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愛滋病患之B型肝炎病毒的基因漸進性改變
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行政院國家科學委員會補助專題研究計畫 成果報告

（計畫名稱）

愛滋病患之 B 型肝炎病毒的基因漸進性改變

Genetic Drift of Hepatitis B Virus in AIDS Patients After HAART

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ABSTRACT

Genetic drift of hepatitis B virus (HBV) after lamivudine-containing highly active anti-retroviral therapy (HAART) was studied retrospectively in patients with human immunodeficiency virus type 1 (HIV-1) infection. Paired sera were available from 16 HIV-1/HBV co-infected patients, nine of them had CD4 cell count less than 200/mm^3. Full-length HBV sequence was available by performing nested PCR and direct sequencing on six paired sera. In one patient, the HBV sequence after treatment with HAART for 15 months was identical to the pre-treatment dominant strain. His CD4 cell count was 2/mm^3 before treatment and he had very poor drug adherence. The rtM204V+rtL180M lamivudine-resistant mutations were found in the post-treatment HBV isolates from two patients. The CD4 cell count of both patients was less than 200/mm^3. The characterization of additional substitutions in these two drug-resistant isolates was not different from those lamivudine-resistant isolates of non-HIV infected patients reported in the literature. The other three post-treatment isolates did not have lamivudine-resistant mutations. Still, genetic drift of HBV was found. The number of genetic changes was 7 and 6 respectively in two isolates. The CD4 cell count of both patients was >200/mm^3. In contrast, 22 genetic changes of HBV were found in one patient after HAART, most of them clustered in core and pre-S1 region. His CD4 cell count was 45/mm^3. Nucleotide hypervariability in core gene had been observed in both the molecular evolution of HBV over 25 years and the genetic changes of HBV after exacerbation of chronic infection. The core gene may be an important immunological target was further suggested by the lowest d_S/d_N ratio in core gene. Our finding that AIDS patient but not HIV-1 infected patients with higher CD4 cell count had extensive genetic drift is compatible with the hypothesis of greater selective pressure imposed by the reconstituted immunity in AIDS patient. However, more data are required to support the hypothesis.

Background

In the era of highly active anti-retroviral therapy (HAART), many HIV-1 infected patients still remained undiagnosed until they had AIDS-defining events. The use of HAART at the stage of profound immunodeficiency is frequently associated with complications. One of them is the inflammatory reactions, termed immune reconstitution inflammatory syndrome (IRIS), that occur

within a few weeks following HAART and mimic new infection [1,2]. According to our own experience, paradoxical reaction due to concomitant *Mycobacterium* tuberculosis (TB) infection [3] is the most frequent IRIS after HAART in Taiwan. Through immune reconstitution, the rebuilt TB-specific immune response may cause inflammatory reaction at the sites where abundant *Mycobacteria tuberculosis* bacilli reside. The host immunity in the initial few months after HAART is not functioning adequately because we had experienced one AIDS patient who was not able to clear B19 viremia for as long as 6 months after HAART [4].

Parvovirus B19 is generally believed to have great genetic stability, genetic drift of B19 is therefore thought to be rare in persistent infection. Contrary to this belief, genetic drift of parvovirus B19 was found in our AIDS patients after treatment with HAART [5]. In contrast, genetic drift of B19 was absent in another AIDS patient not treated with HAART. Consider the very nature of conservation in B19 genome at amino acid level [6], it is surprising that 12 of the 18 nucleotide substitutions in B19 genome were nonsynonymous. This observation suggests that the genetic drift of B19 seen after HAART is positively selected. It is speculated that the driving selective pressure is the partially reconstituted host immunity after HAART.

