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Sequence Variation in the *Gp*120 region of SHIV-CN97001 during *in vivo* Passage*

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Abstract: SHIV-CN97001 played an important role in assessing the immune effect and strategy of the AIDS vaccine which included genes of the predominant prevalent HIV-1 strain in China. In this study, SHIV-CN97001 was *in vivo* passaged serially to construct pathogenic SHIV-CN97001/rhesus macaques model. To identify variation in the *gp*120 region of SHIV-CN97001 during passage, the fragments of *gp*120 gene were amplified by RT-PCR from the plasma of SHIV-CN97001 infected animals at the peak viral load time point and the gene distances (divergence, diversity) were calculated using DISTANCE. The analysis revealed that the genetic distances of SHIV-CN97001 in the third passage animals were the highest during *in vivo* passage. It had a relationship between viral divergence from the founder strain and viral replication ability. The nucleic acid sequence of the V3 region was highly conservative. All of the SHIV-CN97001 strains had V3 loop central motif (GPGQ) and were predicted to be using CCR5 co-receptor on the basis of the critical amino acids within V3 loop. These results show that there was no significant increase in the genetic distance during serial passage, and SHIV-CN97001 *gp*120 gene evolved toward ancestral states upon transmission to a new host. This could partly explain why there was no pathogenic viral strain obtained during *in vivo* passage.

Key words: SHIV; Passage; *Gp*120; Variation; Sequence analysis

Chimeric simian–human immunodeficiency viruses (SHIVs) that contain the HIV-1 *tat, rev, vpu,* and *env* genes inserted into the proviral genome of the pathogenic SIVmac239 clone have been constructed to test the functions of HIV-1 gene products and constitute a valuable model system in the assessment of candidate vaccines directed against the HIV-1 envelope glycoproteins in macaque models (4, 6, 10). However, SHIV constructs require *in vivo* adaptation and serial *in vivo* passage to acquire the ability of depleting $CD4^+$ T cells and inducing simian AIDS. Balfe（2004）found that the animals which later received passage virus had more diverse quasispecies, which supported the model of quasispecies diversity

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as a predictor of pathogenesis (1).

SHIV-CN97001 was constructed by replacing the gp120 and partial gp41 region of SHIV-KB9 which was used as backbone with corresponding region of HIV-1 B'/C recombinant virus strain HIV-CN97001, the predominant prevalent strain in China. SHIV-CN97001 was rapidly and serially passaged five times in Chinese origin rhesus macaques. It was found that the virus replication ability reached the peak in the third passage, then declined. At the same time, no obvious $CD4^+$ lymphocytes decline was detected in all infected animals, indicating that there was no pathogenic virus strain during passage. In this study, we tried to explore the molecular basis why the biological phenotype such as virulence did not change during passage.

MATERIALS AND METHODS

Samples and RNA extraction

Thirteen healthy adult Chinese origin rhesus macaques were selected, which were tested negative for prior exposure to SIV, simian retrovirus type D, simian T-cell leukemia virus and herpes B virus. The two monkeys of first passage were intravenously infected with 64 MID_{50} SHIV-CN97001 (Kindly provided by Dr. Ruth M Ruprecht). 10 mL blood sampled from the SHIV-CN97001 infected monkey 1 to 3 months post infection was injected into the next passage by intravenous route (Fig. 1). Quantitative assays for the measurement of SHIV-CN97001 RNA were performed by real time RT-PCR using primers and probe specific for SIV gag in an ABI Prism 7700 sequence detection system (PE Applied Biosystems, Foster City, CA, USA) as described (8). The EDTA anticoagulant blood of peak viral load time point was collected, separated as soon as possible and freezed in -80℃ refrigerator. Virus RNA was isolated with QIAgen Viral RNA Mini Kit.

