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Novel Mutations L228I and Y232H Cause Nonnucleoside Reverse Transcriptase Inhibitor Resistance in Combinational Pattern

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Abstract

The emergence of drug resistance mutations is increasing after the implementation of highly active antiretroviral therapy. To characterize two novel mutations L228I and Y232H in the primer grip of reverse transcriptase (RT) of HIV-1 circulating recombination form 08_BC (CRF08_BC) subtype, both mutant clones were constructed to determine their impacts on viral phenotypic susceptibility and replication capacity (RC). Results showed that the novel mutation, L228I, conferred a low-level resistance to etravirine by itself. L228I in combination with Y188C displayed a high level of cross-resistance to both nevirapine (NVP) and efavirenz (EFV). The copresence of A139V and Y232H induced a moderate level of resistance to NVP and EFV. Mutations Y188C/L228I, A139V, Y232H, and A139V/Y232H reduced more than 55% of viral RC compared with that of the wild-type (WT) reference virus. Modeling study suggested that the copresence of Y188C/L228I or A139V/Y232H might induce conformational changes to RT, which might result in reduced drug susceptibility and viral RC due to abolished hydrogen bonding or complex interaction with vicinal residues. Our results demonstrated that L228I and Y232H were novel accessory nonnucleoside reverse transcriptase inhibitor resistance–related mutations and provided valuable information for clinicians to design more effective treatment to patients infected with HIV-1 subtype CRF08_BC.

Introduction

To control HIV/AIDS-related morbidity and mortality and improve the life quality of HIV-1–infected patients, highly active antiretroviral therapy (HAART) was introduced and has achieved remarkable progress.[1,2] Currently, the first-line HAART treatment for HIV-infected adults includes two nucleoside reverse transcriptase inhibitors (NRTIs), such as tenofovir (TDF) with lamivudine (3TC) or emtricitabine (FTC), plus one nonnucleoside reverse transcriptase inhibitor (NNRTI), such as efavirenz (EFV). If the regimen above is contraindicated or not available, one of the following options is recommended, including zidovudine (AZT) +3TC+EFV, AZT +3TC+ nevirapine (NVP), or TDF +3TC (or FTC)+NVP.[3] Although HAART can control the viral load to a clinically undetectable level in most HIV cases, a large hindrance for extended effective treatments is the emergence of drug resistance mutations. Patients acquiring or infected with drug-resistant mutant viruses have fewer treatment options and are at higher risk of morbidity and mortality, especially in developing countries where choices of HIV inhibitors are limited.[4] Thus, identifying and characterizing the drug resistance mutations and their impacts on drug susceptibility as well as viral replication can help clinicians offer more effective treatment to HIV patients.

To date, most drug resistance data focused on HIV-1 subtype B. Specifically, 21 major NNRTI resistance mutations at nine sites have been reported by the HIV Drug Resistance Database (http://hivdb.stanford.edu/DR/NNRTIResiNote.html, last updated on March 2, 2014). However, subtype B is mainly prevalent in the Western world, accounting for only 11% of global HIV-1 infections,[5] while non-B subtypes are playing more important roles in the whole pandemic. Limited data are available concerning the genetic mechanisms of drug resistance in non-B subtype viruses. In contrast, it has been observed that different HIV-1 subtypes may develop various

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mutations to certain HIV inhibitors in both *in vitro* and *in vivo* studies.[6–11] Viral replication capacity (RC) also differs among various subtypes, which may be magnified under high selective pressure.[12] Thus, it is important and valuable to evaluate the impacts of mutations in non-B subtypes on drug susceptibility and viral RC.

Circulating recombination form 08_BC (CRF08_BC) is one of the current predominant subtypes in China containing a recombinant reverse transcriptase (RT) gene derived from subtypes Thailand B and Indian C.[13,14] More than 40% of HIV infections in Chinese blood donors were found to be the CRF08_BC subtype from 2007 to 2010.[15] However, few data are available for the drug resistance mutations in this subtype in treatment-exposed patients. Previously, to select the potentially emerging drug resistance mutations during NVP treatment, we propagated a clinical isolate of CRF08_BC subtype (2007CNGX-HK) in human peripheral blood mononuclear cells with increasing NVP concentration for 40 passages (about 400 days).[14] Several novel mutations (L228I, Y232H, and D404N) in RT were observed in that study to confer moderate-to-high-level NVP resistance either alone or in combination.[14] In a later study, we demonstrated that D404N in the RT connection subdomain was an NNRTI resistance–related mutation that displayed cross-resistance to NVP, EFV, rilpivirine (RPV), and AZT.[16]

In this study, we extended our research to the other two novel mutations, L228I and Y232H. Both these mutations locate in the $\beta12$–$\beta13$ hairpin of the palm subdomain, which contains the residues 227–235 (FLWMGYELH) and forms the so-called primer grip in RT.[17,18] The function of the primer grip is to maintain the primer terminus in correct orientation for nucleophilic attack on an incoming dNTP during DNA strand extension.[17] Mutations in this region, such as F227A, G231A, Y232A, E233A, and H235A, have been reported to result in the loss of RNase H function.[19] L234A was confirmed to affect the dimerization of p51 and p66 subunits.[20] Of note, mutations at L228 and Y232 have been reported to be related to drug resistance in subtype B or C in many studies.[18,19,21–24] It is very interesting to further identify whether these previously selected novel mutations (L228I and Y232H) affect drug susceptibility and viral replication in subtype CRF08_BC. This study intensively characterized L228I and Y232H either alone or in combination with the other associated mutations through site-directed mutagenesis based on an infectious clone of the CRF08_BC subtype.

