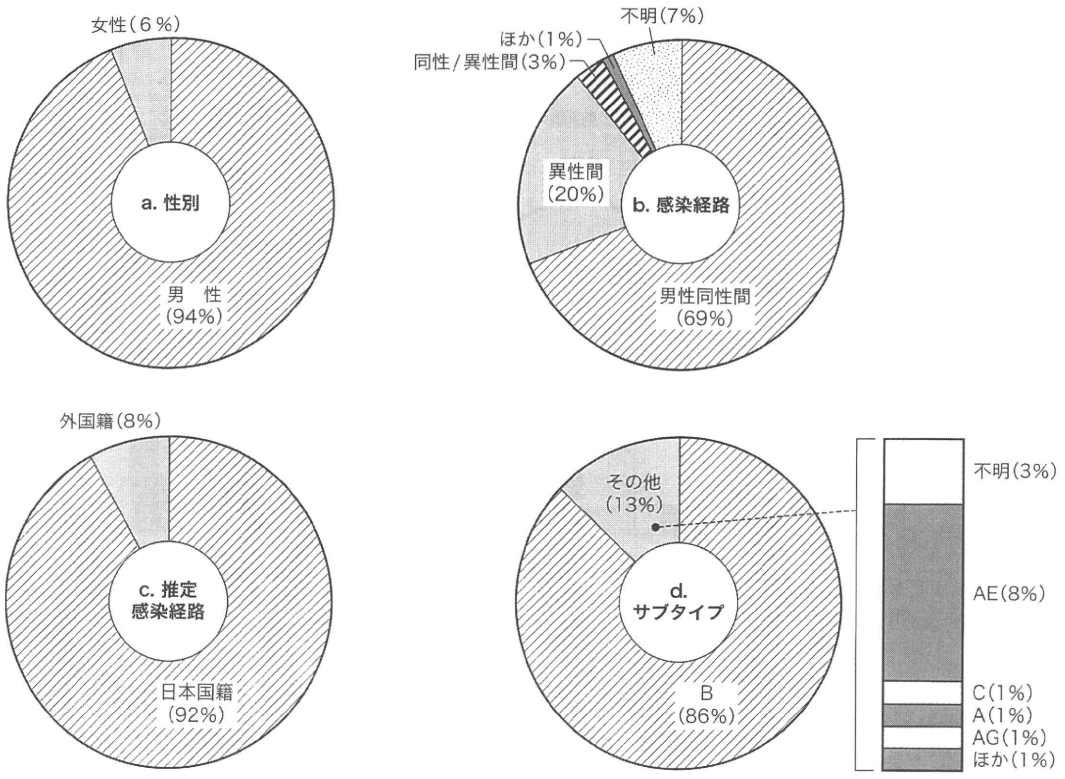

Fig. 2. 日本における HIV 感染の動向

厚生労働省研究班「薬剤耐性 HIV の動向把握のための調査体制確立及びその対策に関する研究(以下薬剤耐性班)」では 2003 年より新規 HIV/AIDS 診断症例を対象に疫学調査を実施しているが，調査報告によればわが国の HIV 感染流行の主体は「日本人」，「男性」，「男性同性間性的接触(MSM)」そして「サブタイプ B」であり(Fig. 2)，この傾向は調査を開始してからの 7 年間変わっていない．一方，異性間性的接触による感染は動向委員会の報告をみても多くなく，本邦では HIV-1 感染症が MSM における疾病となりつつあるといえる．

また薬剤耐性班では新規 HIV/AIDS 診断症例における薬剤耐性 HIV の保有率を調査しているが，Fig. 3 に示すように 2003 年の 5.9% 以降徐々に検出率は増加しており 2007 年には約 1 割に達している．その後 2008～2009 年は顕著な増減はなく 8.5% 前後を推移している[9]．観察される薬剤耐性変異の種類としては核酸系逆転写酵素阻害薬(nucleoside analogue RT inhibitor：NRTI)に関するものがもっとも多く，次いでプロテアーゼ阻害薬(protease inhibitor：PI)，そして非核酸系逆転写酵素阻害薬(non-nucleoside RT inhibitor：NNRTI)となっている．2008 年からは PI に対する耐性が増加し，NRTI に対する変異は減少の傾向を示している．個別の変異についてみていくとその種類は多いが，毎年必ず検出される変異が存在する．NRTI 耐性の T215X, NNRTI 耐性の K103N, そして PI 耐性の M46I/L は毎年必ず検出されており，これらの変異を有する株はすでに流行株として定着していると危惧される．このように薬剤耐性 HIV の拡散が懸念されるが，幸いなことに多くの場合耐性変異は他の変異を伴わない単一の変異として発見されており，いくつかの高度耐性変

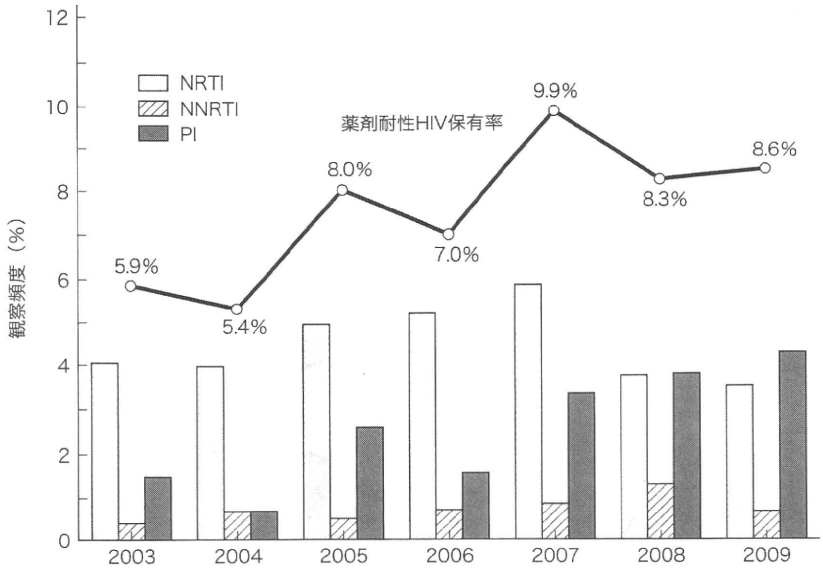

Fig. 3. 新規 HIV/AIDS 診断症例に観察される薬剤耐性変異獲得症例の頻度
NRTI：核酸系逆転写酵素阻害薬，NNRTI：非核酸系逆転写酵素阻害薬，PI：プロテアーゼ阻害薬．［文献 9）より引用］

異を除き治療の妨げになるものではないと思われる．

治療の現状

HIV/AIDS の治療は 1996 年以来多剤併用療法（highly active antiretroviral therapy：HAART）が基本となっているが，抗 HIV 薬剤（anti-retroviral：ARV）の抗ウイルス活性の増強，薬物体内動態の改善，剤型の改良などに伴い大きく進歩している．近年登場した ARV としては，薬剤耐性を獲得しにくい PI darunavir（2007），既存の NNRTI である nevirapine と efavirenz 耐性株にも有効な elvitegravir，最初のインテグラーゼ阻害薬（integrase inhibitor：INI）raltegravir（2008），そして宿主因子 CCR5 を狙った最初の薬剤である maraviroc（2008）があげられる（Table 1）．これらの新薬は当初既存の ARV では治療が困難な症例に対するサルベージ薬として承認された．薬剤耐性班ではこれらの新薬の処方動向と HIV/AIDS 患者の治療状況を把握するために 2009 年に全国 377 の施設に対してアンケート調査を行った．その結果，回答を得た 211 施設（56.0%）における通院症例数は 9,040 名，総服薬症例数 6,296 名（69.6%），そのうち新規 ARV 使用症例数は 280 症例（4.4%）だった．この 280 症例における新規 ARV の使用理由は，副作用による変更が 125 症例（125/280＝44.6%），ウイルス学的失敗による変更が 97 症例（97/280＝34.6%）であり，治療集団全体に対してみた場合はウイルス学的失敗の頻度は（97/6296＝1.54%）となる[10]．

現在日本で推奨されている HAART のレジメは 2 種類の NRTI に PI/NNRTI/INI のいずれか 1 剤を加えることとなっている．2 種類の NRTI としては，2 種類が合剤となっている tenofovir＋emtricitabine（FTC）もしくは abacavir＋lamivudine（3TC）の組み合わせが推奨されている[11]．

抗 HIV 治療を巡る今後の動向

zidovudine（AZT）開発以来 20 年にわたる治療経験の蓄積，そして ARV の進歩により，治療戦略は変貌しつつある．大きな変化は治療の開始時期であり，従来慢性毒性のリスクを下げるために末

Table 1. 2007 年以降承認された新成分を含む抗 HIV 薬(2010 年 8 月現在)

