequencing to be an accurate and reliable technique [13]. This technique is designed for detecting methylation at thousands of CpG loci through the use of one set of conserved primers in each IRS. The primers for all four IRS-qCOBRA derived nucleotide sequences from GenBank: M80343 for LINE-1, GenBank: M10976 for HERV-E, GenBank: M14123 for HERV-K; and the sequence primers related to the nucleotides of the AluSx subfamily sequence [13]. All the PCR reactions were pre-denatured at 95 °C for 15 min followed by denaturing at 95 °C for 1 min, annealing for 1 min, extension at 72 °C for 1 min for 35 cycles, and final extension at 72 °C for 7 min. The details of all primer sequences and annealing temperatures are described in Table S1.

Then, the PCR products of LINE-1 (160bp), Alu (133bp), HERV-E (126bp), and HERV-K (156 bp) were digested with the restriction enzyme 2 U (Fermentas Int. Inc., Burlington, Canada). Subsequently, with the incubation of each reaction overnight at 65 °C, DNA fragments were separated in 8% non-denaturing polyacrylamide gels. Next, the gel was stained through the use of the SYBR green nucleic-acid stain (Invitrogen, Waltham, MA, USA). DNA fragment intensity was assessed using a Phosphoimager and ImageQuant software (Molecular Dynamics, GE Healthcare, Slough, UK). The LINE-1 methylated bands were 80 bp, whereas the LINE-1 unmethylated bands were 98 and 62 bp. The Alu methylated bands were 58, 43, and 32 bp; and the Alu unmethylated bands were 133 bp. The HERV-E methylated bands were 91, 82, 72, and 53 bp; while the HERV-E unmethylated bands were 126 bp. The HERV-K methylated bands were 112 and 44 bp, whereas the HERV-K unmethylated bands

were 156 bp. For inter-assay variation normalization between each experiment, we utilized DNA templates from HeLa, Jurkat, and Daudi cell line as a control. The methylation levels of each cell line were standardized. Therefore, all experiments were adjusted with the same control methylation levels. The details of restriction enzymes and PCR product fragments are demonstrated in Table S1. Representative gel electrophoreses of IRS qCOBRA were demonstrated in Fig. S1. All of the IRS qCOBRA experiments were performed in duplicate.

IRS methylation analysis

We calculated the methylation levels and the percentages of LINE-1 and Alu using the intensity of qCOBRA digested LINE-1 and Alu products, in accordance with previous studies [22,23,24]. DNA fragments from enzymatic digestion of qCOBRA LINE-1 and qCOBRA Alu were separated into five and six fragments, i.e., 160, 98, 80, 62, and 18 bp; and 133, 90, 75, 58, 43, and 32 bp, respectively. The methylation products were calculated from the band intensity of individual bands by the number of double-stranded bp of DNA sequence. LINE-1 was as follows: A = intensity of 160 bp fragment divided by 160; B = intensity of 98 bp fragment divided by 94; C = intensity of 80 bp fragment divided by 79; and D = intensity of 62 bp fragment divided by 62. Next, the methylation level of LINE-1 was computed as a percentage of 100(C+A)/(C+A+A+B+D). Similar to LINE-1, the methylation products of Alu were determined as follows: A = intensity of 133 bp fragment divided by 133; B = intensity of 58 bp fragment divided by 58; C = intensity of 75 bp fragment divided by 75; D = intensity of 90 bp fragment divided by 90; E = intensity of 43 bp fragment divided by 43; and F = intensity of 32 bp fragment divided by 32. Then, the amounts of Alu methylation were determined through the use of the following formula: % Alu methylation = 100(E+B)/(2A+E+B+C+D).

The qCOBRA HERV-E and qCOBRA HERV-K were divided into methylated bands and unmethylated bands according to the HERV-E and HERV-K loci each amplicon yielded [12]. The methylation percentage was calculated as follows: (% methylation = the magnitude of the digested methylated fragment divided by the sum of the digested and undigested amplicons, multiplied by 100).

