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Subtype Identification and Drug-Resistant Point Mutation of Human Immunodeficiency Virus Type I in Taiwan

Abstract

Human immunodeficiency virus (HIV) is the agent resulting in acquired immunodeficiency syndrome (AIDS). After its initial isolation in 1983, HIV has spread worldwide and resulted in many deaths in an astonishing rate. In 1996, Dr. Ho Da-I proposed the highly active antiretroviral therapy (HAART), also called the combination therapy. Currently HIV infection is treated with this method worldwide. Nevertheless, treatment failure has been reported. The most important reason for this is the emergence of drug resistant strains. The mechanism of resistance is mainly gene mutation: changes in genes of reverse transcriptase and protease, essential enzymes for HIV replication. Recently, drug-resistant mutations (natural polymorphism) were found in reverse transcriptase and protease genes in HIV infected patients who never take antiretroviral medications, and this will result in mild to severe resistance to HAART. To understand the prevalence of HIV subtype infection and drug resistance-related mutation in recent year, this study selected 440 HIV-I positive specimens in the country from

1996 to 2004 to analyze there subtype and mutations in reverse transcriptase and protease genes. According to primers specifically designed for C2V3, gag, and pol regions, and with the use of reverse transcriptase PCR (RT-PCR), we amplified gene fragments of viral capsule, protease, and reverse transcriptase and analyzed their subtype and drug resistance-related mutation. In subtype analysis, 376 (85.6%) were subtype B, 39 (8.8%) were subtype CRF01_AE, 16 (3.6%) were subtype CRF07_BC, 2 (0.4%) were subtype CRF02_AG, and 7 (1.5%) were subtype C. In protease gene mutation analysis, 304 (73%) had L63 mutation, 173 (42%) had V77 mutation, and 134 (32%) had M36 mutation. As to the gene of reverse transcriptase, 30 (44.7%) had K70 mutation, 12 (17.9%) had S68 mutation, 7 (10.44%) had T215 mutation, and 4 (5.9%) had M184 mutation. from the aspect of drug resistance, the incidence of primary mutation was low in these specimens, and in genes of protease and reverse transcriptase. The other mutations were mainly secondary mutation.

Introduction

Structure and subtypes of HIV

HIV is member of lentivirus in retroviridae. Under electron microscope, HIV is a spheroid virus with a diameter around 110 nm. It has glycoprotein outer membrane, and double strand RNA as well as enzymes, such as reverse transcriptase, integrase, viral protease, and regulatory proteins, required for replication within its caspid. RNA of HIV is about 9.2 kb, with 9 genes on it. Among them, gag, pol, and env are viral structure proteins. The proteins translated from gag gene are p24, p17, p2, p7, p1, and p6 [1]. The products of pol are reverse transcriptase, protease, and integrase. The products of env gene are outer membrane viral proteins, including gp120 and gp41; these proteins are receptors of lymphocyte CD4, and are required for the virus to enter its host cell.

The rest 6 non-structural proteins are related to viral replication, regulation, hostility, and maturation. Among them, products rev and tat are regulatory proteins required for viral replication; products of nef, vpr, vpu, and vif are related to viral hostility.

HIV is divided into HIV-1 and HIV-2. They are from eastern and western Africa respectively, and have many differences serologically. HIV-2 is more similar to simian immunodeficiency virus (SIV), and HIV-1 is more similar to immunodeficiency virus of chimpanzee. HIV-1 is further divided into two groups: the major (M) group and the outlier (O) group. The M group is again divided into ten subtypes (A to K) according to variations in the env gene. The differences between each subgroup are more than 20% [1, 3]. The O group does not have subgroups. New (N) group was later found in a woman from Cameroon in May 1995. [4] the differences between M, O, and N groups are more than 50%. The distribution of HIV subgroups is geographical. For example, B subgroup is often found in North America and Western Europe, C subgroup is often found in mainland china, and B as well as E subgroups are often found in Taiwan and Thailand. According to previous studies, subgroups are prevalent in different races, and are associated the gender and pattern of sexual behavior [5].

Subgroups of HIV

Subgroup identification of HIV-1 gene is very important in epidemiology, because we can understand the evolution and distribution of HIV-1 in the globe, and regional impact and interaction between subgroups. Subgroups of HIV-1 are related to routes of infection and dissemination, and therefore have vital influence in vaccine development. Subgroups of HIV-1 also have different spontaneous mutations susceptibility to drug [3, 6].

HIV viral protease

The gene of HIV protease is 297 bp. The protease is a monomer consist of 99 amino acids with a molecular weight about 11 KD. While releasing from gag-pol polyprotein, two proteases will bind non-covalently and symmetrically to from a homodimer with two active centers formed by Asp26-Thr26-Gly27. If the Asp was replaced with other amino acids with point mutation, the enzyme activity would lose. Therefore, it is an aspartic protease [7, 8]. Protease is very important in the life cycle of HIV. It is required to cleave reverse transcriptase from the polyprotein, and process the p66/p66 reverse transcriptase homodimer to a mature and functional heterodimer. It is also required to cleave capsid proteins p24, p17, p7, and p6 from the gag p55.

HIV viral reverse transcriptase (RT)

RT plays a vital role in HIV replication. It reverse transcripts viral single strand RNA to single strand DNA, and then double strand DNA. The double strand DNA the can integrate into host chromosomes to continue transcription and translation. RT is a heterodimer consisting to subunits, p66 and p51. p51 is cleaved from p66 by protease. They have the same N-terminal sequence. The C-terminal of p66 has RNase H activity [9, 10].

