Genotyping of TRIM5 locus in northern pig-tailed macaques (Macaca leonina), a primate species susceptible to Human Immunodeficiency Virus type 1 infection

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Abstract

Background: The pig-tailed macaques are the only Old World monkeys known to be susceptible to human immunodeficiency virus type 1 (HIV-1) infection. We have previously reported that the TRIM5-Cyclophilin A (TRIMCyp) fusion in pig-tailed macaques (Macaca nemestrina) is dysfunctional in restricting HIV-1, which may explain why pig-tailed macaques are susceptible to HIV-1 infection. Similar results have also been reported by other groups. However, according to the current primate taxonomy, the previously reported M. nemestrina are further classified into three species, which all belong to the Macaca spp. This calls for the need to look into the previous studies in more details.

Results: The local species Northern pig-tailed macaque (M. leonina) was analyzed for the correlation of TRIM5 structure and HIV-1 infection. Eleven M. leonina animals were analyzed, and all of them were found to possess TRIM5-CypA fusion at the TRIM5 locus. The transcripts encoding the dysfunctional TRIM5-CypA should result from the G-to-T mutation in the 3’-splicing site of intron 6. Polymorphism in the putative TRIMCyp recognition domain was observed. The peripheral blood mononuclear cells (PBMCs) of M. leonina were susceptible to HIV-1 infection. Consistent with the previous results, expression of the M. leonina TRIMCyp in HeLa-T4 cells rendered the cells resistant to HIV-2ROD but not to SIVmac239 infection.

Conclusion: The susceptibility of M. leonina to HIV-1 infection is due to the dysfunctional TRIM5-CypA fusion in the TRIM5 locus. This finding should broaden our perspective in developing better HIV/AIDS non-human primate animal models.
Background

Human immunodeficiency virus type 1 (HIV-1) originated from cross-species transmission from chimpanzees to humans and is the major causative agent of human acquired immunodeficiency syndrome (AIDS) pandemic [1-3]. Although HIV-1 infects human CD4+ cells, it does not infect most non-human primates (NHP). Studies using Vesicular Stomatitis virus G-glycoprotein (VSV-G) pseudotyped viruses, which bypass the receptor restriction, revealed that species-specific host factors restrict HIV-1 infection [4]. For example, the host restriction factor tripartite motif protein 5α (TRIM5α) potently blocks HIV-1 replication in rhesus macaque (M. mulatta) through species-specific post-entry restriction in Old World monkeys [5].

TRIM5α is a member of the TRIM family, which contains the RING, B-Box2 and coiled-coil domains, and a C-terminal B30.2/SPRY domain. TRIM5α interacts with the capsid (CA) portion of HIV-1 Gag protein through its B30.2/SPRY domain, which determines the specificity and potency of TRIM5α restriction to retroviruses [5,6]. Host protein cyclophilin A (CypA) interacts with the CA through incorporation into HIV-1 particles, and modulates HIV-1 replication in host cells [7-9]. It has been documented that in Old World monkey cells, CypA is required for TRIM5α-mediated resistance to HIV-1 [10]. The New World primate owl monkey (Aotus) expresses a TRIM5-CypA (TRIMCyp) fusion protein, in which the B30.2/SPRY domain of TRIM5α is replaced by CypA resulting from retrotransposition of the CypA pseudogene cDNA into the seventh intron at the TRIM5 locus. The owl monkey TRIM5-CypA (omTRIMCyp) restricts several retroviruses including HIV-1, simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV) [11,12]. Recently, we and others reported that pig-tailed macaques may contain individuals of different species. Here, we analyzed the susceptibility of the local species Macaca leonina in Yunnan to HIV-1 infection and the TRIM5 locus. The fusion pattern of TRIMCyp and the polymorphism of the TRIMCyp recognition domain in Macaca leonina were characterized.

