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Expression of RNA-m^{6}A-related genes correlates with the HIV latent reservoir level and the CD4$^+$ and CD8$^+$T cell profiles of patients with AIDS

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ABSTRACT

The HIV latent reservoir is the main obstacle to the eradication of AIDS. Recent studies have shown that the RNA m^{6}A is involved in the regulation of HIV-1 replication. However, no relevant study has reported the relationship between RNA m^{6}A and HIV latent reservoir. For this purpose, peripheral blood mononuclear cell (PBMC) was collected from 36 HIV-infected patients at 1, 24, and 48 weeks after treatment initiation. The number of CD4+ and CD8+ T cells was detected by flow cytometry. Amount of HIV DNA in the PBMC samples one week after treatment initiation was detected by Q-PCR. The expression levels of 23 RNA-m^{6}A-related genes were detected by Q-PCR and Pearson's correlation analysis was performed. Results showed that there was a negative correlation between HIV DNA concentration and the number of CD4+ T cells (r=−0.32, p=0.05; r=−0.32, p=0.06) and a positive correlation with the number of CD8+ T cells (r=0.48, p=0.003; r=0.37, p=0.03). Furthermore, a negative correlation was observed between HIV DNA concentration and the CD4+/CD8+ T cell ratio (r=−0.53, p=0.001; r=-0.51, p=0.001). RNAm6A related genes which correlated with HIV DNA concentration included*ALKBH5* (r=−0.45, p=-0.006), *METTL3* (r=0.73, p=2.76e−7), *METTL16* (r=0.71, p=1.21e2.76e−06), *YTHDF1* (r=0.47, p=0.004). Moreover, they have different degrees of correlation with numbers ofCD4$^+$ and CD8$^+$ T cell subsets, and the CD4$^+$/CD8$^+$T cell ratio. In addition, the expression of *RBM15* was not correlated with HIV DNA concentration but was significantly negatively correlated with the number of CD4$^+$T cells (r=−0.40, p=0.02). In conclusion, the expression of *ALKBH5*, *METTL3,* and *METTL16* is correlated with the HIV DNA level, the levels of CD4$^+$ and CD8$^+$ T cell counts, and the CD4+/CD8+ T cell ratio. *RBM15* is independent of HIV DNA level and negatively correlated with the number of CD4$^+$T cells.

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Introduction

Acquired immune deficiency syndrome (AIDS) is an infectious disease caused by the human immunodeficiency virus (HIV). At present, Highly Active Anti-Retroviral Therapy (HAART) can strongly inhibit the replication of HIV-1, which can prevent or reverse the immunodeficiency of people infected with this virus (1). However, viral replication resumes soon after HAART treatment is interrupted. The main reason for this is the existence of an HIV latent reservoir. HIV can sequester its DNA within human chromosomes and remain dormant, thus evading the assault from antiviral drugs or the immune system. Consequently, even in patients receiving the HAART regimen, latent viruses persist in resting CD4$^+$ T cells (2-6).

The composition and development of the HIV latent reservoir in AIDS patients depend on factors such as viral characteristics, the immune system, and treatment strategies (7,8). The replication ability of the virus is related to its virulence and predicts the speed of disease progression. In the early stage of infection, the level of unintegrated HIV-1 DNA is related to the efficacy of virus replication. Thus, there is a positive correlation between the viral load and the level of HIV-1 DNA expression (9). In addition to virological factors, the host's immune background is also related to the HIV latent reservoir. Studies on the sexual or mother-to-child transmission of HIV have highlighted the important role of human leukocyte antigen (HLA) and the immune response in controlling disease progression (10). In a similar immune context, the type and number of T cell subsets are closely related to inflammation and HIV persistence. The initial HIV-1 DNA load can be controlled by the breadth and size of the HIV-1-specific CD4$_+$ T cell response. In addition, antibody-mediated cytotoxicity is another important factor affecting the level of HIV-1 DNA in host cells. Patients infected with HIV-1 may also be co-infected with other viral or bacterial pathogens (11). Current studies have shown that the asymptomatic replication of human herpesvirus affects immune activation and is associated with high levels of HIV-1 DNA during HAART treatment. The reason that co-infection can affect HIV-1 DNA is related to the fact that infection with these viruses activates antigen-specific CD4$^+$ T cells, which proliferate and provide new target cells for HIV-1 infection (12).