Drug resistance and HAART

Drug resistance of HIV is an enormous obstacle in its therapy. From current studies, the resistance is the results of genetic diversity. Because of the high inherent error rate in reverse transcription, lacking of proof reading ability of RT, and high rate of replication in vivo, multiple variants are easily emerged. Since HAART been proposed by Dr. Ho, its clinical efficacy in inhibit virus replication and viral load have been proofed in clinical studies. The therapy combines two RT inhibitors (RTI) and one protease inhibitor (PI) [13, 14] to terminate and inhibit their enzyme activities and thus viral replication. This therapy has fewer

side effects, and the survival rates and life quality of patients have been raised significantly. Nevertheless, if resistant stains emerge because of prolong medication or decreased compliance, doctors have to consider revise the medication prescribed. Therefore, periodic monitoring the viral load to reflect the sensitivity of virus to drugs is important. In Taiwan, infection rate of HIV continues to rise, and the age group also continues to get lower. It has been reported that drug-resistant stains have been found in HIV patients never took antiretroviral medications. This will lead to decreased efficacy of HAART. Therefore, if the resistance of virus in patients can be determined, we can thus understand the spontaneous mutation of virus in vivo and also provide important information for HIV prevention, treatments of patient, and vaccine development.

Materials and methods

Specimen gathering

440 HIV-positive serum and plasma collected between 1996 and 2004 were gathered from virus laboratory of CDC Taiwan.

Viral RNA extraction

QIAmp Viral RNA kit from QIAGEN was used for RNA purification. 140 ul of plasma or serum was added into 560 ul of Buffer AVL and incubated for 10 min at room temperature. 560 ul of absolute alcohol was then added and vortexed. The mixture was filtered through QIAmp spin column. The column was then washed twice with Buffer AW. RNA was then dissolved with 80℃ RNase free water. The viral RNA was used for RT-PCR later.

Subtype and resistance analysis

Primers specifically for HIV-1 C2V3, PR, and RT genes are used for subtype analysis (table 1 and 2). The RNA extracted with Qiagen ViralAmp was amplified

with RT-PCR and Nest-PCR for the specific fragments, and then for sequence analysis.

a. Reverse Transcription

10 ul viral RNA was added with 75mM KCl, 50 mM Tris-HCL, 3mM $MgCl_2$, 10 mM DTT, dNTP mixture 0.5 mM, RNasin 38U/ul, and anti-sense primer (35R, 1-R-RT21, R-PR-probe 50 pmole) and reacted in 70℃ for 10 min. 100 units of MuLV-reverse transcriptase was than added and reacted at 37℃ for 90 min.

b. Polymerase Chain Reaction

5 ul of cDNA was used as template and added into a mixture containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.1% Triton-X 100, dNTP mixture 1 mM, and 50 pmole of primers (44F/35R, F-MAW26/R-PR-probe, 1-F-RT18/1-R-RT21). 5 units of Taq polymerase (Invitrogen) were then added. After denature for 5 min at 94℃, 40 cycles of reaction were taken at 94℃ for 30sec, 50℃ for 45sec, and 72℃ for 1 min, and ended at 72℃ for 15 min.

c. Nest-PCR

5 ul of 1^{st} PCR products was used as template and added into mixture containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.1% Triton-X 100, dNTP mixture 1 mM, and 50 pmole of primers (33F/48R, Nest-F-PR/Nest-R-PR, 2-F-RT19/2-R-RT20). 5 units of Taq polymerase (Promega) were then added. After denature for 3 min at 94℃, 40 cycles of reaction were taken at 94℃ for 30 sec, 50℃ for 45 sec, and 72℃ for 1min, and then stopped at 72℃ for 15 min.

d. Electrophoresis and sequencing

Products of Nest-PCR were stained with EtBr after electrophoresis in agarose gels. The fragments of C2V3 was 525bp, PR 369bp, and RT 967 bp. Sequencing was done with ABI 3730 with ABI PRISM(™) BigDye(™) Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). High quality

($OD_{260/280}$ >1.8) PCR products was used for sequencing. The nucleotide amount for double strain DNA was 200~500 ng, single strain DNA 50~100 ng, and PCR products 30~90 ng. the nucleotide template was mixed with 3μl premix (including Tris-HCl buffer, pH 9.0, $MgCl_2$, dNTP mix, labeled A-dye terminator, C-dye terminator, G-dye terminator, T-dye terminator, AmpliTaq DNA Polymerase FS with thermally stable pyrophosphatase), 3.2~5.0 pmole primers (Nest-F-PR/Nest-R-PR, 33F/48R, 2-R-RT20/2-F-RT19), and water to a final volume of 10 ul. Mineral oil was then added into the tubes. 25 cycles of reaction were taken at 94℃ for 30sec, 55℃ for 15sec, and 60℃ for 4min, and stopped at 4℃.

e. Drug resistance and evolution tree analysis

Results of sequencing were compared on NCBI website (http://www.ncbi.nlm. nih.gov/BLAST/) and Stanford HIV Drug Resistance Database (http://hivdb. stanford.edu/) for subgroup and drug resistance analysis. Evolution tree was analyzed with Molecular Evolutionary Genetics Analysis (MEGA) version 2.0. The sequences obtained form RT-PCR were compared with subtype reference strains including TW20, TW71, TW115, TW8637,W112,TW8623 ,HIVMN, HIVSF2, TW78, THAI-B, TW8602, TW8610, TW31, HIVJY1, IVNDK, W61, TW8616, THAI-E, TW8604, TW8604, TW8629, TW98, TW8621, IVD747, HIVNOF, BV217, I525A, HIVKENY, HIVZ321, HIVB 7944, VI557, HIVMP51, HIVAN70C, and HIV-1 recombinant reference strain CRF_01AE~CRF_14BG. “Neighbor-joining” method was used with Bootstrap analysis for 1000 times.

