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ORIGINAL ARTICLE

Polymorphisms in the *APOBEC3G* gene of Chinese rhesus macaques affect resistance to ubiquitination and degradation mediated by HIV-2 Vif

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Abstract

Animal cells have multiple innate effector mechanisms that inhibit viral replication. For the pathogenic retrovirus human immunodeficiency virus 1 (HIV-1), there are widely expressed restriction factors, such as APOBEC3 proteins, tetherin/BST2, SAMHD1 and MX2, as well as TRIM5α. We previously found that the *TRIM5α* gene clearly affects SIVmac or HIV-2 replication, but the major determinant of the combinatorial effect caused by multiple host restriction factors is still not fully clear. APOBEC3G (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G), a host restriction factor that restricts HIV replication by causing cytosine deamination, can be targeted and degraded by the SIV/HIV-1/HIV-2 accessory protein Vif. Although rhesus macaques are widely used in HIV/AIDS research, little is known regarding the impact of *APOBEC3G* gene polymorphisms on viral Vif-mediated ubiquitin degradation in Chinese-origin rhesus macaques. In this study, we therefore genotyped *APOBEC3G* in 35 Chinese rhesus macaques. We identified a novel transcript and 27 *APOBEC3G* polymorphisms, including 20 non-synonymous variants and 7 synonymous mutation sites, of which 10 were novel. According to the predicted structure of the A3G protein, we predicted that the E88K and G212D mutations, both on the surface of the A3G protein, would have a significant effect on Vif-induced A3G degradation. However, an *in vitro* overexpression assay showed that these mutations did not influence HIV-2-Vif-mediated degradation of APOBEC3G. Unexpectedly, another polymorphism L71R, conferred resistance to Vif-mediated ubiquitin degradation, strongly suggesting that L71R might play an important role in antiviral defense mechanisms.

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Introduction

Immune defense of animals involves multiple innate effector mechanisms that inhibit viral replication. For the pathogenic retrovirus human immunodeficiency virus 1 (HIV-1), there are widely expressed restriction factors [12], such as APOBEC3 proteins [17], tetherin/*BST2* [14], SAMHD1 [7, 10], MX2 [5], HCG22 [25], and TRIM5α [19]. Also, many genes have previously been reported to be involved in the control of simian immunodeficiency virus (SIV) replication. These include selected MHC class I and II [15, 24] alleles and loci, including *Mamu-A*01*, *-B*08*, and *-B*17*.

Another important restriction factor, APOBEC3G (A3G), a member of the APOBEC3 family, is widely present in primates [6]. In rhesus macaques, the *A3G* gene is located on chromosome 10, with eight exons and seven introns. The protein A3G has two conserved zinc-binding domains: one at the N-terminus, which facilitates binding to viral RNA and HIV gag, and one at the C-terminus, which renders the enzyme

active. However, most lentiviruses, including HIV-1, HIV-2 and SIV, encode an accessory protein called the "viral infectivity factor" (Vif), which binds the C-terminus of the SOCS-box domain of A3G, causing the ubiquitination and degradation of A3G by proteasomes [13, 18, 27]. In the absence of the HIV accessory protein Vif, A3G is encapsidated in virions via the N-terminal domain [12], which facilitates RNA binding. Meanwhile, the C-terminal domain facilitates cytidine-deaminase-dependent dG-to-dA hypermutation on the plus strand during reverse transcription of viral RNA [4, 21, 22]. The result of this induced hypermutation is that several stop codons are introduced in the cDNA, which prevents transcription and subsequent viral replication [26, 28, 29]. Previous studies have suggested that the β4-α4 loop of human A3G (amino acids 122–130) is involved in its interaction with and degradation by Vif and that the residues D128 and P129 are crucial for the binding of Vif to human A3G. The mutation D128K was found to reverse the specificity of A3G for Vif in African green monkeys [1, 16].