分類/商品名/一般名	適応症	使用条件
プロテアーゼ阻害薬 darunavir(DRV) プリジスタナイーブ錠 400 mg (2009 年 9 月承認)	HIV 感染症	治療経験のない HIV 感染患者に使用すること(治療経験のない HIV 感染患者以外に対する有効性および安全性は確立していない) 小児 HIV 感染症に対しては,本剤投与による有効性および安全性が確立していない
プリジスタ錠 300 mg (2007 年 11 月承認)	HIV 感染症	本剤は抗 HIV 薬の治療経験がある HIV 感染患者に使用すること 本剤による治療にあたっては,患者の治療歴および可能な場合には薬剤耐性検査(遺伝子型解析および表現型解析)を参考にすること 初回治療の成人 HIV 感染症および小児 HIV 感染症に対しては,本剤投与による有効性および安全性が確立していない
インテグラーゼ阻害薬 raltegravir(RAL) アイセントレス錠 400 mg (2008 年 6 月承認)	HIV 感染症	本剤による治療にあたっては,患者の治療歴および薬剤耐性検査結果を参考にすること
非核酸系逆転写酵素阻害薬 etravirine(ETV) インテレンス錠 100 mg (2009 年 1 月承認)	HIV-1 感染症	本剤は,NNRTI を含む他の抗 HIV 薬に耐性が認められる場合等に使用すること NNRTI および NRTI を含む併用療法によりウイルス学的効果不十分となった患者には,本剤と NRTI のみの併用はしないこと 本剤による治療にあたっては,患者の治療歴および可能な場合には薬剤耐性検査(遺伝子型解析あるいは表現型解析)を参考にすること 抗 HIV 薬による治療経験のない成人 HIV 感染症および小児 HIV 感染症に対しては,本剤投与による有効性および安全性は確立していない
CCR-5 阻害薬 maraviroc(MVC) シーエルセントリ錠 150 mg (2009 年 1 月承認)	CCR5 指向性 HIV-1 感染症	他の抗 HIV 薬にて十分な効果が期待できない場合,または忍容性に問題があると考えられる場合に限り使用すること 本剤による治療にあたっては,指向性検査を実施すること CXCR4 指向性 HIV-1 感染患者,CCR5/CXCR4 二重または混合指向性 HIV-1 感染患者には,投与しないこと.なお,急性期および無症候期の患者では主に CCR5 指向性ウイルスが検出されるが,進行した HIV-1 感染症では CXCR4 指向性および二重/混合指向性ウイルスが検出される患者の割合が増加することが知られている 抗 HIV 薬による治療経験のない成人 HIV-1 感染症および小児 HIV-1 感染症に対する有効性および安全性は確立していない

梢血中 $CD4^+$T 細胞数値が 250 個/μl を切るまで治療を保留していたが,最近そこまで疾病の進行を許すと,ウイルス自体の有害性および種々の合併症の発生率が高くなり予後が増悪することが明らかになり,350 個/μl の時点まで開始を早めることが推奨されている.一部ではさらに早く,500 個/μl ではじめるべきだという意見もあるが,その有益性に関してはまだ十分なデータはなく,今後の議論の推移と結論を待ちたい[12].

治療のプロトコルも 1996 年の HAART 導入時来の NRTI 2 剤+PI/NNRTI 1 剤という基本からの見直しの動きが出はじめている.興味深いのは

治療導入と維持という考え方であり，従来の3剤併用療法でうまくコントロールされている症例においてboosted PI 1剤のみでの維持が試みられており(MONET，MONARK)，短期的には成功している[13,14]．これは薬剤による慢性毒性や医療費削減の点で魅力的であるが，安全性についてはまだ疑問が残り，さらなる検証が必要である．

感染予防戦略における抗HIV薬

HIVの発見以来四半世紀にわたる研究と多額の資金投入にもかかわらず，予防ワクチンの実現には依然として希望がみえてこない．その一方で止まらない感染拡大の現実があることから，予防ワクチンに替わって抗HIV薬を感染予防に使用するという戦略が登場してきた．すでにサルを用いた実験ではウイルス曝露前後での抗HIV薬の投与により感染予防が可能であることが報告されている[15]．さらに南アフリカにおいて889人の女性を被験者に実施された1% tenofovirゲルの膣内投与臨床試験(CAPRISA004)では，tenofovirゲルを使用した被験者群でHIV感染率が有意に低い結果が得られた．これはヒトを対象とした大規模感染予防試験としてははじめての成功例である[16]．もちろん抗HIV薬による予防には，いつ誰を対象にどのような手段で投与をするのか，薬剤の副作用のリスク，薬剤耐性ウイルスを選択してしまうのではないかという恐れ，などまだ解決すべき問題は多いが，この予防手段は実現に向けて舵が切られつつある．また，Granichらは南アフリカの統計データをもとに数理モデルを構築し，仮に「15歳以上が全員年1回のHIV抗体検査を受け，HIV陽性の場合には即座に治療」すると，50年以内にHIV感染率を大幅に下げることが可能だと推測し，大きな反響をよんでいる[17]．これについては単に数理モデルであるという批判の一方，決して絵空事ではなく，私たちがすでに手にしている薬剤やシステムで十分に実現可能な戦略であり，今後の展開が気になるところである．

おわりに

HIV/AIDSの治療は大きく進歩し，今ある抗HIV薬だけでHIV感染者の多くは寿命を全うできるところまで到達している．そして今，抗HIV薬は「治療」から「予防」へとその活用領域を広げつつある．また，最近になり，かつて封印された「cure(根治)」という言葉をしばしば耳にするようになってきた[18]．これは強力な抗HIV薬を手にし，HIVの複製機構と病態研究の進展に伴い，「根治」をするための標的である「潜伏感染細胞」の姿が解明されつつあり射程に入ってきたと認識しているからである．「根治」という夢の実現に向けての今後のHIV/AIDS研究の進展に期待したい．

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②薬剤投与なし＋選択的帝切分娩，③薬剤投与あり＋経腟分娩，④薬剤投与なし＋経腟分娩の各群の母子感染率は，それぞれ１％，4.2%，０％，36.0%であった。2000年以降の228例では85.5%の例で抗ウイルス薬が投与され，しかもほとんどがHAARTであることから，①薬剤投与あり＋選択的帝切分娩群と③薬剤投与あり＋経腟分娩群の母子感染率はともに０％であった。わが国では選択的帝王切開が推奨されてきたので選択される場合が多く，80～90%に及ぶ。経腟分娩数は極端に少ないが，HAART導入下では分娩様式で母子感染率に差はない可能性がある。

最近では妊娠初期のHIV検査では陰性だったのに後期にHIV感染が疑われる症例も報告されている。充分なHIV母子感染予防対策が取られなかった場合や，小児の問題など課題が残されている。

＊平成21年度厚生労働省エイズ対策研究事業「HIV感染妊婦とその出生児の調査・解析および診療・支援体制の整備に関する総合的研究」班（主任研究者：和田祐一），妊婦HIV検査実施率およびHIV感染妊婦とその出生児の動向に関する全国調査（分担研究者：吉野直人），HIV感染女性から出生した子どもの実態調査と子どもの健康と発達支援（分担研究者：外川正生），HIV感染妊婦とその出生児に関するデータベースの構築およびHIV感染妊婦の疫学的・臨床的情報解析（分担研究者：喜多恒和）

＊＊「HIV母子感染予防対策マニュアル第５版」（分担研究者：塚原優己）エイズ予防情報ネット http://api-net.jfap.or.jp/library/guideLine/boshi/index.html

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産婦人科　谷口晴記

＜特集関連情報＞

本邦におけるHIV-2の疫学動向と新たな組換え流行株CRF01_ABの同定

１．はじめに

ヒト免疫不全ウイルス２型（human immunodeficiency virus type 2: HIV-2）は，HIV-1に次いで1986年に後天性免疫不全症候群（acquired immunodeficiency syndrome: AIDS）の原因ウイルスとして同定された。本邦においてHIV-2感染症例数は少ないものの，近年国内感染が強く疑われる日本人女性感染２例を含む計５例が同定されたことに伴い，2009（平成21）年２月に周知のための通知がなされた[1]。これら５例から分離同定されたHIV-2の分子疫学的解析を実施した結果，３例が世界で最初のHIV-2組換え流行株（circulating recombinant form: CRF）による感染例と判明した。本稿ではその詳細について報告する。

２．HIV-2の分子疫学的特徴

今日HIV-1が世界規模で流行し，約3,300万人の感染者が存在するのに対し，HIV-2の流行は西アフリカと関連するいくつかのヨーロッパおよびアジア諸国に限定しており，その感染者数は約100万人と推定されている。これまでの研究により，HIV-2はHIV-1よりも病原性が低く，HIV-2感染者の多く（75%以上）は無症候のまま生涯を全うすることが知られている。遺伝子学的に，現在，HIV-1は４つのグループ（M, N, O, P）に分類でき，グループM株が世界的規模の流行の主体となっている。一方，HIV-2は８つのグループ（A～H）が同定されており，このうちグループA株とB株が主要な流行株として知られている。これらに加えて，グループA株とB株の組換えウイルスが２種類各１例ずつ同定されてきたが[2,3]，これらの組換えウイルスの流行実態は未確認であった。

３．本邦におけるHIV-2感染報告

本邦における正確なHIV-2感染例数は現在まで分かっていないが，これまでの報告によると，その数は10例に満たない。2002（平成14）年10月に最初のHIV-2感染例の同定に伴う通知がなされ[4]，2006（平成18）年８月には最初の日本人HIV-2感染例の同定に伴う通知がなされてきた[5]が，いずれも日本国内で感染したものではなく，海外における感染事例の国内発見であった。これに対し我々が2009（平成21）年２月に同定した日本人女性のHIV-2感染例では，海外渡航歴が無いことと，HIV-2の疫学的流行地域出身者との交際歴を有していたことから日本国内での感染が強く疑われるものであった[1]。その後，新たなHIV-2感染事例は同定されていないが，本邦におけるHIV-2の疫学動向を把握するためには，より詳細な疫学調査が必要であると思われる。

４．最初のHIV-2組換え流行株CRF01_ABの発見

我々は同定した５例のHIV-2感染症例のHIV-2グループを決定するために遺伝子配列解析を実施した。採取した末梢血単核球からDNAを抽出し，nested PCR法にてHIV-2の*gag*および*env*遺伝子の増幅を試みた。その結果，５例中４例（いずれもAIDS発症例）の増幅に成功した。増幅できなかった１例（無症候例）は複数の異なるプライマーセットを用いて増幅を試みたが成功しなかった。遺伝子増幅に成功した４例については，塩基配列の決定および系統樹解析を実施した。その結果，NMC786はグループA株と判定されたが，残りの３例（NMC307, NMC716, NMC842）は，1990年に西アフリカのコートジボワールより報告されたAB組換えウイルス7312Aとクラスターを形成した。この結果から，NMC307, NMC716, NMC842がAB組換えウイルスである可能性が示唆され，組換え構造を明らかにするためにウイルスの全長ゲノム塩基配列解析を行った。ウイルスゲノム構造を分析した結果，３株とも４つの組換えポイントが確認され，さ