Sequence registration

The results of subtype analysis of positive specimens and spontaneous drug-resistant mutations obtained by CDC Taiwan were registered for Accession

numb on the NCBI database (http://www.ncbi.nlm.nih.gov/Bankit/) to build the gene database of HIV subtype and spontaneous resistant mutation of Taiwan.

Results

For HIV subtype analysis, 440 ELISA or PA positive (in two separate analyses) and Western blotting confirmed HIV-positive specimens from 1996 to 2004 were used (table 3). The primers for RT-PCR were 44F/35R, and the primers used for Nest-PCR were 33F/48R. The fragment of C2V3 gene after two PCR amplifications was 525bp. The sequencing results were compared on NCBI website for subtype analysis. Among them, 376 were subtype B (85.6%), 39 (8.8%) were subtype CRF_01AE, 16 (3.6%) were subtype CRF07_BC, 2 (0.4%) were subtype CRF02_AG, and 7 (1.5%) were subtype C (table 4). The results of subtype analysis showed that subtype B predominant in these specimen followed by subtype CRF_01AE. Subtype CRF07_BC and CRF02_AG recombinant virus was also found (Fig 2). To confirm the recombination, we used HIV-1 gag and pol genes for evolution tree analysis. The results showed that CRF02_AG and CRF07_BC form an isolated cluster (Fig. 4, 5). To understand the recombinant fragments and regions, we need to sequence the whole genome of HIV and find out the break point with software [25].

Currently, HIV-1 infection is mainly treated with HAART with PI and RTI to inhibit to vital enzymes for HIV replication simultaneously. Nevertheless, if the patients have poor compliance or the same medication has been used for too long or spontaneous mutation occurs in reverse transcription or translation, mild to severe resistance to HAART may emerge. While analyzing viral protease, among the 440 specimens, 414 (94%) have PI resistance-related mutations. Among them, 39 (9.4) were at L10, 27 (6.1%) at K20, 134 (32.5%) at M36, 304 (73.8%) at L63,

37 (8.8%) at A71, 173 (42%) at V77, 3 (0.7%) at V82, 2 (0.48%) at I84, and 5 (1.21%) at L90 (table 5). The primary mutations for PI resistance are V82, I84, and L90, and the rest mutations are secondary mutations (table 8).

There were 67 (15.2%) RTI resistance-related mutations. Among them, 3 (4.4%) were at M41, 12 (17.9%) at S68, 2 (2.9%) at T69, 30 (44.7%) at K70, 3 (4.4%) at V75, 4 (5.9%) at M184, 4 (5.9%) at L210, and 7 (10.44%) at T215 (table 6). The primary mutations associated with RTI resistance are T69S, K70R, V75T, T215Y, Y181C, and M184V. We only found two T69S, four M184V and 7 T215Y mutations, indicating low incidence of resistance-related mutations (table 7).

Discussion

Dr. Da-I Ho proposed HAART (cocktail therapy) in 1996, using PI and RTI simultaneously to inhibit enzymes requiring for HIV replication. According to HIV kinetics in patients taking HAART, if HIV are completely inhibited and there are no other unknown host cells, it is estimated to take 3 years to let T (latent HIV in T cells), and V (plasma HIV) to become undetectable, and thus completely eradicate the virus (Fig 3). Nevertheless, while plasma HIV could be inhibited rapidly under HAART, latent HIV in T cells can not be eradicated [15]. Therefore, scientists proposed new therapy to combine interleukin-2 (IL-2) with HAART. Since IL-2 is a T cell mitogen, they hope to stimulate latent HIV to replicate and then suppress by HAART. The method has been proven not to completely eradicate HIV [16-18]. Thus, the most widely used method is to use HAART combined with monitoring CD4 count and viral load.

Drug resistance of HIV raises great clinical obstacles. Because of the high error rate of RT and the high replication rate of HIV in vivo, mutants and

quasispecies are therefore emerging. Selective pressure such as antiretroviral agents will fasten the emergence of resistant strains, and the emergence of resistant strain will be showed by the phenomenon called viral rebounding [19, 20]. This study was to understand the prevalence and HIV subgroups in treatment-naïve patients in Taiwan. In our results, 414/440 (94%) of specimens had PI-related spontaneous mutations, and only 3/414 (0.72%) had V82A mutation, 2/414 (0.48%) had I84V mutation, and 5/414 had L90M mutation. These sites are at the substrate binding site. The rest were secondary mutation in parts outside the active region. [21]. Among the 67 RTI-related spontaneous mutation, only 2 (17.9%) had T69S mutation, 4 (5.9%) had M184V mutation, and 7 (10.44%) had T215Y mutation. These are the primary mutations, and the rest were secondary mutations.

