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Chronic hepatitis B and C co-infection increased all-cause mortality in HAART-naive HIV patients in northern Thailand

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SUMMARY

A total of 755 highly active antiretroviral therapy (HAART)-naive HIV-infected patients were enrolled at a government hospital in Thailand from 1 June 2000 to 15 October 2002. Census date of survival was on 31 October 2004 or the date of HAART initiation. Of 700 (92·6%) patients with complete data, the prevalence of hepatitis B virus (HBV) surface antigen and anti-hepatitis C virus (HCV) antibody positivity was 11·9% and 3·3%, respectively. Eight (9·6%) HBV co-infected patients did not have anti-HBV core antibody (anti-HBcAb). During 1166·7 person-years of observation (pyo), 258 (36·9%) patients died [22·1/100 pyo, 95% confidence interval (CI) 16·7–27·8]. HBV and probably HCV co-infection was associated with a higher mortality with adjusted hazard ratios (aHRs) of 1·81 (95% CI 1·30–2·53) and 1·90 (95% CI 0·98–3·69), respectively. Interestingly, HBV co-infection without anti-HBc Ab was strongly associated with death (aHR 6·34, 95% CI 3·99–10·3). The influence of hepatitis co-infection on the natural history of HAART-naive HIV patients requires greater attention.

Key words: Co-infection, hepatitis B, hepatitis C, mortality, resource-limited settings.

INTRODUCTION

In resource-limited countries, the prevalence of chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection is often high [1], and populations with a high prevalence of HBV and HCV usually overlap with those seriously affected by HIV. In a study from northern India, the reported prevalence of HBV and HCV co-infection in HIV-infected patients was 5·3% and 2·4%, respectively [2]. A study in Tanzania reported 17·3% and 18·1% of HIV-infected patients were co-infected with HBV and HCV, respectively [3], and an earlier report from Thailand showed that the prevalence of HBV infection was 8·7% and HCV infection 7·8% in HIV-infected patients [4].

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Accumulating evidence suggests that HIV co-infection adversely affects the clinical course of hepatitis. Increased HBV carriage rates, greater levels of HBV viraemia, more rapid decline in HBV surface antibody, increased reactivation episodes, and faster progression to liver cirrhosis are all characteristic of HIV/HBV co-infected patients [5, 6]. In HIV/HCV co-infected patients, faster progression of fibrosis resulting in decompensated cirrhosis have been shown in previous studies [7, 8]. As patients on highly active antiretroviral therapy (HAART) survive much longer, liver failure is becoming the major cause of death in patients with hepatitis co-infection [9].

Current International AIDS Society guidelines for the management of HIV recommend that HIV/viral hepatitis co-infected patients should start HAART, the same as HIV mono-infected patients. Moreover, initiation of HAART is recommended regardless of CD4 cell count when treatment for HBV is considered [10]. However, there is a big gap between the recommendation of the guidelines and the real-life situation in resource-limited countries. While access to HAART has markedly increased, even in resource-limited countries [11], overall HAART coverage remains as low as 36% (95% CI 33–39%) [11] based on 2010 WHO guidelines (treatment initiation at CD4 cell count <350 cells/μl. Moreover, monitoring (viral load testing and genotyping) and treatment for hepatitis are not available due to the cost in most resource-limited countries. Thus, we assume that there are still large groups of patients with HIV/chronic hepatitis co-infection who are not receiving HAART in resource-limited countries. For better management of these patients, it is important to know the association between hepatitis co-infection and the natural history of HIV infection.

The effect of viral hepatitis co-infection on HIV progression and all-cause mortality before initiating HAART remains uncertain. Most studies conducted in the late 1990s and early 2000s did not include HIV viral load in their analysis. Some studies presented a more rapid progression to AIDS and reduced survival in patients who have chronic HBV or HCV infection [12–15] while others have shown conflicting results [16–19]. In the majority of previous studies examining HBV and HCV co-infection, the main transmission mode of HIV in participants was homosexual intercourse or injecting drug use (IDU) and none were conducted with a substantial sample size in Asian or African countries where the majority of HIV-infected individuals with HBV vertical transmission reside. The present study aims to evaluate the impact of hepatitis co-infection on all-cause mortality in HAART-naive HIV-infected individuals in northern Thailand.

METHODS

Study site and population

To address the current research question, we re-analysed our previously conducted natural history cohort of HIV-infected patients in northern Thailand [20, 21]. This patient cohort was assembled from volunteers at the HIV centre of a government referral hospital with about 800 beds situated in the centre of Lampang province in upper northern Thailand. The centre was established in October 1995 as an out-patient clinic providing treatment, care and support for HIV-infected patients. The recruitment of this cohort was from 1 July 2000 to 15 October 2002 before the national antiretroviral treatment programme was launched. All adult (aged >18 years) HIV-infected individuals attending the HIV clinic who were HAART-naive at the first visit were approached by the research team and enrolled if written consent were obtained. All participants were requested to visit the clinic at least once every 3 months regardless of the presence of clinical symptoms and were followed up from the date of study enrolment until 15 October, 2004. This study was approved by the Thai Government Ethics Committee in December 1999 and December 2005.

Data collection

Demographic (gender and age at enrolment) data and medical history [HIV-related symptoms, history of antiretroviral therapy (ART) and mode of transmission] of patients were obtained at the study enrolment by well-trained research staff through face-to-face interviews based upon a structured questionnaire. In addition to physical examination by two research physicians, complete blood count (CBC), platelet count, CD4 cell count and HIV viral load (copies/ml) were measured. CD4 cell count was determined by flow cytometric technique FACScan (BD Biosciences, USA) and HIV viral load was measured using a commercial kit (Amplicor HIV-1 Monitor Test, Roche Molecular Systems Inc., USA). To address the present research question, the remaining freeze-stored plasma samples from our previous study were retrospectively tested for hepatitis B surface

antigen (HBsAg), anti-hepatitis B core antibody (anti-HBcAb) and antibody to hepatitis C virus (anti-HCV) were retrospectively tested using commercially kits: Cobas Core HBsAg II EIA, Anti-HBc EIA, and ETI-AB-HCVK-4 (DiaSorin S.p.A., Italy). HBsAg positivity and anti-HCV positivity were determined to define HBV and HCV co-infection, respectively. Cohort patient survival was assessed on 15 October 2004. Survival status for each patient was ascertained by hospital records, death certificates, mailing letters, and contacting families or relatives. Causes of death in HIV/hepatitis co-infected patients were investigated by reviewing hospital records.

Analysis

In survival analysis, patients who started HAART before 15 October 2004 were regarded as censored on the date of starting HAART. Kaplan–Meier survival analysis was performed to estimate survival in relation to the existence of HIV/HBV or HCV co-infection. HBV co-infected patients were divided into two subgroups according to the existence of anti-HBcAb. We used the log-rank test to compare Kaplan–Meier curves. Additionally, Cox's proportional hazard model was conducted to evaluate the influence of HBV or HCV co-infection on survival adjusted by several factors. In multivariate models, other than hepatitis co-infection status and existence of anti-HBcAb, we included all variables with $P<0{\cdot}1$ in univariate analysis. Results were presented as hazard ratios (HRs) with 95% confidence intervals (CIs). The proportional hazard assumption was explored using Nelson–Aalen plots and the likelihood ratio test. Statistical analyses were conducted using SPSS version 17.0 (SPSS Inc., USA) and Stata version 11.0 (StataCorp., USA).

Ethical approval

This study was conducted as part of the Lampang HIV Cohort Phase I and Lampang & Phayao HIV Cohort Phase II, which were approved by Research Ethics Committee of the Thai Ministry of Public Health.

RESULTS

Characteristics of participants

Seven hundred and fifty-five patients (over 95% of patients who attended the clinic during the targeted period) were enrolled in the study. Of 755 HIV-infected persons, 55 patients were excluded (32 patients had received HAART in private clinic or other clinical trial before recruitment, 22 patients had incomplete testing for hepatitis co-infection, and four patients had HBV/HCV dual co-infection). Baseline characteristics of patients are summarized in Table 1. Male:female ratio was 0·75. Median age at enrolment was 33 years (95% CI 29–37), and 21·8% had received mono or dual ART. The major transmission route was heterosexual (96·7%) and half of participants were asymptomatic. The CD4 cell count was >200 cells/μl in 299 (41·5%) patients and <50 cells/μl in 261 (36·3%) patients. The prevalence of HBV and HCV co-infection was 11·9% and 3·3%, respectively. Of 700 patients, 681 (97·3%) had both HBsAg and anti-HBcAb status. HCV co-infection was strongly associated with IDU ($P<0{\cdot}001$). All IDU cases were co-infected with HCV. The baseline CD4 cell count was significantly lower in HBV *vs.* HCV co-infected patients whereas there was no difference between these patient groups in their baseline viral load. Patients with HBV co-infection were more likely to have AIDS symptoms compared to HIV mono-infected patients ($P=0{\cdot}02$). Platelet counts were lowest in HCV co-infected individuals followed by HBV co-infected patients ($P=0{\cdot}03$). Table 2 shows the status of HBsAg and anti-HBcAb. Of 86 HBV co-infected patients, 78 (90·7%) had anti-HBcAb. Interestingly, eight (9·3%) patients did not have anti-HBcAb despite being HBsAg positive.

