Molecular cloning and anti-HIV-1 activities of APOBEC3s from northern pig-tailed macaques (*Macaca leonina*)

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ABSTRACT

Northern pig-tailed macaques (NPMs, *Macaca leonina*) are susceptible to HIV-1 infection largely due to the loss of HIV-1-restricting factor TRIM5α. However, great impediments still exist in the persistent replication of HIV-1 *in vivo*, suggesting some viral restriction factors are reserved in this host. The APOBEC3 proteins have demonstrated a capacity to restrict HIV-1 replication, but their inhibitory effects in NPMs remain elusive. In this study, we cloned the NPM A3A-A3H genes, and determined by BLAST searching that their coding sequences (CDSs) showed 99% identity to the corresponding counterparts from rhesus and southern pig-tailed macaques. We further analyzed the anti-HIV-1 activities of the A3A-A3H genes, and found that A3G and A3F had the greatest anti-HIV-1 activity compared with that of other members. The results of this study indicate that A3G and A3F might play critical roles in limiting HIV-1 replication in NPMs *in vivo*. Furthermore, this research provides valuable information for the optimization of monkey models of HIV-1 infection.

Keywords: *Macaca leonina*; Northern pig-tailed macaques; APOBEC3; HIV-1

INTRODUCTION

The lack of effective HIV-1-infected animal AIDS models hinders our understanding of HIV-1 pathogenesis and the development of AIDS vaccines and drugs (Hatziioannou & Evans, 2012; Zhang et al., 2007). The ideal animal model is one that can be infected by HIV-1 and progress to an AIDS-like disease. However, HIV-1 shows a narrow host range and only infects humans and a handful of nonhuman primates (Kuang et al., 2009). Among them, the pig-tailed macaques (PTMs), which consist of northern pig-tailed macaques (NPMs, *Macaca leonina*), southern pig-tailed macaques (*M. nemestrina*) and Mentawai macaques (*M. pagensis*) (Groves, 2001), are the only Old World monkeys susceptible to HIV-1 infection. To date, however, the mechanism of this susceptibility remains largely unclear.

TRIM5-Cyclophilin A (*TRIMCyp*) fusion in NPMs, identified in our previous research, might account for the susceptibility of NPMs to HIV-1 infection (Kuang et al., 2009; Liao et al., 2007). Thus, NPMs might present fewer impediments to HIV-1 infection and replication than that of other macaques, such as the widely used rhesus and cynomolgus macaques. In addition, we previously reported on many basic biological parameters of NPMs (Lian et al., 2016; Pang et al., 2013; Zhang et al., 2014, 2016; Zheng et al., 2014; Zhu et al., 2015), which will help in the promotion of NPMs in AIDS and biomedical research.

Though HIV-1 can infect PTMs, considerable obstacles exist in regards to persistent replication in these hosts (Agy et al., 1992; Bosch et al., 2000; Hu, 2005). This has limited the application of the PTM animal model of HIV-1 infection in the field of vaccine and drug testing. Furthermore, although APOBEC3 proteins have the capacity to restrict HIV-1 replication (Jia et al., 2015; Simon et al., 2015; Stavrou & Ross, 2015), it is unclear whether such proteins play an important role

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in restricting the replication of HIV-1 in NPMs.

The APOBEC3 family, comprised of seven members (APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H) in primates (Prohaska et al., 2014), can inhibit the replication and spread of various retroviruses by inducing C-to-U hypermutation in newly synthesized viral minus DNA, ultimately leading to G-to-A hypermutation in the viral genome. The activity of APOBEC3 proteins, particularly that of APOBEC3G, is inhibited by vif, an accessory protein encoded by lentiviruses. Interestingly, the degradation of APOBEC3 proteins by different vifs shows pronounced species-specificity (Zennou & Bieniasz, 2006), although this is not absolute. The species-specific interaction between vifs and APOBEC3s suggests that APOBEC3s are important obstacles to cross-species transmission of lentiviruses.

The rate of viral hypermutation mediated by APOBEC3s is associated with viral transmission and disease progression. In recent years APOBEC3s have been treated as potential targets of future therapeutic strategies against HIV-1 (Pillai et al., 2012).

Recently, PTMs were shown to develop AIDS following infection with adapted macaque-tropic HIV-1 (Hatziioannou et al., 2014), which is considered a major step forwards in AIDS research. To explore whether APOBEC3 proteins play a role in restricting the replication of HIV-1 in NPMs, we cloned A3A-A3H genes from NPMs and analyzed their anti-HIV-1 activity. The aim of this study was to provide valuable information for optimizing nonhuman primate models for AIDS research.