Recent studies have shown that the N6-methyladenine (m^{6}A) modification of RNA exists in viral DNA genomes and their RNA transcripts. This m^{6}A modification has pre-viral or antiviral effects on viral replication and involves

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the methylation of the amino group at the 6th position of adenine in RNA.M^{6}A is a common modification in various types of RNA, including the messenger (m)RNA and long non-coding (lnc)RNA of higher biological organisms, as well as micro (mi)RNA, circular (circ)RNA, ribosomal (r)RNA, transfer (t)RNA, and small nuclear (sno)RNA (13,14). m6A is reversible, and its function is mainly determined by "writers", "readers", and “erasers” (15), which regulate the metabolic processing of mRNA. As a dynamic and reversible modification process in organisms, RNA m6A has complex and diverse biological functions and participates in the regulation of many physiological processes, such as cell differentiation, tumor occurrence and development, and viral replication. A study of HIV-1 viral replication showed that m6A was involved in the replication of the HIV-1 virus in CD4$^+$T cells, 293T cells, and HeLa cells. M6A was enriched at the 3 'UTR of the HIV-1 genome RNA, and additional m6A sites were located along the whole viral genome (16,17). Studies have shown that knocking out *METTL3/METTL14* reduced HIV-1 virus replication while knocking out *ALKBH5* had the opposite effect. *ALKBH5* silencing increases the methylation of the Rev response element (RRE) on RNA, thus promoting Rev binding, increasing the nuclear output of viral RNA and ultimately viral replication. Conversely, silencing *METTL3/METTL14* reduces the methylation of RNA RRE and the subsequent recruitment of Rev to the RRE, thereby reducing the nuclear output of viral RNA and inhibiting HIV-1 replication. In addition, the three YTH-domain family (YTHDF1-3) proteins have been shown to bind to HIV-1 RNA and facilitate the replication of HIV-1 (18) Furthermore, Lichinchi et al. also described the function of two potential m^{6}A sites in the RRE of HIV-1. The presence of an m^{6}A site on RRE enhances the binding of Rev to viral RNA, thereby promoting the output of viral RNA.

Collectively, the above studies show that m^{6}A plays an important role in HIV-1viral replication. However, the role of m^{6}A RNA in the HIV latent reservoir is not clear. In this study, we investigated the level of the HIV latent reservoir in patients with AIDS, by: (i) analyzing the expression of m6A-related regulatory factors in their peripheral blood mononuclear cells (PBMCs); and (ii) evaluating the correlation between the expression of m6A-associated regulatory factors and the patient’s immune function and the extent of the HIV latent reservoir.

Materials and Methods

The collection of blood samples and pathological information from patients with AIDS was approved by the Ethics Committee of Mengchao Hepatobiliary Hospital of Fujian Medical University. The HIV-1 clinical samples used in this project were all collected from patients with AIDS attending the Mengchao Hepatobiliary Hospital of Fujian Medical University, Fujian Province, China. After collection, the samples were frozen and stored at−80 °C. The patient was adequately briefed on the purpose of the sample collection, and the patients' informed consent was obtained. The CD4+ and CD8+ counts as well as other medical information were derived from clinical testing. Blood samples were collected from 36 AIDS patients typically at 24 weeks and 48 weeks after HAART treatment initiation. These samples were mainly used for real-time fluorescent quantitative PCR (Q-PCR) to verify the relative expression of m6A-related genes, and HIV-1 DNA levels (i.e. the HIV latent reservoir level) and to perform some clinical correlation analysis.

Isolation of human peripheral blood mononuclear cells (PBMC)

To isolate PBMCs from blood samples, 2mL of anticoagulant and the equivalent volume of phosphate-buffered saline (PBS) solution were added to a centrifuge tube containing blood. The blood and PBS solution were gently mixed by tube inversion or with a disposable sterile straw to dilute the blood evenly.3 mL ofFicoll-Paquewere carefully placed into a 15mL centrifuge tube with a 5mL syringe. The diluted blood was then slowly layered onto the Ficoll-Paque liquid along the tube wall. The tube was centrifugated at 400×*g* for 30min. The upper layer containing plasma and platelets was removed with a pipette, and the PBMC layer was transferred to a new sterile centrifuge tube. The PBMCs were washed in PBS as follows: PBMCs were resuspended in PBS, centrifugated at 500×g for 10min, and the supernatant was discarded. 1mL of erythrocyte lysate was added to the PBMCs, prior to centrifugation at 500×*g* for 10min (at 18–20°C). The supernatant was once again discarded and PBS was used to resuspend the PBMC cells. Following a final round of washing, the PBMCs were ready to be used in experiments.