Impact of chronic hepatitis infection on all-cause mortality

Complete follow-up data were available for 694 (99·1%) patients in the evaluated cohort. Total follow-up time was 1166·7 person-years of observation (pyo) with a median patient follow-up of 588 days [interquartile range (IQR), 317–967]. During the observation period, 258 (36·9%) patients died, resulting in a mortality rate of 22·1/100 pyo (95% CI 16·7–27·8). When stratified according to baseline CD4 cell count, mortality was 64·6/100 pyo (95% CI 59·1–70·9) in patients with CD4 <50 cells/μl compared to 22·9/100 pyo (95% CI 16·3–29·7) in the group with CD4 <50–200 cells/μl and 5·17/100 pyo (95% CI 2·57–7·63) in patients with CD4 >200 cells/μl. When stratified with baseline clinical status, mortality was 8·71/100 pyo (95% CI 5·76–11·6) in asymptomatic patients, 27·5/100 pyo (95% CI 19·6–34·4) in

Table 1. *Baseline characteristics of HIV mono-infected patients and hepatitis co-infected patients*

Characteristics ($N=700$)	HIV mono-infection ($n=594$)	HBV co-infection ($n=83$)	HCV co-infection ($n=23$)
Age (median, IQR)*	33 (29–37)	32 (29–35)	30 (26–34)
Male gender (%)	244 (41·1)	42 (50·6)	14 (50·9)
Mode of transmission (%)*†‡			
Heterosexual	583 (98·1)	79 (95·2)	15 (65·2)
Homosexual	6 (1·0)	1 (1·2)	1 (4·3)
IDU	0 (0·0)	0 (0·0)	5 (21·7)
Others or multiple	5 (0·8)	2 (2·4)	2 (8·6)
Unknown	0 (0·0)	1 (1·2)	0 (0·0)
Previous ART (%)§	129 (21·7)	17 (20·5)	4 (17·4)
Clinical status (%)†‖			
Asymptomatic	312 (52·5)	32 (38·6)	8 (34·8)
Non-AIDS symptomatic	115 (19·4)	15 (18·1)	7 (30·4)
AIDS symptomatic	167 (28·1)	36 (43·4)	8 (34·8)
Baseline CD4 cell count (cells/μl) (median, IQR)*†‡	150 (22–351)	64 (19–219)	334 (31–459)
Baseline vial load (copies/ml) (median, IQR)	157431 (34891–446396)	169773 (69806–437116)	112334 (21415–515515)
Baseline platelet count (median, IQR)†	261000 (215000–319000)	237000 (191000–300000)	213000 (170000–328000)

IQR, Interquartile range; IDU, injecting drug user; ART, antiretroviral therapy; AIDS, acquired immunodeficiency syndrome.

* $P<0·05$, HIV mono-infection *vs.* HCV co-infection.

† $P<0·05$, HIV mono-infection *vs.* HBV co-infection.

‡ $P<0·05$, HBV co-infection *vs.* HCV co-infection.

§ Experience with antiretroviral therapy is limited to monotherapy or dual therapy.

‖ Definition of AIDS according to the National guidelines for the clinical management of HIV infection in children and adults, 6th edn. Thailand: Ministry of Public Health, 2000.

Other than *, †, ‡ there were not any significant differences between the groups.

Table 2. *Status of HBsAg and anti-HBcAb*

	anti-HBcAb(+)	anti-HBcAb(–)	Total
HBsAg(+)	78	8	86
HBsAg(–)	318	277	595
Total	396	285	681

HBsAg, Hepatitis B surface antigen; anti-HBcAb, anti-hepatitis B core antibody.

Both HBsAg and anti-HBcAb status data were available for 681 patients.

symptomatic but non-AIDS patients and 60·6/100 pyo (95% CI 54·4–66·7) in symptomatic AIDS patients. Kaplan–Meier survival analysis (Fig. 1) revealed that HBV co-infection significantly increased mortality ($P<0·001$, log-rank test). HCV co-infection also tended to increase mortality, but the statistical significance was marginal. The curves for HIV/HBV and HCV/HIV co-infection converge at about 500 days. The likelihood ratio test for interaction by time band with a cut-point at 500 days revealed a P value of 0·2, confirming lack of evidence for a relevant violation of the proportional hazard assumption, allowing the use of Cox regression analysis. The influence of hepatitis co-infection on survival analysed by the Cox proportional hazard model is presented in Table 3*a*. In univariate analysis, factors associated with death were male gender, previous ART treatment, clinical symptom at enrolment, baseline CD4 cell count, baseline viral load, HBV co-infection and existence of anti-HBcAb. Patients with a low platelet count (<150 000) were more likely to die than those with a higher platelet count. In multivariate analysis, there was no significant association between platelet count and death. HCV co-infection showed a tendency towards association with decreased survival.

HBV serology and mortality

Survival estimates focused on HBV serology interestingly showed that HBV co-infected individuals without anti-HBcAb had the poorest survival compared

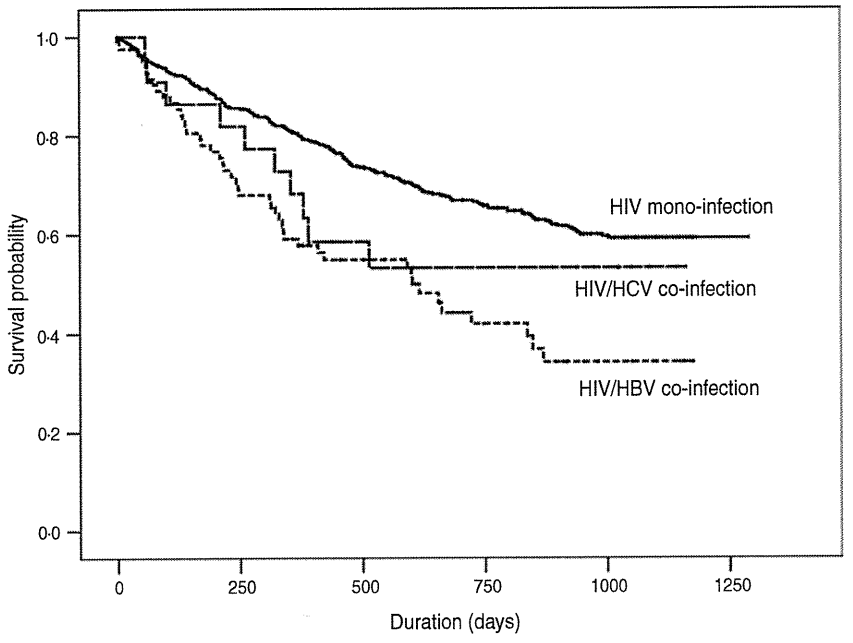

Fig. 1. Kaplan–Meier survival probability estimate of co-infected and mono-infected individuals showing that HBV co-infection significantly increased mortality.

to HIV mono-infected or HBV co-infected patients with anti-HBcAb ($P < 0{\cdot}0001$ by log-rank test, Fig. 2). In multivariate analysis (Table 3*b*), in addition to symptomatic AIDS and low CD4 cell count, HBV co-infection without anti-HbcAb was a strong risk factor for death (adjusted HR 6·34, 95% CI 3·99–10·3).

Cause of death in hepatitis co-infected patients

Although primary causes of death were unknown for nine out of 56 HIV/hepatitis co-infected patients, the majority of deaths (46/56, 82·1%) were attributed to AIDS-related diseases. No patients were diagnosed with liver failure before death except for one patient who was hospitalized for 6 days due to newly diagnosed cirrhosis with portal hypertension. This patient was lost to follow-up after discharge with a CD4 cell count of 22 cells/μl and died 3 months later.

DISCUSSION

In the present study with a substantial sample size, we have clearly demonstrated that HBV co-infection significantly increased all-cause mortality of HAART-naive HIV patients. It was only after the advent of HAART substantially increased life expectancy of HIV-infected individuals that the importance of liver-related death due to hepatitis co-infection was highlighted. Our data indicate that the influence of hepatitis co-infection on the natural history of HAART-naive HIV patients should not be ignored. We also discovered an increased mortality of HCV co-infected patients compared to HIV mono-infected patients. The results of multivariate analysis showed only a trend, but this is probably due to the small number of HCV co-infection in this study.

There are some limitations of the present study. First, the lack of data on HBV DNA and HCV RNA viral load is a limitation. Patients spontaneously clear HCV infection after acquisition. The absence of HCV viraemia in anti-HCV-positive patients might explain why HCV co-infection did not show the strong association with death. Second, cause of death was determined only by reviewing medical charts while the information of survival status was ascertained by contacting families and relatives in addition to reviewing hospital records. It is possible that cause of death might have been misclassified in some cases. However, we focused on all-cause mortality and these limitations do not alter the main results of the study.

To our knowledge, this is the first study from a resource-limited country to address the adverse influence of hepatitis co-infection on survival in HAART-naive HIV patients. We also found a higher mortality rate in this Thai population compared to white patients in New York in the 1980s [22]. Our finding

Table 3 (*a*). *Influence of HBV or HCV co-infection on survival*

Variables	HR (95% CI)	*P* value	aHR (95% CI)	*P* value
Male sex	2·59 (2·02–3·33)	<0·001	1·23 (0·94–1·60)	0·14
Age <30 years*	0·79 (0·61–1·01)	0·06	0·81 (0·63–1·05)	0·11
Previous ART†	0·67 (0·49–0·92)	0·01	1·16 (0·84–1·61)	0·37
Transmission mode				
IDU	Ref.	—	—	—
Homosexual	0·36 (0·05–2·58)	0·31	—	—
Heterosexual	0·68 (0·17–2·72)	0·58	—	—
Others	1·12 (0·21–6·13)	0·89	—	—
Clinical symptom*‡				
Asymptomatic	Ref.	—	Ref.	—
Symptomatic, non-AIDS	3·06 (2·16–4·33)	<0·001	1·42 (0·96–2·08)	0·08
AIDS, symptomatic	6·65 (4·93–8·98)	<0·001	2·05 (1·44–2·93)	<0·001
Baseline CD4 cell count† (cells/μl)				
⩾200	Ref.	—	Ref.	—
50–199	4·46 (2·91–6·84)	<0·001	2·98 (1·85–4·79)	<0·001
<50	12·7 (8·72–18·6)	<0·001	6·44 (4·04–10·3)	<0·001
Baseline viral load (copies/ml)				
<10000	Ref.	—	Ref.	—
10000–49999	2·44 (1·04–5·75)	0·04	1·20 (0·48–3·01)	0·70
50000–99999	3·30 (1·39–7·85)	0·007	1·67 (0·70–4·00)	0·25
⩾100000	8·79 (4·13–18·7)	<0·001	2·19 (0·96–5·00)	0·06
Baseline platelet count				
<150000	1·52 (1·00–2·29)	0·05	1·16 (0·76–1·78)	0·50
HBV co-infection	2·05 (1·49–2·82)	<0·001	1·81 (1·30–2·53)	<0·001
HCV co-infection	1·26 (0·67–2·38)	0·47	1·90 (0·98–3·69)	0·06

HR, Hazard ratio; CI, confidence interval; aHR, adjusted hazard ratio; ART, antiretroviral therapy; IDU, injecting drug user; AIDS, acquired immunodeficiency syndrome.

* At the time of enrolment.