Results

Characterization of the TRIMCyp fusion gene in M. leonina

To investigate the correlation between the TRIM5α sequence and the susceptibility to infection by HIV-1 in M. leonina, the genomic sequence of the TRIM5 locus of 11 animals from several different populations was analyzed (Table 1). A pair of specific PCR primers was designed based on the human TRIM5 genomic sequence, with the forward primer in the TRIM5 exon 8 and the reverse primer in the adjacent genomic region after TRIM5 3'-UTR (Table 2). A fragment of about 2,800 bp was amplified (Fig. 1A), indicating that the TRIM5 locus is longer than normal and thus the TRIM5-CypA pattern might exist. To confirm this notion, another pair of primers was designed, with the forward one in exon 8 and the

Table 1: The information of M. leonina samples used in this study.

| Sample Number # | Sex  | Weight | Origin of Macaque | Sampling Time   | Population Location |
|------------------|------|--------|-------------------|-----------------|---------------------|
| 524              | Male | ND     | Yunnan, China     | 1998-4-15       | KIZ, CAS            |
| 528              | Female | ND    | Yunnan, China     | 1998-4-15       | KIZ, CAS            |
| 551              | Female | ND    | Yunnan, China     | 2000-11         | KIZ, CAS            |
| 87015            | Male  | 11 kg  | Yunnan, China     | 2008-4-11       | KIZ, CAS            |
| 93201            | Male  | 12 kg  | Yunnan, China     | 2008-4-11       | KIZ, CAS            |
| 97203            | Male  | 9.5 kg | Yunnan, China     | 2008-4-11       | KIZ, CAS            |
| 99201            | Male  | 10 kg  | Yunnan, China     | 2008-4-11       | KIZ, CAS            |
| KMZ-1            | Male  | 14.5 kg| Yunnan, China     | 2008-3-28       | KMZ                 |
| KMZ-2            | Male  | ND     | Yunnan, China     | 2008-3-28       | KMZ                 |
| KMZ-4            | Female | 6.4 kg| Yunnan, China     | 2008-3-28       | KMZ                 |
| KMZ-5            | Male  | ND     | Yunnan, China     | 2008-3-28       | KMZ                 |

ND: not determined
reverse one in the CypA sequence (Table 2). Indeed, a CypA cDNA sequence is inserted in the TRIM5 locus in all M. leonina (Fig. 1B, C), which disrupts the normal TRIM5.

To further characterize the CypA insertion in the TRIM5 locus, we performed several other PCR reactions with different pairs of primers (Table 2). The PCR products were recovered and sequenced. The sequencing results revealed that the CypA pseudogene cDNA insertion in the TRIM5 locus resulted from a LINE (long interspersed nuclear element)-1-mediated retrotransposition (Fig. 1D), which is very common in mammals [22,23].

**Expression of TRIMCyp fusion gene in M. leonina**

To test whether the TRIMCyp fusion gene in M. leonina is transcribed to produce mature transcripts, the RNAs from 8 M. leonina samples were reverse transcribed and PCR amplified using specific primers TRIMCypF and TRIM-CypR (Table 2). Multiple mature TRIMCyp transcripts with different lengths were detected in eight M. leonina samples, but not in the Chinese rhesus macaque samples (data not shown). Sequencing analysis of the PCR products revealed that both exon 7 and exon 8 were spliced out in all major isoforms leaving exon 6 fused to the CypA cDNA in frame (data not shown), as previously reported [13-16].

To understand why exons 7 and 8 were not included in the mature transcripts, the sequences of introns 6 and 7 were analyzed for aberrant splicing sites. The Nsi I restriction site upstream the 3' splicing site of intron 6 has been reported to be closely linked to the mutation within the site [17], which allowed a convenient screening of the G-to-T substitution in the splicing site (Fig. 2B). Analysis of the PCR product flanking intron 6 by the Nsi I restriction digestion revealed that all M. leonina were homozygous for the Nsi I site (Fig. 2A). The G-to-T substitution in the 3' splicing site of intron 6 was confirmed by sequencing analysis of the PCR products in all the M. leonina samples (Fig. 2B). The G-to-T substitution in the 3' splicing site of intron 6 should prevent the inclusion of exon 7 during splicing. Sequencing analysis revealed that the 3' splicing site of intron 7 was normal (data not shown). The exclusion of exon 8 in the mature transcript is likely the result of alternative splicing, as previously observed [13].