RNA extraction

The nucleozol (Article No.: 740404.200) reagent was used to extract total RNA. A thawed 200μLPBMC sample was placed into a 1.5mL EP tube with 500 μLnucleosol, mixed by pipetting, and left to sit at room temperature (RT) for 15 min. 200 μL of diethyl pyrocarbonate (DEPC)-treated water (RNase free) was then added to the cell lysate, and mixed vigorously by shaking for 15sec, prior to incubation at RT for 15 min. The sample was next centrifugated at 12,000×g for 15 min, and the supernatant was collected into a new centrifuge tube. The supernatant was then diluted 1:2 in isopropanol, mixed well and left at RT for 10min, prior to centrifugation at 12,000×*g* for 10 min at RT. The supernatant was discarded and replaced with 500 μL of 75% ethanol. After another centrifugation step at 8,000×*g* for 3min at RT, the supernatant was discarded, and the washing step was repeated. After discarding the supernatant for a final time, the pellet was allowed to air dry and the RNA was resuspended with an appropriate amount of DEPC water and stored at −80°C.

DNA extraction

The ezup column blood genomic DNA Extraction Kit v2.0 (product number: b518253; Shanghai SANGON Biotech) was used to extract genomic DNA. A 200 μL thawed PBMC sample was added to a new 1.5mL EP tube containing 200 μL DLbuffer, mixed by shaking and incubated in a 56°C water bath for 10min. 200 μL of absolute ethanol was applied to an adsorption column to prep the membrane. The cell mixture produced in the previous step was then added to the column and incubated at RT for 3min. The column-containing tube was centrifuged at 10,000rpm centrifugation for 1min at RT, and the run-through liquid was discarded. 500 μL GW solution was next added to the adsorption column, which was then centrifuged at 10,000rpm for 1min at RT, and the run-through liquid was decarded. Next, 700 toμL of wash solution was applied to the adsorption column, which was then centrifugated at 10,000rpm for 1min at RT, and the run-through liquid was discarded. The washing step was repeated. The column was dried by centrifugation at 12,000rpm for 2min at RT to remove the residual wash solution. The adsorption column was next placed into a new centrifuge tube and 50 µLof CE buffer was added to the middle of the membrane. Following a 5 min incubation at RT, the tube was centrifugated at 12,000rpm for 2min, and the DNA was collected and stored at −20°C.

Reverse transcription PCR (RT-PCR)

The Hiasen'shifair ® II first strand cDNA synthesis (gDNA digester plus) and reverse transcription (product No.: 11121es60) kits were used for RT-PCR, which was performed according to the manufacturer's instructions. After the RT-PCR reaction, the cDNA products were stored at −20°C or −80°C.

Real-time fluorescent quantitative PCR (Q-PCR)

Q-PCR can not only quantify the relative expression level of genes and non-coding RNA contained in the cDNA template (obtained by RT-PCR), but also directly analyze the relative expression level of DNA in the DNA template. We used the Kang Weishi'sultrasybr mixture (product No.: cw0957h), and performed Q-PCR as follows. The Q-PCR primers were mainly designed using the NCBI website and then sent to Biotechnology Co., Ltd.(Shanghai) for synthesis. The PCR machine and reaction were configured according to the manufacturer's instructions. After the completion of the Q-PCR program, the amplification and fusion curves were observed, the abnormal data values were removed, and the CT values were exported for data analysis.

Quantification of the relative level of HIV DNA

We used the Kang Weishiultrasybr one-step Q-PCR kit (product No.: cw0659s) to quantify the relative level of HIV DNA. Briefly, the primers were designed using the NCBI website and synthesized by Shanghai Biotechnology Co., Ltd. According to the DNA quantitative standard curve, the concentration of *gag* recombinant plasmid constructed using the t-vector was 228.839 ng/µL. The plasmid size was 3,955 kb and the constructed fragment size was 126 kb. To calculate the copy number of the original plasmid DNA solution, the stock DNA was serially diluted to give six concentrations. According to the CT value of the Q-PCR results and the copy number of the DNA stock solution, a standard curve was generated for DNA quantification. In the later experiment, the CT of corresponding Q-PCR experimental results was substituted to obtain the copy number of the stock DNA solution. HIV-1 DNA was quantified using Q-PCR as described in the previous section. After the Q-PCR procedure, the CT values were used together with the standard curve to obtain the copy number of the HIV-1 DNA solution (i.e., the HIV latent reservoir level).