† Experience with antiretroviral therapy is limited to monotherapy or dual therapy.

‡ Definition of AIDS according to the National guidelines for the clinical management of HIV infection in children and adults, 6th edn. Thailand: Ministry of Public Health, 2000.

implies that the high prevalence of hepatitis co-infection is at least partially responsible for the high mortality observed in HIV-infected individuals in resource-limited countries. Several studies have investigated the influence of hepatitis co-infection on the natural course of HIV infection but these studies often include patients receiving HAART and show conflicting findings [12–14, 16, 17, 23]. One of the reasons for this discrepancy appears to be the sample sizes in previous studies [16, 23]. In addition, none of the previous studies has analysed the association between HIV and HBV or HCV co-infection after adjusting for clinical status, viral load, and CD4 cell count in a cohort not receiving HAART with substantial sample size.

Our investigation of hospital records did not identify any patients with liver failure, with one exception. We might have undiagnosed hepatocellular carcinoma in some patients. However, we believe that the majority of chronic HBV or HCV co-infected HIV patients died from AIDS-defined illness rather than liver failure, since they all had significant opportunistic infections with a very low CD4 cell count. These results suggest that hepatitis co-infection accelerates the natural course of HIV infection itself. An *in vitro* study has demonstrated that HBV-X protein super-induces ongoing HIV replication and HIV-1 long-terminal repeat transcription [24]. However, according to our results, HBV co-infection increased the mortality independent of HIV viral load. Thus, the increased HIV viral load *per se* does not fully explain the higher mortality in HBV co-infected patients.

We also considered the possibility that the higher mortality in HBV co-infected patients might have been due to confounding factors. However, our multivariate analysis demonstrated that the association

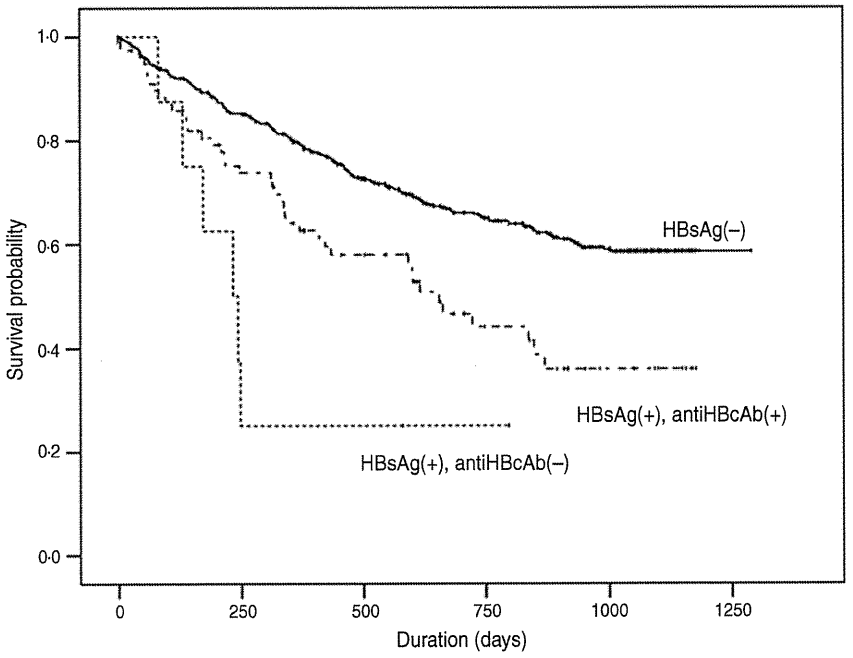

Fig. 2. Kaplan–Meier survival probability estimate showing that HBV co-infected individuals without anti-HBcAb had the poorest survival compared to HIV mono-infected or HBV co-infected patients with anti-HBcAb.

with HBV co-infection was independent of age, gender, transmission route, clinical symptoms and immunological status such as CD4 cell count. Nevertheless, as with most multivariate regression models, residual confounding cannot be fully ruled out. We also analysed some behavioural factors like excessive alcohol consumption. However, we did not find any significant association with HBV co-infection.

It is striking that HBV co-infected patients without anti-HBcAb had the poorest prognosis. Even after adjustment for demographic and clinical factors, the impact on death remained substantially high. Avettand-Fenoel *et al.* suggested three circumstances leading to failure to elicit anti-HBcAb during HBV infection [25]. Our patients may fit two of these circumstances. First, the majority of HBV patients in developing countries were vertically infected. It is hypothesized that infants born to HBeAg-positive carrier mothers may result in the lack of anti-HBcAb production as they have helper T-cell tolerance to HBV core Ag and HBeAg induced by transplacental maternal HBVAg. Another reason for lack of anti-HBcAb production is due to immunocompromised condition like uncontrolled HIV infection. If the former circumstance is true, these patients should be HBVe Ag-positive but such data is not available in this study. Clinical implication of the absence of anti-HBcAb during chronic HBV infection remain largely unknown except that it is not linked to severe hepatic disease course [26] although its impact on HIV progression has never been reported. We found that the frequency of HBV patients without anti-HBcAb is not uncommon in our HIV-infected population. Together with the poor prognosis, our observation suggests that more attention should be given to this group. However, the results should be interpreted with caution because of the small number of HBV patients without anti-HBcAb.

After the initiation of HAART, in both wealthy and resource-limited countries, the proportion of liver-related mortality increased in hepatitis and HIV co-infected patients [27–30]. Unfortunately, adequate treatment for chronic hepatitis is not available in resource-limited countries. A tenofovir-based first-line regimen is now recommended by WHO and is being adopted in many countries. However, the price of tenofovir needs to fall to allow more widespread access to this drug. Currently, the prevailing regimen for chronic hepatitis infection includes lamivudine alone in the most resource-limited countries. Thus, most HIV patients with HBV co-infection are inevitably receiving lamivudine monotherapy for HBV infection. The choice of antiretrovirals for such patients should include at least two drugs effective against HBV such as tenofovir. Last, screening for hepatitis co-infection at the same time as HIV diagnosis should be urgently implemented.

Table 3 (*b*). *Impact of HBs Ag and anti-HBc Ab status on survival*

Variables	HR (95% CI)	*P* value	aHR (95% CI)	*P* value
Male sex	2·59 (2·02–3·33)	<0·001	1·23 (0·96–1·63)	0·11
Age <35 years*	0·79 (0·61–1·01)	0·06	0·79 (0·61–1·02)	0·08
Previous ART†	0·67 (0·49–0·92)	0·01	1·10 (0·79–1·53)	0·59
Transmission mode				
IDU	Ref.	—	—	—
Homosexual	0·36 (0·05–2·58)	0·31	—	—
Heterosexual	0·68 (0·17–2·72)	0·58	—	—
Others	1·12 (0·21–6·13)	0·89	—	—
Clinical symptom*‡				
Asymptomatic	Ref.		Ref.	—
Symptomatic, non AIDS	3·06 (2·16–4·33)	<0·001	1·47 (1·10–2·15)	0·05
AIDS, symptomatic	6·65 (4·93–8·98)	<0·001	2·03 (1·42–2·90)	<0·001
Baseline CD4 cell count† (cells/μl)				
⩾200	Ref.	—	Ref.	—
50–199	4·46 (2·91–6·84)	<0·001	2·03 (1·42–2·90)	<0·001
<50	12·7 (8·72–18·6)	<0·001	6·34 (3·99–10·3)	<0·001
Baseline viral load (copies/ml)				
<10000	Ref.	—	Ref.	—
10000–49999	2·44 (1·04–5·75)	0·04	1·73 (0·72–4·13)	0·22
50000–99999	3·30 (1·39–7·85)	0·007	1·17 (0·47–2·93)	0·74
⩾100000	8·79 (4·13–18·7)	<0·001	2·09 (0·91–4·78)	0·08
Baseline platelet count				
<150000	1·52 (1·00–2·29)	0·05	1·28 (0·84–1·94)	0·25
HBV serology				
HBsAg(–)	Ref.	—	Ref.	—
HBsAg(+)anti-HBcAb(–)	—	—	6·34 (3·99–10·3)	<0·001
HBsAg(+) anti-HBcAb(+)	—	—	1·62 (1·14–2·29)	0·007

IQR, Interquartile range; ART, antiretroviral therapy; AIDS, acquired immunodeficiency syndrome; HR, hazard ratio; aHR, adjusted hazard ratio; CI, confidence interval; HBsAg, hepatitis B surface antigen; anti-HBcAb, anti-hepatitis B core antibody.

* At the time of enrolment.

† Experience with antiretroviral therapy is limited to mono- or dual-therapy.

‡ Definition of AIDS according to the National guidelines for the clinical management of HIV infection in children and adults, 6th edn. Thailand: Ministry of Public Health, 2000.

In summary, whereas HCV co-infection showed marginal association with the survival, HBV co-infection, especially without anti-HBcAb, increased the all-cause mortality in HAART-naive HIV patients in resource-limited countries. In this setting, clinicians and healthcare providers should prioritize HIV/chronic hepatitis co-infected individuals.

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DECLARATION OF INTEREST

None.

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Original article

Gag-CA Q110D mutation elicits TRIM5-independent enhancement of HIV-1mt replication in macaque cells

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Abstract

HIV-1 is strictly adapted to humans, and cause disease-inducing persistent infection only in humans. We have generated a series of macaque-tropic HIV-1 (HIV-1mt) to establish non-human primate models for basic and clinical studies. HIV-1mt clones available to date grow poorly in macaque cells relative to SIVmac239. In this study, viral adaptive mutation in macaque cells, G114E in capsid (CA) helix 6 of HIV-1mt, that enhances viral replication was identified. Computer-assisted structural analysis predicted that another Q110D mutation in CA helix 6 would also increase viral growth potential. A new proviral construct MN4Rh-3 carrying CA-Q110D exhibited exquisitely enhanced growth property specifically in macaque cells. Susceptibility of MN4Rh-3 to macaque TRIM5α/TRIMCyp proteins was examined by their expression systems. HIV-1mt clones so far constructed already completely evaded TRIMCyp restriction, and further enhancement of TRIMCyp resistance by Q110D was not observed. In addition, Q110D did not contribute to evasion from TRIM5α restriction. However, the single-cycle infectivity of MN4Rh-3 in macaque cells was enhanced relative to the other HIV-1mt clones. Our results here indicate that CA-Q110D accelerates viral growth in macaque cells irrelevant to TRIM5 proteins restriction.