Methods

Cells, plasmids, and drugs

The 293FT cells were obtained from the American Type Culture Collection. The TZM-bl cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, from Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme, Inc. Both 293FT and TZM-bl cells were cultured as described previously.[25] HIV-1 CRF08_BC subtype wild-type (WT) infectious plasmid pBRGX was previously constructed.[25] Drugs, including three NNRTIs [NVP, EFV, and etravirine (ETR)] and five NRTIs [AZT, abacavir (ABC), 3TC, FTC, and TDF], were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH.

Site-directed mutagenesis and virus production

Six mutation patterns, including L228I, Y188C, Y188C/L228I, A139V, Y232H, and A139V/Y232H, in RT were introduced into the pBRGX plasmid by site-directed mutagenesis as described previously.[16] Multiple mutations were introduced into pBRGX one by one. The presence of desired mutations in the plasmids was confirmed by DNA sequencing. Mutation-specific primers and primers for amplifying RT in positive clone identification were designed and listed in Table 1.

Viruses were produced by transfection of constructed infectious plasmids into 293FT cells as described previously.[25,26] Viral titer was detected by the measurement of p24 production using the Vironostika Kit (bioMérieux) according to the manufacturer's instructions and by detection of the 50% tissue culture infective dose ($TCID_{50}$) using TZM-bl cells as described previously.[27] Only those with p24 production higher than 50 mg/liter were subjected to phenotypic drug resistance assay and RC analysis.

Phenotypic susceptibility of virus variants

The susceptibility of virus variants to NNRTIs (NVP, EFV, and ETR) and NRTIs (AZT, ABC, 3TC, FTC, and TDF) was measured in a single-cycle cell culture–based phenotypic assay in TZM-bl cells as described previously.[16] In brief, 1×10^4 cells were seeded on a 96-well flat-bottom plate in 100 μl of growth medium (Dulbecco's modified Eagle's medium +10% fetal bovine serum +1% penicillin/streptomycin). Next day, mutant and WT viruses equal to 200–400 blue focus forming unit in serially diluted drugs were added into each well of the plate in triplicate. The wells infected with equal amount of viruses but without drugs were taken as viral controls. Then, the plate was incubated at 37°C in a 5% CO_2 incubator for 48 h. After the cells were fixed and stained, the number of blue cells was calculated using ImmunoSpot 3.2 software after imaged by ELIspot plate reader (Cellular Technology Limited). The 50% inhibitory concentration (IC_{50}) values were determined by SigmaPlot 12.0 software (four-parameter logistic analysis). Viral susceptibility to drugs was expressed as the fold changes in IC_{50} of mutant virus compared to that of the WT reference virus. To compare the results in this study with those of previous work in the same criterion, the levels of resistance are divided into four groups based on fold changes as described previously,[14,27] that is, high-level resistance (>20-folds), moderate-level resistance (4- to 20-folds), low-level resistance (2- to 4-folds), and susceptive (0- to 2-folds).

RC determination of virus variants

The RCs of WT and mutant viruses were evaluated in a noncompetitive RC assay as described previously.[25,28–30] Briefly, 1×10^4 TZM-bl cells per well were seeded on a 96-well plate. Next day, virus stocks were 3- to 10-fold serially diluted and applied to the cells. Each virus was tested in triplicate for each concentration. After 48 h of incubation at 37°C in a 5% CO_2 incubator, cells were fixed and stained. Virus-infected blue cells were counted as described above. RC was determined by normalizing the blue cell number to corresponding p24 production of each mutant virus. Final relative RC levels were expressed as a percentage of mutant viral RC with reference to that of the WT reference virus. RC

Table 1. Primer Sequences for Site-Directed Mutagenesis

Amino acid mutation	*Codon mutation*	*Genetic mutation in BRGX*	*Primer sequences (5′-3′)*	*Position in HXB2*
L228I-F	**C**TT-**A**TT	C3202A	CATTT**A**TTTGGATGGGGTATGAACTCCATCCTGAC	3226–3260
L228I-R	**C**TT-**A**TT	C3202A	ATCCA**A**A**T**AAATGGAGGTTCTTTCTGATGTTTCTTGTCTG	3199–3238
Y188C-F	T**A**T-T**G**T	A3083G	ACTTGT**G**TGTAGGATCTGACTTAGAAATAGGGCAGCAT	3106–3143
Y188C-R	T**A**T-T**G**T	A3083G	ATCCTA**C**A**C**ACAAGTCATCCATGTATTGATAGATAACTATG	3080–3120
A139V-F	G**C**A-G**T**A	C2936T	TGAGG**T**AC**C**AGGGATTAGATATCAGTACAATGTGCTTCCAC	2960–3000
A139V-R	G**C**A-G**T**A	C2936T	CCTGGT**A**CCTCATTGTTTACACTAGGTATGGTAAATGCAG	2932–2971
Y232H-F	**T**AT-**C**AT	T3214C	ATGGGG**C**ATGAACTCCATCCTGACAAATGGACAGTAC	3237–3273
Y232H-R	**T**AT-**C**AT	T3214C	AGTTCAT**G**CCCCATCCAAAGAAATGGAGGTTC	3219–3250
Primers for RT amplification to sequence (5′-3′)				
HI1395			ATGAAGAGGCTGCAGAATGGG	1406–1426
HI4635			GGATTCTACTACTCCCTGACTTTGG	4664–4688

