Published in final edited form as:

J Immunoassay Immunochem. 2017 ; 38(3): 299–307. doi:10.1080/15321819.2016.1260587.

Immunoassay and molecular methods to investigate DNA methylation changes in peripheral blood mononuclear cells in HIV infected patients on cART

Adelina Rosca^{1,2}, Gabriela Anton², Luminita Ene³, Iulia Iancu², Aura Temereanca^{1,2}, Cristian L. Achim^{4,*}, and Simona M. Ruta^{1,2,*}

¹Virology Chair, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania

²Stefan S. Nicolau Virology Institute, Bucharest, Romania

³Victor Babes Hospital for Infectious and Tropical Diseases, Bucharest, Romania

⁴University of California, San Diego, USA

Abstract

This study aimed to investigate the influence of antiretroviral therapy on methylation markers, in a group of HIV infected, heavily treated patients. Immune and molecular methods were used to investigate potential changes in methylation profile in DNA isolated from peripheral blood mononuclear cells collected from antiretroviral-experienced HIV infected patients and healthy controls. The percentage of 5-methylcytosine was inversely correlated with proviral DNA and active replication while DNMT1 ($p=0.01$) and DNMT3A ($p=0.004$) independently correlated with active viral replication. DNMT3A expression increased with total treatment duration ($p=0.03$), number of antiretroviral drugs ever used ($p=0.003$) and cumulative exposure to protease inhibitors ($p=0.02$) even in currently HIV undetectable patients.

Keywords

DNMT1; DNMT3A; HIV; Antiretroviral treatment; DNA methylation; Epigenetics; PBMC

INTRODUCTION

Among the epigenetic mechanisms regulating gene expression, DNA methylation - conversion of cytosine residues to 5-methylcytosine (5-mC) - plays a central role. Maintenance of specific tissue and cell methylation patterns is made through a dynamic process dependent on DNA methyltransferases like DNMT1 (the maintenance enzyme) or DNMT3A/3B (which adds methyl radicals de novo)¹. DNA methylation has been shown to

Corresponding author: Simona Ruta, MD, PhD, Address: 285 Mihai Bravu Avenue, Bucharest 030304, Romania Phone: +40213242590, Fax: +40 213245770, simona@simonaruta.ro.

*Shared senior authorship.

Ethical approval:

All procedures performed on human participants were in accordance with the ethical standards of institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

play an increasingly important role in the normal cellular development ², while the aberrant DNA methylation has been involved in multiple human pathologies. Altered methylation was firstly involved in cancer etiology and progression ³⁴, but recent studies started to explore the role of these epigenetic changes in different pathologies like immune ⁵, neurocognitive ⁶⁷ or viral diseases ⁸⁹. Human viruses change the methylation patterns of host genome DNA ¹⁰¹¹ and in turn, the viral genome itself is prone to methylation. This happens particularly for viruses that integrate their genome into the cellular genome like retroviruses ¹². The Human Immunodeficiency Virus (HIV-1) modifies the cellular epigenome by introducing methyl radicals at the provirus integration sites ¹³ and induces DNMT expression in vitro, which contributes to hypermethylation of cellular genes promoters¹⁴. Moreover, viral proteins are capable to alter the normal methylation profile: in experimental models it was shown that nef uses AP-1 transcription factor to activate DNMT1 promoter ¹⁵ and tat induces DNMT1 and DNMT3A/3B overexpression in B cell lymphomas through specific miRNAs downregulation ¹⁶.

DNA methylation is generally considered a protective mechanism against viral infection ¹⁷, nevertheless methylation of proviral HIV genome contributes to latency establishment and maintenance and helps circumvent the immune response and antiretroviral drugs action ¹⁸.

Based on these observations, it was suggested that combinations of antiretrovirals and drugs targeting epigenetic mechanisms, including DNMT inhibitors, could lead to reactivation of latent viral genomes and elimination of HIV reservoirs ¹⁴. Highlighting new pathways and mechanisms is invaluable for finding new approaches to diagnosis and treatment of human diseases¹⁹.

The aim of the present study was to investigate the influence of antiretroviral therapy on methylation markers in a group of HIV infected, heavily treated patients.