MATERIALS AND METHODS

Animals, cells and viruses

The NPMs used in this study were obtained from the Kunming Institute of Zoology, Chinese Academy of Sciences (KIZ, CAS), and were maintained in accordance with the regulations and recommendations of the Animal Care Committee of KIZ, CAS, and the Guide to the Care and Use of Experimental Animals, as described previously (Zhang et al., 2014).

Peripheral blood mononuclear cells (PBMCs) in NPM blood samples were isolated using Ficoll density centrifugation (Dai et al., 2013). The isolated PBMCs were cultured for 72 h in RPMI 1640 medium (10% fetal bovine serum (FBS) containing interleukin-2 (IL-2, 50 U/mL) and Con A (1 mg/mL). In addition, 293T cells (Type Culture Collection (TCC), CAS) and TZM-bl cells (Medical Research Council, AIDS Reagent Project, UK) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS (Lei et al., 2014). HIV-1_{NL4-3} was made by transfection of 293T cells using Lipofectamine TM 2000 according to the manufacturer's instructions (Invitrogen Carlsbad, CA). HIV-1_{NL4-3} proviral plasmids were kindly donated by Prof. Guang-Xia Gao (Institute of Biophysics, CAS, China).

PCR of APOBEC3 mRNA

Total RNA from PBMCs was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and then reverse transcribed into cDNA using the PrimeScript® RT reagent kit with gDNA Eraser (Takara, Dalian, China). The primers used were synthesized by Generay Biotech (Shanghai, China), and the sequences and PCR conditions are listed in Table 1. The PCR products were analyzed on a 2% agarose gel, visualized by ethidium bromide staining, and purified using a DNA gel extraction kit (Generay Biotech, Shanghai, China). The purified fragments were cloned into pMD19-T simple vector (Takara, Dalian, China) and finally sequenced (Majorbio, Shanghai, China). Sequences were analyzed by MEGA5.0 software and the NCBI's online BLAST server (http: //www.ncbi.nlm.nih.gov/blast/blast.cgi).

Molecular cloning of NPM A3s

For expression studies, FLAG primers were designed based on the CDSs of the NPM APOBEC3s, with the sequences and PCR conditions listed in Table 2. The flag-APOBEC3s

Table 1 Primer sequences and PCR conditions for APOBEC3 mRNA

Primers	Primer DNA sequences	PCR conditions
NPM-A3A-F	5′-CAGGAACCGAGAAGAGACAAGCACAT-3′	94 °C 5 min, (94 °C 30 sec, 59 °C 30 sec, 72 °C 90 sec)×32 cycle, 72 °C 10 min
NPM-A3A-R	5′-CTGCCTTCCTTAGAGACTGAGGC-3′	
NPM-A3B-F	5′-AGAGCGGGACTGGGACAAG-3′	94 °C 5 min, (94 °C 30 sec, 59 °C 30 sec, 72 °C, 2 min) × 32 cycle, 72 °C 10 min
NPM-A3B-R	5′-TTAGAGACTGAGGCCCATCCTTC-3′	
NPM-A3C-F	5′-ACAAGCGTATCTAAGAGGCCG-3′	94 °C 5 min, (94 °C 30 sec, 59 °C 30 sec, 72 °C 90 sec)×32 cycle, 72 °C 10 min
NPM-A3C-R	5′-GGAGGCCCGTGCAGCATG-3′	
NPM-A3D-F	5′-GCGTATCTAAGAGGCTGAACAT-3′	94 °C 5 min, (94 °C 30 sec, 59 °C 30 sec, 72 °C, 2 min) × 32 cycle, 72 °C 10 min
NPM-A3D-R	5′-TTATGTTGGGGAGATGGGAAGAG-3′	
NPM-A3F-F	5′-ACAAAGATCTTAGTCGGGACT-3′	94 °C 5 min, (94 °C 30 sec, 59 °C 30 sec, 72 °C, 2 min) × 32 cycle, 72 °C 10 min
NPM-A3F-R	5′-AGGCTAGAGGAGACAGACCA-3′	
NPM-A3G-F	5′-GTCAGGACTAGCCGGCAAAGGAT-3′	94 °C 5 min, (94 °C 30 sec, 59 °C 30 sec, 72 °C, 2 min) × 32 cycle, 72 °C 10 min
NPM-A3G-R	5′-CTTCCTTAGAGACTGAGGCCCATC-3′	
NPM-A3H-R	5′-GACCAGCAGGCTATGAGGCAA-3′	94 °C 5 min, (94 °C 30 sec, 59 °C 30 sec, 72 °C 90 sec)×32 cycle, 72 °C 10 min
NPM-A3H-F	5′-CCAGAAGCACAGATCAGAAACACGAT-3′	