Mutated nucleotides are *underlined* and in *bold*.
RT, reverse transcriptase.

of test virus (%) = (blue cell number/ng of $p24_{test\ virus}$/blue cell number/ng of $p24_{WT\ reference\ virus}$) × 100.

Molecular modeling of RT

To investigate whether any possible structural changes could be induced by the novel mutations, L228I and Y232H, the models of HIV-1 CRF08_BC RT (1–560 amino acids) were built by homology modeling with HIV-1 RT, NVP, and RNA/DNA hybrid crystal structure 4PUO[31] using the server SWISS-MODEL[32] with default parameters, in which WTs contain L228, A139, and Y232, while mutants contain 228I, 139V, and 232H. The models were further analyzed using Discovery Studio 4.1 Visualizer (Accelrys), and the Ramachandran plot was examined to ensure that the structures of the models are not in the unfavorable regions.

Statistical analysis

The data were evaluated for statistical significance by an unpaired Student's *t*-test using Prism software (version 5.0; GraphPad, Inc.). Values of $p < .05$ were considered significant. Results were given as mean ± standard deviation of triplicate independent experiments.

Results

L228I conferred low-level resistance to ETR and enhanced viral resistance to NVP in the context of Y188C

To define the exact phenotypic effect of a single mutation L228I on NVP and other RT inhibitors, susceptibility assays using TZM-bl cells were performed. Our results showed that a single L228I mutation failed to display a substantial effect on viral susceptibility to the first-generation NNRTIs, NVP (Fig. 1a) or EFV (Fig. 1b) (<2-fold), but exerted a low-level resistance to the second-generation NNRTI, ETR (2.27-fold) (Fig. 1c). Viruses with the primary mutation of Y188C conferred a high-level resistance to NVP (20.86-fold) (Fig. 1a) and a moderate-level resistance to EFV (5.38-fold) (Fig. 1b). Interestingly, the addition of L228I to Y188C significantly increased viral resistance to NVP (35.47-fold) compared to that of Y188C alone (20.86-fold) ($p = .0444$) (Fig. 1a), indicating that L228I could further reduce viral susceptibility to NVP and EFV in the context of Y188C. The viruses above containing mutations Y188C, L228I, and Y188C/L228I were susceptible to all the current clinically available NRTIs (AZT, ABC, 3TC, FTC, and TDF) (<2-fold) (Fig. 1d–h).

To evaluate the effect of the mutation L228I on viral replication, we performed a single-cycle cell culture assay. Compared to the WT virus, L228I retained a 68% RC of WT, which was close to that of Y188C (55% of WT). Y188C/L228I significantly reduced viral RC to 12% of WT compared to that of single Y188C (55%, $p = .0475$) (Fig. 1i). The results suggested that L228I might impair viral replication in the presence of Y188C.

To investigate the possible mechanism behind the effects of the novel mutation L228I, the three-dimensional RT model of HIV-1 subtype CRF08_BC was built by homology modeling with HIV-1 RT, NVP, and the RNA/DNA hybrid crystal structure (4PUO) through the server SWISS-MODEL as described previously.[16] As shown in Figure 2a, the whole structure of WT CRF08_BC subtype RT is a heterodimer of p51 (green) and p66 (blue) subunits, binding an RNA/DNA hybrid (Fig. 2a). The orientation of L228 is in close proximity to the binding pocket of NVP but not interacting with NVP (Fig. 2b). At the same time, a hydrogen bond is formed between L228 and Y188 in the WT RT (Fig. 2b). When L228I and Y188C are introduced, the hydrogen bond will be abolished (Fig. 2c), possibly disrupting the core structure of p66 subunit close to the binding site of NVP. Intriguingly, we also observed that the residue L228 is located at the entrance of the RNA/DNA hybrid together with the hydrophilic residues K65–K70 (Fig. 2d). The mutation L228I with larger side chain might slightly block this entrance site and inhibit the reverse transcription (Fig. 2e).