Statistical analysis

We used the ggplot2 package of R software to draw the scatter diagram and the stat_Cor function to construct correlation curves. Pearson'schi-squared or Spearman rank tests were used to measure the statistical significance of any correlations.

Results

HIV DNA latent reservoir and patients' clinical characteristics

We extracted DNA from the PBMC samples of 36 HIV patients, amplified the HIV *gag* gene, constructed a t-*gag* recombinant vector, and generated a DNA standard curve. The absolute copy number of HIV *gag* in 100 L PBMC samples was detected by Q-PCR to quantify the HIV DNA concentration in the latent reservoir. The latent HIV DNA concentration of most patients was between 1,394-68,320/100 µL, with an average of 12,448 (Table 1). We next divided the study participants into high and low HIV latent reservoir (or *gag* gene expression) groups, according to their latent reservoir HIV DNA concentrations (50%:50%). HIV-infected patients whose log HIV DNA concentration in the latent reservoir was ≥ 5,700 were determined as the high expression group, and those whose log value was<5,700 were determined as the low expression group. We also extracted the relevant clinical information relating to the 36 patients with AIDS and statistically analyzed the correlation between these clinicopathological parameters and the absolute expression of HIV-*gag*in the latent reservoir. The results showed that the HIV DNA concentration in the latent reservoir did not correlate significantly with gender (p=0.7393), age (p=0.462), route of infection (p=0.5491), or body mass index (BMI) (p=0.9329) (Table 1).

General results of the correlation analysis

To study the correlation between RNA-m6A-related genes and HIV latent reservoir, we extracted total RNA from the PBMCs of 36 patients with AIDS and then quantified the relative expression of 23 RNA-m6A-related factors by Q-PCR experiment. In addition, we collected data on the number of $CD4^+$ and $CD8^+$T cells and the $CD4^+$/$CD8^+$T cell ratio from the patients, which were recorded during their clinical visits. Pearson's correlation analysis was used to obtain the correlation between the relative expression level of these 23 genes and the HIV concentration within the latent reservoir, and the number of $CD4^+$ and $CD8^+$T cells and the $CD4^+$/$CD8^+$T cell ratio. A correlation heat map was generated with P≤0.05 as the significance threshold. The results showed that manyRNA-m6A-rela-

Table 1. HIV DNA and clinical characteristics.

clinical characteristics	HIV DNA level		P-value
	high	low	
sexy			
male	12	12	0.8802
female	6	6	
age			
<40years	8	8	0.739
≥40years	10	11	
infection			
homosexuality	4	4	0.2778
heterosexuality	14	14	
BMI			
<18.5	4	3	
18.5≤BMI<24	10	10	0.8908
≥24	4	5	

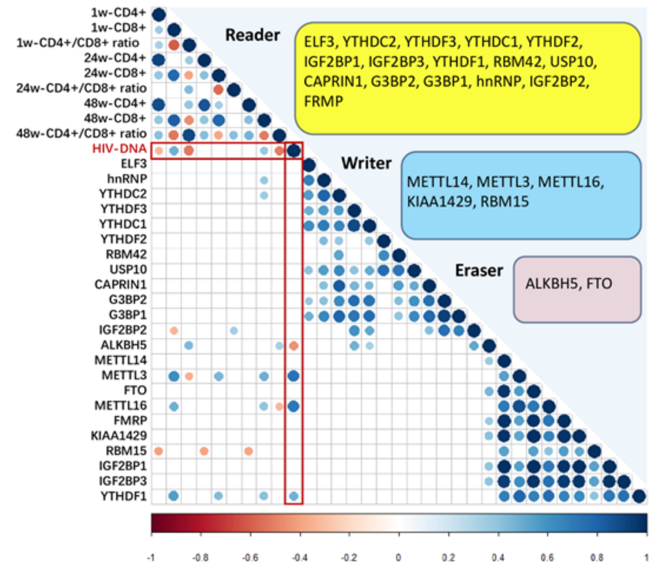

Figure 1. Correlation heatmap of RNA-m6A-related genes, HIV DNA, and $CD4^+$ and $CD8^+$T cell numbers. HIV DNA was isolated from the PBMCs of HIV-infected patients. The patients' $CD4^+$ and $CD8^+$ T cell numbers were obtained from their clinical data, collected at 1, 24, and 48 weeks after HAART initiation. The patients' $CD4^+$/$CD8^+$ T cell ratios were also calculated. RNA-m6A-related genes included writers, readers, and erasers. The Pearson correlation coefficients are indicated by circle size and color. Only significant (P<0.05) correlation coefficients are shown in the heatmap.