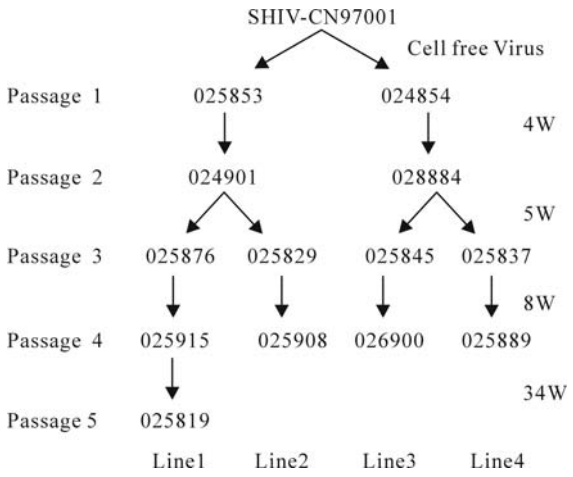

Fig.1. The passage of SHIV-CN97001 *in vivo*

Amplification of SHIV-CN97001 *gp*120 gene

A 1 636 bp fragment that included SHIV-CN 97001 gp120 segment was amplified with one-step RT-PCR method (TaKaRa one step RNA PCR Kit) and with the following primers: forward 5'-CAGAAGACAGTGG CAATGAGA-3' (HIV-HXB2 position: 6 210-6 230), and reverse - 5'- GCCTGTACCGTCAGCGTTATT-3' (HIV-HXB2 position：7 826-7 846). The thermocycler protocol was 50℃ for 30 min and 94℃ for 2 min, then 40 cycles at 94℃ for 30 sec, 62℃ (–0.3℃/cycle) for 30 sec, and 72℃ for 2 min, followed by a final extension at 72℃ for 10 min.

Clone construction

Purified PCR product extracted with QIAquick Gel Extraction Kit was ligated into T vector (pGEM-T Easy Vector system I, Promega) and transformed into JM109 competent cells, 18 clones were selected by PCR method and sequenced in both directions on an ABI 3730 sequencer using the Big Dye Terminator Cycle Sequencing Kit (version 3.1; Applied Biosy-

stems, Foster City, CA).

Sequences analysis

The sequences were edited by SeqED software (ABI) and then analyzed with Wisconsin Package GCG (Version 10) software. The gene distances (divergence, diversity) were calculated using DISTANCE.

RESULTS

Relationship between genetic distance and viral load

The plasma samples from each passage animals were analyzed at peak viral load time point (Day 14) (Fig. 2). Generally, the viral genetic distances (divergence, diversity) reached the peak in the third passage, then declined. When every passage line was analyzed separately, we found there was no significant difference in all the animals of line 3. In line 1 and line 4, the genetic diversity was highest in the third passage. While in line 2 and line 3, it displayed a continuously declined tendency. Noticeably, the divergence and diversity for the fifth passage animal (No.025819) rose remarkably compared with the fourth one in line 1, indicating that the SHIVCN97001 changed obviously after 8 months adaptation in the fourth animal (No.025915). The peak viral load levels were corresponding to the virus replication ability, so that the SHIV-CN97001 replication ability was highest in the third passage in all lines. At the same time, the period of seroconversion was postponed gradually during passage. By comparison, we found that there was relationship between genetic distance and replication ability (Fig. 3).

Gene polymorphism analysis

The variation of different gene in SHIV-CN97001 gp120 was inconsistent (Fig. 4). The greatest variation occurred in V2, C1, V1 and V5 respectively in four lines, While the nucleic acid sequence of the V3 region was highly conservative. In the mass, the gene variation displayed a random evolutionary trend, and the most greatly change occurred in the fourth line.

V3 Loop central motif analysis

Env gene V3 loop tip constituted with characteristic four peptides was the most important neutralizing antibody determinants. There were diverse mutant types of central motif in different subtype HIV viruses. The four peptides in all 197 clones were GPGQ (100%), which was same as the plasmid SHIV-CN97001. No change was found during passage.