Previously, Y232H was selected together with A139V, and the combination of A139V–Y232H was suggested to exhibit a high-level resistance to NVP.[14] Here, the mutant viruses containing Y232H and/or A139V were produced to detect the viral susceptibility to RT inhibitors. As shown in Figure 3, no resistance to NVP, EFV, or ETR was observed in virus containing a single mutation A139V, while Y232H alone induced a low-level resistance to NVP (2.26-fold). Of note, the combination of A139V and Y232H conferred a moderate-level

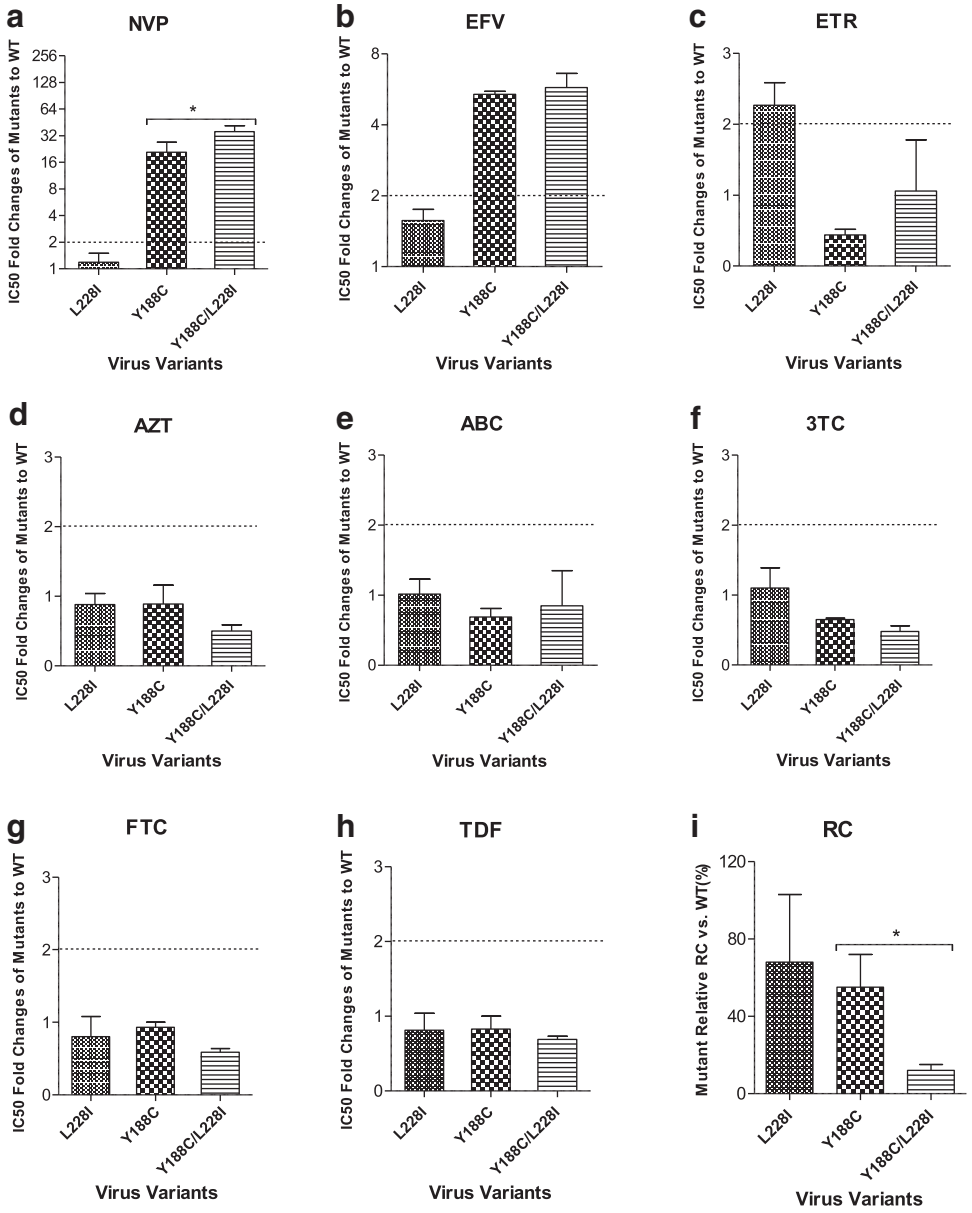

FIG. 1. Phenotypic analysis and viral RC of L228I-associated virus variants. Compared with WT virus, the fold changes of IC_{50} of L228I-associated virus variants against NNRTIs NVP **(a)**, EFV **(b)**, and ETR **(c)**, as well as NRTIs AZT **(d)**, ABC **(e)**, 3TC **(f)**, FTC **(g)**, and TDF **(h)**, were determined by phenotypic assay. Viral RCs of these variants were tested by a single-cycle cell culture assay **(i)**. In phenotypic assay, TZM-bl cells were infected with virus variants and treated with serially diluted RT inhibitors. In RC detection assay, TZM-bl cells were infected with serially diluted virus variants without inhibitors. The *dashed line* is labeled at twofolds of IC_{50} ratio to indicate the drug resistance standard. 3TC, lamivudine; ABC, abacavir; AZT, zidovudine; EFV, efavirenz; ETR, etravirine; FTC, emtricitabine; IC_{50}, 50% inhibitory concentration; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; NVP, nevirapine; RC, replication capacity; RT, reverse transcriptase; TDF, tenofovir; WT, wild type.

resistance to NVP (5.76-fold), which was significantly higher than that of A139V alone ($p = .0045$) (Fig. 3a) and a low-level resistance to EFV (3.55-fold) (Fig. 3b). No resistance to ETR (Fig. 3c) and any of the NRTIs (AZT, ABC, 3TC, FTC, and TDF) (Fig. 3d–h) was detected in either single or the combinatorial pattern of A139V and Y232H. In parallel, viral RCs of A139V and Y232H were also detected (Fig. 3i). The single mutation A139V displayed 41% RC of WT, while Y232H exhibited 26% RC of WT. The combination of A139V–Y232H dramatically reduced viral RC to 5% of WT, which was significantly lower than that of single A139V ($p = .0088$).