In addition to the mutants of A3G mentioned above, there are also many functionally unknown variants of A3G, but according to the current genome-wide association studies (GWAS), there is no single-nucleotide polymorphism (SNP) around *APOBEC3G* that shows a direct association with HIV infection, indicating that the association between the human APOBEC3G polymorphism and HIV infection is still unclear. However, New World monkeys such as owl monkeys are resistant to HIV-1 infection, preventing their use as animal models for HIV-1. However, HIV-2, which is closely related to SIV (smm) suggesting that HIV-2 infected AIDS animal models may more convenient for applications, such as drug testing and pathophysiology studies. Furthermore, cross-infection of HIV-1 and HIV-2 may shorten the course of AIDS, and therefore, understanding the pathogenesis of HIV-2 is necessary for vaccine development against AIDS. Rhesus macaques are widely used as non-human primate models for studying HIV/AIDS pathogenesis and therapeutics [8]. Several groups previously identified *A3G* polymorphisms in Indian rhesus macaques [9, 23] and demonstrated the interaction between Vif and rhesus macaque *A3G* in coexpression experiments [9]. However, few studies have focused on the relationship between *A3G* polymorphisms and their effects on viral infectivity in rhesus macaques. In this study, we identified *A3G* polymorphisms in Chinese rhesus macaques and investigated their effects on HIV-2 and SIV accessory proteins.

Materials and methods

Animals

Thirty-five unrelated Chinese rhesus macaques from China were bred for several generations in Guangdong province, China. All rhesus macaques from which samples were taken were clinically normal with no known diseases.

Peripheral blood mononuclear cell (PBMC) isolation and activation

Whole-blood samples were obtained from 35 Chinese rhesus macaques, and PBMCs were isolated by lymphocyte separation medium (GE Healthcare, Little Chalfont, UK) density gradient centrifugation and then cultured at 1×10^6 cells/ml in RPMI-1640 (Gibco, Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS) and 50 IU of interleukin-2 (IL-2) (Sigma-Aldrich) and 5 μg of concanavalin A (Con A) (Sigma-Aldrich, St. Louis, MO, USA) per ml.

RNA isolation, cDNA synthesis, direct sequencing of PCR products, and analysis

RNA was isolated from peripheral blood samples of rhesus macaques using an E.Z.N.A Blood RNA Kit (Omega Bio-Tek, Guangzhou, China) and subjected to one-step reverse transcription polymerase chain reaction (RT-PCR) using a PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time; TaKaRa Bio, Dalian, China), following the protocols recommended by the manufacturers.

The full-length *A3G* sequence was amplified using two pairs of primers (Table S1), with a 126-bp overlap between the two resulting PCR products. PCR amplification was performed in a 40-μL reaction mixture containing 20 μL of 2× Taq Plus PCR Master Mix, 1 μL (10 pmol/μL) of each pair of primers, 2 μL of template cDNA, and 16 μL of ddH_2O. Amplification was done for 3 min at 94°C followed by 33 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, with a final cycle at 72°C for 10 min. The annealing temperature was adjusted based on the melting temperatures of the primers.

Direct sequencing of PCR samples from each animal was performed by Sangon Biotech (Shanghai) Co., Ltd. and BGI TechSolutions Co., Ltd. The sequence data were analyzed using SeqMan software (DNASTAR). All statistical data were analyzed using SPSS statistical 19.0 software (SPSS Inc., Chicago, IL, USA).

Statistical analysis

Linkage disequilibrium (LD) was calculated between all pairs of allelic loci (except for the deletion variant site) using the R genetics package (??Warnes?? *et al.* 2008). Values of r^2 ranged from 0 for independence to 1 for complete LD between the pairs, and $r^2 > 0.33$ was considered to indicate strong LD.

Structural models of A3G

A model of the rhesus macaque A3G protein was built using Discovery Studio 2.5 software. In a previous report, the A3C structure PDB: 3VOW was selected as a template for the N-terminal domain of A3G [9], and we also followed this strategy. In addition, human A3G_CTD (PDB: 3IQS) served as a template for rhA3G_CTD. The aligned sequences showed relatively high similarity to the templates (37.8% and 38.5% sequence identity, respectively).

