MicroRNA expression profiling of intestinal mucosa tissue predicts multiple crucial regulatory molecules and signaling pathways for gut barrier dysfunction of AIDS patients

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Abstract. Human immunodeficiency virus-1 (HIV-1) infection severely damages the gut-associated lymphoid tissue (GALT), the immune system and the gut barrier, which leads to accelerating the disease progression for patients with acquired immune deficiency syndrome (AIDS). Dysregulation of microRNAs (miRNAs) may contribute to this process. However, few studies have investigated the importance of miRNAs in AIDS pathogenesis and progression. The whole miRNA profile of patients with HIV infection from southwest P.R. China and the mode of interaction between HIV-1 and miRNAs remains to be elucidated. Colon mucosal samples were collected from HIV$^+$ patients and HIV$^-$ healthy individuals, miRNAs were isolated and subjected to array hybridization in the present study. A total of 476 human and virus-derived microRNAs were significantly altered in the HIV$^+$ group when compared with the control group ($P<0.05$), which may be involved in the progression to AIDS. Target genes of the significantly altered miRNAs were predicted using the TargetScan, miRbase and miRanda databases and the 10 shared target genes of upregulated miRNAs and the 391 target genes of downregulated miRNAs were selected. As only 10 target genes were predicted for upregulated miRNAs, subsequent GO and KEGG pathway analyses were focused on the 391 target genes of the downregulated miRNAs. The findings of the present study identified a series of crucial pathways, including cell-extracellular matrix interaction and chemokine regulation, which indicated close affinity with CD4$^+$ T cell activation. These pathways, involving genes such as integrin α5, led to a gut barrier dysfunction of patients with HIV. Important miRNAs include hsa-miRNA-32-5p, hsa-miRNA-195-5p, hsa-miRNA-20b-5p, hsa-miRNA-590-5p. The expression levels of the miRNAs and their target genes were confirmed using RT-qPCR. Taking into previous observations, the findings of the present study identified the importance of miRNAs for regulating gut barrier dysfunction via multiple regulatory molecules and signaling pathways, which elucidated the underlying molecular mechanism of gut barrier dysfunction in patients with HIV.

Introduction

Human immunodeficiency virus (HIV) infection leads to a progressive decline of immunity and eventually results in the initiation and progression of acquired immune deficiency syndrome (AIDS). The progressively weakened immune system of patients with AIDS increases the incidence of opportunistic infections and leads to higher mortality of patients with AIDS. CD4$^+$ T cells are the targets of invading HIV-1; therefore, the counts of CD4$^+$ T cells are used to evaluate the disease stage for patients with AIDS (1). Additionally, gut-associated lymphoid tissue (GALT) is the biggest lymphoid tissue of the human body, which provides the specific anatomical and physiological environment required by CD4$^+$ T cells and other immune cells (2). GALT is also the primary target of HIV-1 infection; therefore, GALT was previously identified as the potential reservoir of HIV-1 due to the depletion of CD4$^+$ T cells observed (3-5). This was followed by damage in intestinal mucosa and disorders of gut micro-ecology, which accelerated disease progression in patients with AIDS (6-9). The intestinal immune dysfunction following HIV infection may injure the gut barrier, leading to gut bacterial translocation into the blood (10,11) and chronic

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immune activation (11-13). This may also increase the risks of other complications, such as cardiovascular disease, osteoporosis, neurodegeneration and metabolic disease (13). In view of the fact that the exhaustion of $CD4^+$ T cells from GALT was much earlier than that of peripheral blood for AIDS patients, $CD4^+$ T cell counts in GALT may be used as potential biomarkers for predicting the mortality rates in patients with AIDS (14). Highly active anti-retroviral therapy may improve $CD4^+$ T cell counts of patients with AIDS; however, the restoration of the immune system is challenging, particularly the counts of $CD4^+$ T cells in gut intestinal mucosa remain at low levels (5,15). Concurrently, this chronic inflammation may provide additional activated $CD4^+$ T cells as targets for HIV infection, subsequently accelerating AIDS progression and exacerbating the symptoms of the patient (7,14,16). Therefore, the treatment of gut barrier damage in patients with AIDS would improve the reconstructing of the immune system and reduce the diffusion of HIV-1 in patients with AIDS.

MicroRNAs (miRNAs) are a type of endogenous non-coding single-stranded RNAs with a length of 21 to 23 nucleotides. miRNAs regulate the gene expression by employing the mechanism termed RNA interference at the post-transcription level, which may lead to the degradation of the target mRNA or suppression of its translation (17). A previous study has predicted that miRNAs may regulate >50% of human protein-coding genes (18). miRNAs have important regulatory roles in different diseases by participating in various processes, such as cell proliferation and development (19,20). Dysregulation of miRNAs was also detected in the disturbance of cellular processes of diabetes and cancer progression (21). The function of miRNAs in the pathogenesis of AIDS has been previously illustrated in terms of the host cells aspect and the virus aspect (22-24). In the host miRNAs, miR-132 was identified to be upregulated following $CD4^+$ T cell activation, which increased HIV-1 replication in $CD4^+$ T cells (22). Conversely, miR-198 inhibited HIV-1 gene expression and replication (23). The HIV-1 tat protein also regulates miR-217 for the regulation of sirtuin 1, IκB kinase and phosphorylated nuclear factor-κB, subsequently inducing various effects on multiple pathways and cellular responses, such as p65-NFκB and AMPK signaling (24). In addition to the aforementioned endogenous miRNAs of host cells, HIV-1 also encodes two miRNAs. One is miR-N367, which suppresses the HIV-1 nef protein and inhibits the transcription of HIV-1, thus maintaining the virus at a latent stage in patients with HIV infection where they are termed long-term non-progressors (LTNPs) (25). The other miRNA is HIV1-miR-H1, which suppresses the expression of the apoptosis antagonizing transcription factor and the downstream B cell leukemia/lymphoma 2 and MYC proto-oncogene and downregulated the expression of cellular miR-149, which targets Viral Protein R of HIV-1, facilitating HIV-1 replication and impairing cellular responses to infection (26). Therefore, previous studies have identified the complicated networks of miRNAs in HIV-1 and host cells that influenced the pathogenesis and progression of AIDS. However, the interactions between the miRNAs that have been identified, remain to be elucidated. Therefore, identifying the miRNAs active in the intestinal mucosa of patients with AIDS may elucidate the networks of miRNAs for HIV-infected patients and identify potential novel mechanisms of miRNA regulation in patients with HIV.