**Polymorphism analysis in the TRIMCyp recognition domain**

A fragment of 3348 bp from intron 6 to the 3' genomic adjacent region was analyzed for polymorphisms via DnaSP 4.5 program [24,25]. In the 11 Northern pig-tailed macaques, a total of 46 polymorphic nucleotide sites were identified, including 40 Singleton variable sites and 6 Par-simony informative sites (Fig. 3A). Among these sites, 16 sites (site 579, 592, 613, 796, 803, 883, 925, 1026, 1087, 2134, 2251, 2303, 2415, 2494, 2506 and 2529) are in the coding region (39%), and the others (site 169, 248, 252, 350, 364, 462, 1262, 1265, 1440, 1718, 1719, 1721, 1723, 1759, 1765, 1912, 2034, 2037, 2057, 2075, 2740, 2749, 2802, 2868, 2907, 2950, 3016, 3031, 3277 and 3282) are in the noncoding region. There are 15 nonsynonymous variation sites in the coding region, except for the synonymous site 2303. In KMZ-4, we identified an insertion-mutation, which results in frame shift relative to the coding region (data no shown). Next, we sought to determine whether the observed polymorphisms are consonant with the real status. Linkage disequilibrium (LD) describes a situation in which some combinations of alleles or genetic markers occur more or less frequently in a population than would be expected from a random formation of haplotypes from alleles based on their frequencies. The occurrence of LD permits the construction of Haplotype. The LD analysis (Fisher's exact test and Chi-square test) showed that no recombinant event occurred, and the degree of LD is strong (P < 0.001) (Fig. 3B). Additionally, the Neutrality theory was used to detect the intra-specific polymorphism level, and two approaches (Tajama’s D test and Fu and Li’s D test) based on different algorithm models were employed. The Neutrality test of Tajama’s D test demonstrated no statistical significance in
Formation of TRIMCyp fusion gene in *M. leonina*. (A and B) The genomic sequences spanning from the 5' end of exon 8 to the 3' end of exon 8 (A) or to the 3'-UTR of CypA cDNA (B) were PCR amplified and subject to electrophoresis analysis. MW: DNA molecular weight marker DL-2000; Lane 1–11: *M. leonina* samples 524, 528, 551, KMZ-1, KMZ-2, KMZ-4, KMZ-5, 87015, 93201, 97203, and 99201. (C) Schematic structure of the TRIM5Cyp fusion. The exons are represented as boxes with the coding region being shaded, and the sequence of the inserted CypA cDNA is denoted below. (D) The CypA pseudogene cDNA retrotransposed into TRIM5 locus. The asterisk (*) indicates the splicing acceptor, CypA pseudogene cDNA sequence is underlined, target site duplication (TSD) is in bold italic, and the start or stop codon of inserted CypA cDNA is in bold-type.

Figure 1
polymorphism along the sequences (P < 0.005), while Fu and Li's D test were significant (P < 0.005) (Fig. 3C).