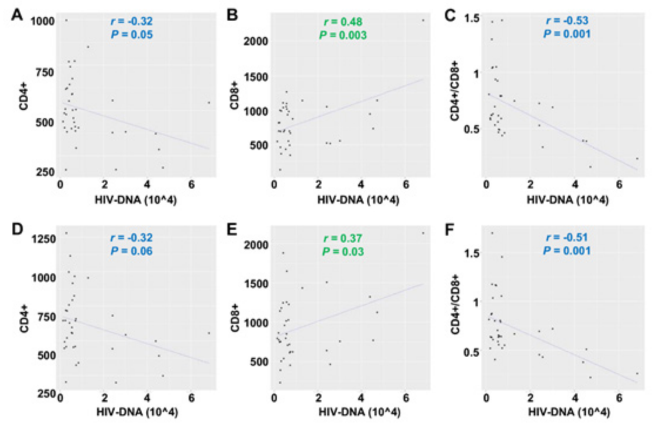

Figure 2. Patient PBMC-derived HIV DNA concentration correlated with their $CD4^+$ and $CD8^+$T cell counts. (A to C) Cell numbers at 1 week after HAART initiation. (D to F) Cell numbers at 48 weeks after HAART initiation. (A and D) $CD4^+$ T cell counts, (B and E) $CD8^+$ T cell counts, and (C and F) $CD4^+$/$CD8^+$ T cell ratios were analyzed. Note that positive correlation coefficients are shown in green and negative ones are shown in blue.

ted genes had no significant correlation with the number of HIV latent reservoirs and T cell numbers (Figure 1). However, *ALKBH5, METTL3, METTL16,* and *YTHDF1* were associated with HIV latent reservoir and T cell numbers. *RBM15* expression was not related to the latent HIV reservoir, only to the number of T cells.

Correlation between the HIV DNA concentration in the latent reservoir and$CD4^+$and $CD8^+$ T cell counts and the $CD4^+$/$CD8^+$ ratio

The experimental results obtained by analyzing the correlation between the HIV DNA concentration in the latent reservoir and the clinical indicators such as T cell subsets are shown in Figure 2. From the results, it can be seen that HIV DNA concentration is significantly correlated with the three clinical indicators: $CD4^+$T cell number, $CD8^+$T cell number, and the $CD4^+$/$CD8^+$T cell ratio in PBMC samples taken 48 weeks after diagnosis and HAART initiation (Figure 2A to 2F).HIV DNA concentrationwas negatively correlated with CD4+ T cell number (r=−0.32, r=−0.32) (Figure 2A and 2D), and positively correlated with CD8+ T cell number (r=0.48, r=0.37) (Figure 2B and 2E). Pearson's correlation coefficient showed that the CD4+/CD8+ T cell ratio displayed the largest negative correlation with HIV DNA concentration (r=−0.53, r=−0.51) (Figure 2C and 2F).

Correlation between RNA-m6A-related genes and the HIV latent reservoir

According to the correlation results between RNA-m6A-related genes and HIV latent reservoir, only the expression of *ALKBH5*, *METTL3*, *METTL16*, and *YTHDF1* was statistically significantly correlated with HIV latent reservoir (Figure 3). There was a significant negative correlation between demethylase *ALKBH5* and the level of