Homology models were built using the PROTEIN MODELING program: 30 models were calculated, and the one with the best PDF total energy and DOPE scores was selected. Subsequently, the energy of the model was minimized using the MINIMIZATION program of Discovery Studio 2.5. The model was evaluated using a Ramachandran plot.

Cells lines

Human embryonic kidney 293T (HEK293T) cells were maintained in Dulbecco's high-glucose modified Eagle's medium (DMEM, Thermo Fisher Scientific, Guangzhou, China) supplemented with 10% FBS and 1% penicillin and streptomycin (Thermo Fisher Scientific, Guangzhou, China).

Plasmids

For the *APOBEC3G* clones, DNA was amplified using the primers A3G-F and A3G-R, (Supplementary Table S1) which contained recognition sites for the restriction enzymes *Kpn*I and *Eco*RI, resulting in the *APOBEC3G* sequence overlapping with a synthesized Flag tag at the C-terminus. The PCR product was cloned into pcDNA3.1(+) to generate an *A3G* control (the genotype was 71L, 88K, 212G). PCR-based one-step site-directed mutagenesis was performed using mutagenic primers (Table S1). The full-length plasmids with the L71R, E88K, G212D mutations were designated as $A3G_{L71R}$ (71R, 88K, 212G), $A3G_{E88K}$ (71L, 88E, 212G), and $A3G_{G212D}$ (71L, 88K, 212D), respectively. These template plasmids were digested with *Dpn*I and cloned in *E. coli* DH5α. The resulting plasmid was sequenced to ensure that the L71R, E88K, G212D mutations were introduced. HIV-2 Vif expression plasmids were obtained from the Clinic for Gastroenterology, Hepatology, and Infectiology, Medical Faculty, Heinrich-Heine-University Düsseldorf [30].

Immunoblotting

A3G degradation experiments were performed in 6-well plates. 0.25×10^6 293T cells were cotransfected with 850 ng of A3G expression plasmid and 2500 ng of HIV-2 Vif expression plasmid. To maintain equivalent DNA amounts, pcDNA3.1(+) empty vector was used instead of Vif plasmids as a control, using Lipofectamine 2000 Reagent (Invitrogen; Shanghai, China) following the manufacturer's recommendations. The cells were harvested 48 hours post transfection and lysed in radioimmunoprecipitation assay (RIPA) buffer (Biotech Well; Shanghai, China) with 1 mM PMSF. For each well, 300 µl of lysis buffer was used. The total protein concentration was determined by bicinchoninic acid (BCA) assay (Biotech Well; Shanghai, China), and samples were normalized with lysis buffer and mixed with an equal volume of 2×Laemmli sample buffer and solubilized by boiling for 10 min at 99°C. Proteins were separated by SDS/PAGE, and tagged proteins were detected with either mouse monoclonal anti-FLAG antibody (Thermo Fisher Scientific; Rockford, USA) or mouse monoclonal anti-V5 antibody (Invitrogen; Shanghai, China) using dilutions recommended by the manufacturer. β-actin was detected with mouse monoclonal anti-beta actin antibody (EarthOx; San Francisco, CA), followed by HRP affinipure goat anti-mouse IgG antibody (EarthOx; San Francisco, CA) and developed with Western BloT Hyper HRP Substrate (Takara; Dalian, China). The expression levels of the engineered A3G mutants were assessed by transfecting 293 cells in a 6-well plate with 3 µg of the A3G control, $A3G_{L71R}$, $A3G_{E88K}$, or $A3G_{G212D}$ construct as described above.