The coding sequences of TRIMCyp exon 7, exon 8 and CypA from *M. leonina* were assembled to deduce the putative amino acid sequences. The phylogenetic tree based on the putative amino acid sequences demonstrated that the 11 *M. leonina* are divided into several major subgroups (Fig. 4A), which may explain the high polymorphic in this region. In the *Aotus* and *Macaca* TRIMCyp proteins, the CypA part is the recognition domain mediating the binding of the fusion protein to the incoming viral capsids. The putative amino acid sequences of the CypA domain from various species were aligned. No major differences were found in sequences of CypA inserted in TRIMCyp from diverse *M. leonina*. The results clearly show that the sequences are homologous among the Old World macaque *M. leonina, M. nemestrina* and *M. mulatta* species, while the *M. fascicularis* and the New World monkey *A. trivirgatus* were much less homologous (Fig. 4B). In addition, some amino acids critical for the restriction of HIV-1 in *A. trivirgatus*, such as N66 and H69, were observed in *M. leonina*. The phylogenetic tree among these primates was constructed based on the inserted CypA amino acid sequences. The result revealed that the *Macaca spp* species members *M. leonina, M. nemestrina* and *M. mulatta* were
Genetic polymorphism analysis of the TRIMCyp recognition domain. (A) Polymorphic sites of the nucleotide sequence of the TRIMCyp recognition domain. The boxed numbers indicate the polymorphic sites, with 6 Parsimony informative positions in italic. The boxed sequences showed the Haplotype of the *M. leonina* samples. Dots present the identical nucleotides. (B) The linkage disequilibrium analysis of all sequenced sites. The histogram X axis plots the $D'$ value, the Y axis plots the DNA sequence nucleotide positions. (C) Tajima's or Fu and Li's $D$ test of the total number of mutations for neutrality test through DnaSP program.
Figure 4
Phylogenetic analysis of primate TRIMCyp recognition domain. (A) Neighbor-joining nucleotide tree of the 11 Northern pig-tailed macaques based on TRIMCyp. Bootstrap values are based on 1,000 replicates. (B) Alignment of putative CypA amino acid sequences of primate TRIMCyp. The gray bar indicates mutation sites in Old World primates, and the bold bar indicates deletion mutations, as compared to A. trivirgatus. (C) Phylogenetic analysis of the primate TRIMCyp recognition domain (based on CypA amino acid sequence) with the A. trivirgatus as outgroup. Bootstrap values are based on 1,000 replicates.
were challenged with HIV-2ROD (MOI = 0.02). Replication confirmed by Western immunoblotting (Fig. 5B). The cells aregatus terminally HA-tagged TRIMCyp of To test whether the infection. established that the magnitude of susceptibility to HIV-GFP-VSVG infection compared to the human PBMCs (Fig. 5A). These results were relatively resistant to HIV-1 infection (Fig. 5A). In comparison, the M. leonina PBMCs demonstrated a magnitude of susceptibility to HIV-GFP-VSVG infection comparable to the human PBMCs (Fig. 5A). These results established that the M. leonina are susceptible to HIV-1 infection.

To test whether the M. leonina TRIMCyp can restrict other retroviruses, we generated HeLa-T4 cell lines expressing C-terminally HA-tagged TRIMCyp of M. leonina and A. trivirgatus. The expression of the TRIMCyp fusion proteins (npmTRIMCyp for northern pigtailed macaque TRIMCyp and omTRIMCyp for owl monkey TRIMCyp) was confirmed by Western immunoblotting (Fig. 5B). The cells were challenged with HIV-2ROD (MOI = 0.02). Replication of the virus in these cells was evaluated by measuring the capsid p27 antigen levels. The results demonstrated that both npmTRIMCyp and omTRIMCyp actively restricted HIV-2ROD by about 16-fold (Fig. 5C). The cells were also assayed for their restriction to SIVmac239 replication. The npmTRIMCyp demonstrated very moderate restriction activity, while the omTRIMCyp could not inhibit SIVmac239 replication (Fig. 5C).

Discussion
Because of their close evolutionary relationship to humans, NHPs are of vital importance in biomedical research and are often the best or only animal models for controlled experiments relevant to a range of human diseases and disorders. Macaque infection models provide unique opportunities for generating discoveries that may lead to new therapeutic options, improved vaccine strategies, and increased preparedness for future disease outbreaks. Among Old World monkeys, the pig-tailed macaques (M. nemestrina) were reported to be prone to HIV-1 infection with AIDS-like symptoms [26-29]. We and other groups identified TRIMCyp fusion protein expression in pig-tailed macaques. However, the fusion protein failed to restrict HIV-1 replication when expressed in some non-restrictive human or macaque cell lines [13-17].