The present study isolated RNA from colon biopsy samples of HIV^+ antiretroviral therapy-naïve (without therapeutic intervention) patients and HIV^- healthy individuals. The samples were subjected to miRNA array hybridization. MiRNAs with significantly different expression levels were identified between the HIV/AIDS and control groups with a threshold of $P<0.05$. The target genes of the significantly different miRNAs were predicted and subsequent GO and KEGG pathway analyses were used to predict the potential signal pathways that may be associated with gut barrier dysfunction of patients with HIV infection and the potential disease progression. RT-qPCR was performed in order to verify the expression of the significantly expressed genes, which modulated the gut barrier dysfunction of patients with HIV infection.

Materials and methods

Patients. Colon biopsy samples from a total of 26 male participants aged 27-53 years were collected by electron endoscopy from the First Affiliated Hospital of Kunming Medical University (Kunming, China) between January 2013 and January 2014 and informed consent was obtained. The HIV-infected antiretroviral therapy-naïve group contained 3 participants and other 3 healthy individuals were assigned to control group. Additional colon biopsies from 10 HIV-infected patients and 10 healthy control individuals were performed. The samples were immediately cryopreserved for transcriptional analysis. Peripheral blood samples were also collected at the time of the endoscopy. Detailed information on the clinical characteristics of the participants are presented in Table I. HIV-infected individuals enrolled in the current study if they adhered to the following criteria: Antiretroviral therapy naïve; viral load of >10,000 HIV-1 RNA copies/ml of plasma; and period for infection with HIV-1 >1 year. The HIV-1 patients with current opportunistic or other infections were excluded from the present study. The procedures in the current study were approved by the Ethics Committee Review Board of Kunming Medical University.

Total RNA extraction and quality inspection. Total RNA from each colon tissue sample was isolated with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and purified with miRNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocols. RNA quality and quantity were quantified using a NanoDrop spectrophotometer at a wavelength of 280 nm (ND-1000; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). RNA integrity was determined by gel electrophoresis (1% agarose). Subsequently, the isolated RNA was purified using RNeasy Mini kit (Qiagen, Inc.) and the purity was examined with NanoDrop ND-1000.

RNA labeling and array hybridization. Following quality control, the miRCURY Hy3/Hy5 Power labeling kit (Exiqon, Vedbæk, Denmark) was used for miRNA labeling according to the manufacturer's protocol. Subsequently, Hy3-labeled samples were hybridized on the miRCURY LNA Array (v.18.0; Exiqon) according to the array manual. The total mixture with hybridization buffer was hybridized to the microarray in a 12-Bay Hybridization system (hybridization

Table I. Patient data and clinical parameters of male Chinese HIV+ patients and normal individuals.

ID	HIV status	CD4+ cell count, cells/μl)	Viral load, copies/ml	Age, years	Duration of infection, years
N78	HIV-	836	NA	29	NA
N121	HIV-	921	NA	47	NA
N324	HIV-	865	NA	31	NA
Y130	HIV+	217	1790	36	4
G45	HIV+	414	1326	30	1
W72	HIV+	205	2204	30	2
N-1	HIV-	664	NA	27	NA
N-2	HIV-	567	NA	27	NA
N-3	HIV-	681	NA	29	NA
N-4	HIV-	688	NA	32	NA
N-5	HIV-	696	NA	28	NA
N-6	HIV-	718	NA	34	NA
N-7	HIV-	643	NA	33	NA
N-8	HIV-	1089	NA	30	NA
N-9	HIV-	810	NA	31	NA
N-10	HIV-	769	NA	38	NA
P-1	HIV+	106	2088	33	1
P-2	HIV+	201	1557	41	5
P-3	HIV+	206	1432	38	3
P-4	HIV+	368	2421	33	3
P-5	HIV+	140	1805	38	2
P-6	HIV+	222	1717	37	2
P-7	HIV+	231	1314	43	5
P-8	HIV+	440	1022	51	4
P-9	HIV+	117	1840	53	3
P-10	HIV+	225	1950	45	3

NA, not applicable; HIV, human immunodeficiency virus.

system; NimbleGen Systems, Inc., Madison, WI, USA), which provided an active mixing action and a constant incubation temperature to improve hybridization uniformity and enhanced the signal. Following hybridization, the slides were washed three times using Wash buffer kit (Exiqon) and scanned using the Axon GenePix 4000B microarray scanner (Molecular Devices, LLC, Sunnyvale, CA, USA). GeneChip microarray experiments were conducted by KangChen Biotech (Shanghai, China).

Microarray data analysis. Scanned images were imported into GenePix Pro version 6.0 software package (Molecular Devices, LLC) for grid alignment and data extraction. Replicated miRNAs were averaged and miRNAs with intensity ≥30 in all samples were selected for the calculation of the normalization factor. Expressed data were normalized using the median normalization. Significantly differentially-expressed miRNAs between the two groups were identified through fold-change and P<0.05. A cut-off of >2.0 fold-change of gene expression was used to identify miRNAs for analysis of hierarchical clustering patterns and downstream biofunctional assessment. The difference between two groups, evaluated by P-value was calculated based on a Student's t-test. Hierarchical clustering was performed to identify distinguishable miRNA expression profiling among samples. A >2.0 fold-change and P<0.05 cut-offs were selected to analyze gene expression data (27), obtain sufficient information and generate a large data set to be used for downstream pathway analysis.

Bioinformatics analysis. To predict the target genes of the differentially-expressed miRNAs, the 3 most popular databases, TargetScan (28), miRanda (29) and miRDB (30) were fully referenced. In order to reduce false positive results, genes which were predicted by all 3 databases were selected as differential miRNA targets for further analysis. The miRNA expression changes were arranged from high to low according to the standardized intensity and fold-changes. The biological functions, including biological processes (BP, pathways and larger processes made up of the activities of multiple gene products), cellular compounds (CC, where gene products are active) and molecular function (MF, molecular activities of gene products) for the potential target genes, were analyzed using Gene Ontology (GO; www.geneontology.org) terms. GO defines concepts/classes used to describe gene function and

