Expression of RNA-m6A-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 m6A is involved in the regulation of HIV-1 replication. However, no relevant study has reported the relationship between RNA m6A 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-m6A-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). RNA6mA 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−6), YTHDF1 (r=0.47, p=0.004). Moreover, they have different degrees of correlation with numbers of CD4+ 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.

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 (m6A) modification of RNA exists in viral DNA genomes and their RNA transcripts. This m6A modification has pre-viral or antiviral effects on viral replication and involves
the methylation of the amino group at the 6th position of adenine in RNA. m6A 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 (sn)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 m6A sites in the RRE of HIV-1. The presence of an m6A site on RRE enhances the binding of Rev to viral RNA, thereby promoting the output of viral RNA.

Collectively, the above studies show that m6A plays an important role in HIV-1 viral replication. However, the role of m6A 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.

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 with 500 μL nucleosol, 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 centrifuged 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 DNA was resuspended with an appropriate amount of DEPC water and stored at –80°C.

RNA extraction

The nucleozol (Article No.: 740404.200) reagent was used to extract total RNA. A thawed 200μL PBMC sample was placed into a 1.5mL EP tube with 500 μL nucleosol, 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 then centrifuged 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.
applied to the adsorption column, which was then centrifuged 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 μL of CE buffer was added to the middle of the membrane. Following a 5 min incubation at RT, the tube was centrifuged 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 Weishuultrasybr 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 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-

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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 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, encoding 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


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 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 reservoir of HIV is exterminated as a result of cytotoxicity (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 m6A 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 gpl20 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-m6A-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 m6A. In addition, we found that the expression of the m6A-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 m6A cannot only interfere with the viral replication process but also participate in the regulation of cell growth and differentiation. It has been reported that RNA m6A can promote the proliferation of primary T cells. Among the RNA-m6A-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). Moreover, the expression of the alkbh5 gene was inversely correlated with 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, Qiaowen Wang 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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