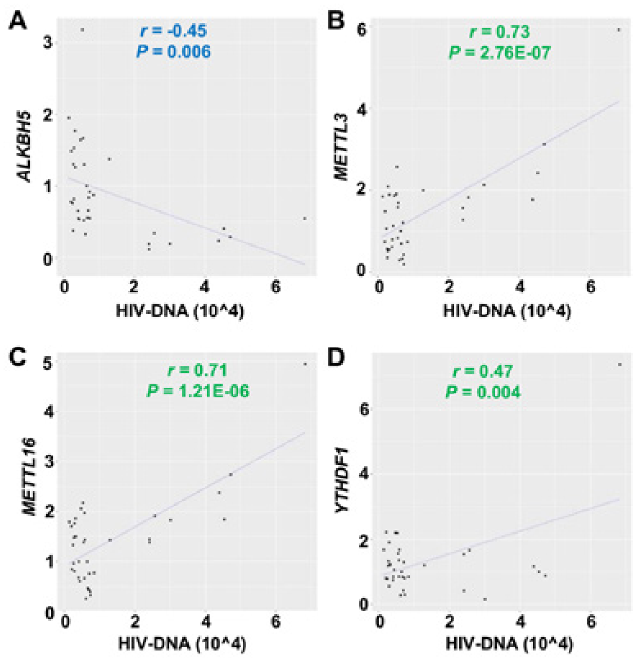

Figure 3. Patient PBMC-derived HIV DNA concentration correlated with expression of RNA-m6A-related genes. (A) *ALKBH5* expression negatively correlated with HIV DNA load. (B) *METTL3*, (C) *METTL16*, and (D) *YTHDF1* expression positively correlated with HIV DNA load. Note that the gene expression results for each patient are presented relative to the expression levels of those genes in patient #1.

the HIV latent reservoir (r=−0.45, and p<0.05) (Figure 3A). As components of methylase complex, *METTL3* and *METTL16* were also significantly positively correlated with the level of HIV latent reservoir (r=0.73 and r=0.71, respectively, and p<0.0001 for both) (Figure 3B and 3C). Meanwhile, there was a significant positive correlation between the expression of *YHDF1*, encoring a reader protein, and the level of the HIV latent reservoir, r=0.47 (Figure 3D).

Expression of ALKBH5 correlates with the $CD4^+$/$CD8^+$T cell ratio

The experimental results obtained by analyzing the

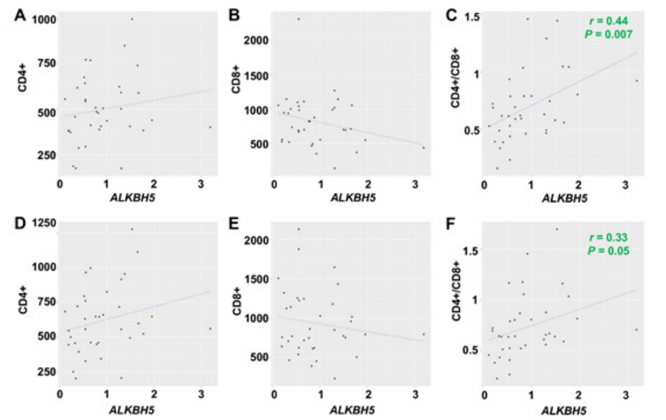

Figure 4. Expression of *ALKBH5* correlated with the CD4+/CD8+ T cell ratio. Cell numbers were counted at 1 week (A to C) or 48 weeks (D to F) after HAART initiation. (A and D) $CD4^+$ T cell counts, (B and E) $CD8^+$ T cell counts, and (C and F) the $CD4^+/CD8^+$ T cell ratios were analyzed. Note that if the P-value is not shown in the image, the correlation was not significant.

correlation between the expression level of *ALKBH5* and the clinical indicators of T cell subsets are shown in Figure 4. We found that the expression level of *ALKBH5* has not significantly correlated with the number of $CD4^+$ T cells or the number of $CD8^+$ T cells in PBMC samples collected 48 weeks after HAART initiation (Figure 4A, 4B, 4D and 4E). In comparison, *ALKBH5* expression was significantly positively correlated with the $CD4^+/CD8^+$ T cell ratio (r=0.44, r=0.33) (Figure 4C and 4F).

Expression of METTL3 and METTL16 correlates with $CD8^+$T cell counts and the $CD4^+/CD8^+$T cell ratio

The experimental results obtained by analyzing the correlation between the expression levels of mettl3 and mettl16 and the clinical indicators of T cell subsets are shown (Figure 5A to 5D). We observed no significant correlation between the expression levels of *METTL3* and *METTL16* and the number of $CD4^+$T cells at 48 weeks after HAART initiation. In comparison, mettl3 expression levels and the number of $CD8^+$ T cells were significantly positively correlated (r=0.61, r=0.49). *METTL16* expression levels were also significantly positively correlated with the number of $CD4^+$ T cells (r=0.49, r=0.40). There was a statistically significant negative correlation between the expression levels of *METTL3* and *METTL16* and the $CD4^+/CD8^+$T cell ratio.