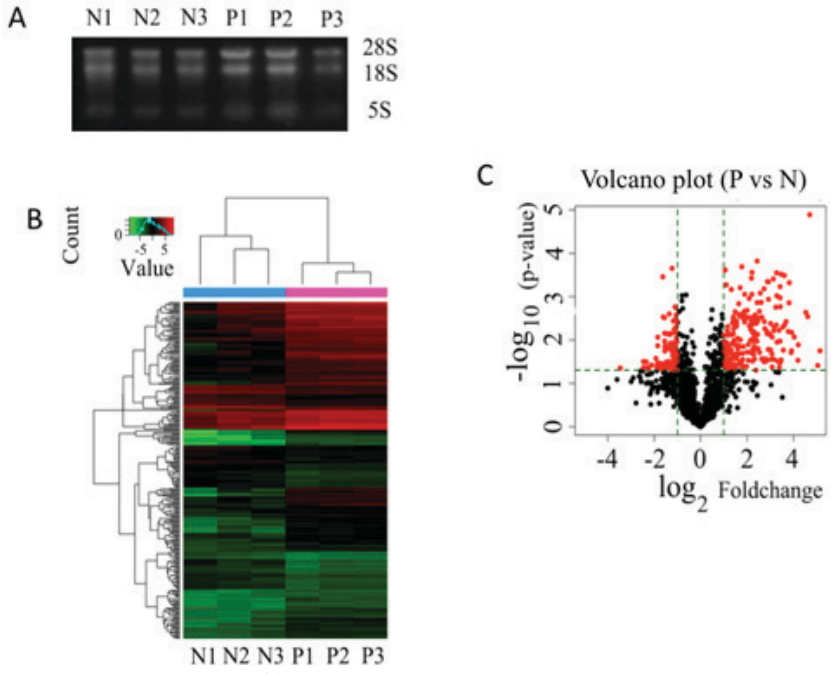


Figure 1. (A) RNA gel electrophoresis revealed clear 28S, 18S and 5S bands for all six samples, indicating good quality isolated RNA. (B) Heat map shows the results of two-way hierarchical clustering of miRNAs and samples. Each row represents a miRNA and each column represents a sample. The color scale shown at the top illustrates the relative expression level of a miRNA. red, high relative expression level; green, low relative expression level; N1, N2 and N3, HIV^- samples; P1, P2 and P3, HIV^+ samples. (C) Volcano plot shows the distribution of the detected miRNAs. The x-axis $\log_2$ (a fold-change) indicates the magnitude of fold change of P group vs. N group, and the y-axis $\log_{10}$ (P-value) indicates the significance. Red dots represent the miRNAs that have a significant increase of decrease in the P group.

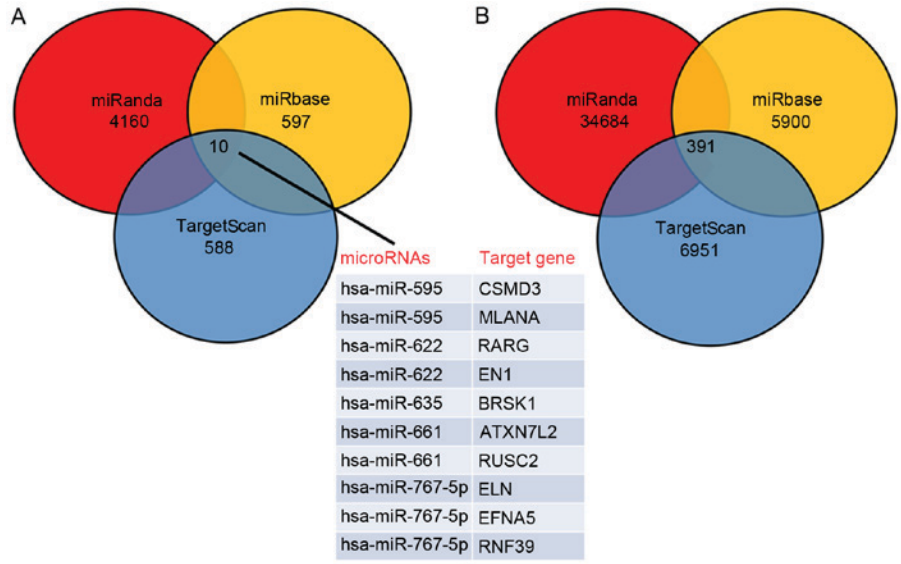
the relationships between these concepts. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was also performed with the target genes, which identified those most likely to be involved in intracellular signal transduction pathways. The major GO terms associated with BP and CC were manually summarized based on gene-term enrichment buttons provided for each functional group at P<0.05.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In order to confirm the expression profiles of key miRNAs, total miRNA was isolated from colon biopsies of 10 additional HIV^+ patients and 10 normal individuals that were used as negative control. Poly-A tails were added to the miRNAs and reverse transcribed into cDNA with miRcute miRNA cDNA First-Strand Synthesis kit (Tiangen Biotech Co., Ltd., Beijing, China). For polyA tail addition, 1 μg RNA was mixed with 0.4 μl *E. coli* poly(A) polymerase (5 U/μl), 2 μl 10X poly(A) polymerase buffer and 4 μl 5X rATP solution for incubation at 37˚C for 60 min. For reverse transcription, the above polyA reaction mix was further mixed with 0.5 μl Quant RTase and other additives for incubation at 37˚C for 60 min. The quantity of miRNA was determined using a miRcute miRNA quantification kit (Tiangen Biotech Co., Ltd.). The forward primers for the miRNAs are as follows (U6 was used as miRNA reference): hsa-miR-199a-3 pF, 5'-CAGACAGTA GTCTGCACATTGGTTA-3'; hsa-miR-20b-5 pF, 5'-GCA AAGTGCTCATAGTGCAGGTAG-3'; hsa-miR-32-5 pF, 5'-CGCAGTATTGCACATTACTAAGTTG-3'; U6-F, 5'-CGA TACAGAGAAGATTAGCATGGC-3'.

The primers for the mRNAs are as follows: (Actin was used as mRNA reference): ITGA5-F, 5'-ACCCAGACCCTG CTCATCCA-3' and ITGA5-R, 5'-TGTGAATCGGCGAGA GTTTGTC-3'; FBN1-F, 5'-CGTGCACCCTATGCCAAG TT-3' and FBN1-R, 5'-GCATTCCTCAGTACCCCAGG-3'. The thermocycling conditions are as follows: 95˚C for 10 min; 45 cycles of 95˚C for 15 sec and 60˚C for 60 sec. The experiments were performed in triplicate with SYBR Green dye (Tiangen Biotech Co., Ltd.). Expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (31) and data were presented as the mean ± standard error. P<0.05 was considered to indicate a statistically significant difference.