To further investigate the possible mechanism behind the reduced drug susceptibility and viral RC by A139V and Y232H, the RT model of subtype CRF08_BC was also built based on 4PUO as prescribed previously.[16] As shown in Figure 4, Y232 in p66 subunit near the NVP binding pocket interacts with the adjacent amino acids W266, Q269, W239, H96, W229, and Q242. When Y232 is substituted into 232H, the primary interactions between Y232 and W266, Q269, W239, H96, and W229 will be abolished, which might induce structural change and disruption in p66 subunit. In addition, residue A139 in p51 subunit locates in a $\beta7$–$\beta8$ loop region, which is in the RT dimerization interface and close to the NVP binding pocket. A hydrogen bond is formed between S134 and A139. The substitution of alanine to valine at residue 139 might influence the hydrogen bond by increasing the hydrophobicity of the side chain as well as the core structure of the loop. Therefore, the combination of A139V–Y232H might cause significant conformational change to the RT structure than each of them alone.

Discussion

In the previous study,[14] we selected two novel mutations, L228I and Y232H, in HIV-1 CRF08_BC subtype RT under

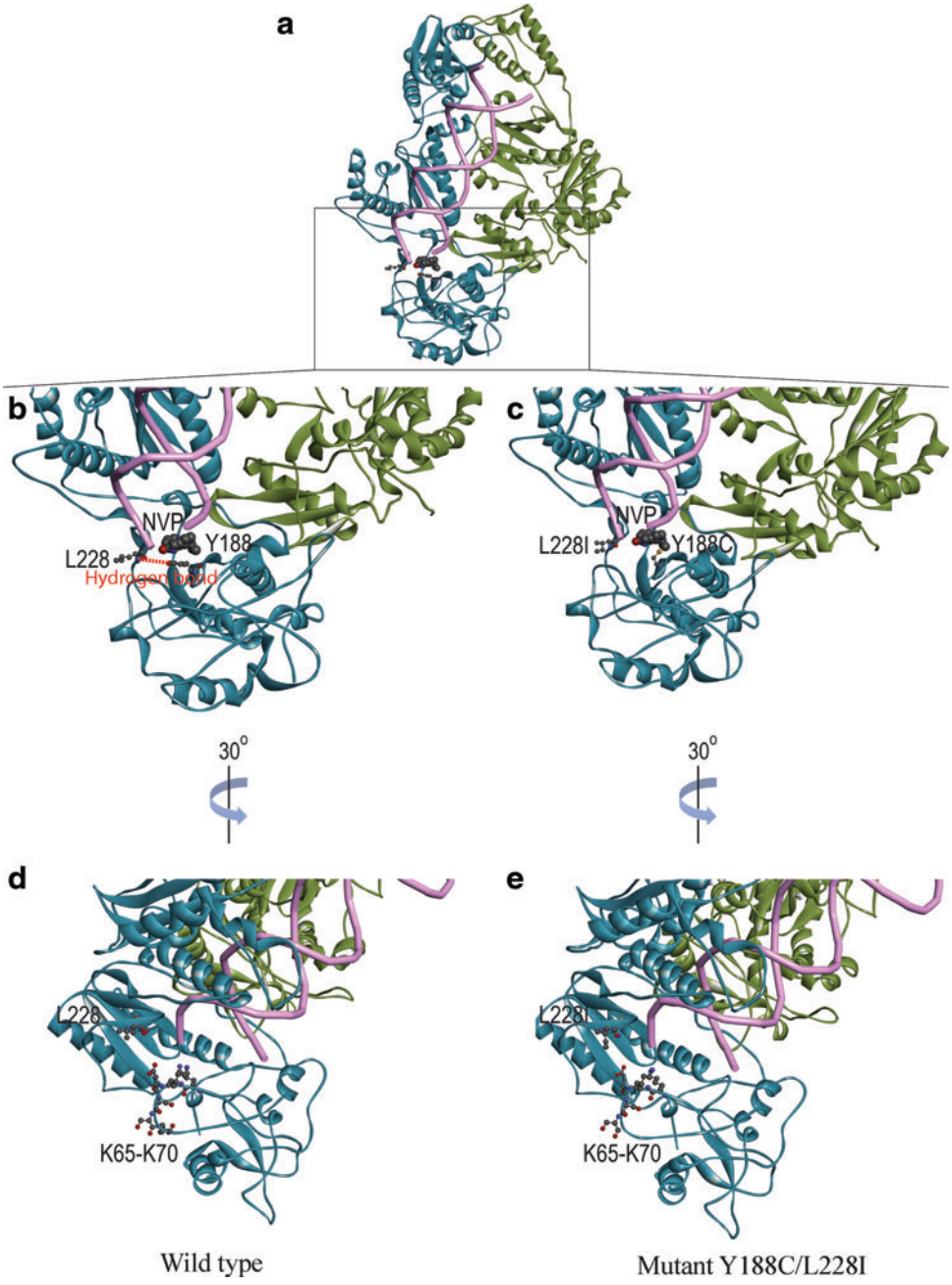

FIG. 2. RT models of WT and mutant Y188C/L228I. The RT models of WT and mutant are shown in ribbon diagram. Subunits of p66 and p51 are colored as *blue* and *green*, respectively **(a)**. The residues at 228, 188 **(b, c)**, and K65–K70 **(d, e)** in p66 subunit are highlighted. Their atoms are labeled in *ball*-and-*stick* format. The hydrogen bond between L228 and Y188 is shown in *red dotted line* **(b)**. The DNA/RNA hybrid is shown in *tube* format in *pink*, while the NNRTI NVP is shown in CPK format **(a)**. The figures were produced using DS Visualizer (Accelrys). Color images available online at www.liebertpub.com/aid

increasing NVP pressure. The results suggested that these two mutations conferred moderate-to-high-level resistance to NVP in combination with other mutations. To further identify the effects of these mutations on viral susceptibility and RC, this study constructed six mutant viruses containing mutations Y188C, L228I, Y188C/L228I, A139V, Y232H, and A139V/Y232H based on an HIV-1 CRF08_BC subtype infectious clone. We found that L228I alone conferred a low-level resistance to ETR. The combinations of Y188C/L228I and A139V/Y232H enabled the virus to be cross-resistant to both NVP and EFV accompanied with severe RC loss. Modeling study suggested that the copresence of L228I and Y188C or A139V and Y232H might lead to significant conformational changes in RT due to abolished hydrogen bonding or amino acid interactions, resulting in drug resistance and reduced viral RC.

At codon 228, the most commonly reported mutation was L228H/R. It was suggested to be associated with treatment containing both NRTIs[22] and NNRTIs[21,33]. Another study reported that L228H/R was associated with the accumulation of both type I and type II thymidine analogue mutation resistance pathways in both subtypes B and C.[34] Recently, L228R was also identified in RT of HIV-1 subtype CRF_BC strains isolated from NVP-containing HAART treatment–experienced patients.[35] However, it was completely absent from those treatment-naive patients in China.[35] That study also found that L228R did not show any significant resistance effect (<2-fold) to NNRTIs, including NVP, EFV,