Results

Identification of a novel *A3G* transcript and *A3G* gene polymorphisms in Chinese rhesus macaques

To investigate the relationship between *A3G* gene polymorphism and HIV-2/SIVmac replication in rhesus macaques, we first amplified *A3G* transcripts from 35 unrelated Chinese rhesus macaques of Vietnamese origin to identify variants of *A3G*. Two pairs of primers were used (A3G-F5/A3G R1-R2 and A3G-F3/A3G-R3, Table S1) to produce 852-bp and 689-bp amplification products, respectively. After sequencing and a BLAST search, we found that one full-length sequence was different from the reference sequence (GenBank: NM_001198693.1) (Fig. 1), suggesting that it was a novel transcript (GenBank: KU058147.2). In addition to the novel transcript, we investigated the other variants of the *APOBEC3G* transcript.

In all, we identified 27 polymorphisms of *APOBEC3G*, including 20 non-synonymous and seven synonymous variants, 10 of which were novel, while 17 had been identified previously by sequencing (Fig. 2a). The individual polymorphisms were detected in at least three individuals. We found that most of the variations were located at the N-terminal domain of A3G, and only two synonymous

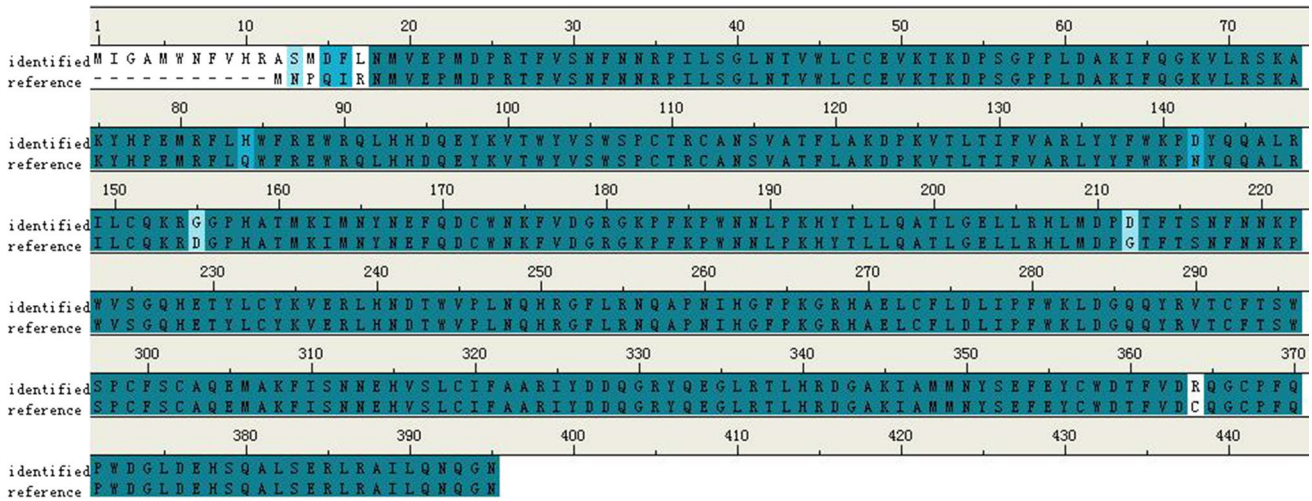

Fig. 1 Sequence alignment of the unexpected main transcript variant and the preferred NCBI-derived sequence

variations were located in the second cytidine deaminase zinc-binding domain (CD2) (Fig. 2b). The top three common variants, F82F (T246C), E88K (G262A), and G212D (A635G) were chosen for subsequent experiments. At the T246C locus, the homozygous C/C genotype (22.9%) occurred less frequently than the genotypes T/T and T/C, while in G262A, G/G (20%) occurred less frequently than the other genotypes. In the case of A635G, the homozygous A/A genotype was the least common (11.4%). Interestingly, nine SNPs (S30F, R35F, W45C, E49K, K51N, K53E, S56S, G57R and K68E) were in strong linkage disequilibrium (LD) (Table S2) as well as clustering in the N-terminus of A3G, indicating that they might play a role in the stability or biological function of the A3G protein. A previous study found that the frequency of the combined 60LR+130D haplotype (71LR+141D in this study) was rare [9], which was consistent with the corresponding haplotype frequency of 5.71% in this study. Analysis of further individuals is needed to obtain a more complete overview of the genetic diversity of *APOBEC3G* in these macaque species.