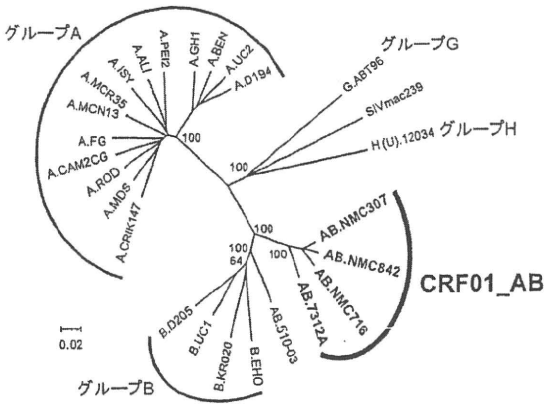

図1. 全長ウイルスゲノム塩基配列を用いて作成したHIV-2系統樹とCRF01_AB
近隣接合法で作成し、1000回の解析により求めたブートストラップ値を主たる節に記した。
各ウイルス株は属するグループと株名で表している。

らに，この4ポイントは7312Aが有する組換えポイントと完全に一致していることが明らかになった。また，ウイルスの全長ゲノム塩基配列を用いた系統樹解析からは，NMC307, NMC716, NMC842と7312Aが系統樹上で一つのクラスターを形成したことから，これらのウイルスは同一の組換えウイルスであることが明確に示された。先に報告されていた7312Aに加えて新たに3つの同一組換えウイルスが確認されたことから，このHIV-2のAB組換え体は米国Los Alamos HIV Sequence Databaseより世界で最初のHIV-2組換え流行株であると認定され，HIV-2 CRF01_AB株と命名された（図1）[6]。

5．終わりに：HIV-2 CRF01_ABの発見から示唆されること

本稿冒頭に述べたように，HIV-2感染例の多くは無症候のまま生涯を全うするが，今回我々が同定した3例のHIV-2 CRF01_AB症例は全例AIDSを発症していたことは特筆に値する。まだ，症例数は少ないものの，HIV-2 CRF01_AB株がゲノム組換えを介して，より高い病原性を獲得した可能性が懸念される。また，HIV-2の流行地域ではない本邦においてHIV-2 CRF01_ABが3例も発見されたことは，このHIV-2組換えウイルス株が既に西アフリカを越えて世界各地へ伝播していることを推測させる。HIV-2 CRF01_ABの疫学的動向を明らかにするためには，今後西アフリカ諸国および関連各国と共同での疫学調査を進めることが必要である。

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＜特集関連情報＞

わが国における抗HIV治療と多剤耐性症例の現状

背　景

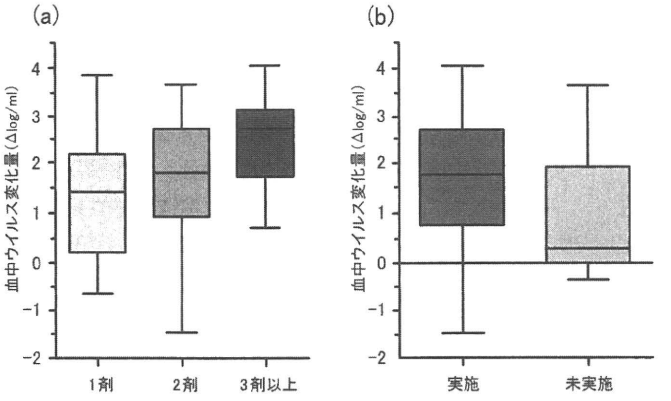
近年開発された抗HIV薬（anti-retroviral drug: ARV）は，耐性を誘導しにくいものへと進歩を遂げ，新規に多剤併用療法（highly active antiretroviral therapy: HAART）を開始したHIV/AIDS症例においては，薬剤耐性の獲得に起因する治療の脱落と臨床的予後の増悪が減少している。これに対し，HAART導入以前の単剤もしくは2剤療法の時期より長期間にわたり治療を継続してきたHIV/AIDS症例においては，薬剤耐性変異の蓄積から多剤耐性（multi drug resistance: MDR）となり，治療に難渋している症例が存在するはずであるが，わが国における実態は掴めていない。

一方，わが国では2007年末～2009年にかけて，薬剤耐性を獲得しにくいプロテアーゼ阻害剤Darunavir（DRV），従来の非ヌクレオシド系逆転写酵素阻害剤に対する薬剤耐性HIVにも有効なEtravirine（ETR），世界初のインテグラーゼ阻害剤Raltegravir（RAL），そしてこれも世界初となる宿主因子CCR5を標的にしたMaraviroc（MVC）が認可された。さらに厚生労働省「エイズ治療薬研究」班を通じて入手可能な未承認薬の融合阻害剤T-20とプロテアーゼ阻害剤Tipranavir（TPV）が選択肢に加わることにより，今までに無い程治療の選択肢が広がり，従来のARVでは治療に難渋していた多くのMDR症例の救済が期待されている。厚生労働科学研究費エイズ対策研究事業「薬剤耐性HIVの動向把握のための調査体制確立及びその対策に関する研究」班では，前述した6種類の新規ARVの使用状況調査を実施し，MDR症例の現状把握と新規ARV導入による治療効果，さらには新規薬剤選択における薬剤耐性検査の有益性について検証を行った。本稿ではこの調査・検証の結果を報告したい。

方　法

日本のエイズ診療拠点病院および首都圏クリニックの計377施設に対して郵送アンケートによる調査を実施した。調査項目は①通院中HIV/AIDS症例数，②治療中の症例数，③新規ARV（DRV, RAL, ETR, MVC, T-20, TPV）を投与中の症例数，④新規ARV

図. 多剤耐性症例の治療に影響を及ぼす因子



多剤耐性症例のサルベージ療法における新規ARVの使用数と治療効果の関係。新規ARVをレジメに加える数を増やすとより高い治療効果を示した。

多剤耐性症例のサルベージ療法で使用するARVの選択に際して薬剤耐性検査を実施した症例のほうがより高い治療効果を示した。

の使用と選択理由，⑤予後，⑥現在の ARV レジメおよびその開始年月，⑦症例の背景（生年月，性別，感染経路），⑧新規 ARV 使用直前および直近の検査値（CD4 陽性細胞数，HIV-RNA 量），⑨新規 ARV 導入直前の ARV レジメ，⑩当該新規 ARV を処方した理由，⑪新規 ARV に際しての耐性検査実施の有無，⑫新規 ARV 導入までに使用したすべての ARV，とした。統計解析は graphPad 社の Prism5 を用いた。

結　果

①対象症例とその治療の現状

調査を依頼した377施設中，回答を得た211施設（56.0％）における通院症例数は9,040名，総服薬症例数6,296名（69.6％），そのうち新規 ARV 使用症例数は280症例（4.4％）だった。この280症例における新規 ARV の使用理由は，副作用による変更が125症例（44.6％），ウイルス学的失敗による変更が（MDR 群）97症例（34.6％），その他の理由による変更31症例（11.1％），初回使用24症例（8.6％），不明 3 症例であった。

②MDR 症例は長い治療歴を有し，CD4 陽性細胞数も有意に低い値を呈していた。

新規 ARV 導入症例中，導入理由が不明な 3 症例を除いた277症例を対象とし，新規 ARV 導入以前の CD4 陽性細胞数（baselineCD4），初回 ARV 開始からの総治療年数，使用経験のある既存 ARV の数，新規 ARV 導入後の治療期間，新規 ARV 導入前後の logΔ HIV-RNA（VL）について解析した。baselineCD4 の中央値（cells/μl）は，MDR 群227，non-MDR 群323と，MDR 群の方が有意に低かった（p=0.0304）。総治療期間の中央値（年）は，MDR 群10.8，non-MDR 群4.3と，MDRグループが有意に長かった（p<0.0001）。使用経験のある既存 ARV 数の中央値（剤）は，MDR 群 7，non-MDR 群 5 と，MDR 群の方が有意に多かった（p<0.0001）。新規 ARV による治療期間の中央値（月）は，MDR 群11，non-MDR 群 7 と，MDR 群の方が有意に長かった（p<0.0001）。

③薬剤耐性検査は至適治療の選択に有効であった。

新規 ARV 導入症例の MDR 群（n=97）を対象とし，導入した新規 ARV 数とlogΔ VL の相関，新規 ARV 導入前の薬剤耐性検査実施の有無とlogΔ VL との相関について解析を行った（図）。新規 ARV 導入剤数とlogΔ VL の中央値（log copies/ml）は，1 剤導入群（n=52）1.4，2 剤導入群（n=33）1.8，3 剤以上導入群（n=11）2.7と，導入剤数が多いほど下げ幅が大きく，1 剤群と 3 剤以上群で有意差が認められた（p=0.0244）。薬剤耐性検査実施の有無とlogΔ VLの中央値（log copies/ml）は，検査実施群（n=81）1.8，非実施群（n=15）0.3と，検査実施群の方が有意に VL を減少させていた（p=0.0223）。

まとめ

既治療 HIV/AIDS 患者における薬剤耐性治療の状況は，薬剤耐性症例自体が少ないこともあり，全体像の把握が難しい。今回我々は，前述新規 ARV の導入を契機に調査を行うことで，わが国の既治療 HIV/AIDS 患者における状況を明らかにすることができた。本調査によって，現在 MDR の獲得により治療に難渋している症例では，新規 ARV 導入時の CD4 陽性細胞数は低く，初回 ART 導入が早く，新規 ARV がより早く導入されていた。MDR 症例では，新規 ARV を単剤より複数剤加えた方が，また，新規 ARV 導入の際に耐性検査を行ってから薬剤選択をした方が，より高い治療効果を示すことが明らかとなった。

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Raltegravir-associated perihepatitis and peritonitis: a single case report

Raltegravir, the first approved HIV integrase inhibitor, has demonstrated an excellent safety and tolerability profile in several clinical trials [1] and is currently used widely as one of the key components of salvage regimens. However, the duration of clinical use is relatively short, and unknown adverse effect may occur. Here, we report one case of peritonitis associated with use of raltegravir. Abdominal symptoms appeared within 2 weeks of commencement of treatment, and raltegravir had to be stopped due to worsening of clinical condition.