Results

Differentially-expressed miRNA profiles in AIDS and healthy groups. In order to identify the miRNAs from the colon biopsy samples of AIDS patients and healthy controls, a 7th generation miRNA array containing 3,100 capture probes, covering all human, mouse and rat miRNAs annotated in miRBase 18.0, all viral miRNAs associated with these species and 25 miRPlus human miRNAs were used. The latter were proprietary miRNAs that were not found in miRBase. RNA quality was inspected prior to performing microarray hybridization. As presented in Fig. 1A, three bands were evident after RNA electrophoresis, denoting 28S, 18S and 5S RNA. Filtering was performed with microarray detection flags (presence or absence of signals), using thresholds of fold-change (≥2) and P<0.05 to identify differentially-expressed miRNAs, 257 miRNAs were upregulated and 219 miRNAs were downregulated (data not shown) in the HIV-infected patients compared with the HIV-negative normal individuals. A heatmap was also generated to display a two-way hierarchical



microRNAs	Target gene
hsa-miR-595	CSMD3
hsa-miR-595	MLANA
hsa-miR-622	RARG
hsa-miR-622	EN1
hsa-miR-635	BRSK1
hsa-miR-661	ATXN7L2
hsa-miR-661	RUSC2
hsa-miR-767-5p	ELN
hsa-miR-767-5p	EFNA5
hsa-miR-767-5p	RNF39

Figure 2. Predicted target genes of (A) downregulated and (B) upregulated miRNAs by overlapping three miRNA databases. miRNA, microRNA.

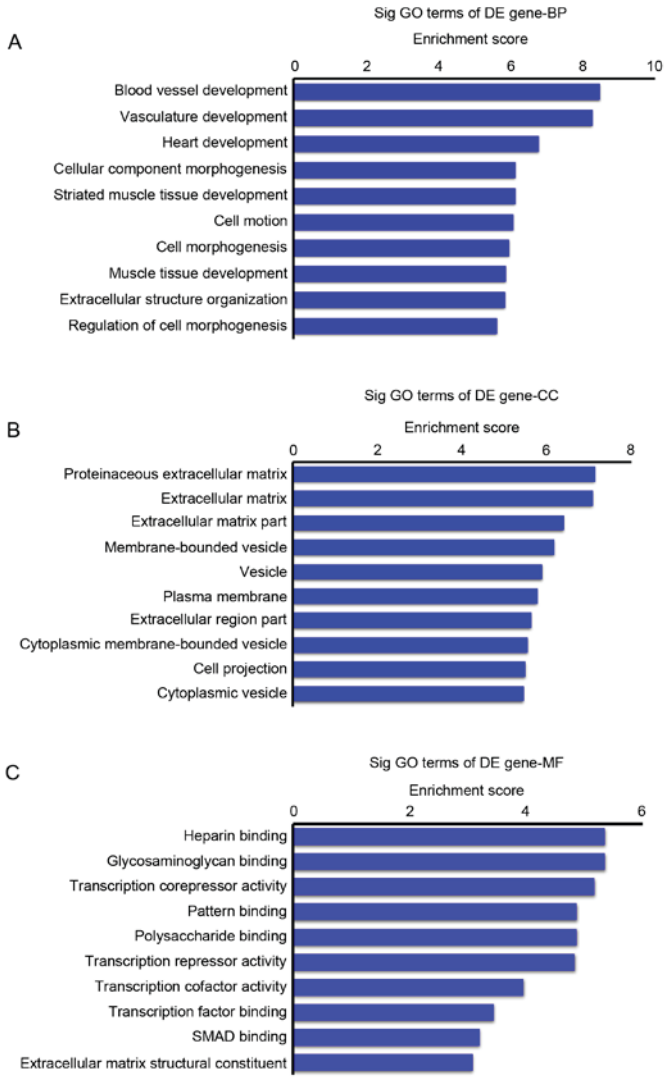


Figure 3. GO analysis of predicted target genes of upregulated microRNAs. Top 10 (A) BP, (B) CC and (C) MF according to enrichment score. GO, gene ontology; BP, biological processes; CC, cellular compounds; MF, molecular functions.

clustering of miRNAs and samples (Fig. 1B). The red color represents upregulated miRNAs, whereas green bars represent downregulated miRNAs. The three patients and the three control samples were grouped as different clusters, due to their similarities in miRNA expression profiles. A Volcano plot was also composed to visually identify the significantly differentially-expressed miRNAs between the two groups, based on the aforementioned thresholds (Fig. 1C). The red dots represent the miRNAs that had significantly altered expression level ($P<0.05$), either upregulated (the red dots to the right) or downregulated (the red dots to the left).

Prediction of target genes from differentially-expressed miRNAs. Functions of miRNAs were predicted by their target mRNAs; therefore, the putative target genes of differentially-expressed miRNAs between AIDS patients and healthy control individuals were predicted using the 3 aforementioned databases and the associated target genes were retrieved from all 3 databases. A total of 10 target genes of significantly downregulated miRNAs were predicted, including CSMD3, MLANA, RARG, EN1, BRSK1, ATXN7L2, RUSC2, ELN, EFNA5 and RNF39 (Fig. 2A). Additionally, 391 target genes of significantly upregulated miRNAs were also predicted (Fig. 2B).

GO analysis for potential target genes of upregulated miRNAs. GO analysis of these target genes, which were predicted according to the differentially-expressed miRNAs, was performed, including BP, CC and MF. As only 10 overlapping target genes of downregulated miRNAs were predicted through the 3 databases, GO analysis for these 10 genes was not performed and the present study focused on GO and KEGG pathway analysis based on the target genes of the upregulated miRNAs.

The target genes were sorted into various BP according to their enrichment scores. The top 10 BP were identified as follows: Blood vessel development, vasculature development, heart development, cellular component morphogenesis,

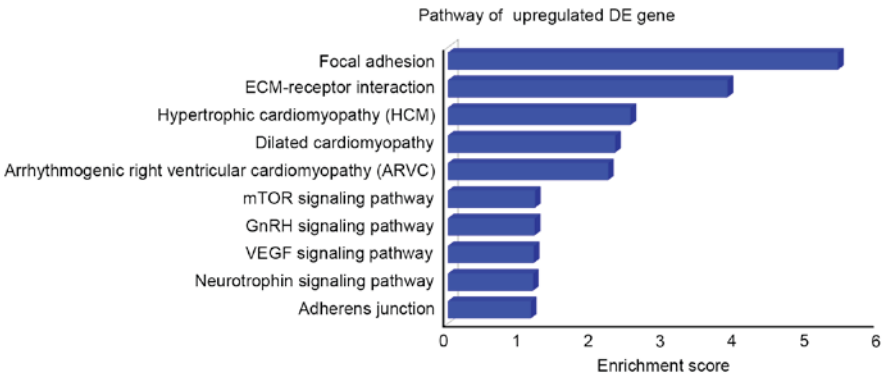


Figure 4. Kyoto Encyclopedia of Genes and Genomes pathway analysis based on the target genes of upregulated miRNAs. Top 10 signaling pathways according to enrichment score. ECM, extracellular matrix; mTOR, mechanistic target of rapamycin; GnRH, gonadotropin releasing hormone; VEGF, vascular endothelial growth factor.