Keywords: HIV-1; HIV-1mt; Gag-CA; Macaque cells; Virus growth; Molecular modeling

1. Introduction

Mammalian cells express a variety of host restriction factors to defend themselves against pathogens. Viruses have evolved countermeasures to subvert their restriction and replicate efficiently in cells [1,2]. HIV-1, a causative agent of human AIDS, evades host restriction factors and replicates well in human cells. However, in macaques for experimental use, e.g. cynomolgus macaques (CyMs) and rhesus macaques (RhMs), HIV-1 replication is completely inhibited by host restriction factors present in their cells [3]. Construction of HIV-1 that overcomes species-barrier contributes much to understand the interaction of HIV-1 and its host as well as the establishment of HIV-1-infected macaque models [4,5].

Extensive molecular biological studies on the HIV-1/host interaction conducted to date have revealed main mechanical bases for the narrow host range exhibited by HIV-1. Macaque cells contain potent antiviral factors that effectively restrict or even abolish HIV-1 replication. These include APOBEC3 proteins (APOs), CyclophilinA (CypA), and TRIM5α/TRIMCyp

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(TRIM5 proteins). HIV-1 can indeed counteract human proteins corresponding to these restriction factors. APOs exhibit cytidine deaminase activity, and introduce lethal mutations into HIV-1 genome. HIV-1 Vif is able to neutralize the antiviral activity of human APOs, but not macaque APOs [6–8]. CypA acts on incoming HIV-1 core to regulate infection positively in human cells but negatively in macaque cells [9–11], though amino acid sequences of CypA from human and macaque are identical. Macaque TRIM5α recognizes and interacts with incoming HIV-1 core, and restricts virus infection in a less-defined mechanism [9–11]. Macaque TRIM5α is polymorphic, and has sequence variation in a C-terminal B30.2/SPRY domain important for capsid (CA) binding. Sequence variation in this domain causes modulation of host susceptibility to retrovirus infection [12,13]. Macaque TRIMCyp is a fusion protein resulted from replacement of a B30.2/SPRY domain with CypA. Both CyM and RhM cells express TRIMCyp, but affinity of these proteins to HIV-1 core is different due to amino acid substitutions in Cyp domains. Thus, CyM TRIMCyp restricts HIV-1 replication, but not RhM TRIMCyp [14,15].

Identification of host restriction factors in macaque cells and their target proteins in HIV-1 has prompted us to generate macaque-tropic HIV-1 (HIV-1mt) with a minimal modification of HIV-1 genome. We successfully constructed prototype HIV-1mt, NL-DT5R, by replacing CypA binding region on a loop between helices 4 and 5 (h4/5L) in *gag*-CA and entire *vif* genes with the corresponding regions of pathogenic SIVmac239 (Fig. 1) [16]. But growth potential of NL-DT5R was inferior to that of SIVmac239 both *in vitro* and *in vivo* [16,17]. These results indicated that genetic modifications in NL-DT5R were insufficient to confer the ability on the virus to grow efficiently in macaque cells [16–18]. In an attempt to improve growth potential of NL-DT5R, we adapted NL-DT5R and its R5-tropic version NL-DT562 to a CyM derived lymphocyte cell line HSC-F, and found a number of genetic substitutions in viral genomes of adapted viruses [19]. We introduced these mutations and CA h6/7L from SIVmac239 into NL-DT5R, and the resultant clone was designated MN4-5S (Fig. 1) [19]. MN4-5S exhibited enhanced growth potential in CyM both *in vitro* and *in vivo* compared to NL-DT5R [19]. But growth ability of MN4-5S was still lower than that of SIVmac239.

In this study, to further improve replication potential of HIV-1mt, we adapted MN4-5S in macaque cells and identified an adaptive mutation in CA that enhances growth ability in the cells. *In silico* structural modeling of the adaptive mutation predicted that Q110D mutation on helix 6 in CA (CA-Q110D) would promote viral replication in macaque cells. Indeed, a proviral clone carrying CA-Q110D, designated MN4Rh-3, exhibited marked enhancement of growth potential in macaque cells relative to all the other HIV-1mt clones we have constructed (Fig. 1). CyM TRIM5α/TRIMCyp susceptibility assays revealed that MN4Rh-3 completely evades from TRIMCyp restriction but not TRIM5α restriction as observed for the other HIV-1mt clones. While CA-Q110D contributed to neither endowment of further resistance to TRIMCyp nor evasion from TRIM5α restriction, CA-Q110D did lead to enhanced single-cycle infectivity to a macaque cell line compared with the other HIV-1mt clones. Our results here indicate that CA-Q110D accelerates viral growth in macaque cells independently of TRIM5 proteins restriction.

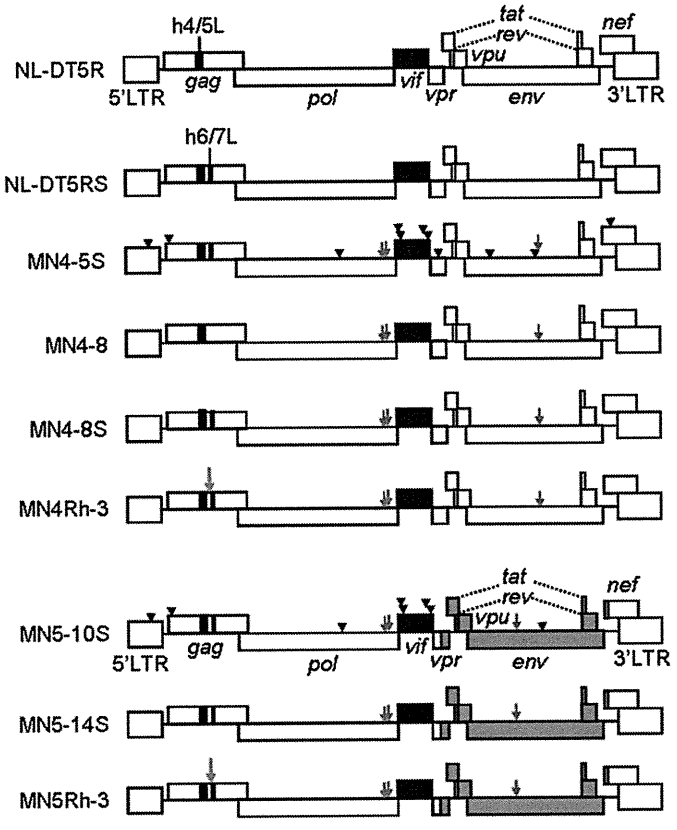


Fig. 1. Proviral genome structure of various HIV-1mt clones used in this study. HIV-1 NL4-3 [26] and SIVmac239 (GenBank: M33262) sequences are indicated by white and black areas, respectively. Gray areas in MN5-10S, MN5-14S and MN5Rh-3 show sequences from NF462 [21]. Blue arrows and black arrowheads show nucleotide substitutions that appeared in viral genomes of NL-DT5R and NL-DT562 during adaptation in HSC-F cells. Among nucleotide substitutions, adaptive mutations that enhance viral growth potential are indicated by blue arrows. Red arrows show the CA-Q110D mutation.

2. Materials and methods

2.1. Plasmid DNA

Construction of NL-DT5R, NL-DT562, NL-DT5RS, and MN4-5S were described previously [16,19–21]. MN4-5S carries all nucleotide substitutions that are present in adapted NL-DT5R and NL-DT562 clones except for mutations in the *env* gene of R5-tropic viruses (MN5-10S, MN5-14S, and MN5Rh-3 in Fig. 1) [19]. MN4-8S contains adaptive (growth-enhancing) mutations in MN4-5S but not the other mutations. MN4Rh-3 was constructed by introduction of the CA-Q110D mutation into MN4-8S. To construct R5-tropic viruses, 3′ halves of viral genomes (*Eco*RI in *vpr* to *Sph*I at the 3′ end of viral genome) of MN4-5S, MN4-8S, and MN4Rh-3 were replaced with the corresponding regions of adapted-NL-DT562,

and were designated MN5-10S, MN5-14S, and MN5Rh-3, respectively. For single-cycle infectivity assays to monitor viral susceptibility to TRIM5 proteins and to determine infectivity for CyM cells, *env*-deficient HIV-1mt variants encoding luciferase gene were constructed. NL-DT5R was cleaved with *Nde*I and *Nhe*I (both sites in *env* gene), blunt ended by T4 DNA polymerase, and resealed by T4 DNA ligase. The resultant clone was designated 5RΔEnv. Luciferase gene was then introduced into *nef* gene of 5RΔEnv as described previously [22], and the resultant clone was designated 5RΔEnv + Luc. A fragment containing the 3′ half genome was cut out from the 5RΔEnv + Luc, and introduced into the corresponding region in HIV-1mt variants (DT5R/4-3, NL-DT5RS, MN4-8, MN4-8S, and MN4Rh-3) to generate 5R/4-3ΔEnv + Luc, 5RSΔEnv + Luc, 4-8ΔEnv + Luc, 4-8SΔEnv + Luc, and 4Rh-3ΔEnv + Luc, respectively.

2.2. Cell culture

A human monolayer cell line 293T [23], a feline kidney cell line CRFK (ATCC CCL-94), and a CyM kidney cell line MK.P3(F) (JCRB 0607) were maintained in Eagles's minimal essential medium (MEM) containing 10% heat-inactivated fetal bovine serum (hiFBS). CRFK cells expressing TRIM5α/TRIMCyp were maintained in MEM containing 10% hiFBS and 400 μg/mL G418 (SIGMA). Macaque lymphocyte cell lines, HSC-F [24] and HSR5.4 [25], were maintained in RPMI-1640 medium containing 10% hiFBS. Recombinant human IL-2 (AbD Serotec) was added to the medium (50 units/mL) for maintenance of HSR5.4 cells. A human lymphocyte cell line MT4/CCR5 (MT4 cells stably expressing CCR5) was maintained in RPMI-1640 medium containing 10% hiFBS and 200 μg/mL hyglomycine (SIGMA).