Statistical analyses

The entire data were analyzed through the use of Kruskal Wallis test and Mann-Whitney U test to compare the methylation levels between groups. A p-value of < 0.05 was regarded as statistical significance. The baseline characteristics were analyzed by descriptive statistics. In order to investigate the relationship between two continuous variables, Spearman's correlation coefficient was utilized. All analyses were conducted using the SPSS software for Windows version 22.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Alteration of HERV-E and HERV-K methylation levels in PLWH but not LINE-1 and Alu

In order to compare the IRS methylation profiles in PLWH and healthy donors, we first isolated CD4+ and CD8+ T cells from PBMC in both PLWH and healthy donors using magnetic beads. The CD4+ and CD8+ T cells were measured for the IRS methylation profile: LINE-1, Alu, HERV-E, and HERV-K, by the COBRA assay. In Line-1 and Alu methylation, no differences were observed in methylation in both CD4+ T cells and CD8+ T cells between PLWH and healthy donors (Fig. 1A and 1B). Interestingly, we found significantly lower HERV-E methylation levels in PLWH in comparison to healthy donors in both CD4+ T cells ($39.80 \pm 4.98\%$ vs. $51.05 \pm 6.23\%$, $p = 0.0011$) and CD8+ T cells ($47.33 \pm 7.23\%$ vs. $52.34 \pm 7.17\%$, $p = 0.0031$), as shown in Fig. 1C. However, PLWH had a significantly higher frequency of HERV-K methylation compared with healthy donors in both CD4+ T cells ($49.78 \pm 1.92\%$ vs. $46.55 \pm 2.43\%$, $p = 0.0051$) and CD8+ T cells ($48.21 \pm 5.16\%$ vs. $45.39 \pm 6.15\%$, $p = 0.0310$), as illustrated in Fig. 1D. Furthermore, correlations between individual IRS methylation levels and HIV-1 viral loads were performed. The findings indicated that there were no correlations between the relationships. Tables S2, S3, and S4 present detailed data on all IRS methylation levels and their correlation with viral loads.

HERV-E and HERV-K methylation levels changing after HIV-1 infection in PBMCs from healthy donors

HIV-1 infection has been related to less methylation of HERV-E and more methylation of HERV-K. We next studied HIV-1 infection in the peripheral blood of healthy donors to determine how the level of HIV-1 affecting HERV-E and HERV-K methylation. Therefore, we performed *ex vivo* HIV-1 infection with three days of PHA-pretreated PBMCs from healthy donors through the use of 100TCID50 of the indicated HIV-1 isolate. Cells were collected on days 0, 4, 7, 10, and 14 for the HERV-E and HERV-K methylation assays and the p24 Ag intracellular staining assay. For the methylation assay, we found that the PBMCs from healthy donors had the same level of HERV-E and HERV-K methylation ($65.16 \pm 8.74\%$ vs. $58.18 \pm 9.02\%$) on day 0 (Fig. 2A and 2B, Table S4). During HIV-1 infection, we found the highest level of HERV-E methylation, whereas the lowest level of HERV-K methylation was on day 4. Moreover, we found that the frequencies of HERV-E methylation levels significantly decreased on days 4 and 14 ($69.39 \pm 5.55\%$ vs. $59.67 \pm 12.06\%$,