Expression of RBM15 correlates with $CD4^+$ T cell counts

There is no statistical correlation between the *RBM15* expression level and the HIV DNA concentration in the HIV latent reservoir. We found that the *RBM15* expression level has not significantly correlated with the number of $CD8^+$T cell or the $CD4^+/CD8^+$T cell ratio in samples from patients with AIDS taken 48 weeks after HAART initiation. In comparison, *RBM15* expression was significantly negatively correlated with the number of $CD4^+$T cells (r=−0.40, r=−0.39).

Discussion

AIDS is a devastating infectious disease. At present, AIDS can be controlled with HAART. HAART drugs

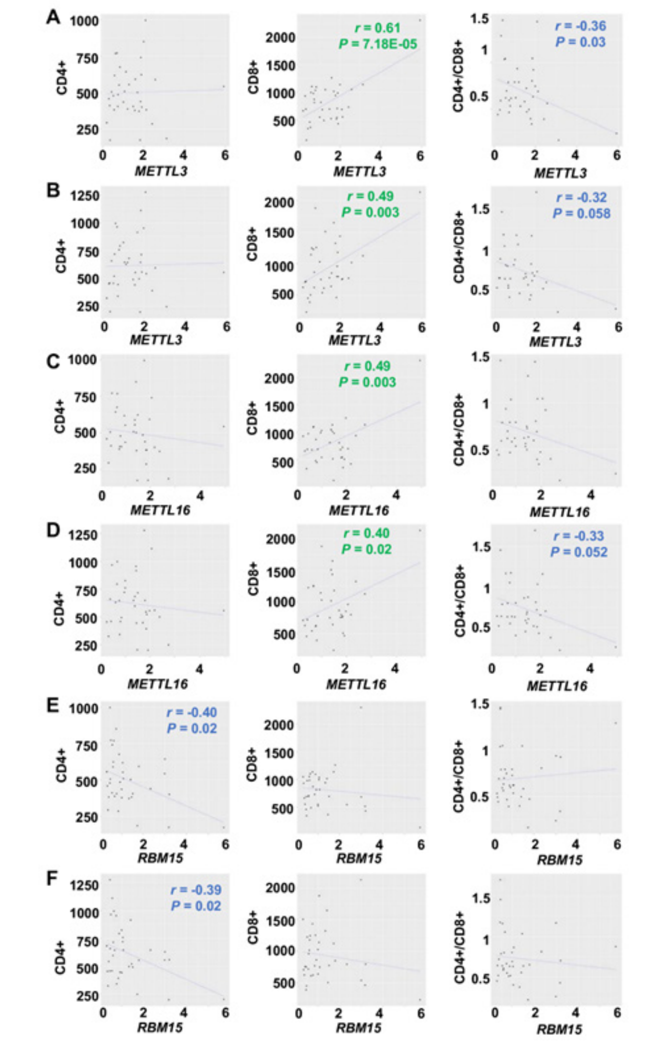

Figure 5. Expression of *METTL3*, *METTL16*, and *RBM15* correlated with certain T cell subsets. (A and B) *METTL3* expression positively correlated with $CD8^+$ T cell numbers and $CD4^+/CD8^+$ T cell ratios at 1 week and 48 weeks after HAART initiation. (C and D) *METTL16* expression correlated positively with $CD8^+$ T cell numbers and $CD4^+/CD8^+$ T cell ratios at 1 week and 48 weeks after HAART initiation. (E and F) *RBM15* expression positively correlated with $CD4^+$ T cell numbers at 1 week and 48 weeks after HAART initiation.

target the HIV replication cycle and include viral entry inhibitors, reverse transcriptase inhibitors, integrase inhibitors, and protease inhibitors (19). These inhibitors can effectively prevent viral replication. However, once the treatment is interrupted, viral replication resumes within a few weeks (6). The reason for this is that HIV DNA integrates into the host cell genome, where it remains in a latent state until conditions are once again favorable for viral replication (20,21). Due to the existence of the HIV latent reservoir, major obstacles to the complete eradication of AIDS remain.

Latent HIV repositories are mainly affected by the following three mechanisms: viral characteristics (replication ability), host immunity, and treatment strategies; among these, viral replication ability is the most important factor. Recently, researchers have developed a strategy to clear the latent HIV reservoir using the "activate and kill" method (20). This involves first using latency reversal agents (LRAs) to activate resting $CD4^+$ T cells and release HIV. The exposed, free HIV can then be eradicated with high doses of anti-HIV drugs, which also serve to induce the apoptosis and lysis of $CD4^+$ T cells. Thus, the latent repository of HIV is exterminated as a result of cytopathy (22,23). However, this technology is not sufficiently developed for clinical application. Therefore, targeting the regulation of HIV replication to eradicate the latent HIV reservoir is another strategy that could employ to improve the efficiency of AIDS treatment (24,25).