Patients with HIV infection may have other coinfected viruses, such as human herpes viruses, hepatitis B and C virus. The impact of HAART on the genetic drift of HCV in HIV infected patients has been described [7]. However, AIDS patients are not enrolled as a separate study group in that study. AIDS patients receiving HAART are unique in having weak and escalating host immunity over time. Therefore, it is wondering that HAART may have different impact on the coinfected viruses between AIDS patients and HIV-infected patients with higher CD4 cell count. In our previous study, 21.7% of HIV infected patients have chronic HBV infection [8]. Interestingly, 35.1% of HIV-HBV coinfected patients developed hepatitis after HAART. The interval between initiation of lamivudine and development of hepatitis was 32 days (range 1-1120days). In our previous report, HAART containing lamivudine suppressed the replication of HBV [9], therefore the early relapse of hepatitis could not be explained by lamivudine-resistant HBV. The possible explanations include drug-induced hepatitis or enhanced inflammatory reaction due to reconstituted immunity. HBV was shown to have viral population heterogeneity [10]. In the presence of quasi-species, it is possible that certain HBV strain may have survival

advantage under the selective pressure of lamivudine and/or reconstituted immunity, therefore genetic drift of HBV may be found in AIDS patients after HAART. Indeed, genetic drift of HBV has been described in one AIDS patients after HAART [11]. It is our goal in this study to detect the genetic drift of HBV in AIDS patients and to find if HIV infected patients also have similar genetic drift.

MATERIALS AND METHODS

Sera

The definition of AIDS patients is HIV infected patients with AIDS-defining events or patients with CD4 cell count less than 200/mm^3. Paired sera were obtained from 10 AIDS patients who were co-infected with hepatitis B virus. For comparison, paired sera were also available from 6 HIV/HBV co-infected patients whose CD4 cell count were over 200/mm^3 before HAART. The first of the paired sera was obtained before the use of HAART and the second was obtained 7-38 months (average 12 months) after HAART. The wide range of interval between two sera was due to the retrospective nature of this study. All the sera were stored at -70^o C.

Polymerase chain reaction (PCR) and nucleotide sequencing

The whole genome of HBV was amplified by nested-PCR and sequenced directly. HBV DNA was extracted from 200 µL sera by using high pure viral nucleic acid kit (Roche, USA). The primers for nested-PCR were listed in Table 1. First round PCR was performed by using 10µL of the eluted DNA sample to make 50µL of PCR reaction solution containing 10 mmol/L Tris HCL, pH 8.3, 50 mmol/L potassium chloride, 2.5 mmol/L magnesium chloride, 200µmol/L of each dNTP, 1 unit of Taq DNA polymerase (Supertherm, Roche, Germany) and 100 ng of each primers. Thirty-five cycles were done using the following conditions: denaturation at 94^{o}C for 30 seconds, annealing at 50 ^{o}C for 30 seconds and extension at 72 ^{o}C for 2 minutes. The condition of second round PCR was similar to the first round. The nested PCR products were purified by using QIAquick PCR purification kit and sequenced by DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems).

Table 1. Primers for nested PCR

Set 1		5’→3’
Outer	sense	ATGCAACTTTTTCACCTCTGC
	Anti-sense	CCCACCTTATGAGTCCAAGG
Inner	sense	TTTTTCCCCTCTGCCTAATCA
	Anti-sense	TTATGAGTCCAAGGGATACTA
Set 2		
Outer	sense	GGCAGGTCCCCTAGAAGAAGAACT
	Anti-sense	CCACTGCATGGCCTGAGGATG
Inner	sense	GAAGAACTCCCCCGCCTCGC
	Anti-sense	CATGGCCTGAGGATGAGTGTCCC
Set 3		
Outer	sense	ACTGTGCCAGCAGCTCCTCCTCC
	Anti-sense	AGAGGACAAACGGGCAACATAC
Inner	sense	GCTCCTCCTCCTGCCTCCACCAA
	Anti-sense	AGAGGACAAACGGGCAACATAC
Set 4		
Outer	sense	CTCATCGAAGACTGGGGACCC
	Anti-sense	TGGAGAGAAAGTAAAAGCCTG
Inner	sense	CTCATCGAAGACTGGGGACCC
	Anti-sense	AGCCTGTTTTGCTTGTATACATGC
Set 5		

Outer	sense	TTGATTGGAAAGTATGTCAACGAA
	Anti-sense	AAAAAGTTGCATGGTGCTGG
Inner	sense	TATGTCAACGAATTGTGGGTCTTT
	Anti-sense	AAAAAGTTGCATGGTGCTGG

Cloning of HBV genomes

Genetic drift of HBV was determined by studying the population frequency of different HBV viral strain. PCR was performed as described above. The PCR products were purified by PCR purification kits and cloned by TA cloning kit (Invitrogen, California USA). The cloned HBV gene was sequenced by SP6 and T7 primers.