Table 2 Primer sequences and PCR conditions for molecular cloning of NPM A3s

Primers	Primer DNA sequences	PCR conditions
NPM-A3A-CD-F	5′-gcgaagcttgccaccATGGACGGCAGCCCAGCATCCAG-3′	94 °C 5 min, (94 °C 30 sec, 58 °C 30 sec, 72 °C 90 sec) ×32 cycle, 72 °C 10 min
NPM-A3A-CD-R	5′-cctctagaggcTCACTTATCGTCGTCATCCTTGTAATCgccgccGTTTCCCTGATTCTGGAG AATGGCCC-3′	
NPM-A3B-CD-F	5′-gcgaagcttgccaccATGAATCCACAGATCAGAAATCCGATGGAGC-3′	94 °C 5 min, (94 °C 30 sec, 58 °C 30 sec, 72 °C 2 min) ×32 cycle, 72 °C 10 min
NPM-A3B-CD-R	5′-cctctagaggcTCACTTATCGTCGTCATCCTTGTAATCgccgccGTTTCCCTGATTCTGGAG AATGGCC-3′	
NPM-A3C-CD-F	5′-gcaagcttgccaccATGAATCCACAGATCAGAAACCCGATGA-3′	94 °C 5 min, (94 °C 30 sec, 58 °C 30 sec, 72 °C 90 sec) ×32 cycle, 72 °C 10 min
NPM-A3C-CD-R	5′-cctctagaggcTCACTTATCGTCGTCATCCTTGTAATCgccgccCTGAAGAATCTCCCGTAG GC-3′	
NPM-A3D-CD-F	5′-gcgaagcttgccaccATGAATCCACAGATCAGAAATCCGATGGAGGG-3′	94 °C 5 min, (94 °C 30 sec, 58 °C 30 sec, 72 °C 2 min) ×32 cycle, 72 °C 10 min
NPM-A3D-CD-R	5′-cctctagaggcTCACTTATCGTCGTCATCCTTGTAATCgccgccCTGAAGAATCTTCCATAG GCGTCTTTCCAGA-3′	
NPM-A3F-CD-F	5′-gcgaagcttgccaccATGAAGCCTCAGTTCAGAAACACAGT-3′	94 °C 5 min, (94 °C 30 sec, 58 °C 30 sec, 72 °C 2 min) ×32 cycle, 72 °C 10 min
NPM-A3F-CD-R	5′-CctctagaggcTCACTTATCGTCGTCATCCTTGTAATCgccgccCTCGAGAATCTCCTGCA GCTTG-3′	
NPM-A3G-CD-F	5′-gcgaagcttgccaccATGAATCCTCAAATCAGAAACATGGTGGA-3′	94 °C 5 min, (94 °C 30 sec, 58 °C 30 sec, 72 ℃ 2 min) ×32 cycle, 72 °C 10 min
NPM-A3G-CD-R	5′-cctctagaggcTCACTTATCGTCGTCATCCTTGTAATCgccgccGTTTCCCTGATTCTGGAG AATGGC-3′	
NPM-A3H-CD-F	5′-gcgaagcttgccaccATGGCTCTGCTAACAGCCAAAACATTC-3′	94 °C 5 min, (94 °C 30 sec, 58 °C 30 sec, 72 °C 90 sec) ×32 cycle, 72 °C 10 min
NPM-A3H-CD-R	5′-cctctagaggcTCACTTATCGTCGTCATCCTTGTAATCgccgccTCTTGAGTTGCGTATTGA CGATGAGGGG-3′	

amplicons were cloned into the pcDNA3.1 (+) vector (Invitrogen, Carlsbad, CA) using the XbaI and HindIII restriction sites and then sequenced (Majorbio, Shanghai, China). Finally, the constructs of these recombinant plasmids were verified by restriction mapping of XbaI and HindIII.