V82A/F/T/S mutations may result in severe resistance to two kinds of PI, Indinavir, and Ritonavir. L90M may results in severe resistance to Saquinavir and Nelfinavir, both are PIs. I84V mutations will produce significant resistance to Amprenavir (table 8) [22]. HIV protease may accumulate up to 5 (L10I + K20R + M36I + L63P + V71)) mutations, and these minor spontaneous mutation will also result in drug resistance phenotype. T215Y mutation will result in significant resistance to Zidovudine, and M184V to Zalcitabine, Lamivudine, and Abacavir. T69S mutation will cause resistance to multiple Nucleoside RT inhibitor (NRTI) (table 7) [23]. There may be up to 3 (S68G+ L210W+T215F or M41L+K70R+V75I) on HIV RT.

Subtype identification are important for understanding the evolution and distribution of HIV. Subtypes are also associated with route of infection, dissemination, and thus have significant effect on vaccine and drug development. Different subtypes may have different susceptibility to drugs and different

drug-resistant strains. In this study, we found subtype B (85%) and subtype A/E (8.8%) (Table 4) are most common in Taiwan, different from that of previous studies. In the study of professor Yi-Ming Chen from Yang-Ming University, subtype B were account for 68.2% of infection, and subtype E account for 27.3%; Males were often infected by subtype B, and females were often infected by non-B subtypes [1, 3]. Professor Jing-Nang Lee from National Taiwan University using vpu gene for subtype study also showed that among 363 patients, 73.8% were subtype B, 23.1% were subtype E, and 11% were subtype C [6]. Our results showed higher ratio of subtype B and lower ratio of subtype E. This may related to our sampling specimens. They were gathered by CDC between 1996 to 2004from screening or confirmed cases with male predominance. The exact ratio of genders could not be determined due to anonymous screening cases. From subtype and sexual behavior pattern analysis, homosexual male or bisexuals are more likely infected by subtype B, male heterosexuals are more likely be infected by subtype E. Subtype E is also prevalent in heterosexual groups [1, 5].

Moreover, two special subtypes, HIV-1 BC and AG circulating recombinant forms, were found in evolution tree analysis (fig. 2). These are rare subgroups in Taiwan. Since we used env region for gene compare and evolution tree analysis and many HIV-1 recombination occur in gag-pol region, we also used these region to confirm our findings (fig. 4, 5). HIV-1 CRF07_BC recombinant virus is more closer at its anterior pol region to subtype B; on the other hand, HIV-1 CRF08_BC recombinant virus is more closer to subtype C. This may be due to HIV-1 CRF07_BC contains more genes of subtype B and the anterior of pol region (fig. 1). In this study, our HIV specimens were at the same cluster with HIV-1 CRF07_BC and closer to subtype B in pol gene evolution tree analysis. Nevertheless, if gag region were used, CRF07_BC and CRF08_BC were closer to

subtype C. Recombinant virus may be produced in a patient infected by two subtypes of HIV, and recombination may occur within cells. HIV-1 CRF02_AG is prevalent in central and western Africa [24]. We found two specimens were CRF02_AG. Tracing there origin, one is number 5927 from a male blood donor in Kaohsiung, and the other one is number 6412 from a female patients in Tao-Yuan General Hospital. HIV-1 BC recombinant virus is prevalent in IV drug abusers in Yunnan Province of China, CRF07_BC in northwestern China, and CRF08_BC in southeastern China [2]. Currently, HIV-BC recombinant virus such as 97CN001, and 97CNGX-6 is form from genome of subtype C interrupted with fragments of subtype B [25] (Fig. 1). Initially, drug abusers in Yunnan were infected by subtype B and Thai-B, but since 1990 subtype C predominant. Coinfection may resulted from needle sharing [2]. HIV-1 BC recombinant virus in our study was from two IV drug abusers in Tainan. To understand the genome structure and recombinant breakpoint, we need the full length of genome and analyze it with bootscanning plots and exploratory tree, and find the origin of infection with questionnaire.

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Authors

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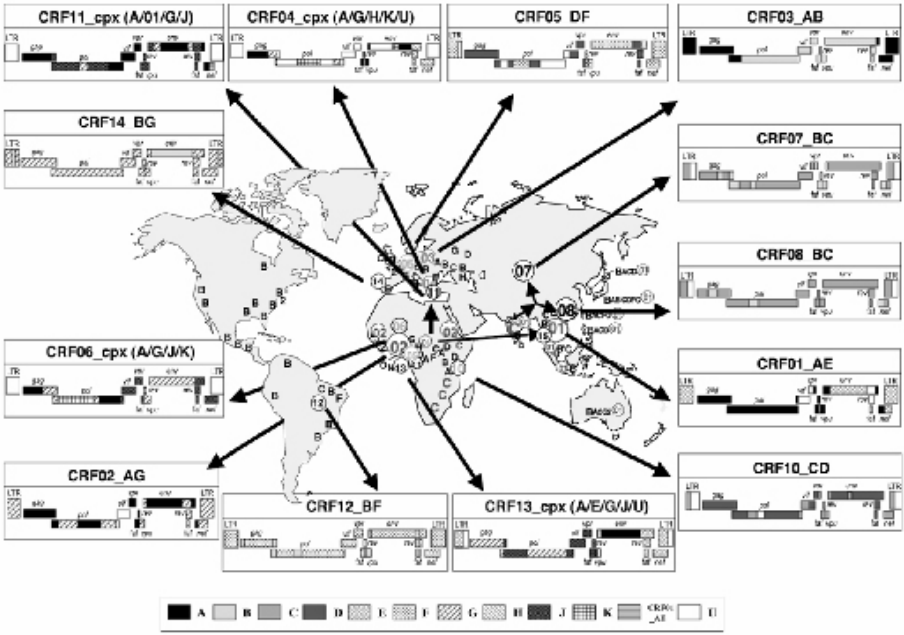
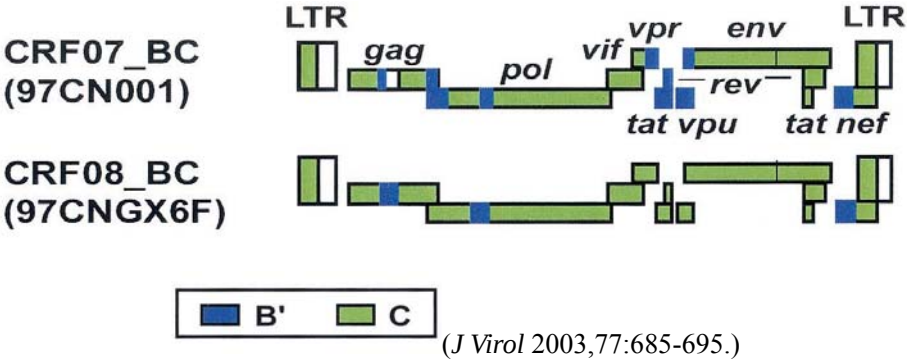
Fig 1 Recombinant Strains of HIV