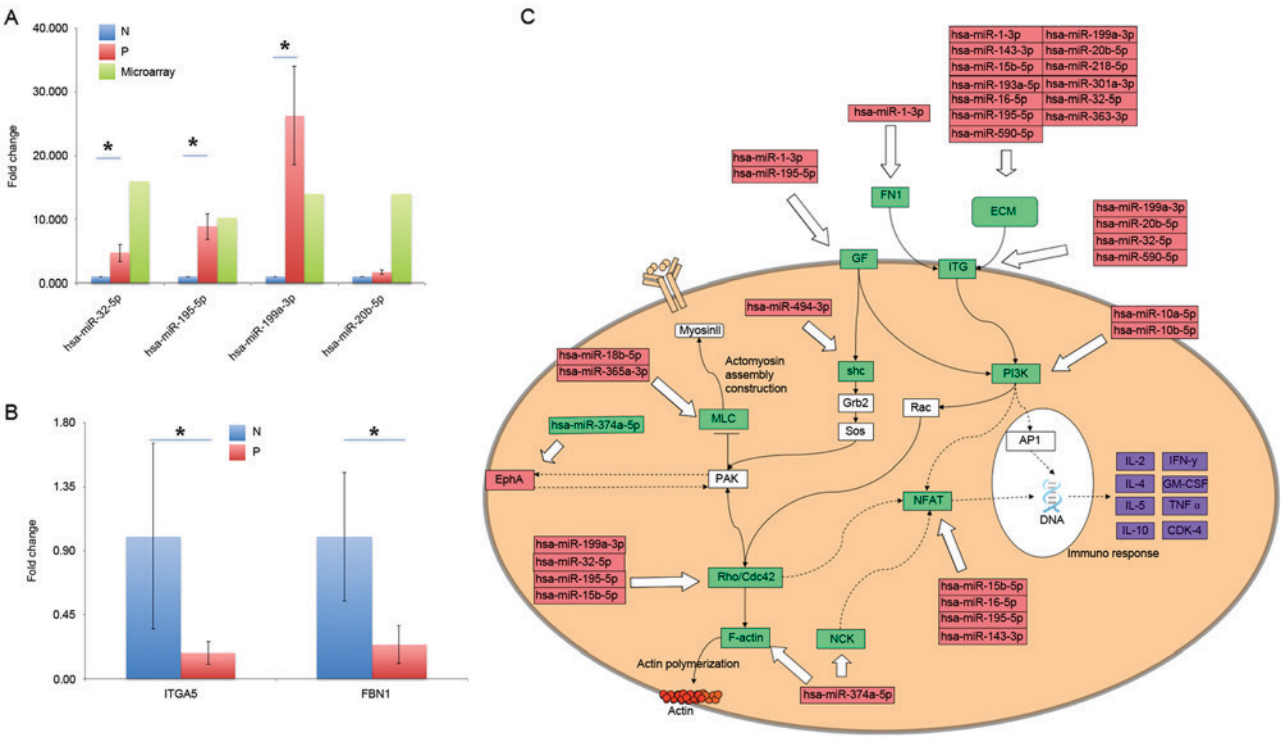


Figure 5. Confirmation of some miRNA and target gene expression and integrative pathway analysis. (A) Total miRNA was isolated from colon biopsies of 10 additional HIV$^+$ patients and 10 normal healthy individuals. The quantity of miRNA was determined using the fold-changes from the microarray also being shown for comparison. *P<0.05. (B) miRNA expression levels of two important putative genes were confirmed by RT-qPCR. Triplicates were performed for each sample. Expression levels were calculated using the $\Delta\Delta C_q$ method, and data are presented as mean ± standard error *P<0.05. (C) An integrative schematic illustration was used to identify the miRNAs, target genes and pathways that are affected or were predicted to be affected in the patients. These pathways involve altered signaling pathways induced by ECM-cell interaction and many of them converge at the regulation of immune response-associated cytokines and chemokines. miRNA, microRNA; ITGA5, integrin subunit a 5; FBN1, fibrillin 1; N, HIV$^-$ samples; P, HIV$^+$ samples.

striated muscle tissue development, cell motion, cell morphogenesis, muscle tissue development, extracellular structure organization and regulation of cell morphogenesis (Fig. 3A).

The predicted target genes of upregulated miRNAs were also sorted into various categories of CC according to their enrichment scores, with the top 10 CC categories being listed as follows: Proteinaceous extracellular matrix, extracellular matrix, extracellular matrix part, membrane-bounded vesicle, vesicle, plasma membrane, extracellular region part, cytoplasmic membrane-bounded vesicle, cell projection and cytoplasmic vesicle (Fig. 3B).

The GO analysis was used to sort the predicted target genes of upregulated miRNAs were into various categories of MF (Fig. 3C). The top 10 categories of MF were: Heparin binding, glycosaminoglycan binding, transcription corepressor activity, pattern binding, polysaccharide binding, transcription repressor activity, transcription cofactor activity, transcription factor binding, SMAD binding and extracellular matrix structural constituent.

KEGG pathway analysis of predicted target genes of upregulated miRNAs. KEGG pathway analysis was used to

analyze the pathways, which were most likely to be altered by upregulated miRNAs (Fig. 4). The top 10 signaling pathways identified for the predicted target genes of upregulated miRNAs according to their enrichment scores were focal adhesion, ECM-receptor interaction, hypertrophic cardiomyopathy, dilated cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy, mechanistic target of rapamycin signaling pathway, gonadotropin releasing hormone signaling pathway, vascular endothelial growth factor signaling pathway, neutrophin signaling pathway and adherens junction.

Confirmation of expression levels of key upregulated miRNAs. To confirm the expression levels of key miRNAs, RT-qPCR was performed with colon biopsies from 10 additional HIV-infected patients and 10 healthy control individuals. Normalized expression levels of AIDS patients, and the fold-changes from microarray data are presented in Fig. 5A. A total of 5 miRNAs were tested, 4 of which produced the consistent results with the microarray data. These miRNAs were hsa-miR-32-5p, hsa-miR-195-5p, hsa-miR-199a-3p and hsa-miR-20b-5p. The other miRNA, hsa-miR-590-5p, was upregulated in the samples of HIV-infected individuals from microarray data, but was demonstrated to be decreased in qPCR data. These miRNAs were selected as they were predicted to regulate some important genes that are implicated in intestinal mucosa dysfunction. For example, hsa-miR-32-5p was predicted to modulate the expression of integrin α5 (ITGA5), which has an important role in colon epithelium functions. All of them had high fold-changes of >10 in the P group, and the expression of some miRNAs, including hsa-miR-32-5p, hsa-miR-195-5p and hsa-miR-20b-5p was confirmed by RT-qPCR, had fold-changes of 4.764, 8.914, 1.712 and 26.290, respectively. Additionally, expression of some putative target genes was also confirmed. As presented in Fig. 5B, expression of ITGA5 and fibrillin 1 (FBN1), the two putative target genes of upregulated hsa-miR-32-5p, were downregulated. Although the error bars are large for some groups due to the variability between the samples, the difference between the groups was significant ($P<0.05$). These findings partially confirmed the accuracy of the microarray data and validated the further analysis performed.