Western blot analysis

The 293T cells were transfected with pcDNA3.1-npmA3s-Flag (pcDNA3.1-npmA3A-Flag, pcDNA3.1-npmA3B-Flag, pcDNA3.1-npmA3C-Flag, pcDNA3.1-npmA3D-Flag, pcDNA3.1-npmA3F-Flag, pcDNA3.1-npmA3G-Flag, pcDNA3.1-npmA3H-Flag), pcDNA3.1-humanA3G-Flag, and pcDNA3.1-empty vector in 6-well plates with Lipo2000 (Invitrogen, Carlsbad, CA), and were lysed with cell lysis buffer (Beyotime, , Shanghai, China after 48 h transfection. The extracted proteins were denatured in SDS/PAGE loading buffer by boiling for 15 min and separated by SDS/PAGE. The flag-tagged proteins were detected using mouse monoclonal anti-flag antibody (Abmart, Shanghai, China) and then HRP-conjugated secondary antibody, followed by light staining with chemiluminescent detection reagents (Millipore, Bedford, MA).

Co-transfection

The HIV-1NL4-3 proviral plasmid (3 μg) was transiently co-transfected with the NPM A3A-A3G and human-A3G expression plasmid (1 μg) and pcDNA3.1 (+) control plasmid (1 μg) in the 293T cells with Lipo2000, according to the manufacturer's protocols (Invitrogen , Carlsbad, CA). The cell culture medium was replaced with fresh medium 8 h after transfection. On day 2 after transfection, the cell supernatant containing the virus was collected, filtered, and then treated by DNase I (Takara, Dalian, China) at 37 °C for 1 h to prevent plasmid carryover.

Single-round viral infectivity assays

The amount of viral particles harvested from the co-transfection supernatants was assayed by p24 antigen enzyme-linked immunosorbent assay (ELISA) (ZeptoMetrix Corp., Buffalo, NY, USA). The TZM-bl cells seeded at a density of 1×10^4 cells per well in 96-well plates were infected with equal amounts of virus. The infection was performed in triplicate for 48 h. After incubation, luminescence was measured by Bright-Glo luciferase assay reagent (Promega , Madison, WI). Statistical differences between mean percentages were compared by Student's *t*-tests (two-tailed, $P<0.05$) using GraphPad Prism software v5.0.

RESULTS

PCR amplification and sequence analysis of NPM A3s

Total RNA was extracted from PBMCs and reverse transcribed into cDNAs, which were used as PCR templates. The sizes of the A3A to A3H amplicons were 665 bp, 1 205 bp, 629 bp, 1 181 bp, 1 178 bp, 1 208 bp, and 689 bp, respectively (Figure 1).

The purified amplicons were cloned into pMD19-T simple vector and finally sequenced. Sequences were analyzed by MEGA5.0 software and BLAST online. BLAST analysis of the NPM A3A-A3H CDSs showed 94%-99% nucleotide identity with those of rhesus, crab-eating, and southern pig-tailed macaques, and 89%-93% of nucleotides were identical to those of humans. Among them, the identities of NPM cDNA sequences with those of rhesus and southern pig-tailed macaques reached 99%

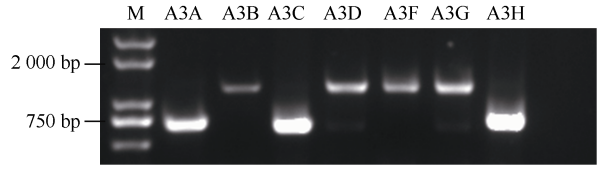

Figure 1 Analysis of PCR products of NPM A3A-A3H with agarose gel electrophoresis

Lane 1: DNA marker; Lane 2-8: PCR product of NPM A3A-A3H genes.

(Table 3). To explore the clustering of NPM A3A-A3H sequences with specific primate lineages, we constructed a phylogenetic tree (Figure 2). Amino acid sequence alignment was subjected to phylogenetic analyses using the neighbor joining method. The results showed that NPM A3A-A3H sequences clustered according to the corresponding A3A-A3H sequences from different primate taxa. These analyses demonstrated that the cloned NPM A3A-A3H gene classifications were correct. GenBank accession numbers of the NPM A3A-A3H CDSs are KX583650, KX583652, KX583655, KX583653, KX583651, KX583654, and KX583656, respectively.