(*J Virol* 2003,77:685-695.)

Fig 2 HIV subtype analysis (env)

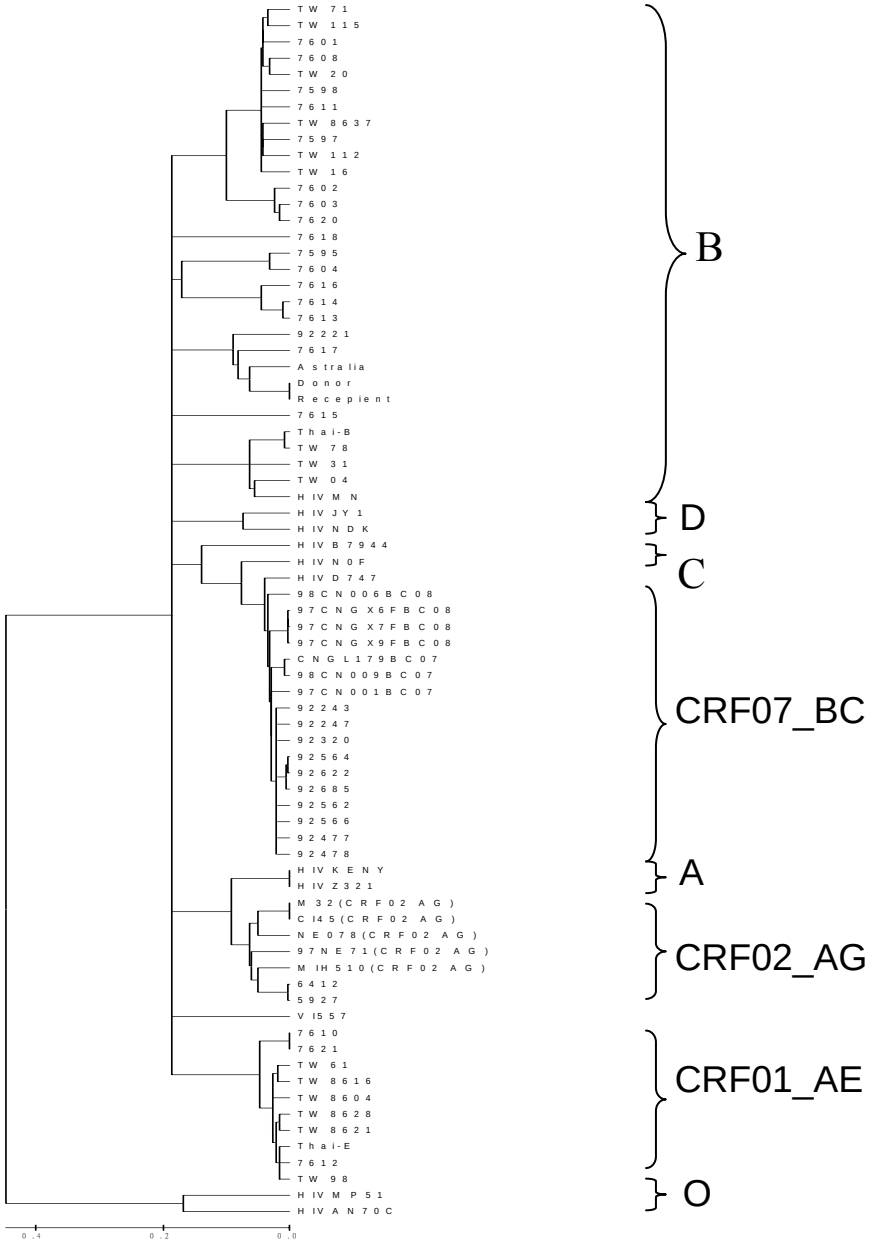
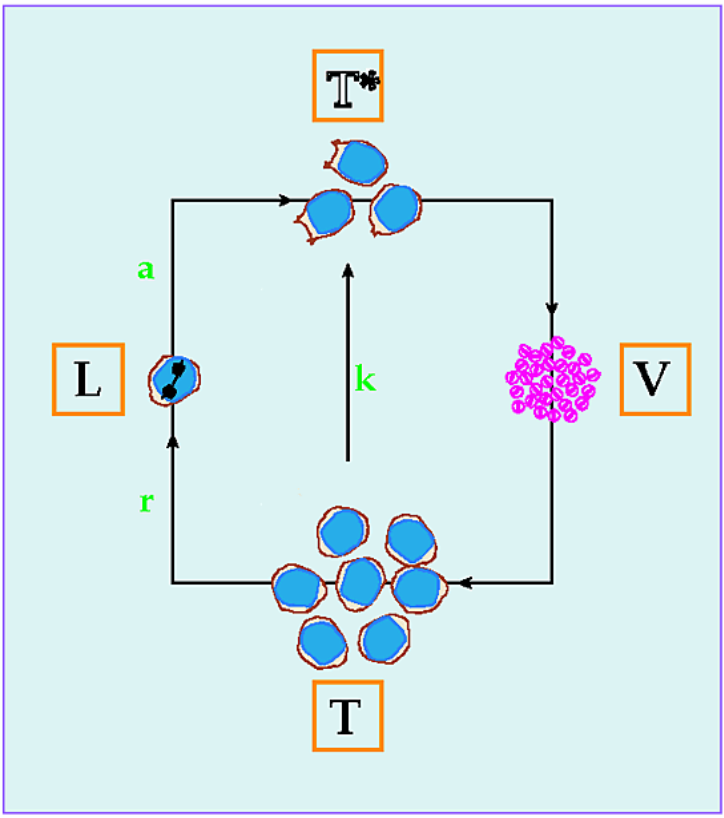