Structural modeling of A3G mutants with variations in potential binding resides

Vif acts as an adaptor protein to connect A3G to E3 ubiquitin ligase, thereby inducing the polyubiquitylation and proteasomal degradation of A3G. Mutations that interfere with the recognition and binding of A3G to Vif could potentially result in resistance to Vif-induced A3G degradation. To identify which mutants might influence the degradation progress induced by HIV-2 Vif, we made a structural model of the A3G protein to find out which mutants affect surface resides of A3G that might be involved in binding of HIV-2 Vif. Using the PROTEIN MODELING program, we constructed the homologous structure models of A3G, and the model with the best score of PDF total energy and DOPE was selected (Fig. 3). In this model, E88K and G212D were located on the surface of the A3G protein, suggesting that these residues might play a role in Vif recognition and interaction. A previous study showed that the L71R mutation identified here was associated with resistance to SIVsm-Vif-mediated A3G degradation [9]. According to our structural model, the L71R mutation, located on the surface of the A3G protein could change the conformation of A3G as well. The E88K mutation represents a change from an acidic residue to an alkaline residue, the G212D mutation represents a change from a hydrophilic residue to an acidic residue, and the L71R mutation represents a change from a hydrophobic residue to an alkaline residue. These mutations might therefore influence the stability or biological functions of *A3G in vivo*.

Resistance of *A3G* mutants to HIV-2-Vif-induced degradation *in vitro*

To confirm our hypothesis, we constructed A3G mutants (E88K and G212D). According to the A3G protein structure model built using the PROTEIN MODELING program, the E88K and G212D substitutions occurred on the surface of the A3G protein, indicating that these two mutants may influence the binding affinity of HIV-2 Vif and thus lead to resistance to protein degradation during virus replication. To investigate whether these variations significantly influence the resistance of A3G to HIV-2 infection, we constructed plasmids encoding wild-type or mutant A3G containing a C-terminal Flag tag. The nucleotide sequence of each plasmid was confirmed by sequencing (Supplemental data). These A3G-encoding plasmids were used to transfect 293T