Discussion

The present study identified a large number of miRNAs with altered expression levels in HIV-infected individuals compared with healthy individuals. The data in the current study revealed that the regulation of some miRNAs was consistent with previous studies, such as miR-132, which was reported to be upregulated in activated $CD4^+$ T cells and enhance HIV-1 replication (22). Some miRNAs activate $CD4^+$ T cells, thereby creating a favorable environment for HIV-1 replication (32,33), such as miR-21, miR-142-3p/5p, miR-155, miR-181a and miR-27b (34-40). Among them, miR-181a was capable of activating $CD4^+$ T cells by enhancing various associated pathways, including ERK and calcium flux (40). The importance of miR-181a was also confirmed by the present study with a 3-fold increase in expression in patients with AIDS, in association with $CD4^+$ T cell activation. Conversely, some inconsistencies were also detected between the current study and previous literature. For example, miR-142-3p/5p were downregulated in $CD4^+$ T cells from patients with systemic lupus erythematosus, which may also lead to the over activation of $CD4^+$ T cells via their targets, such as SAP, CD84, interleukin (IL)-10 (37). However, the higher levels of miR-142-3p in the present study suggested a possible deactivation of $CD4^+$ T cells. Additionally, lower levels of hiv1-miR-TAR-3p, an HIV-1-encoded miRNA, in HIV-infected individuals were observed, which may reveal that HIV-1 infection may trigger apoptosis. This was consistent previous studies of HIV-1 entry, facilitated by CD4 and CCR5 chemokine receptor. This elevated Fas levels in the cells and rendered the cells susceptible to apoptosis induced by the Fas/Fas ligand interaction (41).

Using criterions of fold change ≥2 and $P<0.05$ a total of 476 human and virus-derived miRNAs were identified as significantly altered in HIV/AIDS when compared with the control group ($P<0.05$). Among them, a lower number of target genes of downregulated miRNAs were predicted compared with the target genes of upregulated miRNAs (10 vs. 391). These 10 genes include retinoic acid receptor γ (RARG), an antagonist of RAR signaling. Deficiency in retinoic acid receptors α (RARα) may lead to defects in $CD4^+$ T cell activation (42), which implied that the retinoic acid pathway through RARα was essential for $CD4^+$ T cell effector responses. The reduced expression of some miRNAs, such as miR-622, may predict an increase in RARG. As a nuclear receptor, RARG shares functional similarities with other isoforms, such as RARα and RARβ (43). Therefore, the transcription of associated genes would be altered. Therefore, it is possible that RARG may also contribute to the promotion of $CD4^+$ T cell effector responses and acceleration of disease progression may occur, although definitive evidence is still required.

Although the expression patterns of some miRNAs were confirmed, their roles remain to be fully elucidated. As gene expression may be modulated by a broad range of biological factors, including other gene products, miRNAs, long non-coding RNA, prediction of changes in the expression of target genes only based on their regulator miRNAs may not be entirely reliable. By considering the aforementioned factors, GO and KEGG pathways analyses were performed, where the direction of the expression alterations (upregulation or downregulation) was not required. Therefore, the present study demonstrated pathways that are modulated by the differentially-expressed miRNAs. The GO and KEGG pathway analyses identified a number of pathways involved in heart development and cardiomyopathy development. Previous studies have determined that AIDS patients are more prone to be dilated cardiomyopathy and other heart diseases (44,45). The actin, a, cardiac muscle 1 (ACTC1) gene, which encodes cardiac muscle α actin and is responsible for heart muscle contraction, was regulated by miR-30a-5p, miR-32-5p and miR-363-3p. Some of the validated miRNAs had important functions in AIDS and associated diseases. For example, nuclear factor of activated T-cells 3 (NFATC3) was a predicted target of hsa-miR-195-5p. Upregulation of hsa-miR-195-5p reduced the NFATC3 mRNA level, which confirmed by the mRNA microarray data (not shown). This protein is a member of the nuclear factors of activated T cells and regulates the expression of various cytokines, including IL-2 and tumor necrosis factor-a (TNF-α; Fig. 5C. Therefore, NFATC3 and

its regulator hsa-miR-195-5p, are important for the immune response. Another important molecule is hsa-miR-32-5p, whose putative targets include RAB23, member RAS oncogene family, FBN1, Kruppel like factor 2 and ITGA5. These genes modulate multiple biological processes and components, such as the extracellular matrix. The expression for parts of these putative target genes were confirmed by RT-qPCR as presented in Fig. 5B.

Using the significantly changed miRNAs, the potential genes that may have crucial roles on regulating gut barrier dysfunction for HIV-infected patients were predicated using the DAVID comprehensive functional classification. Three pathways and an important immune response mediator, NFAT (Fig. 5C) were identified. A number of significantly upregulated miRNAs that may alter NFAT expression were identified, including miR-10a-5p (46). NFAT, present in the cytosol, was translocated to the nucleus by T cell receptor stimulation and became a member of the nuclear factors for activating T cells transcription complex (47). Due to the reduced NFAT expression, its translocation and regulatory functions may be severely impaired, leading to reduced levels of target cytokine expression, including IL-2, IL-4, IL-5, IL-10, interferon-γ, TNF-α. Their reduced expression may lead to autoimmune diseases and immune deficiency (48).

It is of note that various altered miRNAs were identified to affect components of the extracellular matrix (ECM). These ECM molecules, including fibronectin, bind to integrin subtypes and affect multiple cellular processes, including cell-cell and cell-matrix interaction, cell motility and signaling pathways (49). Actin polymerization was a prerequisite of highly concentrated CD4 and CXCR4 membrane receptors, which was determined to be essential for HIV propagation (50,51). Therefore, actin polymerization and cytoskeleton remodeling was hijacked by infected cells to facilitate HIV attack and AIDS progression. Conditions may be worsened with weakened adherens junctions regulated by some upregulated miRNAs, such as miR-10b-5p (52).