Fig 3 HAART Kinetics



(David Ho , Nature Medicine Vol.6 No.7 July2000)

HAART : V , T* undetectable ; k , r zero

V: cell free virions ; T: CD4 T lymphocyte ; T: latently infected reservoir ; L: productively infected CD4 ; T lymphocyte ; k : equally fast rate ; r: replacement rate ; a: decay rate

Fig 4 HIV subgroup analysis (pol)

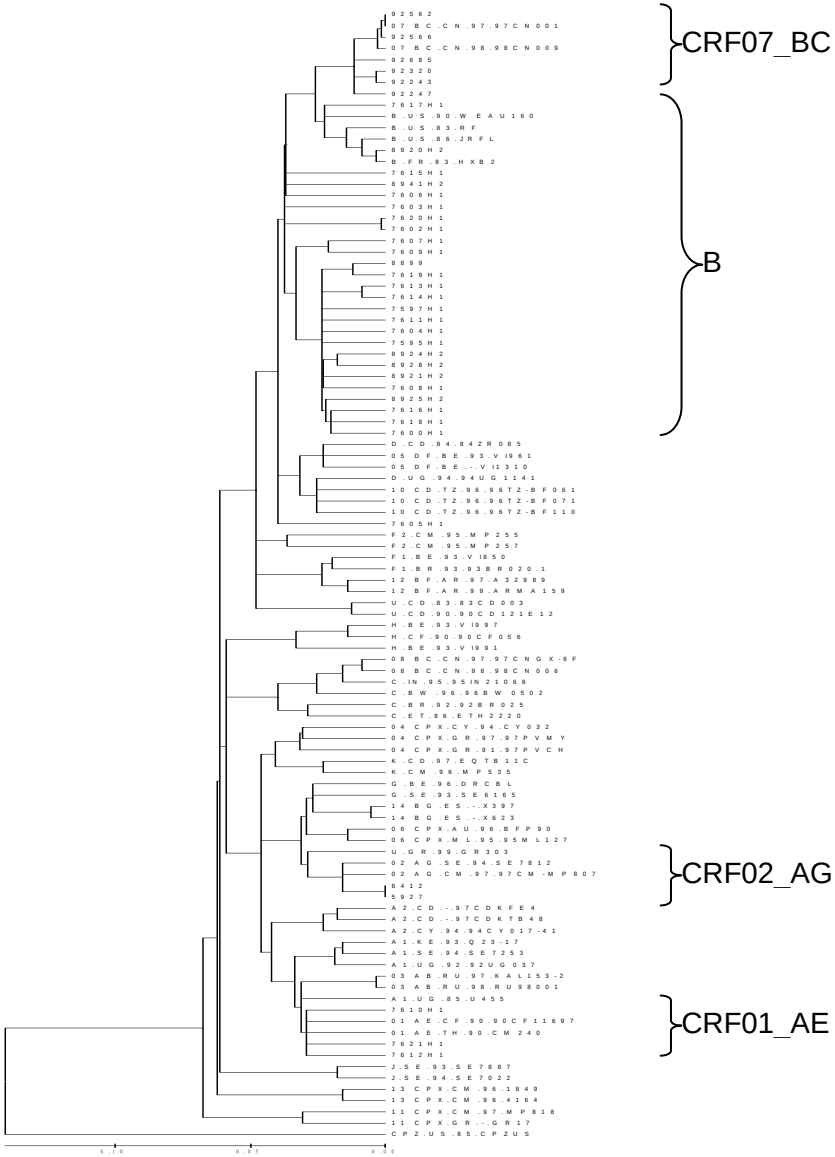


Fig 5 HIV subgroup analysis (gag)