Interestingly, the aforementioned pig-tailed macaques are divided into three macaque species according to current primate taxonomy [18]. Understanding the relationship between the TRIM5 locus and the susceptibility to HIV-1 in these species is urgently required. Here, we surveyed the M. leonina in Yunnan province, China. The results showed that the PBMCs of M. leonina are susceptible to HIV-1 infection, a finding which is consistent with previous results [13-17]. Moreover, the M. leonina fusion protein npmTRIMCyp can potently block HIV-2ROD, which may account for the different modulatory roles of host cell CypA, as the CypA is incorporated into HIV-1 but not HIV-2. Some other mechanisms may also exist, and one needs more work to dissect these possibilities. The same fusion pattern of TRIM5-CypA gene was observed in all the 11 surveyed M. leonina animals. Although it has been reported that both M. nemestrina and M. mulatta express TRIMCyp fusion proteins [15-17], our results suggest that the frequency of TRIM5-CypA fusion is higher in M. leonina than in M. mulatta. Further studies suggest that the fusion results from the insertion of the CypA pseudogene cDNA into the 3'-UTR of TRIM5 through the LINE-1-element-mediated retrotransposition. The transcription products of TRIM5-CypA were also detected in M. leonina PBMCs, and the transcript formation was attributed to the G-to-T substitution in the 3'-splicing site of TRIM5 intron 6 in macaques 17. In the human genome, more than 60 processed CypA pseudogenes were reported across the genome by retrotransposition [30]. In New World primates, the TRIM5-CypA fusion gene was only identified in Aotus species, and the CypA exposed positive selection in the evolutionary history [31]. However, what drove the CypA pseudogene cDNA retrotransposition into the TRIM5 locus twice in the New World and Old World primates independently? These questions call for more genetic research to delineate the mechanistic details.

Host genetic variations have important impact on the susceptibility to HIV-1 infection. TRIM5α restricts HIV-1 through the B30.2/SPRY domain specifically recognizing and interacting with the CA protein. In owl monkey, the CypA copy in the TRIMCyp fusion protein can bind lentiviral CA and block their replication [11,12]. In pig-tailed macaques, CypA pseudogene cDNA substituted the B30.2/SPRY in the TRIM5α, resulting in the formation of the TRIMCyp fusion gene. Virgen et al. suggested that the TRIMCyp protein from M. nemestrina does not bind to HIV-1 CA because of the single amino acid (R69H) muta-
Figure 5 (see legend on next page)
In conclusion, the HIV-1 infection.

broaden the perspective in developing better HIV/AIDS animal models. Our results should establish optimal HIV/AIDS NHP models in primates to HIV-1 infection. It might be possible to establish optimal HIV/AIDS NHP models in M. leonina through screening macaques that possess SNPs more susceptible to HIV-1 infection.

In conclusion, the TRIM5-CypA fusion in M. leonina maybe a pivotal factor associated with their susceptibility to HIV-1. The M. leonina appears to be a good candidate for an HIV/AIDS animal model. Our results should broaden the perspective in developing better HIV/AIDS NHP animal models.

Materials and methods

Animals
Northern pig-tailed macaques KMZ-1, KMZ-2, KMZ-4 and KMZ-5 were bred in the Kunming Zoo (KMZ), animals 524, 528, 551, 87015, 93201, 97203 and 99201 were raised in the Kunming Institute of Zoology (KIZ), Chinese Academy of Sciences (CAS) (Table 1). The Chinese rhesus macaque 95005 was raised in the KIZ, CAS. Whole blood from these animals was collected in EDTAK2 Blood Collection Tubes following the National Experimental Animal Handling Ordinance.

RNA/DNA samples
PBMCs were isolated from EDTA K2-treated whole blood by EZ-Sep™ Monkey 9× (Dakewe Biotech) or Ficoll-Hypaque density centrifugation. Total RNA was isolated from 8 × 10⁶ PBMCs using the RNAprep Cell Kit (Tiangen) following the manufacturer’s instruction. Genomic DNA was isolated from 300 μL whole blood using the Puregene DNA Purification Kit (Qiagen) following the manufacturer’s handbook.

Genomic DNA Amplification and Sequencing
The PCR primers for amplifying the genomic DNA from intron 6 to the 3’ flanking sequence (Table 2) were designed based on the sequences published in Genbank (accession numbers EU371641 and NT_009232). The fragments were amplified using LA Taq-PCR or Ex Taq-PCR kit (TaKaRa), purified with DNA Gel Extraction Kit (Watson Biotech), and cloned into the pMD 19-T Simple vector (TaKaRa), clones were picked up for sequencing analysis.