2.3. Virus replication assays

Virus stocks for infection were prepared from 293T cells transfected with proviral clones as described previously [16,19,26]. Virion-associated reverse transcriptase (RT) activity was measured as described previously [16]. HSC-F cells (10^6) were infected with equal RT units of viruses in the presence of IL-2. For infection of MT4/CCR5 cells (10^6), the spinoculation method [27] was used. Viral growth was monitored by RT activity released into the culture supernatants. We assessed the viral growth potential by the peak day of virus production, and if the viral growth kinetics are similar, by the production level on the peak day.

2.4. Generation and characterization of adapted viral clones

MN4-5S and MN5-10S viruses (Fig. 1) prepared from transfected 293T cells were inoculated into HSR5.4 cells (3×10^6) with an equal amount of viruses (5×10^7 RT units). The cultures were maintained in the presence of IL-2, and HSC-F cells were added on day 34 post-infection. The culture supernatants (collected on day 18 post-cocultivation, the peak day of virus production) were inoculated into fresh HSR5.4 cells, and total DNA was extracted from the cells on day 15 post-infection. Integrated proviruses were amplified from total DNA as two overlapping fragments by the polymerase chain reaction (PCR), and amplified products were cloned into MN5-10S as described previously [16]. Viruses were prepared from 293T cells transfected with the resultant clones, and inoculated into HSR5.4 cells. Only one clone exhibited a rapid growth kinetics compared to MN5-10S, and was designated Ad clone-25. To identify an adaptive mutation that enhances growth potential, each mutation found in the genome of Ad clone-25 was introduced into MN5-14S by site-directed mutagenesis (STRATAGENE). For screening, viruses prepared from transfected 293T cells were inoculated into HSC-F cells, and virus replication was monitored by RT activity released into the culture supernatants.

2.5. Molecular modeling of HIV CA N-terminal domain (NTD)

The crystal structure of HIV-1 CA NTD at a resolution of 2.00 Å (PDB code: 1M9C [28]) was taken from the RCSB Protein Data Bank [29]. The three-dimensional (3-D) models of HIV-1 CA NTD were constructed by the homology modeling technique using 'MOE-Align' and 'MOE-Homology' in the Molecular Operating Environment (MOE) (Chemical Computing Group Inc., Quebec, Canada) as described [30–32]. We obtained 25 intermediate models per one homology modeling in MOE, and selected the 3-D models which were the intermediate models with best scores according to the generalized Born/volume integral methodology [33]. The final 3-D models were thermodynamically optimized by energy minimization using an AMBER99 force field [34] combined with the generalized Born model of aqueous solvation implemented in MOE [35]. Physically unacceptable local structures of the optimized 3-D models were further refined on the basis of evaluation by the Ramachandran plot using MOE.

2.6. Single-cycle infectivity assays

To generate CRFK cells expressing CyM TRIMCyp, the cDNA was isolated from HSC-F cells, and expression vector of FLAG-tagged CyM TRIMCyp was constructed as described previously [18]. The sequence of TRIMCyp from HSC-F cells was identical with Mafa TRIMCyp2 (GenBank: FJ609415). CRFK cell lines expressing CyM TRIMCyp were selected by G418 as described previously [18]. Expression and inhibitory effect of the selected cell clones were verified by Western blotting with anti-FLAG antibody (SIGMA) and by infection with vesicular stomatitis virus G protein (VSV-G) pseudotyped 5R/4-3ΔEnv + Luc, respectively. Assays using naïve CRFK, CRFK expressing CyM TRIM5α [18] or CyM TRIMCyp, and MK.P3(F) cells were similarly performed as described previously [36]. VSV-G pseudotyped virus stocks were prepared from 293T cells transfected with individual HIV-1mtΔEnv + Luc clones and pCMV-G (GenBank: AJ318514)

at a molar ratio of 1:1. Naïve CRFK, CRFK expressing TRIM5α/TRIMCyp and MK.P3(F) cells were infected with an equal titer of viruses (to generate approximately 10^7 relative luminescence (RLU) for naïve CRFK cells), and on day 2 post-infection, cells were analyzed for luciferase activity. Assays using recombinant Sendai virus (SeV)-CyM TRIM5α/TRIMCyp expression system were performed as described previously [31].

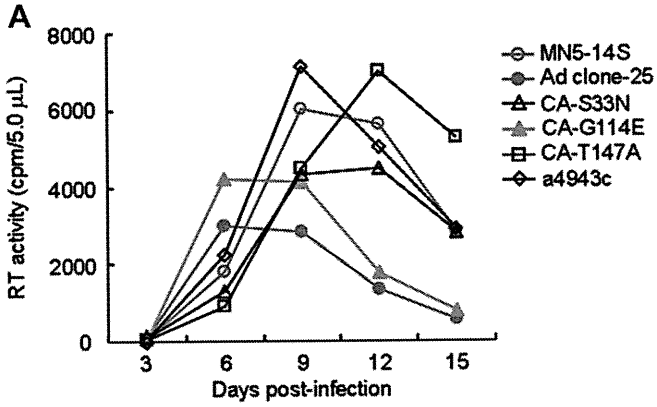
3. Results

3.1. An adaptive mutation G114E on helix 6 in CA (CA-G114E) enhances viral growth potential in macaque cells

An HIV-1mt variant MN4-5S replicated more slowly than SIVmac239 in macaque cells. In order to improve its growth potential, we carried out virus adaptation in a macaque lymphocyte cell line HSR5.4. Virus adaptation was performed by long-term culture of HSR5.4 cells infected with MN4-5S (X4-tropic) or its R5-tropic version MN5-10S (Fig. 1). Construction of proviral clones from adapted viruses was described in Materials and methods. We obtained only one clone (Ad clone-25) with enhanced growth potential from 100 proviral clones constructed and tested. We sequenced the entire genome of Ad clone-25, and found three non-synonymous mutations in CA (S33N, G114E, and T147A in Fig. 2A) and one synonymous mutation in integrase (IN)(a4943c in Fig. 2A). To identify an adaptive mutation that enhances growth potential, each mutation found in Ad clone-25 was introduced into a parental clone MN5-14S (Fig. 1). MN5-14S carries only growth-promoting mutations in MN5-10S, and the two clones exhibit similar growth potential in macaque cells. Viruses were prepared from 293T cells transfected with MN5-14S, Ad clone-25, or clones carrying individual mutations, and inoculated into HSC-F cells (Fig. 2A). Only one clone carrying CA-G114E exhibited similar growth kinetics to that of Ad clone-25 but not the others. This result indicates that CA-G114E is an adaptive mutation enhancing growth potential of HIV-1mt in macaque cells. This mutation is exactly the same as the previously found adaptive mutation, which enhanced growth of NL-4/5S6/7SvifS virus in human CEM-SS cells [37]. NL-4/5S6/7SvifS virus is a prototype HIV-1mt bearing the same CA with that of MN4-5S.

3.2. Molecular modeling of the CA NTD of HIV-1mt variants suggests that CA-G114E and CA-Q110D mutations have a similar positive effect on viral replication

The amino acid at position 114 is located in CA NTD. To obtain structural insights into impacts of the G114E substitution in order to improve growth capability of HIV-1mt variants in macaque cells, we conducted computer-assisted structural study: we constructed 3-D models of CA NTD of three HIV-1mt variants, CA-G114E, CA-G114Q, and MN4-5S, using homology-modeling technique (see Materials and methods). Main chain folds of the three models were indistinguishable, suggesting that 3-D position and type of side chain are critical



Nucleotide change	Region	Amino acid change in the region
g1283a	CA	S33N
g1526a	CA	G114E
a1624g	CA	T147A
a4943c	IN	None

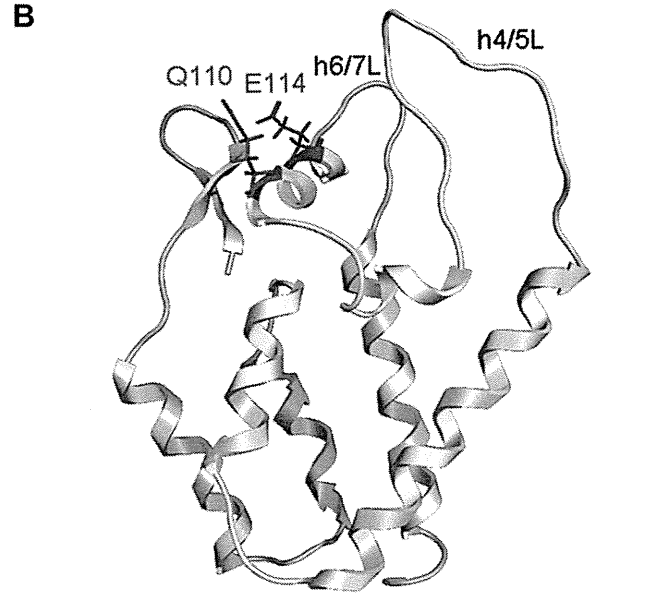


Fig. 2. Mutations in Gag-CA. (A) Identification of an adaptive mutation that enhances viral growth. Nucleotide substitutions found in the genome of Ad clone-25 are indicated at the bottom. Virus samples were prepared from 293T cells transfected with the indicated proviral clones, and equal RT units were inoculated into HSC-F cells. MN5-14S and Ad clone-25 served as controls. Virus replication was monitored by RT activity released into the culture supernatants. (B) 3-D structural models for CA NTD of HIV-1mt variants. Structural models of CA NTD of HIV-1mt variants were constructed by homology-modeling using "MOE-Align" and "MOE-Homology" in MOE as described previously [30–32]. Crystal structure of HIV-1 CA NTD at a resolution of 2.00 Å (PDB code: 1M9C [28]) was used as template for homology modeling. Main chain folds were indistinguishable among the models, and only the model of G114E CA is shown as a representative. Magenta and red sticks: side chains of 110th and 114th amino acid residues, respectively, of the G114E CA NTD.

for the phenotypic change. The modeling study revealed that 114th residue of G114E CA NTD is located on helix 6 in CA NTD such that its side chain protrudes into the exposed surface of CA (Fig. 2B). A charged amino acid residue on a protein surface participates in determining physicochemical properties of interaction surface of the protein and thus influences its structural and functional properties. Therefore, we assumed that the protrusion of a negatively charged side chain from helix 6 into exposed surface could have somehow a positive effect on growth capability of the HIV-1mt variants in macaque cells. In this regard, especially worth noting is that 110th amino acid residue on helix 6 of the HIV-1mt variant CAs was positioned on the same helical face with 114th amino acid residue (Fig. 2B). Therefore, we predicted that substitution of glutamine (Q) at position 110 by acidic amino acid such as aspartic acid (D) and glutamic acid (E) may also have a positive effect on growth capability of the HIV-1mt variants in macaque cells as G114E does. SIVmac239 has aspartic acid and glutamine at the positions 110 and 114, respectively.