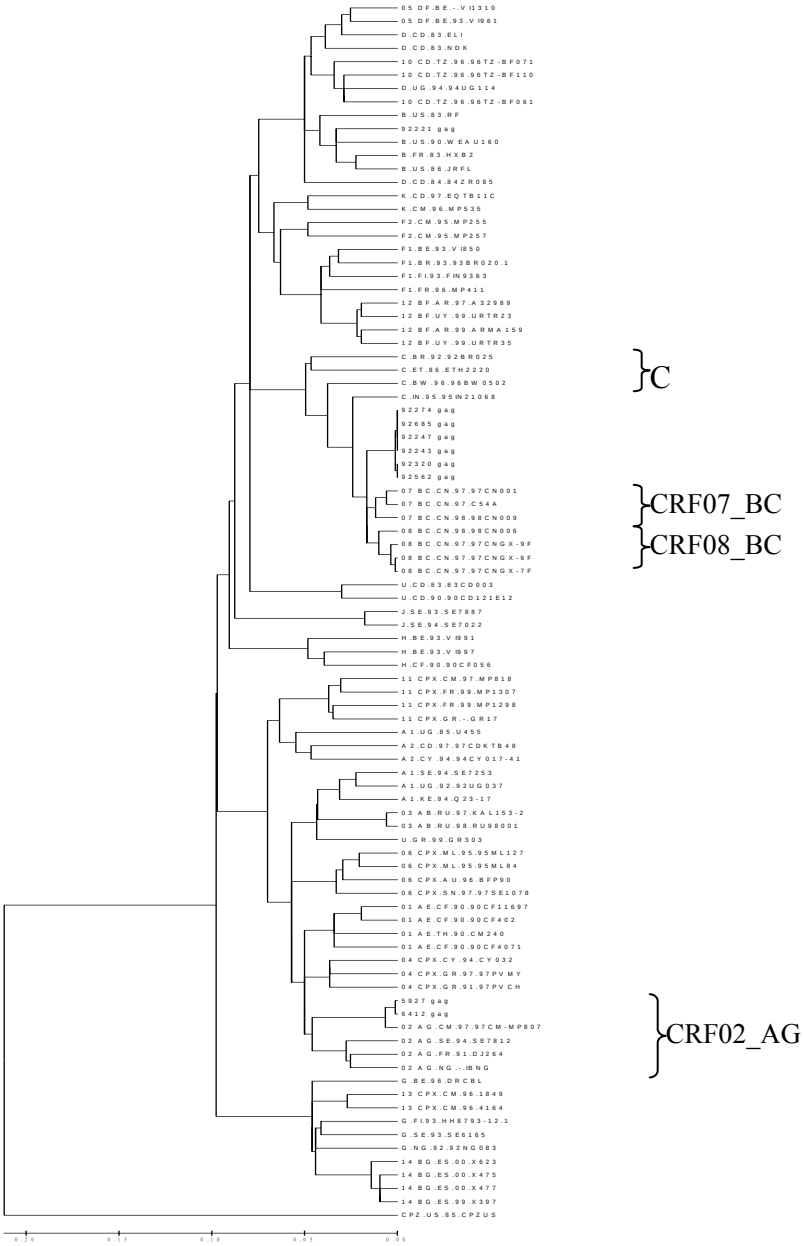


Table 1 Primer sequences used in RT-PCR and Nest-PCR for HIV protease and reverse transcriptase

Primer	Sequence[a]	Gene	Position
1-F-RT1	GGAAACCAAAAATGATAGGGG	Pol	2376-2407
8	GAATTGGAGG	Pol	3088-3119
1-R-RT2	CTGTATTTCTGCTATTAAGTCTT	Pol	2453-2475
1	TTGATGGG	Pol	3021-3051
2-F-RT1	GGACATAAAGCTATAGGTACAG	Gag	1991-2013
9	CTGCCAGTTCVAGCTCTGCTTC	Pol	2734-2711
2-R-RT2	TTGGAAATGTGGAAAGGAAGG	Gag	2197-22302600-256
0	AC	Pol	6
F-MAW2	GGCAAATACTGGAGTATTGTAT		
6	GG		
R-PR-pro	CAACTCCCCCTCAGAAGCAGG		
be	AGCCGATAGACAA		
Nest-F-P	CATCCATTCCTGGCTTTAATTTT		
R	ACTGGTACAGT		
Nest-R-P			
R			

Table 2 Primer sequences used in RT-PCR and Nest-PCR for HIV C2V3 gene

Primer	sequence	polarity
44F	5’-ACAGTRCARTGYACACATGG-3’	Sense
35R	5’-CACTTCTCCAATTGTCCITCA-3’	Anti-sense
33F	5’-CTGTTIAATGGCAGICTAGC-3’	Sense
48R	5’-RATGGGAGGRGYATACAT-3’	Anti-sense

Table 3 Specimen amount in individual year

year	1996	1998	1999	2001	2002	2003	2004
specimen (no.)	2	30	68	28	88	125	99

Table 4 Prevalence of HIV subtypes

Subtype	Quantities	Percentage (%)
B	377	85.6
CRF01_AE	39	8.8
CRF07_BC	16	3.6
CRF02_AG	2	0.4
C	7	1.5
Total	440	100