Table 3 Nucleotide identity of A3A-A3H genes from NPMs compared with other primate species

Gene	Species	Percentage identified with NPM (%)
NPM-A3A	*Macaca mulatta*	99
	Homo sapiens	90
NPM-A3B	*Macaca mulatta*	96
	Homo sapiens	92
NPM-A3C	*Macaca mulatta*	98
	Macaca fascicularis	98
	Homo sapiens	91
NPM-A3D	*Macaca mulatta*	94
	Homo sapiens	90
NPM-A3F	*Macaca nemestrina*	99
	Macaca mulatta	99
	Homo sapiens	93
NPM-A3G	*Macaca nemestrina*	99
	Macaca mulatta	99
	Macaca fascicularis	99
	Homo sapiens	89
NPM-A3H	*Macaca mulatta*	99
	Homo sapiens	91

Enzyme digestion and expression product identification of NPM A3A-A3H expression plasmids

Positive colonies containing non-mutated target fragments were picked and identified by double digestion with restriction enzymes HindIII and XbaI. Agarose gel electrophoresis showed a series of DNA bands, which were in accordance with the expected sizes (Figure 3).

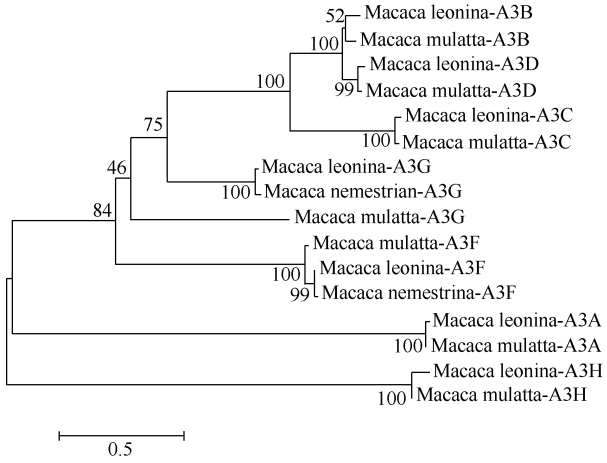

Figure 2 Phylogenetic tree of APOBEC3 proteins

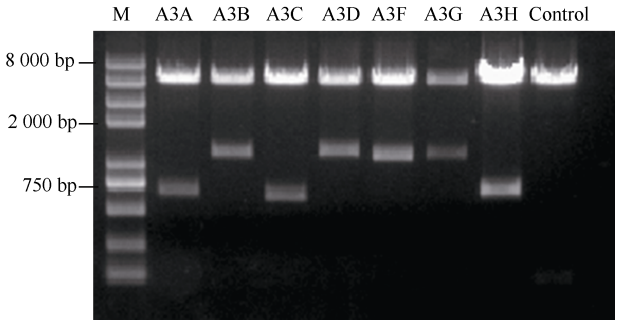

Figure 3 Identification of pcDNA3.1-NPM A3A-A3H by restriction analysis

Lane 1: DNA marker; Lane 2-8: Restriction product of pcDNA3.1-NPM; A3A-A3H; Lane 9: Control.

To verify the constructed pcDNA3.1-NPM-A3s expressed proteins, the recombinant plasmid and an empty pcDNA3.1 vector as a control were transfected into 293T cells. The cells were then subjected to protein detection by Western blot analysis. Protein expressions were detected and the molecular weights of the products were consistent with the expected values (Figure 4).

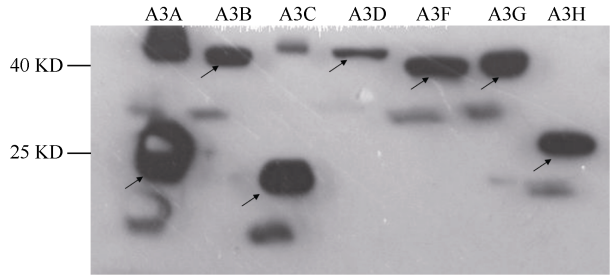

Figure 4 Detection of protein expression in 293T cells by Western blot analysis

In vitro anti-HIV-1 activities of NPM APOBEC3s

To address whether different NPM APOBEC3 isoforms were able to effectively inhibit HIV-1 infectivity, we performed a single-cycle infectivity assay in TZM-bl cells. We observed more efficient inhibition of HIV-1_{NL4-3} infectivity by NPM A3G and A3F. Furthermore, NPM A3B and A3D showed less efficient inhibition than A3G/F, A3H showed relatively weaker inhibition compared with that of A3B/D, and the HIV-1 produced in the presence of A3A and A3C expression vector did not show any reduction in relative infectivity (Figure 5).