Case report

The patient was a 49-year-old Japanese hemophiliac coinfected with HIV and hepatitis C virus (HCV). HIV-RNA was undetectable, and $CD4^+$ cell count was above 500 cells/μl for more than 5 years under the combination of abacavir, nevirapine and lopinavir/ritonavir. In January 2009, lopinavir/ritonavir was replaced with raltegravir because of bleeding tendency related to the use of a protease inhibitor. Abacavir and nevirapine were continued, and no other drugs were modified. The patient visited the hospital on day 18 after the use of raltegravir, complaining of a gradually worsening pain in the right upper abdomen and lower chest wall for 3 days. A nonsteroidal anti-inflammatory drug was not effective, and a computed tomography (CT) scan performed 11 days after the onset of the symptom revealed contrast enhancement of the liver surface (Fig. 1a) and fatty stranding of the greater omentum (Fig. 1b), which are findings compatible with perihepatitis and peritonitis. Oral prednisone (60 mg/day for 3 days, then 30 mg/day for 3 days) was prescribed, and all the symptoms resolved immediately. However, abdominal symptoms developed again after withdrawal of prednisone, necessitating its reintroduction on day 31 at 30 mg/day. Attempts to taper prednisone led to worsening of abdominal pain and development of stomatitis, resulting in continuation of treatment at 20 mg/day. Raltegravir was switched to lopinavir/ritonavir 11 weeks after the onset of abdominal pain and, finally, all antiretroviral drugs were terminated 4 days later because of diarrhea and bleeding related to lopinavir/ritonavir. Abdominal symptoms gradually improved, and prednisone could be tapered to 10 mg/day within 2 weeks. A CT scan performed 10 days after cessation of antiretroviral therapy showed an improvement of perihepatic enhancement. C-reactive protein levels increased to 1.42 mg/dl during raltegravir use and fell to normal levels 6 days after discontinuation of raltegravir. Other laboratory data including transaminase levels showed no changes, and $CD4^+$ cell count and HIV-RNA were stable throughout the course.

This is the first reported case of severe peritonitis associated with raltegravir use. Although not described here, we have experienced several other cases with similar abdominal symptoms that disappeared after raltegravir termination. Several case reports have recently described previously unknown adverse effects related to raltegravir, such as rhabdomyolysis [2] and exacerbation of depression [3]. However, to our knowledge, raltegravir-associated peritonitis has not been reported. In the BENCHMRK

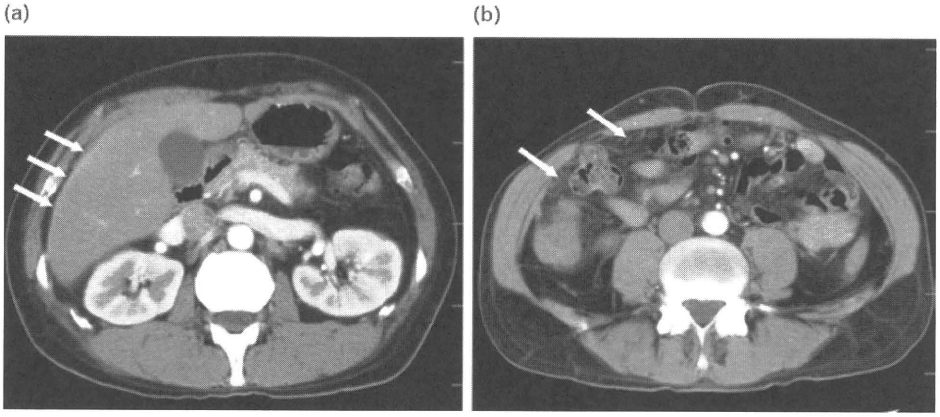

Fig. 1. A computed tomography scan performed 11 days after the onset of the symptoms. A computed tomography scan shows contrast enhancement around the liver surface (a) and fatty stranding of the greater omentum (b).

(Blocking integrase in treatment Experienced patients with a Novel Compound against HIV: MeRcK, MK-0518) study [1], abdominal symptoms, such as diarrhea and nausea, were noted in patients on raltegravir, and some of which might be associated with mild peritonitis.

Fortunately, raltegravir-associated peritonitis seemed reversible, at least to some extent. However, the longer use of raltegravir after onset of symptoms may lead to irreversible and lethal sequelae. Cessation of antiretroviral therapy as a result of severe abdominal symptoms is a potential risk for re-emergence of acute retroviral syndrome or the further accumulation of HIV-resistant mutations.

Whether the described side effects are universal or related to Asians, hemophiliacs or those who have underlying liver disease is unknown at present. Careful monitoring of abdominal symptoms and the consideration of an appropriate radiographic examination are warranted after commencement of raltegravir-containing regimens.

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FAST TRACK

Impact of human leukocyte antigen-B*51-restricted cytotoxic T-lymphocyte pressure on mutation patterns of nonnucleoside reverse transcriptase inhibitor resistance

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Objective: The objective of this study is to determine the impact of human leukocyte antigen (HLA)-B*51-restricted cytotoxic T-lymphocyte (CTL) pressure on the development of nonnucleoside reverse transcriptase inhibitor (NNRTI) resistance.

Design: The prevalence of HIV-1 harboring an escape mutation, I135X, in a major epitope of HLA-B*51-restricted CTL located in reverse transcriptase is increasing worldwide. We analyzed the effects of escape mutations on the emerging mutation patterns of NNRTI resistance.

Methods: Monoclonal HIV-1 sequences harboring each of the escape mutations, including I135L (HIV-1_{I135L}), I135V (HIV-1_{I135V}), I135T (HIV-1_{I135T}), and I135R (HIV-1_{I135R}) in reverse transcriptase, and a wild-type monoclonal HIV-1 (HIV-1_{WT}) were cultured in the presence of increasing concentrations of efavirenz. Induced mutations during culture passages of the culture were analyzed.

Results: E138K emerged during the cultural passages of HIV-1_{I135V}, HIV-1_{I135T}, and HIV-1_{I135R}, but not during the passages of HIV-1_{WT}. The combination of I135T, the most frequent escape mutation, and E138K (HIV-$1_{I135T/E138K}$) conferred significant resistance to efavirenz, nevirapine, and etravirine. The HIV-$1_{I135L/E138K}$ and HIV-$1_{I135R/E138K}$ were significantly resistant to nevirapine and etravirine, respectively, though each solo of escape mutations and E138K did not confer significant resistance to NNRTI. Computational analysis indicated that I135T and E138K cooperatively extend the gap between the binding site of reverse transcriptase and NNRTI.

Conclusion: HLA-B*51-restricted CTL can induce novel mutation patterns of NNRTI resistance by selecting escape mutations. The spread of CTL escape variants may alter the mutation patterns of drug resistance.

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Keywords: antiretroviral therapy, cellular immunity, cytotoxic T-lymphocyte escape variant, E138K, major epitope

Introduction

Cytotoxic T lymphocytes (CTLs) are one of the antiretroviral host factors that can modify the clinical course of HIV-1 infection [1]. However, HIV-1 evades these cells by acquiring escape mutations in recognized epitopes, and some of the CTL-escape variants remain stable without reversion even in the absence of such selective pressure [2]. TAFTIPSI (reverse transcriptase 128–135) is a major epitope recognized by human leukocyte antigen (HLA)-B*51-restricted CTL [3], and we recently reported that its escape mutation, I135X, is detected in the majority of HLA-B*51-positive infected individuals and also in a significant proportion of HLA-B*51-negative individuals, and that I135X can exist persistently even in HLA-B*51-negative individuals probably because it does not cause a significant fitness cost [4]. Consequently, I135X can spread as a polymorphic mutation among infected individuals and has in fact accumulated in the HIV-positive populations, especially among the Japanese, in whom HLA-B*51 is highly prevalent. Previous studies reported that I135X was associated with low-level resistance to nonnucleoside reverse transcriptase inhibitors (NNRTIs) [5–7] and suggested that I135X may be a determinant of evolutional patterns of NNRTI resistance [8,9], though it has also been reported that there is no correlation between the presence of I135X at baseline and efficacy of NNRTI [10]. To determine whether CTL escape mutations alter the development of drug resistance, we focused on I135X and induced NNRTI resistance from I135X-harboring HIV-1s by cultural passages in the presence of increasing concentrations of efavirenz (EFV).

Materials and methods

HIV-1 sequences and human leukocyte antigen types in treatment-naive patients

We recently reported the frequent prevalence of I135X mutations in Japan [4]. To confirm the same and to determine the frequency of each mutation, we used another cohort that included 575 treatment-naive newly diagnosed HIV/AIDS patients recruited from across Japan between January 2003 and December 2004 [11]. Among them, data of HLA typing were available for 97 patients.

Generation of recombinant HIV-1 sequences

The desired mutations were introduced into the *Xma*I-*Nhe*I region of pTZNX, which encodes Gly-15 to Ala-267 of HIV-1 reverse transcriptase (strain BH10) [12]. The *Xma*I-*Nhe*I fragment was inserted into pNL_{H219Q}, which was modified from pNL101 and encoded the full genome of HIV-1. Each molecular clone was transfected into COS-7 cells, and the obtained virions were harvested 48 h after transfection and stored at −80°C until use.