Table 5 Resistance-related mutation analysis in viral protease

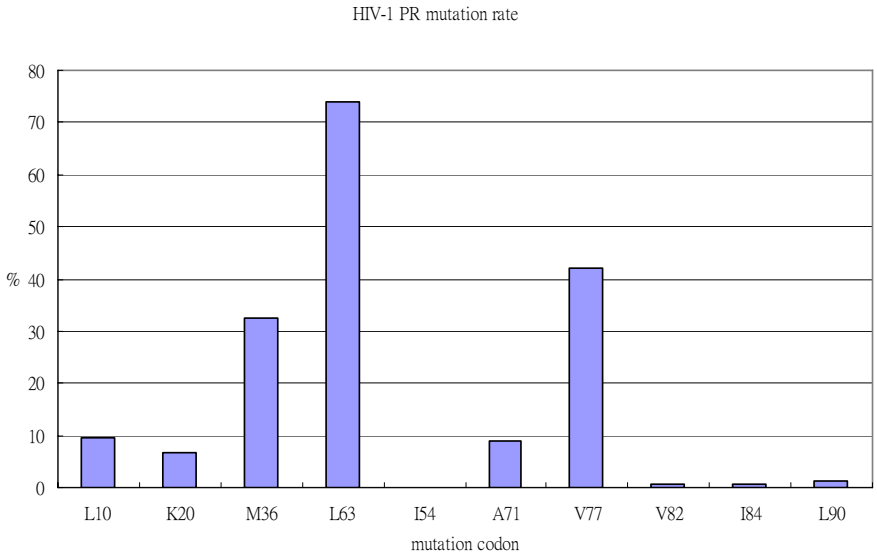


Table 6 Resistance-related mutation analysis in viral RT

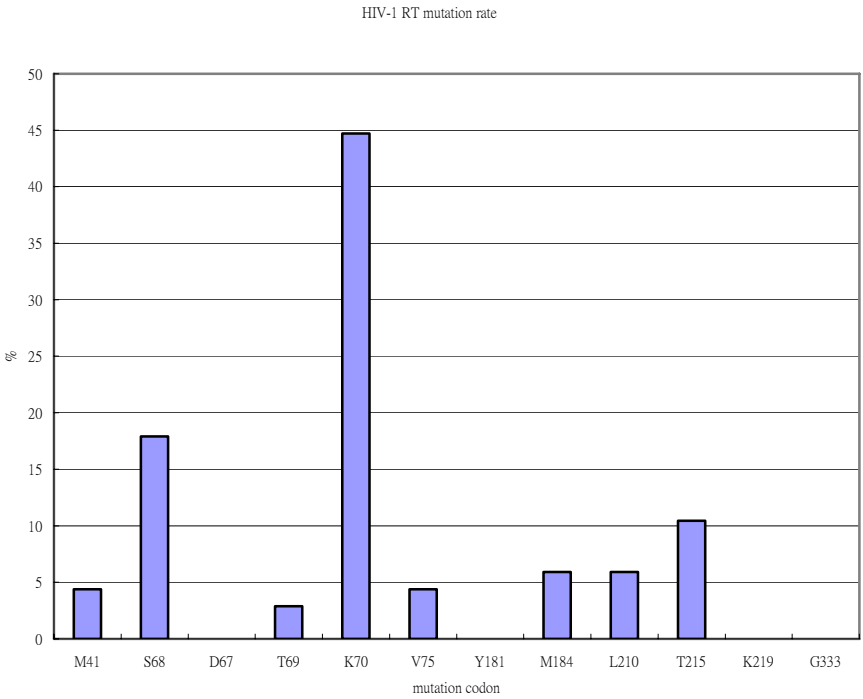


Table 7 Primary and secondary mutations of HIV-1 RT

RT inhibitor	Primary mutations	Secondary mutations
Nucleoside RT inhibitors	K70R， T215Y/F	M41L， D67N，L210W，K219Q，
Zidovudine	L74V	K65R，M184V/I
Didanosine	K65R ， T69D ，L74V，M184V/I	
Zalcitabine	E44D，V118I，M184V	
Lamivudine	E44D，V118I，M184V	
Stavudine	V75T	
Abacavir	K65R， L74V，M184V	M41L，D67N，K70RY115F，L210W，K219Q
Multinucleoside resistance （a.）	Q151M	A62V，V75I，F77L F116Y
Multinucleoside resistance （b.）	T69S-SA/G/S	M41L，A62V，D67NK70R，L210W，T215Y K219Q
Non-nucleoside RT inhibitors		
Nevirapine	K103N，V106A，V108I，Y181C，Y188C，G190A	L100I
Delavirdine	K103N，Y181C	P236L
Efavirenz	K103N，Y188L，G190A	L100I，V108I，P225H

Table 8 Primary and secondary mutations of HIV-1 protease

Protease inhibitor	Primary mutations	Secondary mutations
Indinavir	M46I, V82A/F/T/S	L10I/R/V, K20M/R, L24I, V32I, M36I, I54V, A71V/T, G73S/A, V77I, I84V,L90M
Ritonavir	V82A/F/T/S	K20M/R, V32I, L33F, M36I, M46I/L, I54V/L, A71V/T, V77I, I84V, L90M
Saquinavir	G48V , L90M	L10I/R/V ,I54V, A71V/T, G73S, V77I, V82A, I84V
Nelfinavir	D30N , L90M	L10F/I, M36I , M46I/L A71V ,V77I V82A/F/T/S, I84V, N88D
Amprenavir	I50V , I84V	L10F/I/R/V, V32I , M46I, I47V, I54V