MATERIALS AND METHODS

Study population

Peripheral blood samples were collected on EDTA from 81 HIV-positive individuals parenterally infected in their early childhood with F1 subtype and 18 healthy age and sex matched controls. The demographic and clinical characteristics of study participants are presented in Table 1. Total duration of therapy was expressed in months from the first exposure to antiretroviral treatment (including mono- or dual therapy). Treatment interruptions were not included in the total duration of antiretroviral therapy (ART).

No opportunistic infections were registered at the time of the study. HCV co-infection rate was very low, only 3 (3.7%) patients had anti-HCV antibodies, 30 (37%) were HBsAg chronic carriers but only 5 (5.74%) had HBV DNA over 20,000 IU/mL.

Written informed consent was obtained from all participants and the study was approved by the ethics committee of Stefan S Nicolau Institute of Virology.

PBMC isolation and DNA isolation

Peripheral blood mononuclear cells (PBMC) were isolated within 4 hours of blood collection by density gradient centrifugation (Biochrome AG, Berlin, Germany). Genomic DNA was extracted from PBMC using a method adapted from Miller et al.²⁰ and stored at -20°C until further use. Nucleic acids quality (DNA and RNA) was evaluated by BioSpectrometer measurement (Eppendorf, Hamburg, Germany) at 260 and 280 nm.

HIV RNA and CD4+ cells number quantification

HIV plasma viral load was determined with a commercial kit for nucleic acid amplification (COBAS TaqMan HIV-1 Test Version 2.0, Roche Molecular Systems, Branchburg, NJ, USA) with a linear range between 34 and 10⁶ copies HIV RNA/mL.

CD4+ cell numbers were assessed by flow cytometry (BD Tritest CD4/CD8/CD3, Becton Dickinson, San Jose, CA, USA).

HIV DNA measurement

Integrated HIV DNA load was measured by ddPCR, using HIV-gag specific primers, in total DNA extracted from PBMC collected from HIV+ participants with undetectable viremia. Results were expressed as number of HIV DNA copies/10⁶ PBMC.

Quantification of 5-Methylcytosine in DNA

The percent of 5-methylcytosine (5-mC) was determined using the 5-mC DNA ELISA kit (Zymo Research Corp., Irvine, CA, USA) as per the manufacturer's instruction. Briefly, the protocol consists in thermic denaturation of 100 ng of genomic DNA, incubation with anti-5-mC antibodies followed by incubation with secondary antibody- HRP labelled and color development in the presence of substrate. Absorbance was measured at 450 nm using a HumaReader HS microtiter plate reader (Human, Wiesbaden, Germany). The percent of 5-mC for each sample was calculated using the regression equation of the second order obtained from the standard curve constructed by using positive and negative controls - provided by the manufacturer - processed in the same manner as the samples.

Gene expression quantification

Total RNA was extracted from PBMC using the PureLink RNA Mini kit (Ambion, Life Technologies, Darmstadt, Germany) and cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to producers instructions. cDNA amplification was performed on an Applied Biosystems 7300 cyclor (Applied Biosystems, Foster City, CA, USA) with FastStart Universal SYBR Green Master (Roche Diagnostics, Mannheim, Germany) under the following conditions: 95 °C for 10 minutes, and 40 cycles at 95 °C for 15 seconds and 60 seconds at 60 °C, then a dissociation melting curve was constructed in the range of 60–95 °C. Relative gene expression was calculated by the Ct method. Ct value of GAPDH was subtracted from the gene of interest Ct value for each sample (Ct), then the average of healthy controls was subtracted (Ct) and the relative expression ratio was calculated (2^{-Ct}). The used primers were: DNMT1 (Fw 5'-CGGTATACCCACCATGACA-3'; R 5'-

AGGCTTTGCCGCTTCC-3'), DNMT3A (Fw 5'-GGGAGGCACTTGACACCG-3'; R 5'-GCTCCACCTGGCGCTG-3') and GAPDH (Fw 5'-CATCTTCCAGGAGCGAGATCCCT-3'; R 5'-TGAGCCCCAGCCTTCTCCATGGT-3').

Statistical analysis

All statistical analyses were performed using SPSS v.20.0.0 for Windows. Correlations between non-normally distributed continuous variables were assessed by Spearman's rho. Differences between groups were evaluated using Mann-Whitney test. Linear regression was used to test for independent association between continuous variables. Binary regression was used to find independent predictors for dichotomized variables (ART duration under and above group median; HIV plasma RNA detectable and undetectable). $P < 0.05$ was considered statistically significant for all analyses.