Induction of efavirenz-resistant HIV-1

The infectious HIV-1 clones were propagated in MT-2 cells in the presence of increasing concentrations of EFV [12]. Briefly, MT-2 cells (1×10^5) were exposed to 500 blue cell-forming units (BFUs) in MAGIC-5 cells (CCR5-expressing and CD4-expressing HeLa-LTR-β-D-gal cells) of each monoclonal HIV-1 and cultured in the presence of EFV at an initial concentration of 3 nmol/l. The culture supernatant was harvested on day 7 of culture and used to infect fresh MT-2 cells for the next round of culture. When the virus began to propagate in the presence of the drug, the drug concentration was increased by half-log fold. This selection was carried out until the EFV concentration reached 1000 nmol/l. Proviral HIV-1 reverse transcriptase gene in the infected MT-2 cells was amplified and sequenced at several passages.

Drug susceptibility assay

EFV and nevirapine (NVP) were generously provided by Merck Co., Inc. (Rahway, New Jersey, USA) and Boehringer Ingelheim Pharmaceutics Inc. (Ridgefield, Connecticut, USA), respectively. Etravirine (ETR) was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Recombinant HIV-1 susceptibility to EFV, NVP, and ETR was determined in triplicate using MAGIC-5 cells [12]. The drug susceptibility assay was performed in triplicate and repeated three times. Fold resistance was calculated by comparing viral IC_{50} with that of monoclonal wild-type HIV-1 ($HIV\text{-}1_{WT}$). Drug resistance was considered significant when it was higher than three-fold.

Structural modeling

We constructed structural models of the HIV-1 reverse transcriptase and NNRTI complex by computational analysis. First, we constructed the initial models of wild-type reverse transcriptase with one of the three NNRTIs by homology modeling using Molecular Operating Environment (MOE) 2007.09.02 (http://www.chemcomp.com/). The crystal structures of reverse transcriptase with NNRTI (PDB code: 1IKW [13], 1VRT [14], and 1SV5 [15]) were used for template structures. The ff94 force field and distance-dependent electrostatic energy function were applied in the modeling. Next,

we refined the initial models by energy minimization using sander module of AMBER9 software package through two steps. In the first step, energies for the NNRTI in the complex models were minimized at the gas phase by the conjugated gradient method. In the second step, energies of whole structures were converged up to 0.5 kcal/mol/Å by 50 steps of the steepest descent method and the subsequent conjugated gradient method at implicit water solvent condition. In each minimization, the AMBER ff03 [16,17], the general AMBER force field (gaff) [18], and the generalized Born implicit solvent surface area (GBSA) method (IGB = 2) [19] were applied for potential energy calculations. The charges and atom types of every atom in NNRTI were automatically assigned using the AMBER9 Antechamber module. We also constructed the respective mutant reverse transciptases with the NNRTI by considering every possible conformer of the respective mutant models. The possible conformers were generated from the wild-type homology models using PyMOL version 0.99rc6 (http://www.pymol.org). The structural model of each conformer was refined by a method similar to that used in the wild-type models. Among the refined conformers, we selected those with the lowest energy as each mutant model.

Results

The 135th amino acid in HIV-1 reverse transcriptase and human leukocyte antigen-B*51

We analyzed the relationship between HLA-B*51 and the 135th amino acid of HIV-1 reverse transciptase in 97 infected individuals newly diagnosed in Japan between January 2003 and December 2004 (Table 1). As expected, CTL escape mutations I135X, including I135T, I135L, and I135V, were observed in all but one HLA-B*51-positive patient (94.1%), representing a significantly higher prevalence than in the HLA-B*51-negative patients (Fisher's exact test; $P = 0.01$). However, in the HLA-B*51-negative patients, escape mutations were still observed at a high frequency (62.5%), indicating that I135X variants can transmit from HLA-B*51-positive patients to HLA-B*51negative individuals and can persist even in the absence of HLA-B*51-restricted CTL pressure. Overall, I135X mutations were observed at a high frequency in the treatment-naive patients in Japan, and the most frequent amino acid was I135T (35.1%), which was more frequent than the wild-type I135 (32.0%).

Induction of efavirenz-resistant HIV-1

As described above, I135L, I135V, I135T, and I135R mutations were detected in treatment-naive patients. In order to analyze their effects on the mutation pattern for NNRTI resistance, EFV resistance was induced from monoclonal HIV-1s harboring each of these mutations by culturing them in the presence of increasing concentrations of EFV. These induction experiments were performed independently in triplicate. In one of the three induction experiments on HIV-1_{I135L}, V179D emerged when EFV concentration reached 100 nmol/l, as well as emergence of K103R in the presence of EFV at 1000 nmol/l (Fig. 1a). We previously reported that the combination of K103R and V179D confers significant resistance to NNRTIs [12]. In another experiment, V108I emerged at an EFV concentration of 100 nmol/l and L100I at an EFV of 1000 nmol/l (Fig. 1b). Both L100I and V108I are listed in the International AIDS Society (IAS)-USA Resistance Table [20] as EFV resistance mutations. In the last experiment on HIV-1_{I135L}, G190A emerged followed by V106A (Fig. 1c). The latter two are also listed in the IAS-USA Table. In one of the three induction experiments on HIV-1_{I135V}, E138K emerged at an EFV of 100 nmol/l and L100I at an EFV of 1000 nmol/l (Fig. 1d). E138K is a rare mutation and not listed as a resistance mutation in the IAS-USA Table. It was reported that E138K alone did not alter drug susceptibility significantly, though it emerged during resistance induction experiments with ETR and other experimental NNRTIs (Brillant *et al.* 13th International HIV Drug Resistance Workshop, 2004; Su *et al.* 16th International HIV Drug Resistance Workshop, 2007) [21–23]. L100I emerged first followed by Y188H in another experiment, and L100I emerged first followed by V108I in the last experiment (Figure 1e and f). In one of the three induction experiments on HIV-1_{I135T}, V108I emerged at an EFV of 100 nmol/l and K101E at an EFV of 1000 nmol/l (Fig. 2a). In another experiment, V106I emerged first followed by V179D (Fig. 2b). The combination of V106I and V179D was confirmed to confer a significant NNRTI resistance by our group (unpublished data). In the last experiment, V108I emerged first followed by E138K and L100I (Fig. 2c). In one of the three induction experiments on HIV-1_{I135R}, L100I emerged at an EFV of 100 nmol/l followed

Table 1. Frequency of amino acids at codon 135 of HIV-1 reverse transcriptase in human leukocyte antigen-B*51-positive and human leukocyte antigen-B*51-negative patients.

135th amino acid	I	L	V	T	R
B*51 (+)/17 (%)	1 (5.9)	3 (17.6)	1 (5.9)	12 (70.6)	0 (0)
B*51 (−)/80 (%)	30 (37.5)	13 (16.3)	11 (13.8)	22 (27.5)	4 (5.0)
Total/97 (%)	31 (32.0)	16 (16.5)	12 (12.4)	34 (35.1)	4 (4.1)

HLA type was determined by standard sequence-based genotyping. HLA, human leukocyte antigen.

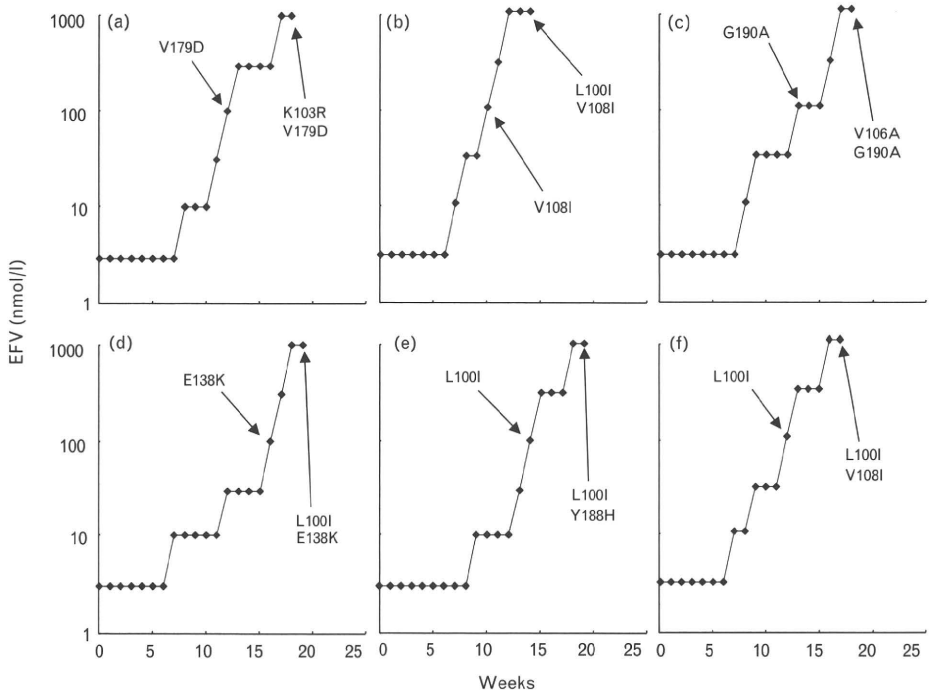

Fig. 1. Induction of efavirenz resistance from HIV-1_{I135L} and HIV-1_{I135V}. HIV-1_{I135L} (a–c) and HIV-1_{I135V} (d–f) were propagated in MT-2 cells in the presence of increasing concentrations of EFV. The induced amino acid substitutions were analyzed at several passages by sequencing proviral HIV-1 *RT* gene in MT-2 cells. EFV, efavirenz; RT, reverse transcriptase.