3.3. CA-Q110D promotes viral growth more efficiently in macaque cells than CA-G114E mutation but its enhancing effect is species-specific

To confirm our prediction described above, CA-Q110D mutation was introduced into MN5-14S (designated MN5Rh-3), and the growth property in HSC-F cells of MN5Rh-3 and a viral clone carrying G114E (CA-G114E in Fig. 2A) was compared. As shown in Fig. 3A, MN5Rh-3 grew better than CA-G114E, indicating that CA-Q110D further accelerates HIV-1mt replication in macaque cells compared with an adaptive CA-G114E mutation. We next constructed an X4-tropic proviral clone carrying the CA-Q110D (designated MN4Rh-3) (Fig. 1), and compared its growth property with MN5Rh-3 in HSC-F cells (Fig. 3B). MN4Rh-3 was found to exhibit higher growth ability than MN5Rh-3, and was therefore used for infection experiments hereafter.

While CypA and TRIM5α have inhibitory effect on HIV-1 replication in macaque cells, CypA promotes HIV-1 infection in human cells and human TRIM5α only weakly inhibits HIV-1 replication [38–40]. Since the CA-Q110D mutation (acquisition of negatively charged side chain), as predicted by structural modeling, could impact on the interaction of HIV-1 CA and its binding factor(s) by altering physicochemical properties of CA binding surface, it can be speculated that CA-Q110D may promote viral replication specifically in macaque cells. Thus, we analyzed the effect of CA-Q110D on viral growth in macaque and human cells. In this experiment, we used HIV-1mt variants (MN4-8, MN4-8S, and MN4Rh-3) that have distinct CA structures (Fig. 1). Viruses prepared from transfected 293T cells were inoculated into macaque HSC-F and human MT4/CCR5 cells, and examined for growth property (Fig. 3C). The introduction of SIVmac239 CA h6/7L (MN4-8S) resulted in enhanced and reduced viral growth in macaque and human cells, respectively, relative to MN4-8. MN4Rh-3 grew clearly better in macaque cells relative to MN4-8 and MN4-8S, but more poorly in human cells than the other twos. These results demonstrate that the CA-Q110D mutation enhances viral replication in a host cell species-specific manner.

3.4. CA-Q110D does not contribute to evasion from CyM TRIM5 proteins restriction

We predicted that the growth enhancement by CA-Q110D may come from the increased resistance to CyM TRIM5 proteins, and therefore examined the susceptibility of HIV-1mt variants to them by two independent assays.

First, assays were performed in feline kidney CRFK cells expressing TRIM5α or TRIMCyp by using VSV-G pseudotyped viruses encoding the luciferase gene (Fig. 4A–C). The sequence differences between HIV-1mt variants reside only in CA and IN (Figs. 1 and 4). Since adaptive mutations in IN contribute to enhancement of virion production but not early replication phase (manuscript in preparation), only the difference in CA affects the relative single cycle infectivity in this assay. A pseudotyped virus 5R/4-3 carries HIV-1 (NL4-3) CA without any modifications and served as negative control. While 5R and 4-8 have an identical CA structure carrying h4/5L from SIVmac239, 5RS and 4-8S have both h4/5L and h6/7L from SIVmac239 CA. 4Rh-3 carries CA-Q110D mutation in addition to h4/5L and h6/7L from SIVmac239 CA. Viral infectivity was measured by luciferase activity in infected cells and presented as RLU. Naïve CRFK and CRFK cells expressing TRIM5α were infected with an equal amount of viruses generating 10^7 RLU in naïve cells. As shown in Fig. 4B, the infectivity of 5R and 4-8 for cells expressing CyM TRIM5α was similar to that of a negative control 5R/4-3. However, higher infectivity was observed for 5RS and 4-8S relative to 5R and 4-8. These results were consistent with previous reports that h4/5L and h6/7L in HIV-1 CA are a part of determinant for TRIM5α restriction [20,36]. The sensitivity of 4Rh-3 to TRIM5α was similar to that of 5RS and 4-8S. This indicates that CA-Q110D did not contribute to increase the resistance to TRIM5α. It has been reported that CyM TRIMCyp has the ability to restrict HIV-1 replication [15]. To examine the susceptibility of HIV-1mt variants to TRIMCyp, we generated feline CRFK cells expressing TRIMCyp, and the cells were infected with pseudotyped viruses as described above. As shown in Fig. 4C, all the clones tested were more resistant to a similar extent to TRIMCyp than the control 5R/4-3. In agreement with a previous study showing that elimination of alanine at position 88 within h4/5L of HIV-1 CA confers the resistance on the virus to TRIMCyp [15], our results indicate that the replacement of HIV-1 CA h4/5L with that of SIVmac239 is sufficient for HIV-1mt to evade from the TRIMCyp restriction. Second, we performed another susceptibility assay using the recombinant SeV expression system. This system assures a very high expression level of target proteins in cells infected with the recombinant SeV. Therefore, the ability of viruses to completely counteract the restriction effect of TRIM5 proteins could be determined by MT4/SeV-TRIM5 expression system. Human MT4 cells were infected with recombinant SeV expressing CyM TRIM5α, TRIMCyp, or SPRY(−)TRIM5, and then super-infected with HIV-1

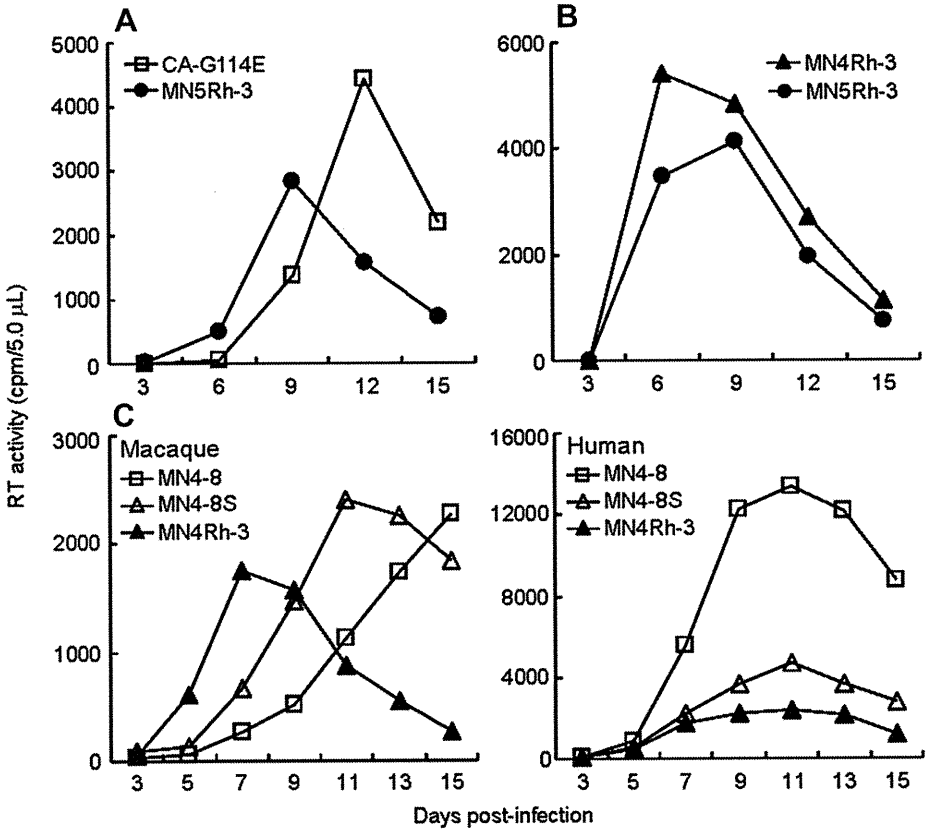


Fig. 3. Effect of CA modification on viral growth in macaque and human lymphocyte cell lines. (A and B) Growth kinetics of HIV-1mt clones carrying CA-G114E or CA-Q110D (MN5Rh-3 and MN4Rh-3) in CyM HSC-F cells. Virus samples were prepared from 293T cells transfected with the indicated proviral clones, and equal amounts (5×10^5 RT units) were inoculated into HSC-F cells (10^6). Virus replication was monitored by RT activity released into the culture supernatants. (C) Growth kinetics of MN4-8, MN4-8S, and MN4Rh-3 in HSC-F (Macaque) and MT4/CCR5 (Human) cells. Virus samples were prepared from 293T cells transfected with the indicated proviral clones, and equal amounts (10^6 RT units) were inoculated into HSC-F cells (10^6). For spinoculation of MT4/CCR5 cells (10^6), 6×10^5 RT units were used as inocula. Virus replication was monitored by RT activity released into the culture supernatants.

(NL4-3), SIVmac239, or HIV-1mt variants. SPRY(−)TRIM5 which can not bind to viral CA served as control. NL4-3 and SIVmac239 also served as controls for viral replication. As shown in Fig. 4D, NL4-3 replicated in cells expressing SPRY(−)TRIM5, but not in TRIM5α and TRIMCyp expressing cells. SIVmac239 exhibited similar growth kinetics in SPRY(−)TRIM5, TRIM5α and TRIMCyp expressing cells. All HIV-1mt variants replicated in TRIMCyp expressing cells similarly well in SPRY(−)TRIM5 cells. Together with assays in CRFK cells, these results showed that all HIV-1mt variants except for 5R/4-3 completely evade from TRIMCyp restriction. In contrast, the growth of all HIV-1mt variants was inhibited in CyM TRIM5α expressing MT4 cells. These results indicate that HIV-1mt variants do not evade from TRIM5α restriction as effectively as SIVmac239.