RESULTS

Global methylation in healthy controls and HIV positive patients

Although a higher percent of methylation was observed for HIV participants compared to healthy controls (median [IQR]: 14.65% [13.07–14.65] vs. 19% [16.47–23.76]), the difference in 5-mC % was not statistically significant ($p = 0.23$).

In HIV undetectable study participants, higher 5-mC level was correlated with lower number of gag copies/ 10^6 cells ($p = 0.03$, $\rho = -0.52$) and less time with detectable HIV viremia ($p = 0.03$, $\rho = -0.46$) measured in number of days with detectable HIV RNA in plasma.

A significant ($p = 0.003$) linear regression analysis showed that both cDNA level in PBMC ($p = 0.005$, OR: -0.60; 95% CI: -0.03; -0.008) and days with detectable HIV RNA ($p = 0.006$; OR: -0.58; 95% CI: -0.006; -0.001) were independent predictors for 5-mC% in currently undetectable HIV viremia participants.

HIV viral load influence on expression of enzymes involved in methylation patterns

Significant differences in DNMT1 and DNMT3A expression were found between HIV patients with and without active viral replication. DNMT3A expression was higher (0.32 [0.05; 0.72] vs. 0.03 [-0.24; 0.28], $p = 0.006$), while DNMT1 was lower (-0.88 [-1.51; 0.19] vs. 0.14 [-0.64; 0.36], $p = 0.03$) in patients with detectable viral load (>34 HIV RNA copies/mL) compared with those with undetectable viremia (Figure 1a).

A highly significant ($p < 0.0001$) binary logistic model revealed that both DNMT3A ($p = 0.004$; OR: 6.30; 95% CI: 1.79–22.15) and DNMT1 ($p = 0.01$; OR: 0.44; 95% CI: 0.22–0.85) were independently correlated with active viral replication.

For those participants who had currently undetectable viremia, DNMT3A expression was correlated with the time spent with detectable HIV RNA ($p = 0.04$, $\rho = 0.27$).

ART influence on methylation markers

Higher DNMT3A levels were present in patients treated for a duration exceeding the group median- 122.87 months- (0.27 [0.02; 0.42] vs. -0.001 [-0.26; 0.15], $p = 0.005$) (Figure 1b)

and patients with multiple therapeutic regimens (0.15 [−0.19; 0.42] vs. −0.08 [−0.50; 0.04], $p=0.01$) when compared with patients treated for less than 122.87 months or on first ART regimen.

DNMT3A expression increased with the total ART duration ($p=0.03$, $\rho=0.24$), with the number of ARVs ever used ($p=0.003$, $\rho=0.32$) and with the cumulative exposure to PI ($p=0.02$, $\rho=0.25$). Further, these correlations were maintained in currently undetectable HIV patients.

DNMT1 expression levels, increased with longer duration of current therapeutic regimen ($p=0.02$, $\rho=0.26$), but was not influenced by total treatment duration.

No direct association was found between DNMT1 and DNMT3A or between DNMTs and participant's gender, age, CDC clinical classification or viral infection duration.

DISCUSSION

HIV has been previously reported to increase global methylation in experimental models²¹ and to induce the expression of DNMT1 and DNMT3A both in vitro¹⁵ and in vivo¹⁶. While methylation of the integrated viral genome and its relation to host gene expression has been extensively studied in experimental models¹⁸, there are few in vivo studies centered on methylation patterns and the results are often contradictory, mostly because of the great diversity of the clinical samples.

We analyzed the relationship between expression of genes that regulate DNA methylation (DNMT 1 and 3A) and HIV infection markers in patients with long-term exposure to antiretrovirals. To our knowledge this is the first study to evaluate the global DNA methylation in PBMCs (one of the main targets of HIV infection in latency compartments), collected from subjects with well-documented ART history. We found an inverse correlation between 5-mC% and the amount of HIV integrated DNA and the total time spent with detectable viral load, that was maintained in participants without current active viral replication, probably reflecting the link between methylation and maintenance of viral latency, described in previous studies²². We found a trend toward higher methylation in HIV patients compared with healthy controls, in accord with previous studies²¹, but without statistical significance, likely due to the small sample size of controls, as well as to the high number of HIV infected patients with undetectable viral load and good immune status.