In conclusion, the present study provided a complete network of miRNAs with altered expression in HIV-infected individuals. Some of the alterations were consistent with those previously observed in literature (22,40). Target genes of the significantly different miRNAs were predicted using the TargetScan, mirBase and miRanda databases, and the shared target genes from the three databases were selected. GO and KEGG pathway analyses focused on the 391 downregulated target genes and predicted important pathways including cell-EMC interaction and chemokine regulation. Alterations in these pathways are closely associated with $CD4^+$T cell activation and reduction in chemokine levels. They also lead to gut barrier dysfunction of patients with HIV infection. miRNAs that potentially influence these target genes and pathways included hsa-miRNA-32-5p, hsa-miRNA-195-5p, hsa-miRNA-20b-5p and hsa-miRNA-590-5p. Expression of some miRNAs and target genes was confirmed with RT-qPCR. The present study identified the crucial roles of miRNAs in the regulation of the gut barrier dysfunction via multiple regulatory molecules and signaling pathways, which elucidated the underlying molecular mechanism for gut barrier dysfunction of HIV infection. The specific roles of these miRNAs in disease progression remain to be further investigated.

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References

1. Kelleher AD and Zaunders JJ: Decimated or missing in action: $CD4^+$ T cells as targets and effectors in the pathogenesis of primary HIV infection. Curr HIV/AIDS Rep 3: 5-12, 2006.
2. Vergnon-Miszczycha D, Lucht F, Roblin X, Pozzetto B, Paul S and Bourlet T: Key role played by the gut associated lymphoid tissue during human immunodeficiency virus infection. Med Sci (Paris) 31: 1092-1101, 2015 (In French).
3. Estes JD, Harris LD, Klatt NR, Tabb B, Pittaluga S, Paiardini M, Barclay GR, Smedley J, Pung R, Oliveira KM, *et al*: Damaged intestinal epithelial integrity linked to microbial translocation in pathogenic simian immunodeficiency virus infections. PLoS Pathog 6: e1001052, 2010.
4. Andrew AL, Mahesh M and Ronald SV: The gastrointestinal tract and AIDS pathogenesis. Gastroenterology 136: 1966-1978, 2009.
5. Chun TW, Nickle DC, Justement JS, Meyers JH, Roby G, Hallahan CW, Kottilil S, Moir S, Mican JM, Mullins JI, *et al*: Persistence of HIV in gut-associated lymphoid tissue despite long-term antiretroviral therapy. J Infect Dis 197: 714-720, 2008.
6. Heise C, Miller CJ, Lackner A and Dandekar S: Primary acute simian immunodeficiency virus infection of intestinal lymphoid tissue is associated with gastrointestinal dysfunction. J Infect Dis 169: 1116-1120, 1994.
7. Vyboh K, Jenabian MA, Mehraj V and Routy JP: HIV and the gut microbiota, partners in crime: Breaking the vicious cycle to unearth new therapeutic targets. J Immunol Res 2015: 614127, 2015.
8. Vujkovic-Cvijin i, Dunham RM, Iwai S, Maher MC, Albright RG, Broadhurst MJ, Hernandez RD, Lederman MM, Huang Y, Somsouk M, *et al*: Dysbiosis of the gut microbiota is associated with HIV disease progression and tryptophan catabolism. Sci Transl Med 5: 193ra91, 2013.
9. Lozupone CA, Li M, Campbell TB, Flores SC, Linderman D, Gebert MJ, Knight R, Fontenot AP and Palmer BE: Alterations in the gut microbiota associated with HIV-1 infection. Cell Host Microbe 14: 329-339, 2013.
10. Novati S, Sacchi P, Cima S, Zuccaro V, Columpsi P, Pagani L, Filice G and Bruno R: General issues on microbial translocation in HIV-infected patients. Eur Rev Med Pharmacol Sci 19: 866-878, 2015.
11. Vassallo M, Mercié P, Cottalorda J, Ticchioni M and Dellamonica P: The role of lipopolysaccharide as a marker of immune activation in HIV-1 infected patients: A systematic literature review. Virol J 9: 174, 2012.
12. Steele AK, Lee EJ, Vestal B, Hecht D, Dong Z, Rapaport E, Koeppe J, Campbell TB and Wilson CC: Contribution of intestinal barrier damage, microbial translocation and HIV-1 infection status to an inflammaging signature. PLoS One 9: e97171, 2014.
13. Hunt PW: Role of immune activation in HIV pathogenesis. Curr HIV/AIDS Rep 4: 42-47, 2007.
14. Hunt PW, Sinclair E, Rodriguez B, Shive C, Clagett B, Funderburg N, Robinson J, Huang Y, Epling L, Martin JN, *et al*: Gut epithelial barrier dysfunction and innate immune activation predict mortality in treated HIV infection. J Infect Dis 210: 1228-1238, 2014.
15. Asmuth DM, Pinchuk IV, Wu J, Vargas G, Chen X, Mann S, Albanese A, Ma ZM, Saroufeem R, Melcher GP, *et al*: Role of intestinal myofibroblasts in HIV-associated intestinal collagen deposition and immune reconstitution following combination antiretroviral therapy. AIDS 29: 877-888, 2015.