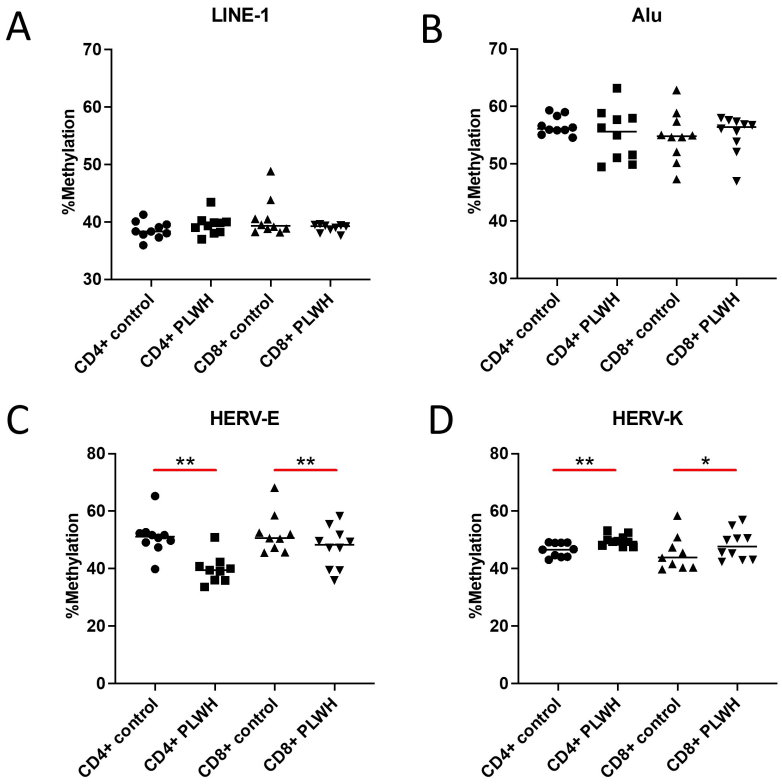

Fig. 1 Comparisons of IRS qCOBRA methylation levels between People living with HIV (PLWH) and healthy donors. No differences were found in LINE-1 (A) and Alu (B). In PLWH, lower HERV-E (C) and higher HERV-K (D) in both CD4+ T cells and CD8+ T cells were observed in PLWH. Denoted: * $p < 0.05$; ** $p < 0.01$.

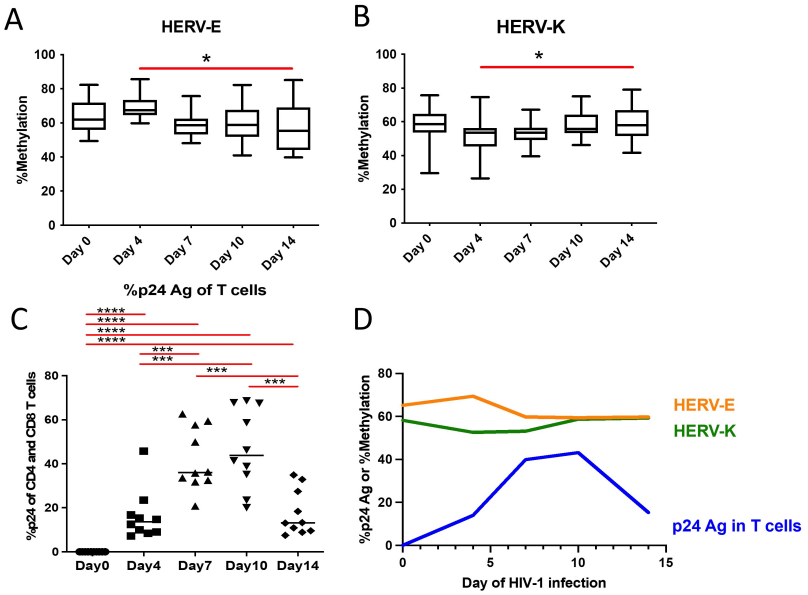

Fig. 2 *Ex vivo* experiment comparisons of p24 Ag levels and HERV methylation in HIV-1-infected cells among different days. Days 7, 10, and 14 showed trends of decreasing HERV-E (A) and increasing HERV-K (B) compared with day 4; p24 Ag was increased, highest on day 10 and then decreased on day 14 (C); comparison graph of HERV-E methylation, HERV-K methylation, and p24 Ag (D). Denoted: * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$.