The prediction of the co-receptor usage

The SHIV-CN97001 co-receptor usage can be predicted on the basis of the critical amino acids

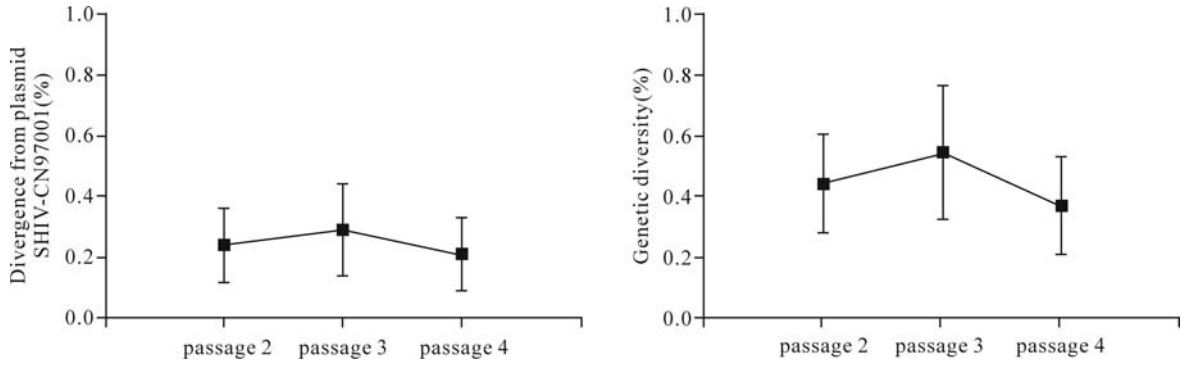

Fig.2. Changes in genetic distance during *in vivo* passage

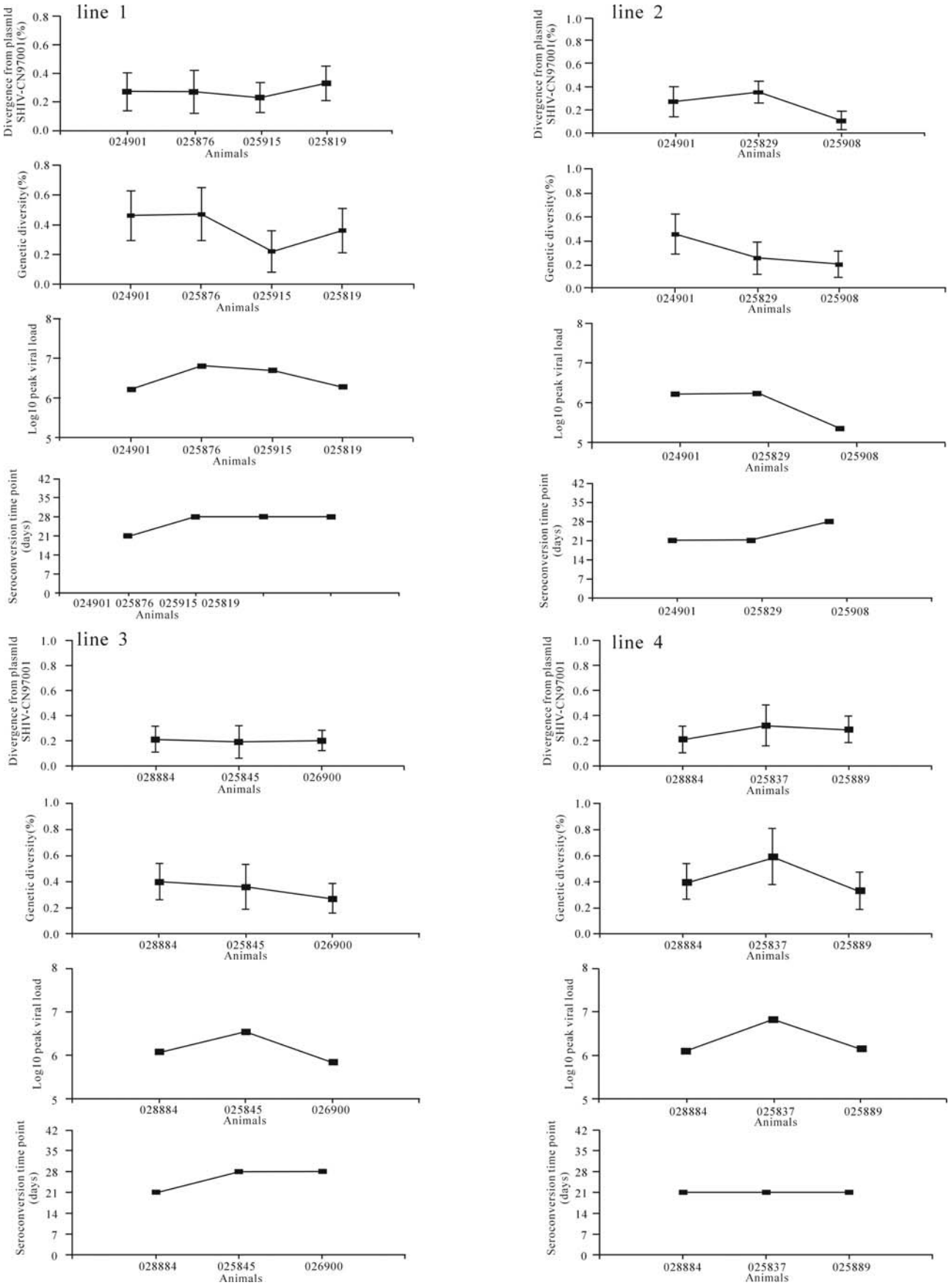

Fig.3. Changes in genetic divergence, diversity, peak viral load and seroconversion time during *in vivo* passage.

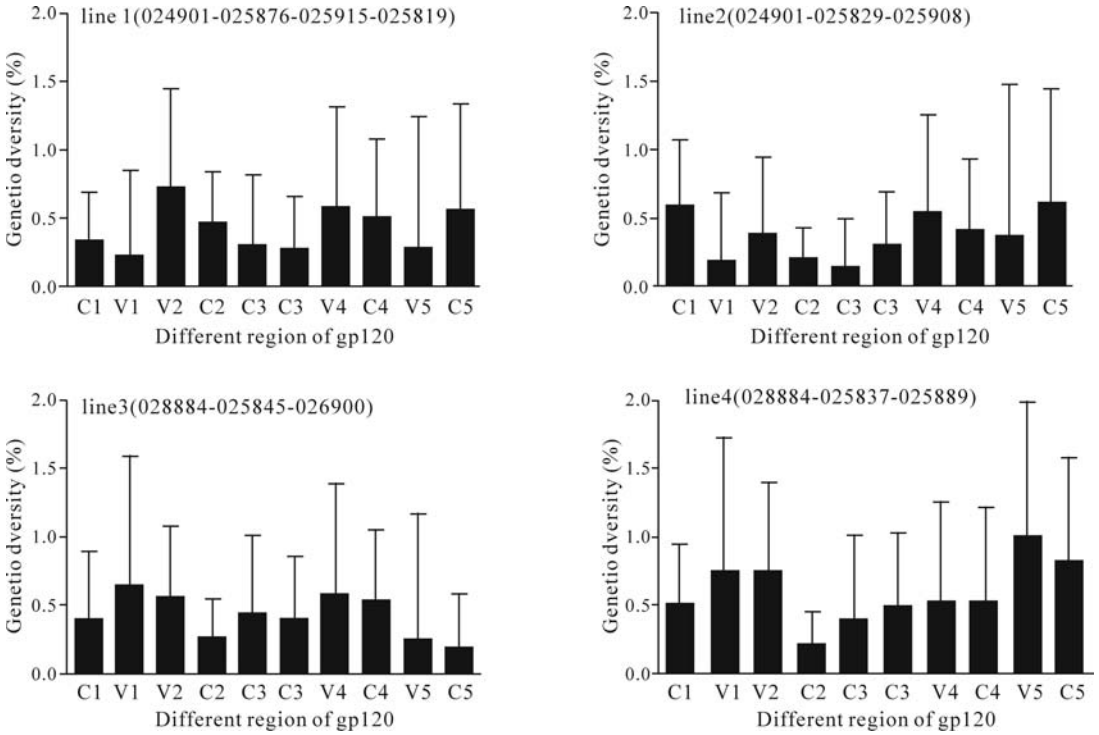

Fig. 4. Genetic diversity in different region of SHIV-CN97001 gp120.

within V3 loop. S/ GXXXGPGXXXXXXXE/ D (V3 loop position: 11-25) was the common prediction motif. If the key nucleic acid sites were replaced with positive charge arginine (R) or glutamine (Q), the SHIV-CN97001 then used CXCR4 as coreceptor to enter into cells. In this study, All the motif in 196 clones were SXXXGPGXXXXXXX D, which was same as the plasmid SHIV-CN97001. Only one clone in the third passage in line 4 had different motif (GXXXGPGXXXXXXXD). All clones were predicted to be using CCR5 by sequence analysis.