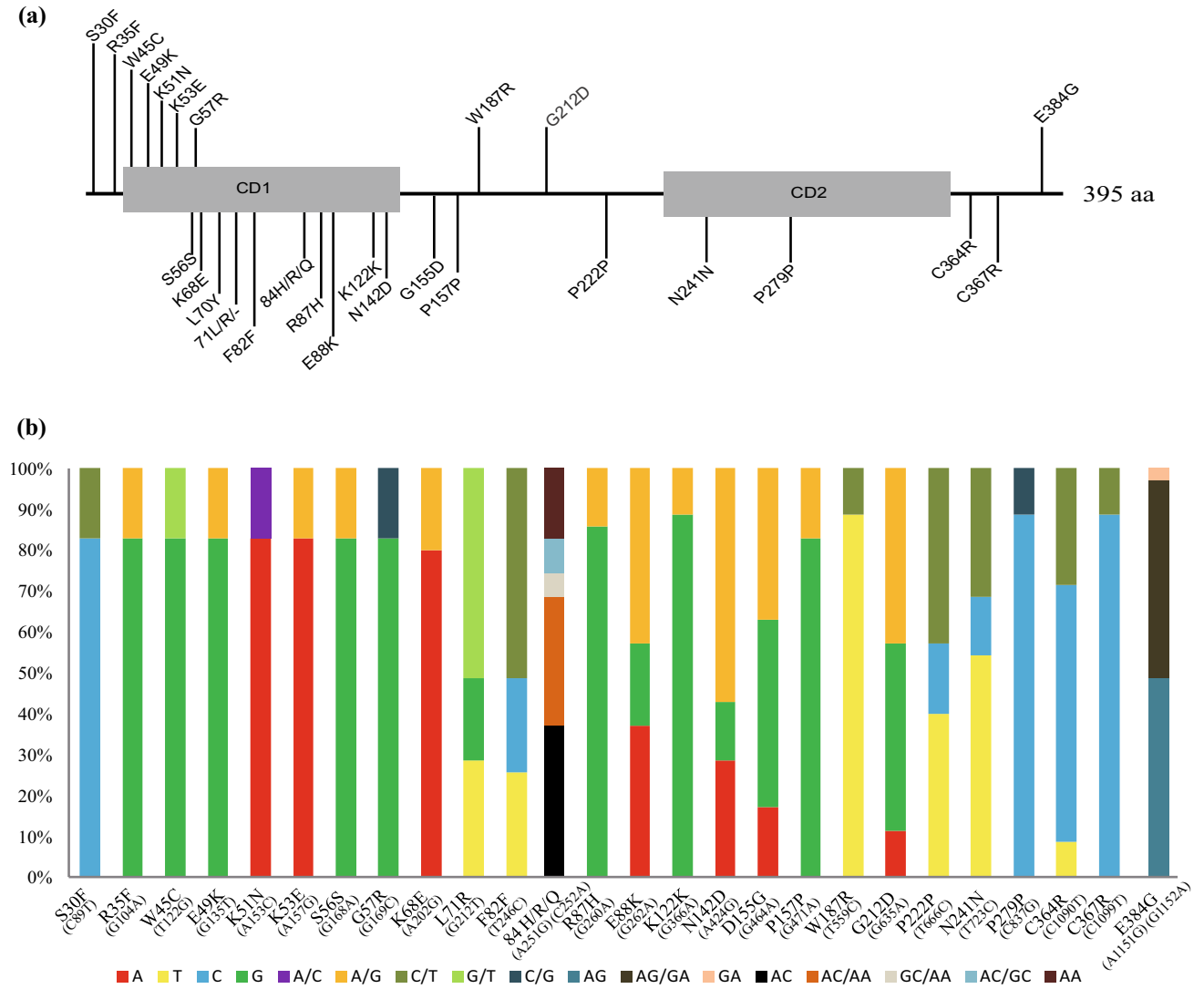

Fig. 2 (a) Sequence variations in APOBEC3G in Chinese rhesus macaques. Variations identified in previous studies are indicated below the diagram, while novel variations identified in this study are indicated above the diagram. CD1, the first cytidine deaminase zinc-binding domain; CD2, the second cytidine deaminase zinc-binding domain; aa, amino acids. (b) Genotype frequency of each SNP

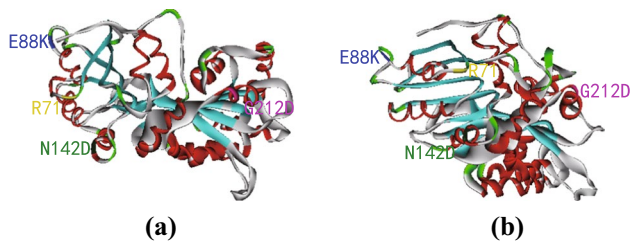

Fig. 3 Model of the full-length Chinese rhesus macaque APOBEC3G protein. A model of the full length of A3G was built using A3C structure PDB: 3VOW and human *A3G*_CTD PDB: 3IQS as a template model. a) The amino acids at each of the variant positions are labeled in the same color; b) The same structure as in panel A, rotated around the *y*-axis

cells for expression of the A3G protein. The amount of the different variants of A3G produced did not differ significantly, indicating that these three variations did not influence the level of translation or rate of degradation of A3G protein (Fig. 4).