16. Nwosu FC, Avershina E, Wilson R and Rudi K: Gut microbiota in HIV infection: Implication for disease progression and management. Gastroenterol Res Pract 2014: 803185, 2014.
17. Rice AP: Roles of microRNAs and long-noncoding RNAs in human immunodeficiency virus replication. Wiley Interdiscip Rev RNA 6: 661-670, 2015.
18. Pasquinelli AE: MicroRNAs and their targets: Recognition, regulation and an emerging reciprocal relationship. Nat Rev Genet 13: 271-282, 2012.
19. Li LM, Wang D and Zen K: MicroRNAs in drug-induced liver injury. J Clin Transl Hepatol 2: 162-169, 2014.
20. Alvarez-Garcia I and Miska EA: MicroRNA functions in animal development and human disease. Development 132: 4653-4662, 2005.
21. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, *et al*: miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci USA 102: 13944-13949, 2005.
22. Chiang K, Liu H and Rice AP: miR-132 enhances HIV-1 replication. Virology 438: 1-4, 2013.
23. Sung TL and Rice AP: miR-198 inhibits HIV-1 gene expression and replication in monocytes and its mechanism of action appears to involve repression of cyclin T1. PLoS Pathog 5: e1000263, 2009.
24. Zhang HS, Wu TC, Sang WW and Ruan Z: MiR-217 is involved in Tat-induced HIV-1 long terminal repeat (LTR) transactivation by down-regulation of SIRT1. Biochim Biophys Acta 1823: 1017-1023, 2012.
25. Omoto S, Ito M, Tsutsumi Y, Ichikawa Y, Okuyama H, Brisibe EA, Saksena NK and Fujii YR: HIV-1 nef suppression by virally encoded microRNA. Retrovirology 1: 44, 2004.
26. Kaul CD, Ahlawat A and Gupta SD: HIV-1 genome-encoded hiv1-mir-H1 impairs cellular responses to infection. Mol Cell Biochem 323: 143-148, 2009.
27. Han ZB, Zhong L, Teng MJ, Fan JW, Tang HM, Wu JY, Chen HY, Wang ZW, Qiu GQ and Peng ZH: Identification of recurrence-related microRNAs in hepatocellular carcinoma following liver transplantation. Mol Oncol 6: 445-457, 2012.
28. Agarwal V, Bell GW, Nam JW and Bartel DP: Predicting effective miRNA target sites in mammalian mRNAs. Elife 4: e05005, 2015.
29. John B, Enright AJ, Aravin A, Tuschl T, Sander C and Marks DS: Human MicroRNA targets. PLoS Biol 2: e363, 2004.
30. Wang X: miRDB: A microRNA target prediction and functional annotation database with a wiki interface. RNA 14: 1012-1017, 2008.
31. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
32. Siliciano RF and Greene WC: HIV latency. Cold Spring Harb Perspect Med 1: a007096, 2011.
33. Huang J, Wang F, Argyris E, Chen K, Liang Z, Tian H, Huang W, Squires K, Verlinghieri G and Zhang H: Cellular microRNAs contribute to HIV-1 latency in resting primary CD4$^+$ T lymphocytes. Nat Med 13: 1241-1247, 2007.
34. Nathans R, Chu CY, Serquina AK, Lu CC, Cao H and Rana TM: Cellular microRNA and P bodies modulate host-HIV-1 interactions. Mol Cell 34: 696-709, 2009.
35. Triboulet R, Mari B, Lin YL, Chable-Bessia C, Bennasser Y, Lebrigand K, Cardinaud B, Maurin T, Barbry P, Baillat V, *et al*: Suppression of microRNA-silencing pathway by HIV-1 during virus replication. Science 315: 1579-1582, 2007.
36. Chiang K, Sung TL and Rice AP: Regulation of cyclin T1 and HIV-1 Replication by microRNAs in resting CD4$^+$ T lymphocytes. J Virol 86: 3244-3252, 2012.
37. Ding S, Liang Y, Zhao M, Liang G, Long H, Zhao S, Wang Y, Yin H, Zhang P, Zhang Q and Lu Q: Decreased microRNA-142-3p/5p expression causes CD4$^+$ T cell activation and B cell hyperstimulation in systemic lupus erythematosus. Arthritis Rheum 64: 2953-2963, 2012.
38. Stahl HF, Fauti T, Ullrich N, Bopp T, Kubach J, Rust W, Labhart P, Alexiadis V, Becker C, Hafner M, *et al*: miR-155 inhibition sensitizes CD4$^+$ Th cells for TREG mediated suppression. PLoS One 4: e7158, 2009.
39. Fenoglio C, Cantoni C, De Riz M, Ridolfi E, Cortini F, Serpente M, Villa C, Comi C, Monaco F, Mellesi L, *et al*: Expression and genetic analysis of miRNAs involved in CD4$^+$ cell activation in patients with multiple sclerosis. Neurosci Lett 504: 9-12, 2011.
40. Palin AC, Ramachandran V, Acharya S and Lewis DB: Human neonatal naive CD4$^+$ T cells have enhanced activation-dependent signaling regulated by the microRNA miR-181a. J Immunol 190: 2682-2691, 2013.
41. Pan Z, Radding W, Zhou T, Hunter E, Mountz J and McDonald JM: Role of calmodulin in HIV-potentiated Fas-mediated apoptosis. Am J Pathol 149: 903-910, 1996.
42. Hall JA, Cannons JL, Grainger JR, Dos Santos LM, Hand TW, Naik S, Wohlfert EA, Chou DB, Oldenhove G, Robinson M, *et al*: Essential role for retinoic acid in the promotion of CD4(+) T cell effector responses via retinoic acid receptor alpha. Immunity 34: 435-447, 2011.
43. Safi R, Muramoto GG, Salter AB, Meadows S, Himburg H, Russell L, Daher P, Doan P, Leibowitz MD, Chao NJ, *et al*: Pharmacological manipulation of the RAR/RXR signaling pathway maintains the repopulating capacity of hematopoietic stem cells in culture. Mol Endocrinol 23: 188-201, 2009.
44. Barbaro G, Di Lorenzo G, Grisorio B and Barbarini G: Incidence of dilated cardiomyopathy and detection of HIV in myocardial cells of HIV-positive patients. Gruppo italiano per lo studio cardiologico dei pazienti affetti da AIDS. N Engl J Med 339: 1093-1099, 1998.
45. Lewis W: Cardiomyopathy in AIDS: A pathophysiological perspective. Prog Cardiovasc Dis 43: 151-170, 2000.
46. Zhou X, Wang H, Burg MB and Ferraris JD: Inhibitory phosphorylation of GSK-3β by AKT, PKA, and PI3K contributes to high NaCl-induced activation of the transcription factor NFAT5 (TonEBP/OREBP). Am J Physiol Renal Physiol 304: F908-F917, 2013.
47. Macián F, López-Rodríguez C and Rao A: Partners in transcription: NFAT and AP-1. Oncogene 20: 2476-2489, 2001.
48. Brocker C, Thompson D, Matsumoto A, Nebert DW and Vasiliou V: Evolutionary divergence and functions of the human interleukin (IL) gene family. Hum Genomics 5: 30-55, 2010.
49. Leyme A, Marivin A, Perez-Gutierrez L, Nguyen LT and Garcia-Marcos M: Integrins activate trimeric G proteins via the nonreceptor protein GIV/Girdin. J Cell Biol 210: 1165-1184, 2015.
50. Liu Y, Belkina NV and Shaw S: HIV infection of T cells: Actin-in and actin-out. Sci Signal 2: pe23, 2009.
51. Blanco J, Bosch B, Fernández-Figueras MT, Barretina J, Clotet B and Esté JA: High level of coreceptor-independent HIV transfer induced by contacts between primary CD4 T cells. J Biol Chem 279: 51305-51314, 2004.
52. Sufiawati I and Tugizov SM: HIV-associated disruption of tight and adherens junctions of oral epithelial cells facilitates HSV-1 infection and spread. PLoS One 9: e88803, 2014.