HIV replication can enhance the expression of DNMT3A, involved in de novo methylation, in agreement with previous studies¹⁶ that showed upregulation of DNMT3A/3B expression in HIV positive tumors due to direct action of specific miRNAs that target DNMTs, like miR-29 family. We have previously shown²³ that increased miR-29a expression correlates with low HIV viral load, so miRNA action could indeed explain the relation we found here between DNMT3A and HIV viremia.

The inverse relation we found between DNMT1 and HIV viral load is in agreement with the reduced DNMTs activity observed in epithelial cells collected from ARV (antiretroviral) treated HIV individuals²⁴ but diverge from previous reports²⁵ of increased DNMT

expression in HIV presence, further supporting the notion that methylation patterns are cell specific and that the mechanisms observed in vitro do not always reflect the in vivo findings.

In our study, the DNMT3A expression was influenced by the total ART duration, as well as by the number of ARV drugs used over time; the main contributor appears to be the exposure to protease inhibitors. These findings are supported by previous studies that observed an accumulation of methylation in integrated viral genomes, in long-term-treated HIV patients ²² or a hypomethylating effect of atazanavir, a protease inhibitor, on AluYb8 repetitive element in infants exposed to ART in utero ²⁶.

Overall, the current study shows long-term antiretroviral therapy alters DNA methylation and the expression of DNMT1 and 3A.

Acknowledgments

Funding

This work was supported through the project entitled “Long term effects of chronic HIV infection on the developing brain”, grant no MH094159 from National Institute of Health

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Figure 1.

Expression of enzymes involved in maintaining methylation patterns in HIV patients. a. Box plot representing DNMT3A and DNMT1 expression in patients with detectable (HIV VL DET), n=24 and undetectable (HIV VL ND), n=57 HIV plasma viral load. Differences between groups were analyzed by Mann-Whitney test. b. Box plot representing DNMT3A and DNMT1 relative expression in patients treated for more (n=41) and less (n=40) than group median ART duration (122.87 months). Differences between groups were analyzed by Mann-Whitney test.

Table 1

Demographic and clinical characteristics of study participants

	HIV positive study participants (n=81)	Healthy controls (n=18)
Median age years; (IQR)	24 (23–25)	21.5 (21–22.75)
Males, n (%)	40 (49.4%)	7 (38.9%)
Median nadir CD4 cells/mm ³ ; (IQR)	85 (23–202)	n/a
Median CD4 cells/mm ³ ; (IQR)	574 (316–779)	n/a
Current CD4<500, n (%)	35 (43.2%)	n/a
Median HIV plasma viral load log ₁₀ copies/mL (IQR)	1.64 (0–3.40)	n/a
Patients with detectable plasma viral load, n (%)	24 (29.6%)	n/a
Median time on detectable HIV RNA, days (IQR)	1132.5 (271.25–2736.25)	n/a
Median HIV infection duration years (IQR)	23.81 (22.83–24.71)	n/a
Median current ART duration months; (IQR)	29.30 (13.37–64.52)	n/a
Median total ART duration months; (IQR)	122.87 (75.41–164.96)	n/a
Participants with more than 122.87 months on ART, n (%)	41 (50.6%)	n/a
Median cumulative exposure to NRTI, days (IQR)	256.91 (194.58–312.37)	n/a
Median cumulative exposure to NNRTI, days (IQR)	50.75 (26.80–77.27)	n/a
Median cumulative exposure to PI, days (IQR)	165 (95.50–221)	n/a
Current ART regimen type		
2 NRTI + PI	46 (56.8%)	n/a
2 NRTI + NNRTI	11 (13.6%)	n/a
NRTI + NNRTI + PI	10 (12.3%)	n/a
Other regimens	14 (17.3%)	n/a
CDC clinical staging, n (%)		
A	1 (1.2%)	n/a
B	34 (42%)	n/a
C	46 (56.8%)	n/a

IQR: interquartile range. NRTI: Nucleoside reverse transcriptase inhibitors. NNRTI: Non-nucleoside reverse transcriptase inhibitors. PI: Protease inhibitors. n/a: not applicable.