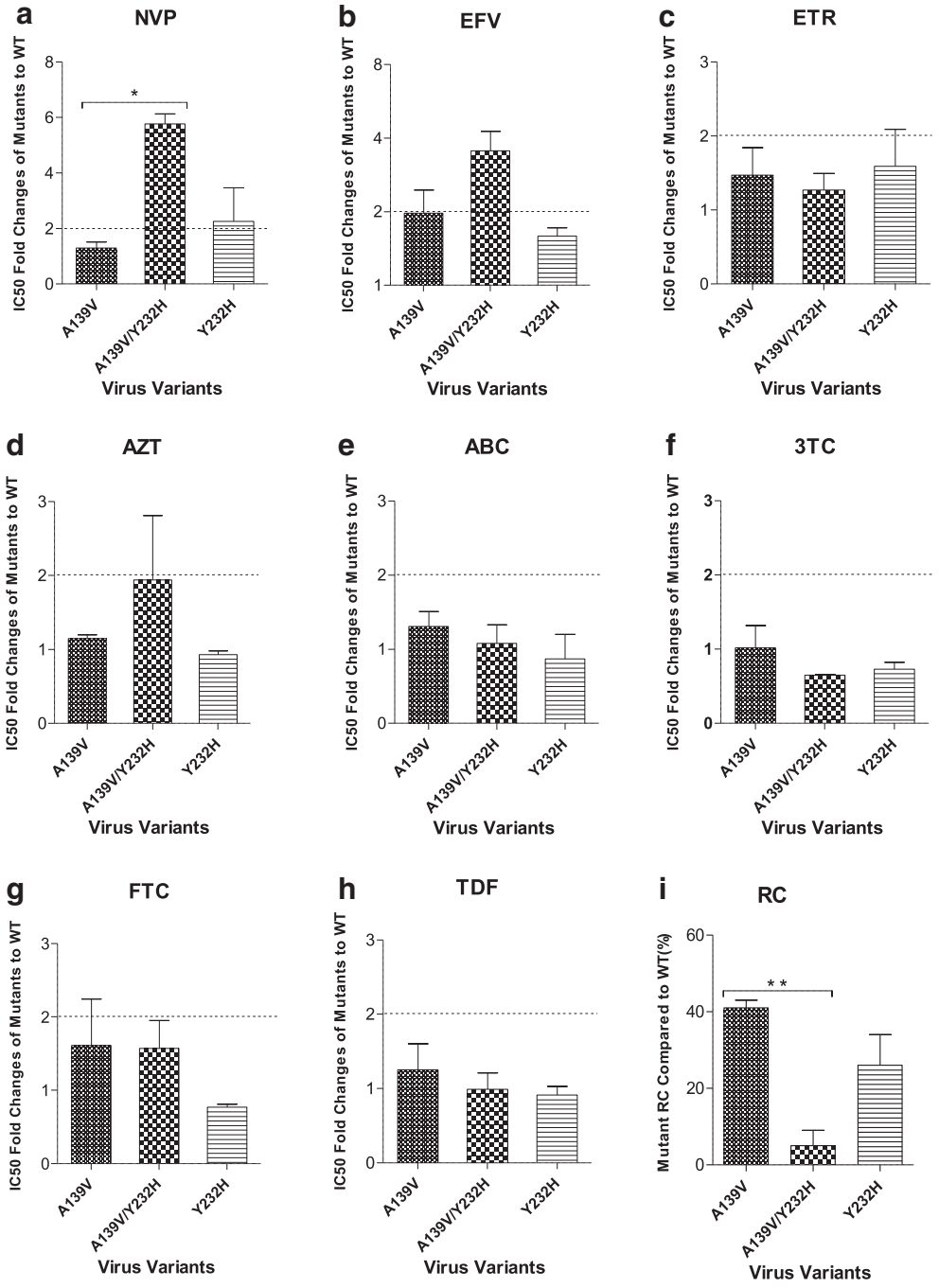

FIG. 3. Phenotypic analysis and viral RC of Y232H-associated virus variants. Compared with WT virus, the fold changes of IC_{50} of Y232H-associated virus variants against NNRTIs NVP **(a)**, EFV **(b)**, and ETR **(c)**, as well as NRTIs AZT **(d)**, ABC **(e)**, 3TC **(f)**, FTC **(g)**, and TDF **(h)**, were determined by phenotypic assay, while viral RCs of these variants were tested by a single-cycle cell culture assay **(i)**. In phenotypic assay, TZM-bl cells were infected with virus variants and treated with serially diluted RT inhibitors. In RC detection assay, TZM-bl cells were infected with serially diluted virus variants without inhibitors. $*p < .05$, $**p < .01$. The *dashed line* is labeled at twofolds of IC_{50} ratio to indicate the drug resistance standard.

delavirdine (DLV), and ETR.[35] To our knowledge, only one study observed L228I in the context of V75M and F227L in one Iranian HIV-1–positive patient who failed to antiretroviral therapy.[36] No further investigation on L228I was reported thereafter. In this study, although viruses with a single L228I mutation remained susceptive to NVP and EFV, the combinations of Y188C–L228I exhibited a high level of cross-resistance to these two first-line HAART drugs, which was in agreement with our previous results.[14] No significant resistance to any NRTIs (AZT, ABC, 3TC, TDF, and FTC) was detected by the L228I-containing viruses. Our results suggested that L228I might be a novel accessory NNRTI resistance–related mutation in subtype CRF08_BC. If L228I and Y188C occur at the same time during the genotypic resistance test