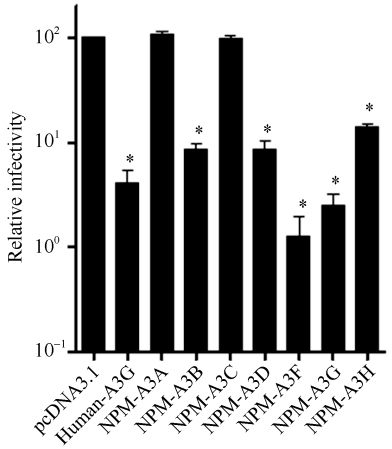

Figure 5 Anti-HIV-1 activities of NPM APOBEC3s

The empty parental vector served as a negative control and human-A3G vector as a positive control; *: $P<0.05$.

DISCUSSION

NPMs express a *TRIM5-Cyclophilin A* (*TRIMCyp*) fusion protein, instead of a *TRIM5α* protein, which makes them more susceptible to HIV-1 infection than other macaques (Kuang et al., 2009; Liao et al., 2007). However, HIV-1 still encounters considerable obstacles in regards to persistent replication in this host (Agy et al., 1992; Bosch et al., 2000; Hu, 2005), suggesting that other restriction factors act as barriers. In previous studies, APOBEC3s have demonstrated a capacity to restrict HIV-1 replication (Jia et al., 2015; Simon et al., 2015; Stavrou & Ross, 2015). However, it is unclear whether the APOBEC3 proteins of NPMs play an important role in restricting the replication of HIV-1.

In the current study, the NPM A3A-A3H genes were cloned, with BLAST analysis of their CDSs showing 94%-99% nucleotide identity with those of rhesus, crab-eating, and southern pig-tailed macaques, and 89%-93% nucleotide identity with those of humans. Among them, the identities of NPM cDNA sequences with those of rhesus and southern pig-tailed macaques reached 99%. Such a high nucleotide identity suggests that APOBEC3 genes might be important for the species to survive in nature. In addition, compared with rodents, which have only one APOBEC3 gene, primates have as many as seven (Jarmuz et al., 2002; Wedekind et al., 2003; Zhang & Webb, 2004), indicating that APOBEC3 gene expansion has been critical for primate survival during evolution.

About 35-50 million years ago, the dramatic decline in retrotransposon activities in primates might have resulted from the successful expansion of the APOBEC3 gene (Zhang & Webb, 2004). Retrotransposon activities increase genomic instability. Frequent retrotransposition can be detrimental for species living in stable environments and adapting to the environment at the same. However, moderate retrotransposon activities promote gene mutation, which is helpful for a species population to adapt to changing living environments. The APOBEC3 gene family has undergone rapid expansion, from one or two genes in non-primate mammals to at least seven in primates. In the past 30 million years, the primate genome has been infected by many viruses (Belshaw et al., 2004; Wolfe et al., 2004). The rapid evolution of the APOBEC3 genes has kept pace with the rapidly evolving viruses (Sawyer et al., 2004), suggesting that the APOBEC3 gene family plays an important role in restricting virus infection and maintaining genomic stability.

We analyzed the anti-HIV-1 activity of the A3A-A3H genes. NPM A3G/F demonstrated very efficient anti-HIV-1 activity; however, NPM A3A/C exhibited no anti-HIV-1 activity, which is consistent with the anti-HIV-1 activity of APOBEC3s in humans and rhesus macaques (Virgen & Hatziioannou, 2007). Our study indicates that A3G and A3F might play critical roles in restricting HIV-1 replication in NPMs *in vivo*.

Although results indicated that NPM A3A and A3C exhibited no anti-HIV-1 activity, their antiviral activity against other viruses and important biological roles cannot be ignored. Some studies have suggested that primate A3A limits the replication of the hepatitis B virus (HBV) and human papillomavirus (HPV) (Suspène et al., 2005; Vartanian et al., 2008) and the retrotransposition of Alu elements (Stenglein et al., 2010). In this study, we only determined the anti-HIV-1 activity of NPM APOBEC3s *in vitro*. Whether NPM A3G and A3F exhibit better anti-HIV-1 activity *in vivo* needs to be further explored.

In conclusion, A3A-A3H genes from NPMs were cloned, and their CDSs were found to be 99% identical to relevant sequences from rhesus and southern pig-tailed macaques. NPM A3G and A3F showed the greatest anti-HIV-1 activity compared with that of the other members, whereas A3A and A3C exhibited no anti-HIV-1 activity at all. This study indicates that A3G and A3F might play critical roles in limiting HIV-1 replication in NPMs *in vivo*. Our study provides valuable information for the optimization of monkey models of HIV-1 infection.

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