by E138K at an EFV of 1000 nmol/l (Fig. 2d). In another experiment, E138K emerged first then G190A and V108I (Fig. 2e). In the last experiment, L100I emerged first followed by K101E (Fig. 2f). In summary, during the induction experiments, all the induced mutations were already known NNRTI-resistance mutations except for E138K, which emerged in one of the three induction experiments on HIV-1_{I135V}, in one of the three induction

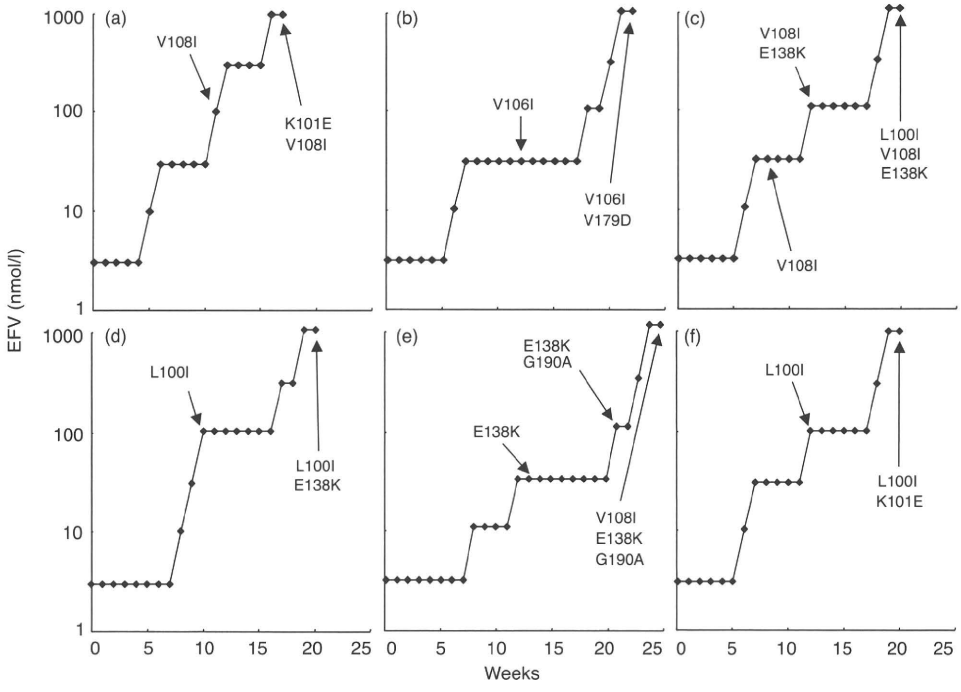

Fig. 2. Induction of efavirenz resistance from HIV-1_{I135T} and HIV-1_{I135R}. HIV-1_{I135T} (a–c) and HIV-1_{I135R} (d–f) were propagated in MT-2 cells in the presence of increasing concentrations of EFV. The induced amino acid substitutions were analyzed at several passages by sequencing proviral HIV-1 *RT* gene in MT-2 cells. EFV, efavirenz; RT, reverse transcriptase.

experiments on HIV-1_{I135T}, and in two of the three induction experiments on HIV-1_{I135R}. We also performed EFV-resistance induction experiments on HIV-1_{WT} in triplicate using the same procedure. All the induced mutations were already known NNRTI-resistance mutations, whereas E138K did not emerge in any of the three induction experiments on HIV-1_{WT} (data not shown).

Nonnucleoside reverse transcriptase inhibitor resistance conferred by E138K combined with I135X

During the induction experiments on HIV-1s harboring I135X, the emergence of E138K, which is usually a rare mutation, was often observed. To analyze the effects of E138K alone and its combination with I135X on NNRTI susceptibility, a panel of recombinant HIV-1 clones was constructed and their IC_{50} values for EFV, NVP, and ETR were determined. As expected, I135X alone did not confer significant NNRTI resistance (Table 2). The combination of I135T and E138K (I135T/E138K) conferred significant resistance to EFV, NVP, and ETR, though E138K alone did not change NNRTI susceptibility as reported previously (Su *et al.* 16th International HIV Drug Resistance Workshop, 2007) [22,23]. I135L/E138K and I135R/E138K conferred significant resistance to NVP and ETR, respectively. In summary, E138K conferred significant resistance when combined with some of the I135X mutations, especially I135T, which is the most prevalent in treatment-naive individuals in Japan (Table 1).

Structural modeling of reverse transcriptase harboring I135T and E138K

The in-vitro drug susceptibility assay described above showed that I135T/E138K conferred the most efficient resistance to EFV and significant resistance to NVP and ETR. To analyze the molecular mechanisms by which E138K combined with I135T alter NNRTI susceptibility, we conducted a structural analysis that included computational methods. A total of 12 structural models of reverse transcriptase-NNRTI complexes were constructed with four reverse transcriptases (wild-type, I135T, E138K, and I135T/E138K) and three NNRTIs (EFV, NVP, and ETR). We first calculated the binding energies between reverse transcriptase and NNRTI. Differences in the binding energies between mutant and wild-type complexes ($\Delta\Delta$Gb) were calculated using the models. The $\Delta\Delta$Gb value correlated positively with the logarithm of fold resistance value obtained by our in-vitro drug susceptibility assay described above: a greater reduction in the binding energy correlated with a greater resistance ($r = 0.77$, $P < 0.02$) [24], suggesting that our modeling appropriately reflects the actual binding mode between the reverse transcriptase molecule and NNRTI. In the 12 models tested, the $\Delta\Delta$Gb value of the I135T/E138K RT-NNRTI complex was persistently larger than wild-type and single mutation reverse transcriptases, indicating that I135T/E138K caused a larger loss of interactions between reverse transcriptase and NNRTI than the single mutations. We then examined the structural changes in the loss of interactions by I135T/E138K. In the wild-type reverse transcriptase, the E138 positioned relatively closely to the EFV, which could contribute to the generation of van der Waals and electrostatic interactions between reverse transcriptase and NNRTI (Fig. 3a). The I135T single substitution caused no significant changes in the steric position of the E138 side chain (Fig. 3b). E138K substitution caused significant changes in the steric position of the E138 side chain (Fig. 3c), whereas the calculated van der Waals energy was similar to that of wild-type reverse transcriptase. I135T/E138K also caused significant changes in the steric position of the K138 side chain, but the orientation of the side chain was different from that of the E138K single mutant reverse transcriptase, possibly due to the interactions between T135 and K138 (Fig. 3d). The K138 conformation in the $RT_{I135T/E138K}$ generated a steric gap between K138 and EFV, and significantly reduced van der Waals energy. In addition, the conformational change necessitated increased electrostatic energy of the reverse transcriptase–EFV complex. These data suggest that an appropriate steric position of the 138th residue is critical for the generation

Table 2. Nonnucleoside reverse transcriptase inhibitor susceptibility of recombinant HIV-1 sequences.

	Mean IC_{50} (μmol/l) ± SD (fold resistance*)		
HIV-1	EFV	NVP	ETR
Wild-type	0.002 ± 0.0007	0.05 ± 0.01	0.0012 ± 0
I135L	0.003 ± 0.0005 (1.5)	0.07 ± 0.01 (1.4)	0.0012 ± 0.0002 (1)
I135V	0.0024 ± 0.0003 (1.2)	0.04 ± 0.01 (0.8)	0.0011 ± 0.0001 (0.9)
I135T	0.002 ± 0.001 (1)	0.06 ± 0.01 (1.2)	0.0016 ± 0.0002 (1.3)
I135R	0.003 ± 0.001 (1.5)	0.03 ± 0.01 (0.6)	0.0012 ± 0.0002 (1)
E138K	0.004 ± 0.0004 (2)	0.08 ± 0.01 (1.6)	0.0026 ± 0.0001 (2.2)
I135L/E138K	0.003 ± 0.001 (1.5)	0.23 ± 0.02 (4.6)	0.0033 ± 0.0006 (2.8)
I135V/E138K	0.006 ± 0.001 (3)	0.04 ± 0.01 (0.8)	0.0033 ± 0.0006 (2.8)
I135T/E138K	0.014 ± 0.002 (7)	0.19 ± 0.06 (3.8)	0.005 ± 0.0002 (4.2)
I135R/E138K	0.005 ± 0.002 (2.5)	0.14 ± 0.04 (2.8)	0.0047 ± 0.0003 (3.9)

The drug susceptibility assay was performed in triplicate and repeated three times. EFV, efavirenz; ETR, etravirine; NVP, nevirapine.
*Fold resistance was calculated by comparing viral IC_{50} with that of wild-type HIV-1.