Results obtained by our two assay systems with respect to the susceptibility of HIV-1mt variants to CyM TRIM5α were apparently different (Fig. 4B and D), but this difference is most likely to be due to the TRIM5α expression level. In MT4 cells infected with recombinant SeV, TRIM5α is expressed at much higher level than that in transduced CRFK cells, masking the increase of resistance to TRIM5α detectable by the transduced CRFK system (Fig. 4B). Indeed, the growth enhancement of 5RS relative to 5R [20] can be explained by the results in Fig. 4B but not those in Fig. 4D. The apparent discrepancy of the sensitivity depending on TRIM5α expression level was also observed between B-LCL cells and transduced CRFK cells [41]. In sum, we can conclude here that MN4Rh-3 exhibits a partial resistance to TRIM5α insufficient for complete evasion as 5RS and 4-8S do, and that the CA-Q110D mutation is irrelevant to this property.

3.5. CA-Q110D enhances viral infectivity for macaque cells

Results so far showed that CA-Q110D does not contribute to evasion from TRIM5 proteins restriction in rather artificial systems using feline and human cells (Fig. 4). To investigate further how CA-Q110D enhances viral replication, we examined single-cycle viral infectivity in macaque cells. CyM kidney MK.P3(F) cells, which have heterozygote for TRIM5α and TRIMCyp, were infected with various VSV-G pseudoviruses and analyzed for their infectivity as described above. As shown in Fig. 5A, viral infectivity was increased by modification of h4/5L (compare 5R/4-3 and 5R&4-8). Modification of h6/7L in addition to h4/5L further augmented viral infectivity (compare 5R&4-8 and 5RS&4-8S). Introduction of the CA-Q110D mutation into 4-8S clone gave the highest infectivity among the viruses tested (see 4Rh-3). The results in

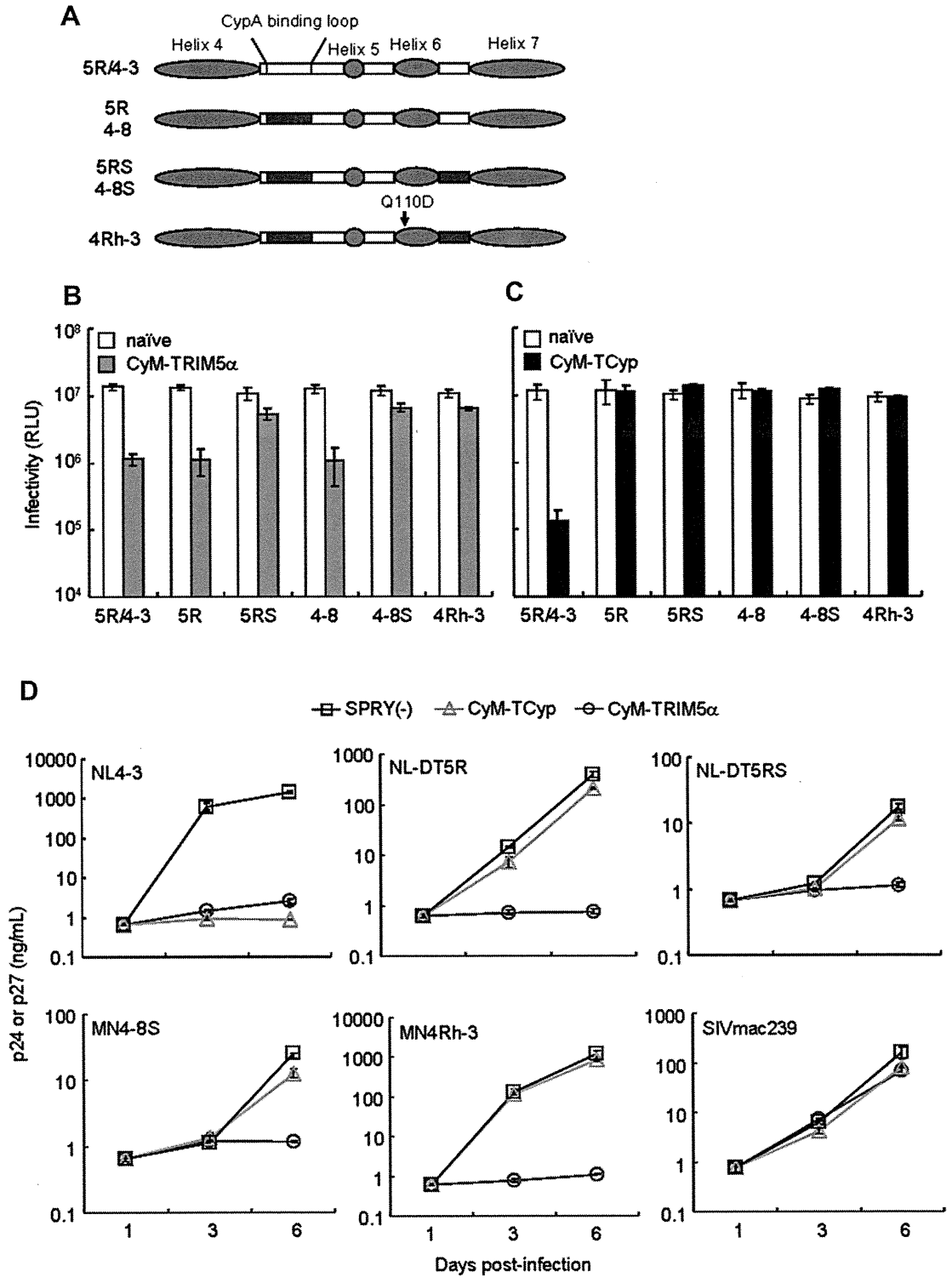


Fig. 4. Effect of CA modification in HIV-1mt variants on viral infectivity. (A) CA structure of viral clones used in TRIM5α/TRIMCyp susceptibility assays. Blue and white areas show helices and loops from HIV-1 NL4-3 CA, respectively. Sequences from SIVmac239 are indicated by black areas. (B and C) Susceptibility of HIV-1mt variants to CyM TRIM5 proteins as examined by CRFK system. Results for CyM TRIM5α (B) and for CyM TRIMCyp (TCyp) (C) are shown. VSV-G pseudotyped viruses were prepared from transfected 293T cells as input samples. Viruses generating 10^7 RLU in CRFK-naïve cells were inoculated into CRFK cells that express CyM TRIM5α or CyM TCyp. On day 2 post-infection, cells were analyzed for luciferase activity by a luminometer. (D) Susceptibility of HIV-1mt variants to CyM TRIM5 proteins as examined by SeV system. Human MT4 cells (10^5) were infected with recombinant SeV expressing CyM TRIM5α, TRIMCyp, or SPRY (−) TRIM5. Nine hours after infection, cells were super-infected with 20 ng (Gag-p24) of HIV-1 NL4-3, various HIV-1mt clones, or 20 ng (Gag-p27) of SIVmac239. Virus replication was monitored by the amount of Gag-p24 from NL4-3 and HIV-1mt clones or Gag-p27 from SIVmac239 in the culture supernatants. Error bars show actual fluctuations between duplicate samples. Data from one representative of three independent experiments are shown.

Fig. 5A show that CA-Q110D uniquely increases viral infectivity in macaque cells not observed in the other experimental systems (Fig. 4), and suggest that some factor(s) in CyM cells other than TRIM5α and TRIMCyp proteins is associated with this enhancement.

As shown in Fig. 5B, MN4Rh-3 displayed slower growth kinetics relative to those of SIVmac239 (note the peak day of virus production), although it grew better than the other HIV-1mt clones in CyM HSC-F cells. Approximately 100-fold more input virus (RT units) compared to SIVmac239 was required for MN4Rh-3 to exhibit similar growth kinetics with SIVmac239 (data not shown). These results have shown that even MN4Rh-3 grows more poorly in macaque cells than a standard SIVmac clone pathogenic for macaque monkeys.

4. Discussion

In this study, we have demonstrated that a single CA mutation (Q110D) greatly promotes HIV-1mt growth in macaque cells (Fig. 3). This enhancing effect was afforded independently of TRIM5 proteins restriction. The virus carrying the CA-Q110D mutation (MN4Rh-3) certainly overcame the anti-viral action of CyM TRIMCyp but not completely CyM TRIM5α. However, the mutation itself (Fig. 1) did not influence anti-TRIMCyp/TRIM5α activity of MN4Rh-3 reported here (Fig. 4). Notably, this mutation exquisitely enhanced viral growth in macaque cells (Fig. 3) by augmenting viral single-cycle infectivity (Fig. 5). The viral growth enhancement reported here is well reproduced in CyM peripheral blood mononuclear cells and in CyMs (manuscript in preparation).

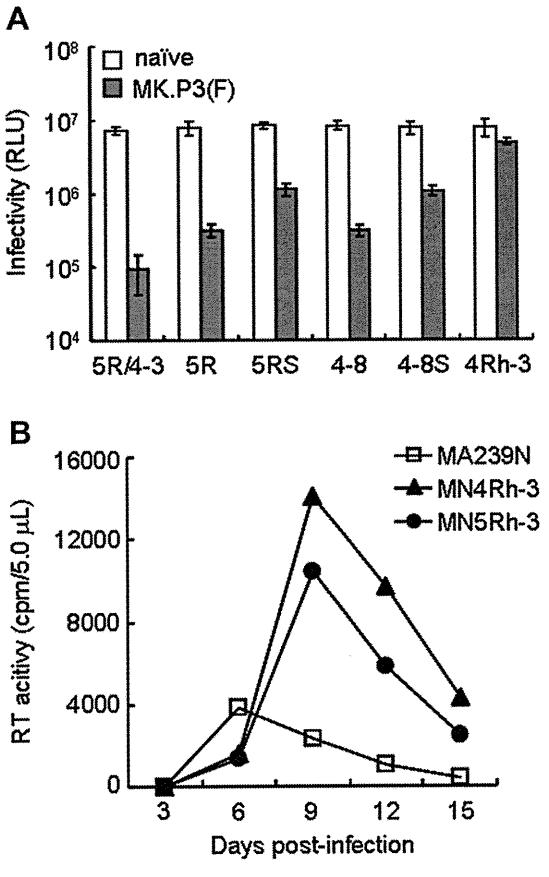
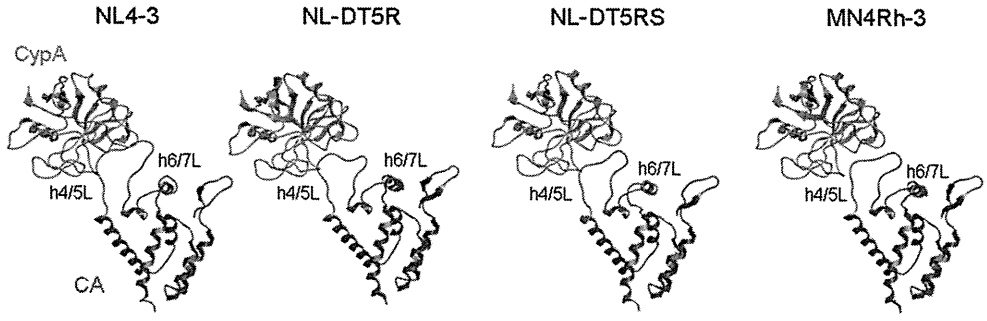