$p = 0.0144$). We also observed significantly increasing frequencies of HERV-K methylation on days 4 and 14 ($52.56 \pm 9.59\%$ vs. $59.20 \pm 6.34\%$, $p = 0.0273$). These results suggested that HIV-1 infection could alter HERV-E and HERV-K methylation in human cells. In order to understand the interaction of the HIV and the T cell populations and its effects on the methylation patterns, we collected the cells from HIV-1 infected PBMC from healthy donors on days 0, 4, 7, 10, and 14; and the p24 antigens of CD4+ T cells and CD8+ T cells were measured by intracellular staining assay to confirm the level of HIV-1 in infected cells. We found that the frequencies of p24 on days 4–14 were significantly higher than those on day 0 ($p < 0.0001$), as shown in Fig. 2C and Table S4. The highest frequencies of p24 were observed on day 10 and were then decreased on days 14. This result confirmed the HIV-1 infection in CD4+ T cells and CD8+ T cells from healthy donors. Furthermore, we examined the correlations between frequencies of HERVs methylation and p24 antigen levels. Meanwhile, no correlation was observed between HERV-E and HERV-K methylation and p24 antigen levels during the HIV-1 infection until day 14 (Fig. 2D). However, our data illustrated that HERV-K methylation levels were negatively correlated with the frequencies of p24 antigen on days 4 and 7 ($R = -0.4424$, $p = 0.2044$; and $R = -0.5152$, $p = 0.1334$; respectively).

DISCUSSION

Epigenetics regulate gene expression without changing the DNA sequence. Our experiments revealed that HERV-E methylation levels in HIV-infected individuals were significantly lower, whereas methylation levels for HERV-K were significantly higher in both CD4+ and CD8+ T cells in comparison with healthy donors. In addition to HERV-K methylation, one previous research demonstrated a significant increase in HERV-K RNA in PLWH [25,26]. The relationship between HERV-E methylation and HERV-E expression, however, had not been mentioned before. According to the study conducted by Vincendeau et al, they reported an elevation in the expression of HERV-E, HERV-T, HERV-K (HML-10), and two subgroups of ERV9 in persistently HIV-1 infected cell lines such as H4-7-5, LC5-HIV, and KE37.1-IIIB. Additionally, they demonstrated that knockdown of HIV-1 in LC5-HIV cells led to a reduction in transcription of HERV-E and HERV-K. These results suggested that HIV-1 infection could be associated with the activation of various HERV types [27]. An interesting study by Zwolinska K et al [28] revealed that HERVs sequences affected HIV infection susceptibility and that HERV-K113 and HERV-K115 in PLWH were higher than those in healthy donors in the Polish population. HERVs are retroviruses similar to HIV, and they may play a major role in HIV pathogenesis by regulating immune responses. Therefore, we could assume that there were complicated connections between two kinds of sequence. In addition, further investigation is necessary to validate the methylation and expression of the two sequences.

Our results suggested that the methylation levels of HERV-E and HERV-K were associated with HIV infection, whereas there was no significant association between LINE-1 and Alu methylation. Taken together, we hypothesized that HIV-1 infected cells would change these behaviors. Our study was the first to determine these specific methylation changes. More research should be done to figure out exactly what these mechanisms were.

In the next experiment, we measured the HERV-E and HERV-K methylation levels in HIV-1 infected PBMC from healthy donors on days 0 to 14. Moreover, we tested the p24 antigen of CD4+ T cells, which are HIV-1 host cells, through the use of intracellular staining assay. Our study demonstrated that the highest levels of HERV-E methylation and the lowest levels of HERV-K methylation were found on day 4. Meanwhile, the HIV-1 p24 Ag level was the highest on day 7 and then decreased until day 14. It could be possible that PBMCs experienced some epigenetic alterations related to HIV-1 before HIV-1 replication. During HIV-infection, HERV-E methylation levels increased on day 4 and then decreased until day 14. However, the HERV-K methylation levels decreased on day 4 and then increased until day 14. These phenomena warranted further investigation to elucidate the underlying mechanisms in details.

Certainly, in our experiment involving HIV-infected PBMCs, we observed a trend of slightly lower methylation of HERV-E and higher methylation of HERV-K. However, it was important to note that this *ex vivo* differences had *p*-value lower than the case-control study. This suggested the presence of potential variations between human samples and HIV-infected samples. To gain a deeper understanding of these differences and their implications, further investigations are required.