Recent studies have shown that RNA m^6A methylation modification is closely related to HIV viral replication. The silencing of methylation-modified genes leads to changes in HIV RNA export and viral replication. For instance, silencing a demethylase-encoding gene *ALKBH5* has been shown to increase the activity of the RRE of HIV RNA and gp120 mRNA, leading to increased HIV replication (16,26,27). However, the role of m6A in the regulation of the HIV latent reservoir has not been documented. In this study, we analyzed the relationship between RNA-m^6A-related factors and the HIV latent reservoir level. We found that the relative expression of the alkbh5 gene in HIV-infected patient PBMCs was significantly negatively correlated with the latent repository of HIV, while the methylase-encoding*METTL3* and *METTL16* genes were positively correlated with the latent repository of HIV. The results suggest that RNA m6A may be involved in the positive regulation of the HIV latent reservoir level. This is consistent with the reported positive regulation of HIV replication by RNA m^6A. In addition, we found that the expression of the m^6A-associated reader-encoding gene, ythdf1, was positively correlated with the latent reservoir. Studies have reported that ythdf1 plays a contrasting positive and negative role in the process of HIV infection by inhibiting the production of viral guide (g)RNA during cell entry and promoting the production and release of proviral DNA in HIV-producing cells (18). The relationship between ythdf1 and the HIV latent reservoir implies that the level of the latent HIV pool is linked to the production of viral DNA in HIV-producing cells (18).

RNA m^6A can not only interfere with the viral replication process but also participate in the regulation of cell growth and differentiation. It has been reported that RNA m^6A can promote the proliferation of primary T cells. Among the RNA-m^6A-related genes that were associated with the level of the HIV latent reservoir, *ALKBH5* expression was only linked to the $CD4^+/CD8^+$T cell ratio, but not to the number of $CD4^+$ or $CD8^+$T cells alone. The expression of *METTL3* and *METTL16* was significantly correlated not only with the number of $CD8^+$ T cells, but also with the $CD4^+/CD8^+$ T cell ratio. The level of latent HIV DNA found in the study was closely related to the number of CD8+ T cells and especially the $CD4^+/CD8^+$T cell ratio, which is related to the immune activation state of HIV-infected patients (22, 28-32). It is also related to the "activate and kill" HIV latent reservoir clearance strategy, which reveals that RNA m6A not only impacts on viral replication but is also likely to regulate HIV latent reservoir via the modulation of $CD8^+$T cells. Moreover, the number of $CD4^+$ cells is an important indicator of immune reconstitution in AIDS patients. In our study, the direct correlation between the number of $CD4^+$T cells and the latent reservoir has not been clear. We found that there was no correlation between the expression of *RBM15* and the HIV DNA concentration in the latent reservoir, but there was a significant negative correlation with the number of $CD4^+$T cells. Thus, *RBM15* may be a new potential target for immune reconstitution therapy.

In conclusion, by studying the HIV latent reservoir in AIDS patients, analyzing the expression of RNA-m6A-related genes and investigating the distribution of $CD4^+$ and $CD8^+$ T cells in patients with AIDS, we found that the level of HIV latent reservoir was significantly negatively correlated with $CD4^+$ T cell counts and the $CD4^+/CD8^+$T cell ratio, and positively correlated with $CD8^+$ T cell numbers. *ALKBH5* expression was positively correlated with the $CD4^+/CD8^+$T cell ratio and negatively correlated with the HIV DNA concentration in the latent reservoir. *METTL3* and *METTL16* were positively correlated with CD8+ T cell counts, negatively correlated with the $CD4^+/CD8^+$ T cell ratio, and positively correlated with the level of the HIV latent reservoir. In addition, *RBM15* did not correlate with the HIV latent reservoir level but was negatively correlated with $CD4^+$ T cell counts. In conclusion, RNA m6A is involved in HIV replication and immune system regulation in patients with AIDS patients through multiple pathways. However, the mechanisms remain to be defined.

Author Contributions

Yahong Chen and Shujin Lin designed the project, performed experiments and wrote the draft manuscript, Jinglan Lai, Jing Lin, QiaowenWang and Wen Ao collected clinical data and processed raw data. Xiao Han and Hanhui Ye designed the project, supervised the experiment and wrote the manuscript.

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