Fig. 5. Replication ability of various viruses in CyM cells. (A) Single-cycle infectivity of various HIV-1mt clones in CyM kidney MK.P3(F) cells. VSV-G pseudotyped viruses indicated were prepared from transfected 293T cells. MK.P3(F) cells were infected with an equal titer of viruses giving 10^7 RLU in CRFK-naïve cells. On day 2 post-infection, cells were analyzed for luciferase activity by a luminometer. (B) Multi-cycle growth kinetics of SIVmac and HIV-1mt viruses in CyM lymphocyte HSC-F cells. Virus samples were prepared from 293T cells transfected with the indicated proviral clones, and equal amounts (10^4 RT units) were inoculated into HSC-F cells (10^6). Virus replication was monitored by RT activity released into the culture supernatants. MA239N, an infectious clone of SIVmac239 with *nef*-open.

Regarding the mechanism for enhancement of viral growth by CA-Q110D, we initially thought a possibility that CA-Q110D compensates the disadvantage in HIV-1mt genome resulted from replacement of HIV-1 CA h4/5L and h6/7L with those of SIVmac239. However, this is highly unlikely because the enhancing effect is macaque cell-dependent (Fig. 3). Most feasible explanation is that CA-Q110D contributes to evade from a negative factor(s) in macaque cells such as CypA. Because HIV-1mt CA was designed not to bind to CypA, and the interaction between the two molecules was indeed undetectable by monitoring CypA virion-incorporation [18,20], we analyzed the binding by computer-assisted structural modeling. Homology modeling of the CA-CypA complexes was performed based on the crystal structure of HIV-1 CA NTD bound to CypA (PDB code: 1M9C [28]), and the binding energies, E_{bind}, were calculated using MOE as described previously [42,43]. As shown in Fig. 6, HIV-1 (NL4-3) CA was predicted to interact with CypA via its h4/5L (binding energy: −64.4 kcal/mol). The binding energy of CA and CypA was decreased by CA modifications, such as h4/5L replacement (NL-DT5R: −31.0 kcal/mol), h4/5L and h6/7L replacement (NL-DT5RS: −36.1 kcal/mol), and Q110D substitution in addition to h4/5L and h6/7L replacement (MN4Rh-3: −30.1 kcal/mol). Decrease in E_{bind} in NL-DT5R is consistent with the result that the h4/5L region directly interacts with CypA [28]. Notably, the E_{bind} for the NL-DT5RS CA was greater than that of the NL-DT5R and MN4Rh-3 CAs. These results suggest that not only h6/7L replacement but also Q110D substitution can influence structure of CypA binding surface of CA. The Q110D substitution is located on the exposed surface of helix 6 connecting to the h6/7L (Fig. 2B). CA helix 6 has been reported to interact with CypA binding region on h4/5L through hydrogen bonding [44,45]. Thereby it is reasonable that the local electrostatic change on the helix 6 by the Q110D substitution influenced structures of h4/5L via changes in fluctuation and conformation of h6/7L. This in turn could lead to reduction in stability of the MN4Rh-3 CA-CypA complex compared with NL-DT5RS CA-CypA complex, as predicted in Fig. 6. Our computer-assisted structural study suggests that the Q110D substitution can induce electrostatic modulation of the overall CA surface structure including h4/5L and h6/7L. Similar modulation mechanism of binding surface structures via charged amino acid substitution at distant site from the binding surface has been reported for Cyp domain of CyM TRIMCyp [15] and CD4 binding site of HIV-1 gp120 outer



	NL4-3	NL-DT5R	NL-DT5RS	MN4Rh-3
CA	HIV-1 CA	h4/5L from SIVmac239	h4/5L and h6/7L from SIVmac239	h4/5L and h6/7L from SIVmac239 and Q110D
Binding energy (kcal/mol)	-64.447	-31.012	-36.071	-30.051

Fig. 6. Structural models of HIV CA NTD bound to CypA. The model of CA NTD bound to CypA was constructed by homology modeling using the crystal structure of HIV-1 CA NTD and CypA complex (PDB code: 1M9C [28]). The binding energies, E_{bind} (kcal/mol), of the complex were calculated using MOE as described previously [42,43]. The formula $E_{bind} = E_{complex} - (E_{CA} + E_{CypA})$ was used for the E_{bind} calculation, where $E_{complex}$ is the energy of the CA/CypA complex models, E_{CA} is the energy of the CA monomer model, and E_{CypA} is the energy of the CypA monomer model.

domain [46]. Thus, it is not unreasonable to assume that the replication of MN4Rh-3 carrying CA-Q110D is enhanced in macaque cells but reduced in human cells by augmenting its dissociation from CypA (Fig. 6). However, it was found to be difficult to experimentally confirm this structural insight by determining the effect of cyclosporine A or of siRNA against CypA on viral infectivity because interaction between the HIV-1mt CA and CypA was so weak. Alternatively, CA-Q110D may contribute to the alteration of the affinity to unknown anti-CA factor(s) other than CypA and TRIM5 proteins. In this case, it is speculated that the factor(s) might act negatively on HIV-1 replication in macaque cells but positively in human cells, and vice versa. Further study is required to elucidate the mechanism for enhancement of viral growth potential by CA-Q110D.

In conclusion, further modification of the HIV-1mt genome is necessary to overcome unconquered replication block(s) present in macaque cells and obtain viral clones similarly replication-competent in macaque cells and pathogenic for animals with SIVmac (Fig. 5). Considering the genome structure of MN4Rh-3 and the results presented here, major targets for modification now are *gag*-CA (against TRIM5α) and *vpu* (against tetherin). Gag-CA is one of the two principal viral determinants (CA and Vif) for the HIV-1 species-tropism. Construction of HIV-1 CA that evades from TRIM5α restriction is also useful for elucidation of the less-defined CA-TRIM5α interaction and antiviral mechanism of TRIM5α. Tetherin, identified as anti-virion release factor, is antagonized by Vpu [47,48], but macaque tetherin was not counteracted by HIV-1 Vpu [49]. Construction of HIV-1 Vpu that down-modulate macaque tetherin may enhance viral replication *in vivo* as well as *in vitro* [50]. Through these approaches, we may be able to precisely analyze HIV-1 replication and pathogenesis *in vivo* and provide new strategies against HIV-1/AIDS.

Acknowledgments

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Polymorphisms in *Fas* Gene Is Associated with HIV-Related Lipoatrophy in Thai Patients

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Abstract

The present study aimed to evaluate the role of genetic polymorphisms in the emergence of lipoatrophy or lipodystrophy in HIV-infected patients with antiretroviral therapy (ART) in Thailand. Position 455 upstream of the *Apolipoprotein C3* gene (*ApoC3* T–455C, rs2854116), codon 64 of the *Beta3 adrenergic receptor* gene (*ARβ3* Tcod64C, rs4994), and position 670 upstream of the *Fas* gene (*Fas* A–670G, rs1800682) were genotyped in 829 HIV-infected Thai patients who had started ART. Crude and adjusted incidence rate ratios (IRR) were calculated using Poisson regression. The serum levels of cholesterol, triglycerides, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) were also analyzed. Multivariate analysis revealed an association between the *Fas* −670AA genotype, but not the *ApoC3* −455 or *ARβ3* cod64 genotypes, with the incidence of lipoatrophy after adjusting for gender and stavudine (d4T)-containing regimens (IRR = 1.72, 95% CI = 1.20–2.45, $p = 0.003$). However, *ApoC3* −455C homozygous patients showed elevated serum levels of triglycerides, while this genotype did not affect serum total cholesterol, HDL, or LDL levels in patients with lipoatrophy or lipodystrophy. In contrast, the *ARβ3* cod64 genotype did not show any significant association with the serum levels of cholesterol, triglycerides, HDL, or LDL. In conclusion, *Fas* −670AA affected the incidence of lipoatrophy in HIV-1-infected Thai patients, while the *ApoC3* −455C allele affected the serum levels of triglycerides. These results confirmed the role of genetics in the development of ART-related metabolic disorders.

Introduction

METABOLIC AND MORPHOLOGICAL alterations observed in human immunodeficiency virus (HIV)-infected patients represent a significant health problem related both to the possible long-term consequences and to the behavioral and psychological well-being of patients. Since the introduction of highly active antiretroviral therapy (HAART), the life expectancy of patients infected with HIV type 1 (HIV-1) has increased remarkably. Despite the clinical benefits, long-term HAART is associated with a complex spectrum of morphological alterations, characterized by body changes that may stigmatize patients and compromise their compliance with therapy, as well as metabolic effects, including dyslipidemia and insulin resistance, which lead to an elevated risk of developing diabetes mellitus and myocardial infarction.[1–3]

Morphological alterations are collectively named lipodystrophy (LD); however, in recent years it has been recognized that fat loss and fat gain represent distinct entities. Clinical features of fat loss include loss of subcutaneous fat in the face, arms, legs, and buttocks, while fat accumulation is characterized by excess fat deposition in visceral adipose tissue in the abdomen, breasts, dorsocervical region, and trunk.[4]

The underlying mechanism of antiretroviral therapy (ART)-related lipoatrophy (LA) has not been fully elucidated. Many mechanisms have been suggested, including mitochondrial toxicity by nucleoside reverse transcriptase inhibitors (NRTIs), inhibition of adipocyte differentiation by protease inhibitors (PIs), increased levels of inflammatory cytokines, and HIV itself.[5–7] Mitochondrial toxicity has been clearly shown to play a pivotal role; in particular, thymidine analogs are strong inhibitors of DNA polymerase-γ and lead

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