HERVs, fossil viruses, began to be integrated into the human genome more than 30 million years ago, and now about 8% was found in the human genome. Since HERV-K is a member of the type C gamma-like retroviruses, some evidence suggested that it, like HIV-1, formed particles at the plasma membrane [26]. In addition, it was believed that HERVs could interfere with the replication cycle of exogenous retroviruses, including HIV. A previous study revealed that HIV-1 could induce HERV transcription, resulting in increased expression of HERV protein [29]. Evidence had also suggested a correlation between the activation of HERV elements and the level of HIV-1 production. Furthermore, Monde K et al studied the effect of HERV-K on HIV-1 replication and found that the high expression of HERV-K Gag could reduce the release efficiency

and infectivity of HIV-1 [26,30]. Our findings indicated a relationship between HIV-1 and specific types of epigenetic changes, highlighting the need for future studies to validate this observation. One postulated that HIV RNA might likely function in RdDM (RNA-directed DNA methylation) process, with the ability to methylate HERV-K, or antagonist to RdDM process of HERV-E. This regulation could affect the corresponding protein, resulting in the phenotypes differences of PLWH [31].

The limitations of this preliminary study included a small number of participants, only ten PLWH and ten healthy donors, and ten *ex vivo* experimented samples. Another limitation was that the study solely examined the IRS methylation profiles in PLWH and HIV-infected PBMCs. Further experiments were necessary to explore the underlying mechanisms in greater details.

CONCLUSION

The current study revealed valuable information about IRS methylation in T cells of PLWH. The findings indicated an alteration in the methylation levels of HERV-E and HERV-K in PLWH. This information needed further in-depth investigation with a larger sample size.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2023.098.

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Appendix A. Supplementary data

Table S1 Details of IRS qCOBRA.

IRS	Primer (5′ − 3′)	Annealing temperature	Restriction enzyme	PCR product (bp)	
				Before	After
LINE-1	Forward: CCGTAAGGGGTTAGGGAGTTTTT Reverse: RTAAAACCCTCCRAACCAAATATAAA	50 °C	*TaqI* and *TasI*	160	160, 98, 80, 62
Alu	Forward: GGRGRGGTGGTTTARGTTTGTAA Reverse: CTAACTTTTTATATTTTTAATAAAAACRAAATTTCACCA	63 °C	*TaqI*	133	133, 90, 75, 58, 43
HERV-E	Forward: TTTTGTTAGTTGATGTRKGTA Reverse: CCCCAAAAAAAAAATTCYTAACC	58 °C	*TaiI*	126	126, 91, 82, 73, 53
HERV-K	Forward: ATATTAAGGGAATTTAGAGGTTGG Reverse: CCCCTACACACCTATAAATATTTC	60 °C	*TaiI*	156	156, 112

Table S2 IRS methylation levels (mean ± SD).

T cell		IRS methylation (%) and p-value							
		LINE-1		Alu		HERV-E		HERV-K	
CD4+	Healthy controls	38.60±1.49	$p = 0.3150$	56.69±1.64	$p = 0.3930$	51.05±6.23	$p = 0.0011$	46.55±2.43	$p = 0.0051$
	HIV-infected patients	39.52±1.73		55.08±4.62		39.80±4.98		49.78±1.92	
CD8+	Healthy controls	40.67±3.31	$p = 0.3154$	54.80±4.39	$p = 0.6842$	52.34±7.17	$p = 0.0031$	45.39±6.15	$p = 0.0310$
	HIV-infected patients	38.94±0.69		55.11±3.39		47.33±7.23		48.21±5.16	

Table S3 Correlation between IRS methylation and viral load (VL); *ex vivo* study.

Spearman	CD4+ T cells		CD8+ T cells	
Correlation	R	p-value	R	p-value
LINE-1 vs. VL	−0.0909	0.8113	0.5333	0.1475
Alu vs. VL	−0.2970	0.4069	0.0909	0.8113
HERV-E vs. VL	−0.7000	0.0433	0.3333	0.3487
HERV-K vs. VL	0.3212	0.3697	0.1636	0.6567

Table S4 HERV methylation (mean ± SD) and p24 Ag in HIV-1 infected PBMCs; *ex vivo* study.