DISCUSSION

Nonpathogenic SHIV-CN97001 contained the gp120 and partial gp41 region of B'/C recombinant HIV-CN97001, and played an important role in assessing the immune effect and strategy of the AIDS vaccine which included genes of the predominant prevalent HIV-1 strain in China. In this study, SHIV-CN97001 was *in vivo* passaged serially to construct pathogenic SHIV-CN97001/rhesus macaques model. After five times of passge for two years, no pathogenic mutant was isolated by viral load and FACS analysis. Since the envelope antigen mutation to escape from surveillance of host immune system was very important for the virus pathogenesis, we focused on the sequence variation in gp120 region of SHIV-CN97001, trying to explore the molecular basis why the biological phenotype such as virulence and tropism did not change during *in vivo* passage. It was found that the SHIV-CN97001 gp120 genetic distances reached the peak in the third passage, then declined. In the 72 clones of fourth passage, there were four clones whose gp120 sequences were identical to the plasmid SHIV-CN97001, but only one identical sequence was found in the third passage. This result was in good agreement with the "evolved toward ancestral states" theory, which pointed out that

HIV recovered certain ancestral features when infecting a new host (3). The convergence of viral sequences suggested that there were strong sequence constraints that were important to viral reproduction across animals. Notablely, there were correlations among the genetic distances, replication ability and humoral immune response.

The variation of various genes in SHIV-CN97001 gp120 were different by gene polymorphism analysis, it may be affected by diverse immune pressure level in different animals. However, the V3 loop was the most conservative in all lines. All the SHIV-CN97001 strains had V3 loop central motif (GPGQ) and were predicted to be using CCR5 on the basis of the critical amino acids within V3 loop, which was in coincident with the finding of gene variation analysis in the V3-V4 region of the HIV-1 C subtype strains (5). These results showed that, in order to maintain the stable V3 loop function, sequence changes in flank region of V3 loop accumulated principally in response to immune pressure exerted by the host, generating viral variants that can persist in the presence of a strong host immune response. The high conservatism of V3 region had relation with the specific biological properties of HIV-1 C subtype strain such as CCR5 tropism.

Nowak (1991) put forward a mathematical model of the dynamic interaction between viral diversity and the human immune system. This model suggested the existence of an antigen diversity threshold, below which the immune system was able to regulate viral population growth but above which the virus population induced the collapse of the $CD4^+$ lymphocyte population (7). Four successive, rapid serial passages of the nonpathogenic, CCR5-tropic $SHIV_{SF162}$ in Indian origin rhesus macaques resulted in the emergence of a pathogenic isolate $SHIV_{SF16P3}$ in one of the passage three transfer animals (2). Balfe (2004) found the animals of later passage had more diverse quasispecies, which supported the model of quasispecies diversity as a predictor of pathogenesis. Maybe, the reason that genetic distance of SHIV-CN97001 did not rise obviously during passage was due to different immune pressure between Chinese origin and Indian origin rhesus macaque. This conclusion was in accordance with Trichel's finding, only one of two major phylogenetic groups found within the SIV/DeltaB670 inoculum was selective amplified in Chinese subspecies, but both phylogenetic groups were commonly identified in Indian subspecies (9). Moreover, because the immune system of selected monkeys in this study had already maturated, maybe we could gain ideal effect if the juvenile rhesus macaques were chosen.

In a word, there was no significant increase in the genetic distance during rapid serial passage, and SHIV-CN97001 gp120 gene evolved toward ancestral states upon transmission to a new host. This could partly explain why there was no pathogenic viral strain obtained during *in vivo* passage.

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