Results

Detection of lamivudine-resistant mutation

Among the paired sera from 16 patients, HBV DNA was amplified successfully from only 6 sera after HAART. The low successful rate could be due to the short interval between the first and second serum acquirement. The interval was usually less than 12 months and HBV replication was presumably still under effective suppression by lamivudine. One of the six patients had poor drug adherence and was excluded from this study. Nucleotide sequence of HBV remained unchanged in this patient 13 months after the initiation of HAART. Two patients were found to have M204V mutation in the YMDD motif of the reverse transcriptase.

Nucleotide substitutions found in the HBV genomes after HAART

The two lamivudine resistant YMDD variants were found in the sera obtained 38 and 9 months after HAART, respectively. Since both strain had the rtM204V mutation, they also had rtL180M mutation. Similar to other studies on resistant strains with and without breakthrough hepatitis [10,12], additional mutations were found (Table 2). Most of them were in the region of

polymerase open reading frame.

Table 2. The position of nucleotide substitutions found in lamivudine-resistant HBV strains from two patients after HAART

Patient A				Patient B			
Position		amino acid change	region	Position		amino acid change	region
667	T→A	L→M	P	1	C→A	L→I	P
739	A→G	M→V	P	330	G→A	S→N	S
		I→M	S			No	P
793	G→G/A	A→A/T	P	667	T→A	L→M	P
814	T→G	L→V	P	739	A→G	M→V	P
		No	S			I→M	S
1032	G→A	no	P	855	A→G	no	P
1230	A/G→G	no	P	930	A→C	Q→H	P
1613	A/G→A	K/R→K	P	2242	T→T/G	no	C
1633	G→A	G→E	X	2724	G→A	V→I	P

No lamivudine-resistant mutations were detected in three patients after treatment with HAART for 12, 8 and 8 months, respectively. Two patients had CD4 cell count >200 before HAART. The position of nucleotide change as compared to the pre-treatment HBV strain was listed in Table 3. Notably, many of these genetic changes had been found in the pre-treatment HBV strain as minor, co-dominant or dominant strain. Other genetic changes were noted only after HAART but did not become the dominant strain.

Table 3. Genetic changes of HBV in two HIV-infected patients whose CD4 cell counts are greater than 200/mm^3

Patient C			**Patient D**		
Position	amino acid change	region	Position	amino acid change	region
286 A>G→G	no	S	793 A/G→G	I/M→M	S
	N>D→D	P		T/A→A	P
987 A/G→A	no	P	1106 C→C>T	T→T>I	P
1090 T→T/A	F→F/I	P	1123 A→A>C	S→S>R	P
1128 C>A→A	D>E→E	P	1128 A→A>C	K→K>N	P
1730 G>A→G	no	X	1134 T→T>C	no	P
1961 T>C→T	S>P→S	C	1139 A→A>C	N→N>T	P
2047 A>T→A	no	C			

In contrast to these two patients with higher CD4 cell count, 22 genetic changes were found in HBV isolate from one AIDS patient after HAART (Table 4). His CD4 cell count was 45/mm^3 before HAART. Moreover, most of these genetic changes were found in C and pre-S1 region while most of the genetic changes observed in patients A to D were in the P region.