Day	% HERV-K methylation	% HERV-E methylation	p24 Ag level of HIV-1 infected PBMCs
0	58.18 ± 9.02	65.16 ± 8.74	0.00
4	52.56 ± 9.59	69.39 ± 5.55	13.95
7	53.13 ± 2.50	59.80 ± 5.94	39.87
10	58.70 ± 4.28	59.35 ± 7.57	43.20
14	59.20 ± 6.34	59.67 ± 12.06	15.30

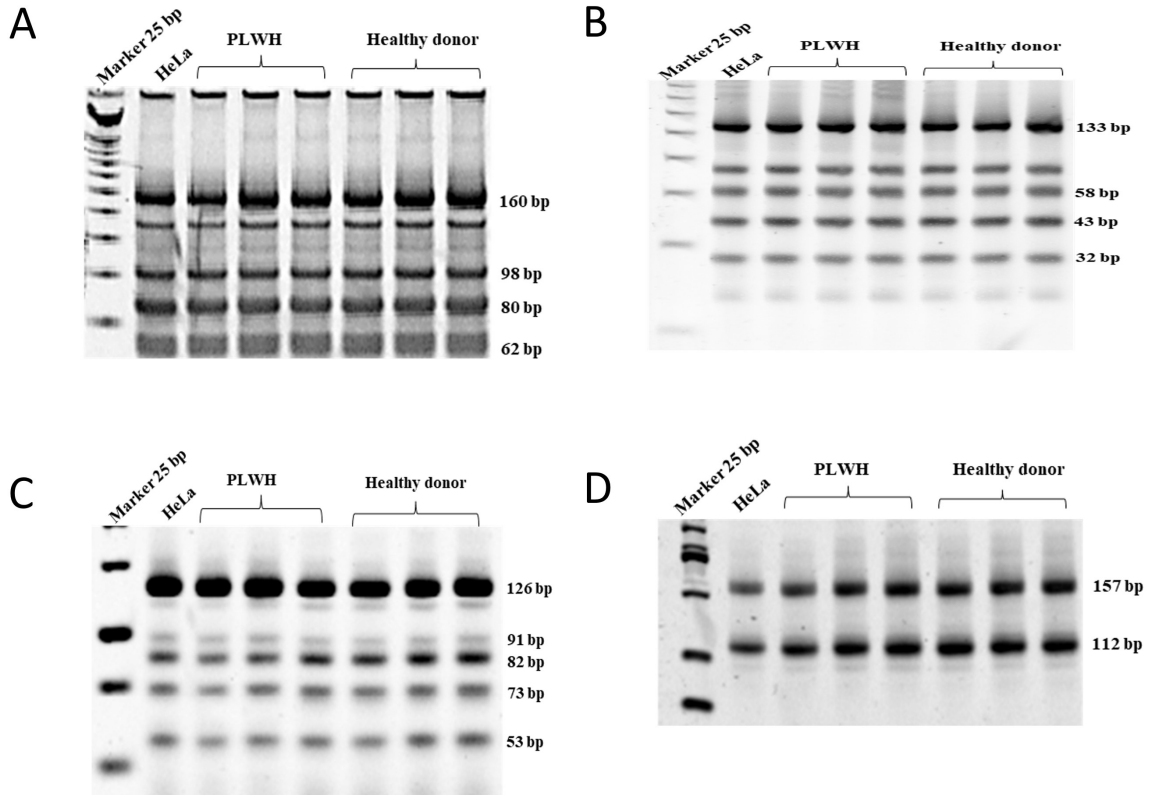

Fig. S1 Representative gel electrophorheses of IRS qCOBRA. The gel images depict enzyme-digested product fragments in LINE-1 (A), Alu (B), HERV-E (C) and HERV-K (D). HeLa DNA was used as control.