before the treatment starts, the first-generation NNRTIs, including NVP and EFV, should be avoided to be prescribed. Additionally, we also assessed the drug susceptibility to NNRTIs of Y188C and found that Y188C conferred a high-level resistance to NVP and a moderate-level resistance to EFV, which was consistent with previous reports from our and others' studies.[37–39]

Modeling study showed that a hydrogen bond was formed between L228 and Y188 in WT RT. When only a single mutation L228I emerged, it would not affect this hydrogen

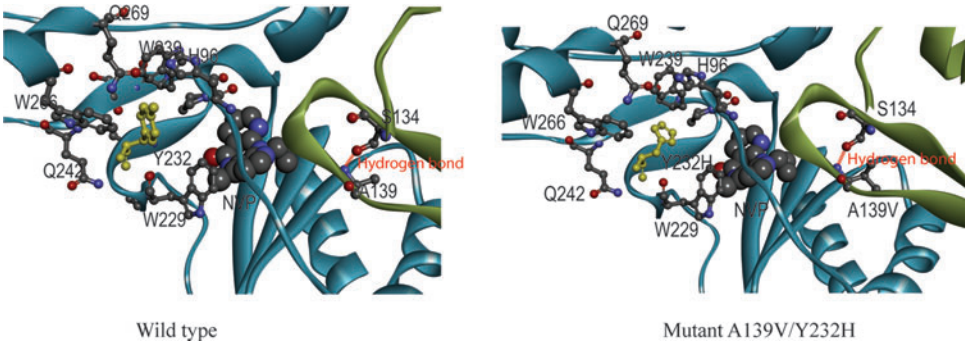

FIG. 4. RT models of WT and mutant A139V/Y232H. The models of WT and mutant are shown in ribbon diagram. Subunits of p66 and p51 are colored as *blue* and *green*, respectively. The residues at 232 and its vicinal sites in p66 subunit, as well as A139 and S134 in p51 subunit, are highlighted. Their atoms are labeled in *ball*-and-*stick* format. Y232 is colored in *yellow*. The hydrogen bond between A(V)139 and S134 is shown in *red dotted line*. The DNA/RNA hybrid is shown in *tube* format in *pink*, and the NNRTI NVP is shown in CPK format. The figures were produced using DS Visualizer (Accelrys). Color images available online at www.liebertpub.com/aid

bond. However, when Y188C and L228I were introduced, the hydrogen bond would be abolished. Since Y188 is in the NNRTI binding pocket,[40] the abolished hydrogen bond might affect the NNRTI binding. This might be helpful to understand why in this study a single mutation L228I did not induce significant drug resistance to NVP and EFV but Y188C could confer high- or moderate-level resistance to these two NNRTIs.