Table 4. The genetic changes of HBV found in one AIDS patient after treatment with HAART for 8 months

Patient E			**Patient E**		
Position	amino acid change	region	Position	amino acid change	region
216 T→T/C	L→L/S	S	2323 A>G→G>A	no	C
	No	P		Q>R→R>Q	P
354 A/C→C	H/P→P	S	2531 A/T→A	K/N→K	P
	No	P			
639 T→T/A	L→L/Q	S	2555 C/T→T	no	P
	No	P			
705 T/C→C	V/A→A	S	2666 A/T→A	K/N→K	P
	No	P			
1909 T/C→T>C	no	C	3002 A/G→G	N/S→D	pre-S1
			3003 A/G→A	E→G	P
2038 A/T→T	E/D→D	C	3010 C>A→C	P/Q/K→Q	pre-S1
			3011 C>A→A	A/D/E→A	P
2045 A/T→T	T/S→S	C	3017 C/T→T	A/V→V	pre-S1

				No	P
2131 A/C→C	E/D→D	C	3073 A>C→C	M>L→L N>T→T	pre-S1 P
2160 C>G→G>C	T>S→S>T	C	3081 G/A→G	no V/M→V	pre-S1 P
2198 C/A→A	L/I→I	C			
2241 C/T→C	T/I→T	C			

Discussion

The mechanism of genetic drift of viruses seen in chronic viral infection is complex because both the virological factors and the host environment must be considered. In chronic viral infections such as HIV, HBV and HCV infection, the situation is further complicated by the selective pressure of antiviral drugs. These three viral infections all require reverse transcription during viral replication and higher rate of nucleotide changes is anticipated. Similar to HIV and HCV, HBV quasispecies are commonly found in patients with chronic HBV infection [10,12]. It is speculated that the drug-resistant mutant may have the chance to be present in the pre-treatment vial population as a minor strain and may evolve to become the dominant strain under the selective pressure of antiviral drug treatment. Accordingly, it was found that the population of drug-resistant HBV variants was rather homogeneous [10,12]. The emergence of lamivudine-resistant mutations may or may not associate with breakthrough hepatitis. No evidence was found to support a significant role of viral factor or host immunity in such breakthrough hepatitis [10,12]. In the study of breakthrough hepatitis after lamivudine treatment,

the mean number of nucleotide and amino acid substitutions per genome pair was equivalent in post-transplantation or immunocompetent patients [12]. The number and position of amino acid substitutions in two of our AIDS patients (CD4< 200/mm^3) who developed lamivudine-resistant mutations without breakthrough hepatitis were also in accordance with the conclusion of the two reports [10,12]. However, it must be stressed that there is a dynamic escalating change in host immunity after HAART which is different from patients after organ transplantation.

Two of our patients whose initial CD4 was higher than 200/mm^3 did not have lamivudine-resistant mutations 12 and 8 months after treatment with HAART, respectively. In patient C, genetic drift of HBV was found as evidenced by the changes at nucleotide position 286 and 1128. Only one genetic change was noted in HBV from patient D after HAART at nt. 739. It is not known that the genetic drift is driven by reconstituted host immunity (unidentified immunogenic epitope), selective pressure of lamivudine (confer relative lamivudine-resistance) or natural evolution [13]. Interestingly, several low frequency mutations were clustered in a short region of Pol from nt.1106 to 1139. Whether these mutations exist in the same minor viral strain will be studied by cloning.

The finding of extensive genetic drifts in the HBV from patient E after HAART was not expected. In addition, their positions tend to cluster in C and pre-S1 region and most of them are non-synonymous. In the study of molecular evolution of hepatitis B over 25 years, the nucleotide hypervariability was observed within the polymerase and pre-S/S overlap region and within the core gene [13]. Positive selection of nucleotide changes in core gene possible due to immune evasion was suggested based on the low d_S/d_N ratio. Accordingly, mutations in the core gene had been frequently detected in patients with chronic HBV infection [14]. Furthermore, mutations tended to appear at the beginning of the immune clearance phase. The analysis of genetic changes after acute exacerbation of chronic HBV infection also found some patients had multiple amino acid substitutions in core or surface gene [15]. Based on the published data of core gene mutations and the greater number of genetic changes, it is very possible that the genetic drift of HBV seen in patient E might be driven by reconstituted immunity after HAART. The absence of

similar finding in two AIDS patients who harbored drug-resistant HBV mutants may indicate the stronger selective pressure imposed by lamivudine.

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