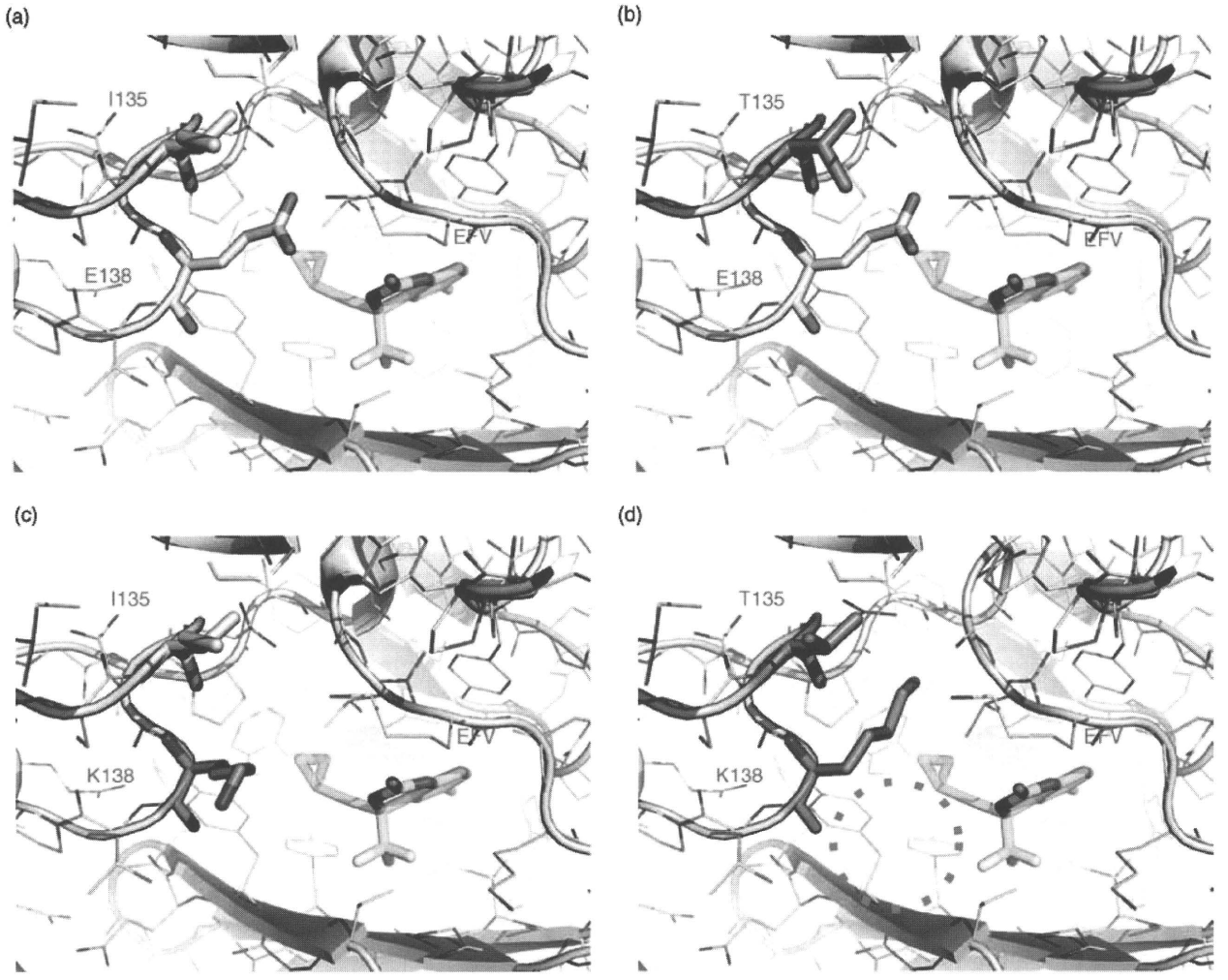

Fig. 3. Structural models of HIV-1 reverse transcriptase–efavirenz complexes. The binding clefts of four complex models are shown. (a) $RT_{wild\text{-}type}$, (b) RT_{I135T}, (c) RT_{E138K} and (d) $RT_{I135T/E138K}$. Sticks indicate the amino acids at positions 135 and 138 of RT, and the atoms of EFV. The mutated residues (I135T and E138K) and the EFV atoms are highlighted with orange and cyan sticks, respectively. The dotted circle in panel D indicated the enlarged gap by I135T/E138K mutations. EFV, efavirenz; RT, reverse transcriptase.

of an optimal EFV binding pocket, and that I135T/E138K, but not the single mutations, effectively break the binding pocket for EFV.

Discussion

As the HIV-1 pandemic progresses, viral genetic diversity is increasing and becoming geographically heterogeneous [25,26]. We recently indicated that HIV-1 adapts to CTL by acquiring escape mutations in the CTL epitopes, and that such escape variants are increasing in the populations at an alarming high rate of corresponding HLA alleles [4]. When escape mutations occur in drug target proteins, they may alter the mutation patterns of drug resistance even if they do not confer drug resistance themselves. In this study, we focused on I135X in reverse transcriptase, which are escape mutations of HLA-B*51-restricted CTL, because I135X are the prevailing mutations and accumulating in Japan, where the frequency of HLA-B*51 is high (~20%). Cultural passages of HIV-1 sequences harboring I135X in the presence of increasing concentrations of EFV induced the emergence of E138K, which is not listed as a resistance mutation in the IAS-USA Table. The analysis of recombinant HIV-1 sequences showed that the combination of E138K and some of the I135X, especially I135T, which is most frequent, conferred significant resistance to NNRTI, though solo E138K did not alter drug susceptibility significantly. However, E138K did not always emerge in triplicate experiments of EFV-resistance induction from HIV-1 sequences harboring I135X, whereas the already known NNRTI-resistance mutations emerged. Importantly, variable mutation patterns emerged under the same conditions of resistance induction experiments,

indicating that the drug selective pressure is one of the driving forces making the genetic diversity of HIV-1 at population levels as CTL pressure does (HLA-B*51-restricted CTL pressure selects not only I135T but also other I135Xs).

In clinical data, Richard *et al.* [27] examined HIV-1 reverse transcriptase sequences in treated Ugandans. In their longitudinal cohort, the HIV-1 infecting one patient (JLT05) acquired I135T/E138K during EFV-containing treatment without any other NNRTI resistance-associated mutations (GenBank: AY556834). Marconi *et al.* [28] performed genotypic resistance testing in patients who experienced virologic failure during their first antiretroviral therapy, and the HIV-1 in one patient (SW065) was found to have I135T/E138K after the failure of EFV-containing treatment (EU308076). In tipranavir clinical trials, the HIV-1s of seven cases who experienced NNRTI treatment failure harbored I135T/E138K (DQ880123, DQ880358, DQ879290, DQ880378, DQ877823, DQ878145, and DQ878874) [29]. These data indicate that I135T/E138K confers significant NNRTI resistance *in vivo* also, suggesting that HLA-B*51-restricted pressure may alter the mutation patterns of NNRTI resistance by inducing escape mutations.

Evidences for the interactions between CTL and drug resistance mutations are accumulating [30–34]. Considering that HIV-1 adapts to particular human HLA alleles and evolves among infected individuals, drug mutation patterns may be affected and altered in currently prevailing viruses. Analysis of drug resistance mutations and development of new antiretroviral agents against laboratory HIV-1 strains derived from isolates obtained decades ago may not always be a suitable strategy. The use of recently obtained clinical isolates may be critical and indispensable in some studies.

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H.G. designed and executed the study, analyzed the data and wrote the manuscript. H.O. and H.S. performed computational analysis and wrote the manuscript. A.H. and T.H. executed the study and collected data. M.T. provided the hypothesis and participated in discussion and review. S.O. participated in discussion and review and supervised the study.

There are no conflicts of interest.

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Combination of V106I and V179D Polymorphic Mutations in Human Immunodeficiency Virus Type 1 Reverse Transcriptase Confers Resistance to Efavirenz and Nevirapine but Not Etravirine[▽]†

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Etravirine (ETV) is a second-generation nonnucleoside reverse transcriptase (RT) inhibitor (NNRTI) introduced recently for salvage antiretroviral treatment after the emergence of NNRTI-resistant human immunodeficiency virus type 1 (HIV-1). Following its introduction, two naturally occurring mutations in HIV-1 RT, V106I and V179D, were listed as ETV resistance-associated mutations. However, the effect of these mutations on the development of NNRTI resistance has not been analyzed yet. To select highly NNRTI-resistant HIV-1 *in vitro*, monoclonal HIV-1 strains harboring V106I and V179D (HIV-1_{V106I} and HIV-1_{V179D}) were propagated in the presence of increasing concentrations of efavirenz (EFV). Interestingly, V179D emerged in one of three selection experiments from HIV-1_{V106I} and V106I emerged in two of three experiments from HIV-1_{V179D}. Analysis of recombinant HIV-1 clones showed that the combination of V106I and V179D conferred significant resistance to EFV and nevirapine (NVP) but not to ETV. Structural analysis indicated that ETV can overcome the repulsive interactions caused by the combination of V106I and V179D through fine-tuning of its binding module to RT facilitated by its plastic structure, whereas EFV and NVP cannot because of their rigid structures. Analysis of clinical isolates showed comparable drug susceptibilities, and the same combination of mutations was found in some database patients who experienced virologic NNRTI-based treatment failure. The combination of V106I and V179D is a newly identified NNRTI resistance pattern of mutations. The combination of polymorphic and minor resistance-associated mutations should be interpreted carefully.

Human immunodeficiency virus type 1 (HIV-1) sequences differ among infected individuals, and there are a number of naturally occurring amino acid changes commonly found in treatment-naïve patients (3, 23, 28). These polymorphic changes can occur even in genes that encode drug target proteins, and in fact, some drug resistance-associated mutations in protease genes are often present in treatment-naïve individuals, especially in non-subtype B clade-infected individuals (13, 15, 22). Minor resistance mutations, which are considered to compensate for the impaired replication fitness of viruses containing major resistance mutations, do not have a substantial effect on the viral phenotype by themselves (14, 27). In the reverse transcriptase (RT) coding region, drug resistance-associated mutations were detected at a low frequency in treatment-naïve individuals regardless of the HIV-1 clade. However, etravirine (ETV), a second-generation nonnucleoside RT inhibitor (NNRTI), has been available in the clinical setting and the following have been listed as ETV resistance-associated mutations in RT: V90I, A98G, L100I, K101E/H/P, V106I, E138A, V179D/F/T, Y181C/I/V, G190S/A, and M230L (14). V106I and V179D are often found in treatment-naïve individuals but are considered to have no substantial impact on NNRTI-containing treatment by themselves. ETV exhibits activity against many viruses that are resistant to first-line NNRTIs, including efavirenz (EFV) and nevirapine (NVP), and shows clinical efficacy in salvage treatment after NNRTI treatment failure (18, 21). However, it is possible that EFV- and NVP-resistant viruses derived from HIV-1 harboring V106I or V179D could compromise the efficacy of ETV. To determine the impact of these polymorphic mutations on the mutation patterns of NNRTI resistance, EFV-resistant HIV-1 strains were selected *in vitro* from monoclonal viruses harboring V106I and V179D, respectively. The virologic effects of selected specific mutation patterns were analyzed by constructing recombinant HIV-1 clones, and their clinical relevance was confirmed by analysis of isolates from infected individuals.