To determine the impacts of Y188C and L228I on the viral RC, a single-cycle cell culture assay was performed in this study. Results showed that the single mutation Y188C or L228I slightly impaired viral RC. The copresence of Y188C and L228I conferred significant lower RC than the single mutation Y188C, which indicated that there was a synergistic effect between L228I and Y188C on reducing viral RC. Modeling study showed that L228 locates in the primer grip and the lager side chain of L228I might block the entrance site of RNA/DNA hybrid during reverse transcription, which might also lead to impaired viral replication.

In addition to L228I, our previous study also selected another novel mutation Y232H in the primer grip region, which exhibited a high-level resistance to NVP together with A139V.[14] In this study, the phenotypic results showed that a single Y232H mutation only showed a low-level resistance to ETR but kept susceptive to NVP and EFV. A single mutation A139V also did not impact viral susceptibility to NVP and EFV, which is consistent with the results of another study in subtype B infection.[41] Nevertheless, the combination of A139V and Y232H displayed moderate and low levels of resistance to NVP and EFV, respectively. This observation suggested that there was a synergistic drug resistance effect between A139V and Y232H. The first-line HAART regimen should be considered to include ETR instead of NVP or EFV in the context of the combinational pattern of A139V and Y232H. Of note, the resistance level to NVP induced by A139V/Y232H in this study was a little lower than that suggested previously.[14] This might be associated with the cumulative effect of other additional mutations since in previous study A139V and Y232H were present together with three other mutations (Y181C, H221Y, and E396G).

Furthermore, we also found that both Y232H and A139V had substantial negative impacts on viral replication. This might be associated with their spatial positions. Modeling study suggested that when Y232 in the p66 subunit was substituted into 232H, the previous interactions between Y232 and its vicinal residues in the palm and thumb subdomains might be abolished. This might induce further conformational change to p66 subunit, leading to reduced viral RC. Besides, it has been reported that the $\beta7$–$\beta8$ loop (codons 132–140) in p51 subunit of RT is important for RT dimerization,[41–43] and the integrity of the loop is maintained by hydrogen bonding between the side chains of S134 and T139. In this study, the RT model also showed a hydrogen bond between A139 and S134 in the p51 subunit. However, the substitution of alanine to valine (A139V) at codon 139 would influence the hydrogen bond by increasing the hydrophobicity of the side chain as well as the core structure of the loop. Therefore, we hypothesize that RT dimerization might also be influenced by the mutation A139V, resulting in impaired RT activity. This may explain why A139V could diminish part of viral RC. When both Y232H and A139V are induced simultaneously, the RT configuration changes would be enhanced and result in more severe impairment to viral RC.

TABLE 2. MUTATIONS AT 139, 228, AND 232 IN SUBTYPE B AND CRF08_BC

	Subtype B		*Subtype CRF08_BC*	
Site in RT	*Amino acid*	*Codon*	*Amino acid*	*Codon*
Wild type				
139	Thr (T)	ACA	Ala (A)	GCA
228	Leu (L)	CTT	Leu (L)	CTT
232	Tyr (Y)	TAT	Tyr (Y)	TAT
Mutants				
139	Lys (K)	AAA	Val (V)	GTA
	Arg (R)	AGA		
	Ile (I)	ATA		
228	Arg (R)	CGT	Ile (I)	ATT
	His (H)	CAT		
232	His (H)	CAT	His (H)	CAT

CRF08_BC, circulating recombination form 08_BC.

In addition, other substitutions at codon 139, such as T139K/R, were also reported to be associated with RTI treatment in clinical samples of subtype B patients,[23,35,44] but they did not appear in our previous selection study. This might be related to the subtype-specific codon differences. As shown in Table 2, the WT amino acid at codon 139 in HIV-1 RT is different in subtype CRF08_BC (Ala, A) from subtype B (Thr, T). It is supposed to be easier for Ala (GCA) to be mutated into Val (GTA) than Thr (ACA) since A139V needs one base pair change (C-T), while T139V requires two (AC-GT). Similarly, it should be easier for Thr (T, ACA) to be substituted by Lys (K, AAA) and Arg (R, AGA) for the same reason. That might be helpful to explain why we selected 139V but not 139K/R in subtype CRF08_BC.

In conclusion, we identified and characterized two novel accessory NNRTI resistance–associated mutations L228I and Y232H in the RT of HIV-1 subtype CRF08_BC virus. Attention should be paid to these novel mutations in the genotypic resistance test beside those reported mutations in subtype B. Overall, our study contributed by filling critical gaps in the global profile of HIV-1–induced drug resistance in non-B subtype. The results provide valuable information for drug resistance surveillance, HIV-1 genotypic resistance testing, and the treatment modulation of HIV-1–infected individuals in China.

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Author Contributions

X.M.Z. and B.J.Z. conceived and designed the experiments. X.M.Z., Q.Z., H.W., and K.Z. performed the experiments. T.C.K.L. and X.L. constructed RT modeling study and analyzed the results. X.M.Z., H.C., J.Z., Z.W.C., D.Y.J., and B.J.Z. analyzed results and wrote the article.

Author Disclosure Statement

No competing financial interests exist.

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