We next investigated whether these variations influence the interaction of HIV-2 Vif with the A3G protein *in vitro*. We co-transfected cells with HIV-2 Vif expression plasmids and plasmids encoding wild-type and mutant *A3G* genes, then detected the expression of A3G and Vif by probing the Flag tag and V5 tag in a western blot assay. We found that $A3G_{E88K}$ and $A3G_{G212D}$, were efficiently degraded by HIV-2 Vif (Fig. 4), indicating that these variations do not affect the resistance of A3G to degradation mediated by HIV-2 Vif. However, another mutant, $A3G_{L71R}$, was not

efficiently degraded by HIV-2 Vif, suggesting that the L71R substitution could have affected the interaction of HIV-2 Vif with A3G, resulting resistance to Vif-mediated proteasome degradation. This provides strong evidence that the L71R mutation in the A3G protein has a protective effect during HIV infection in Chinese rhesus macaques. This new insight might be useful for anti-HIV drug research.

Discussion

The *APOBEC3G* gene has been widely reported to potentially affect retroviral infection. To study the molecular interaction between the APOBEC3G protein and HIV, nonhuman primate APOBEC3G has been widely used to investigate its interaction with HIV/SIV Vif [2, 3, 9]. Weiler *et al.* previously identified 15 SNPs and an insertion/deletion in their cohort of SIV-infected Indian rhesus macaques, but none of these were significantly correlated with SIVmac239 replication [23]. Similarly, Krupp *et al.* found seven nonsynonymous *APOBEC3G* SNPs and one deletion. However, *APOBEC3G* gene polymorphisms and their effect on viral infection in Chinese rhesus macaques have not been thoroughly investigated.

CD1 in A3G is responsible for binding HIV/SIV *Vifs*, while CD2 facilitates cytidine deamination. In this study, we found several polymorphisms in CD1 but only two synonymous mutations in CD2, which is consistent with previous research showing that CD2 is conserved to maintain its enzymatic activity. HIV-1/HIV-2 and other lentiviral Vifs bind a conserved region (residues 128–130) within A3G. HIV-1 and SIVagm Vifs bind residues 128 and 129, while HIV-2 and SIVsm Vifs interact only with residue 129 [11]. It has recently been hypothesized that residue 59 or 60 interacts with residues16–19 in Vif and that residues 42–46 in Vif interact with residues 128–130 in A3G. A positively charged arginine at residue 60 hinders SIVsm Vif binding to A3G, allowing A3G to continue its function [9]. In study, we also found an insertion at residue 71 (corresponding to residue 60 in other studies) and a polymorphic site at residue 142 (corresponding to residue 130 in other studies), and using a western blot assay, we found that these variations affect the affinity of HIV-2 Vif for A3G (see below). In addition, SIVmac Vif was found to efficiently degrade all CD1 A3G variants independently of the loop containing residues 128 and 129, which led us to speculate that SIVmac Vif binds to a distinct A3G region outside this loop [11]. The presence of a negatively charged glutamate at position 17 of SIVmac239 Vif, which interacts with residues 59 and 60 of A3G, would be expected to neutralize the repelling force between two positive amino acid residues. In human A3G, G48 of HIV-2 Vif is essential for specifically interacting with residues 163–321 of A3G [20], suggesting that in rhesus macaque A3G, G212D (corresponding to residue 201 in other studies) might be located on the binding surface.

To investigate the possible structures of the two nonsynonymous variants, we built a homologous model of the full-length $A3G^{LR}$ (residues 70–71 are LR) (Fig. 5). We used the A3C structure that was used by Krupp *et al.* and added a human A3G_CTD template to restrict folding of the C-terminal domain. The model was in agreement with that of Krupp *et al.* with Arg71 and Asn142 likely located on the same face of A3G as shown previously [9]. E88K was located on the same surface as Arg71 and Asn142. This mutation results in a change from a positively charged lysine to a negatively charge aspartic acid, indicating that it might partly increase the repulsive force against HIV-2 Vif.