MATERIALS AND METHODS

HIV-1 sequences and clinical isolates from treatment-naïve individuals. HIV-1 RT sequences were analyzed in 364 antiretroviral treatment-naïve infected individuals who visited the outpatient clinic of the AIDS Clinical Center, International Medical Center of Japan, in 2007 and 2008 and gave written informed consent to this study. Viral RNA was extracted from stocked plasma samples, and the HIV-1 RT coding region was amplified by RT-PCR and nested PCR using previously published primer pairs (7, 10, 11). Direct sequencing was performed using dye terminators, and the HIV-1 subtypes of the sequences obtained were determined by the neighbor-joining method. Clinical HIV-1 isolates were obtained using MAGIC-5 cells (CCR5- and CD4-expressing HeLa-LTR-β-D-gal cells) from fresh plasma samples collected from seven of the above-mentioned treatment-naïve patients and stored at −80°C until use (9).

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TABLE 1. Frequencies of amino acids at positions associated with NNRTI resistance mutations in HIV-1 RT in treatment-naïve patients

Position	Amino acid, frequency [*n* (%)/364][a]						
90	V, 361 (99.2)	I, 3 (0.8)					
98	A, 344 (94.5)	S, 20 (5.5)					
101	K, 352 (96.7)	Q, 8 (2.2)	R, 3 (0.8)	E, 1 (0.3)			
103	K, 353 (97.0)	R, 7 (1.9)	N, 2 (0.5)	Q, 2 (0.5)			
106	V, 355 (97.5)	I, 9[b] (2.5)					
108	V, 362 (99.5)	I, 2 (0.5)					
138	E, 361 (99.2)	K, 1 (0.3)	A, 1 (0.3)	G, 1 (0.3)			
179	V, 312 (85.7)	D, 21[c] (5.8)	I, 21 (5.8)	E, 4 (1.1)	A, 4 (1.1)	T, 1 (0.3)	N, 1 (0.3)

[a] Only wild-type amino acids (L, Y, Y, G, P, and M, respectively) were identified at the 100th, 181st, 188th, 190th, 225th, and 230th positions of HIV-1 RT.
[b] Including two cases with V90/A98/K101Q/K103/V106I/V108/E138/V179, two cases with V90/A98/K101/K103/V106I/V108/E138/V179I, one case with V90/A98/K101/K103/V106I/V108/E138/V179D, and four cases with V90/A98/K101/K103/V106I/V108/E138/V179.
[c] Including 2 cases with V90/A98S/K101/K103/V106/V108/E138/V179D, 1 case with V90/A98/K101E/K103R/V106/V108/E138/V179D, 1 case with V90/A98/K101/K103R/V106/V108/E138/V179D, 1 case with V90/A98/K101/K103/V106I/V108/E138/V179D, and 16 cases with V90/A98/K101/K103/V106/V108/E138/V179D.

Generation of recombinant HIV-1 strains. The desired mutations were introduced into the XmaI-NheI region of pTZNX, which encodes Gly-15 to Ala-267 of HIV-1 RT (strain BH 10), by the oligonucleotide-based mutagenesis method (10, 16). The XmaI-NheI fragment was inserted into pNL_{H219Q}, which was modified from pNL101 and encoded the full genome of HIV-1 strain BH 10. pNL_{H219Q} harbors the H219Q mutation in the HIV-1 Gag region, which facilitated HIV-1 replication in MT-2 and H9 cells (6, 8). HIV-1 derived from pNL_{H219Q} was used as the wild type. Determination of the nucleotide sequences of the plasmids confirmed that each clone had the desired mutations but was devoid of unintended mutations. Each molecular clone was transfected into COS-7 cells with the GenePORTER Transfection Reagent (Gene Therapy Systems, San Diego, CA), and the virions obtained were harvested 48 h after transfection and stored at −80°C until use.

Selection of EFV-resistant HIV-1. The infectious HIV-1 clones harboring the V106I (HIV-1_{V106I}) and V179D (HIV-1_{V179D}) mutations in their RTs were propagated in MT-2 cells in the presence of increasing concentrations of EFV (7, 8, 31). Briefly, MT-2 cells (1×10^5) were exposed to 500 blue-cell-forming units (BFU) in MAGIC-5 cells containing HIV-1_{V106I} and HIV-1_{V179D} and cultured in the presence of EFV at an initial concentration of 3 nM. Viral replication was monitored by observation of the cytopathic effect in MT-2 cells. The culture supernatant was harvested on day 7 of culture and used to infect fresh MT-2 cells for the next round of culture. When the virus began to propagate in the presence of the drug, the drug concentration was increased by 0.5-log-fold. This selection was carried out for a total of 14 passages. The proviral HIV-1 RT coding region in infected MT-2 cells was amplified and sequenced at several passages.

Drug susceptibility assay. EFV, NVP, and ETV were generously provided by Merck Co., Inc. (Rahway, NJ), Boehringer Ingelheim Pharmaceutics Inc. (Ridgefield, CT), and Tibotec Pharmaceuticals (Little Island, Co., Cork, Ireland), respectively. Recombinant and isolated HIV-1 susceptibility to EFV, NVP, and ETV was determined in triplicate using MAGIC-5 cells (10, 12, 16). Briefly, MAGIC-5 cells were infected with an adjusted virus stock (300 BFU) in various concentrations of NNRTIs, cultured for 48 h, fixed, and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Takara Shuzo, Ohtsu, Japan). The blue-stained cells were counted under a light microscope. The drug concentrations that inhibited 50% of the stained cells of a drug-free control (EC_{50}s) were determined by referring to the dose-response curve. The drug susceptibility assay was performed in triplicate and repeated three times. Fold resistance was calculated by comparing the viral EC_{50} with that of monoclonal wild-type HIV-1 (HIV-1_{WT}). Drug resistance was judged significant when it was higher than threefold.

Viral replication kinetics assay. MT-2 cells (1×10^5) were exposed to each infectious virus preparation (500 BFU) for 2 h, washed twice with phosphate-buffered saline (PBS), and cultured in the presence or absence of 10 nM EFV, 100 nM NVP, or 10 nM ETV. The culture supernatants were harvested every other day, and p24 Gag amounts were determined with a chemiluminescence enzyme immunoassay kit (Fuji-Rebio, Tokyo, Japan). Replication assays were performed in triplicate and repeated three times using independently generated virus preparations (7).

Competitive HIV-1 replication assay. Freshly prepared H9 cells (3×10^5) were exposed to mixtures of paired virus preparations (300 BFU each) to be examined for their replication ability for 2 h, washed twice with phosphate-buffered saline (PBS), and cultured in the absence or presence of 10 nM EFV or 100 nM NVP as described previously (7, 17). On day 1, one-third of the infected H9 cells were harvested and washed twice with PBS, and proviral DNAs were sequenced (0 week). Every 7 days, the supernatant of the virus culture was transferred to new uninfected H9 cells; the cells harvested at the end of each passage were subjected to direct DNA sequencing of the HIV-1 RT coding region, and the change in the viral population was determined by the relative peak height on the sequencing electrogram. The persistence of the original amino acid substitution was confirmed at the end of the assay.

Structure modeling. We constructed 18 structural models of the HIV-1 RT and NNRTI complex by computational analysis. First, we constructed the initial models of wild-type RT with one of three NNRTIs by homology modeling using Molecular Operating Environment 2007.09.02 (Chemical Computing Group, Montreal, Quebec, Canada). The crystal structures of RT with NNRTIs (Protein Data Bank codes 1IKW [20], 1VRT [26], and 1SV5 [4]) were used as template structures. The homology modeling enabled the building of missing atoms in template structures. The ff94 force field and distance-dependent electrostatic energy function were applied in the modeling. Next, we refined the initial models by energy minimization using the sander module of the AMBER9 software package in two steps. In the first step, energies for the NNRTIs in the complex models were minimized in the gas phase by the conjugated gradient method. When the energy was not converged until 10,000 steps, this step was ignored. In the second step, energies of whole structures were converged up to 0.5 kcal/mol/Å by 50 steps of the steepest-descent method and the subsequent conjugated gradient method under implicit water solvent conditions. In each minimization, the AMBER ff03 (5, 19), the general AMBER force field (30), and the generalized Born implicit solvent surface area method (IGB = 2) (24) were applied for potential energy calculations. The cutoff for long-distance interaction energy was set at 15.0 Å. The charge and type of every atom in NNRTIs were automatically assigned using the AMBER9 Antechamber module. We also constructed five mutant RTs with the NNRTIs by considering every possible conformer of the respective mutant models. The possible conformers were generated from the wild-type homology models using PyMOL ver. 0.99rc6 (http://www.pymol.org). V106A, V106I, and V179D mutants had one, three to five, and five possible conformers, respectively. The structural model of each conformer was refined by a method similar to that used in the wild-type models. Among the refined conformers, we selected the conformer with the lowest energy as each mutant model.

RESULTS

Frequencies of NNRTI resistance mutations in treatment-naïve individuals. To determine the frequency of NNRTI resistance-associated mutations, the HIV-1 RT coding region was analyzed and the viral subtype was determined in 364 treatment-naïve infected individuals. The most frequent subtype was clade B (*n* = 334; 91.8%), followed by clade AE (*n* = 20; 5.5%). Clades C and G were also found and at similar low frequencies (*n* = 5; 1.4%). Variable amino acid substitutions were identified at the positions of NNRTI resistance-associated mutations, including the 90th, 98th, 101st, 103rd, 106th, 108th, 138th, and 179th, though only the wild-type amino acids were observed at the 100th, 181st, 188th, 190th, 225th, and 230th positions of HIV-1 RT (Table 1). K103N and V108I are