To screen for the G-to-T mutation associated with the 3’-splicing site within TRIM5 intron 6, the fragment encompassing the 3’-splicing site in intron 6 was PCR amplified with the sense primer T5in6F1 and the anti-sense primer T5ex8R3. The PCR products were digested with restriction endonuclease Nsi 1 (Fermentas), followed by electrophoresis in a 1.4% agarose gel [17]. In addition, the PCR products were purified and cloned for sequencing analysis.

cDNA Amplification
Primers TRIMCypF and TRIMCypR (Table 2) for amplification of the complete coding sequence of Northern pig-tailed macaques TRIM5 have been described previously [13]. The total RNA was reverse transcribed into cDNA using the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa) following the manufacturer’s instruction. The PCR condition was: 94°C for 2 minutes; 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1.5 minutes; held at 72°C for 7 minutes, and stored at 4°C.

Sequence analysis
The sequences were assembled with the Contig program, and aligned by the Clustal X 1.83 or DNASTar 7.1.0 (Lasergene) software. Nucleotide sequence polymorphisms were analyzed by DnaSP 4.50 program. Neighbor-joining nucleotide tree of primate TRIMCyp was constructed via MEGA 3.1 program, bootstrap values were based on 1,000 replicates. Representative species of Aotus trivirgirtas was used as outgroup. The reference sequence accession num-
**Cell culture, transfection, and infection assay**

Primates and human PBMCs were isolated by EZ-Sep monkey 9× or Ficoll-Hypaque PBMCs separation solution. PBMCs were cultured in RPMI 1640 complete medium supplemented with 5 μg/ml Phytohemagglutinin (PHA) (Sigma-Aldrich) and 50 IU/ml Interleukin-2 (IL-2) (Sigma-Aldrich) for 72 hours to activate the cells. Human T lymphocyte MT-4, monocyte U937, and a transformed Chinese rhesus macaque B lymphocyte line were cultured in complete RPMI 1640 medium. Activated PBMCs were seeded at the density of 5 × 10^5 cells/well in the 96-well plate, and the control cell lines were seeded at 4 × 10^5 cells/well. The HIV-GFP-VSVG viral stocks were thawed in a room temperature water bath, diluted with culture medium supplemented with 20 mM pH7.5 HEPES and 8 μg/ml polybrene (Sigma-Aldrich) on the ice. The cells were infected for 4 hours in the 37°C, 5% CO2 chamber, washed twice with phosphate-buffered saline (PBS), and then cultured in fresh RPMI 1640 complete medium. After 48 hours post-infection, the percentage of GFP positive cells was counted by FACS analysis using a FACSCalibur (Becton Dickinson).

HeLa-T4 cells were cultured in DMEM supplemented with 10% fetal calf serum (GIBCO), penicillin (Sigma), and streptomycin (Invitrogen). The C-terminally avian influenza Hemagglutinin (HA)-tagged TRIMCyp cDNA recombinant pLPCX expression plasmids of M. leonina and A. triivirgatus were constructed and transfected as previously described [13], nptTRIMCyp and omTRIMCyp expression in HeLa-T4 cells were detected by Western blot for the HA-tag, β-actin as a loading control. HeLa-T4 cells stably expressing TRIMCyp proteins were seeded in the 24-well plate at a density of 3 × 10^4 cells/well. On the following day, the cells were inoculated with viruses (MOI = 0.02) at 37°C for 2 hours. The cells were washed twice with PBS and cultured in 500 μl of fresh DMEM complete medium at 37°C, 5% CO2. Culture supernatants were quantified by a p27-specific SIV p27 anti-HEPES kit (Zeptometrix).

**Nucleotide sequence accession numbers**

The M. leonina TRIM5-CypA sequences are deposited in the GenBank database, the accession numbers are GQ180913–GQ180923.

**Competing interests**

The authors declare that they have no competing interests.

**Authors' contributions**

YIZ and YQK conceived of the study, and participated in its design. YQK, XT, and FLL carried out the experiments. YQK, YIZ, GG, XLJ and YPZ analyzed the results and drafted the manuscript. All authors read and approved the final manuscript.

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