Another variation, G212D, was located in the linkage region between CD1 and CD2, on the surface of A3G. This position (212) may be located in the HIV-2 Vif binding site, according to the findings of Smith *et al.* Another possible explanation was that residue 212 is not a binding site but helps to form the binding structure of Vif, with the mutation changing the conformation of A3G, which may lead to steric hindrance of Vif.

Based on previous reports and the analysis described above, we suspected that the nonsynonymous variants E88K and G212D disturbed the interaction between A3G

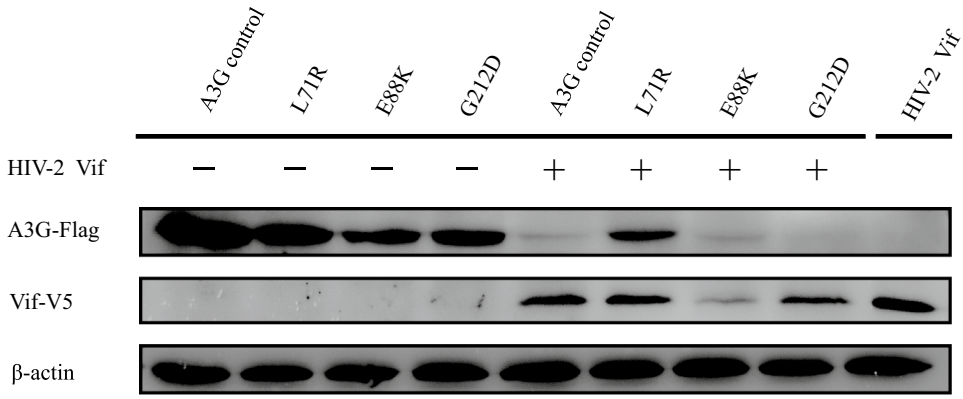

Fig. 4 Expression of A3G and its mutants in 293T cells after transfection with plasmid constructs. The expression of A3G was detected using an anti-FLAG antibody. HIV-2 Vif and A3G or its mutants were coexpressed in 293T cells using plasmid cotransfection. Immunoblots were used to detect the expression of A3G and Vif, using anti-FLAG and anti-V5 antibodies, respectively

and HIV-2 Vif, allowing A3G to escape degradation by proteasomes. To test this hypothesis, we performed an A3G degradation experiment, using a western blot assay for detection. However, the $A3G_{E88K}$ and $A3G_{G212D}$ mutants showed no significant resistance to degradation, indicating that these two residues were not critical for the process of Vif-induced protein degradation. However, another mutant, L71R, showed significant resistance to Vif-induced protein degradation.

Furthermore, we found 10 corresponding human *A3G* mutations based on the dbSNP database. Five of these were SNPs (rs201215107, rs573440801, rs577767262, rs747970268, rs754124415) that were fixed in the human population according to the allele frequency from the 1000 genome project. Notably, the C allele of rs577767262(C/T) was fixed in humans, but not in macaques (T/C). However, there is no corresponding human A3G polymorphism of L71R and G212D. Although human SNP rs1233861728(A/C) corresponds to E88K (G262A), unfortunately, no allele frequency data were found.

Conclusion

We found one novel transcript and 10 variants (F82F, E88K and G212D) of *APOBEC3G*. Among these mutants, according to the western blot assay result, L71R showed resistance to HIV-2-Vif-mediated *APOBEC3G* degradation, strongly suggesting that L71R could play an important role in the host's antiviral defense. Further studies are required to determine the mechanistic basis for APOBEC3G-mediated HIV-2/SIV infection, which will increase our understanding of the pathogenesis of primate lentiviruses.

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Author contributions FL and YTZ conceived and designed the experiments. XRY, HLZ, HY, JTO, and ZMW performed the experiments. YBJ, XRY, and HY analyzed the data. BLL and MZ contributed reagents/materials/analysis tools. XRY and FLL wrote the paper. YEL, YTZ, and FL edited the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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