High resistance to reverse transcriptase inhibitors among persons infected with human immunodeficiency virus type 1 subtype circulating recombinant form 02_AG in Ghana and on antiretroviral therapy

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Abstract
This study sought to determine the dominant circulating human immunodeficiency virus type 1 (HIV-1) subtype and associated drug resistance mutations in Ghana.

This cross-sectional study was conducted with archived samples collected from patients who received care at 2 hospitals in Ghana from 2014 to 2016. Blood samples were earlier processed into plasma and peripheral blood mononuclear cells and stored at –80°C. Ribonucleic acid (RNA) was extracted from the archived plasma. Two HIV-1 genes; protease and reverse transcriptase, were amplified, sequenced using gene-specific primers and analyzed for subtype and drug resistance mutations using the Stanford HIV Database.

Of 16 patient samples successfully sequenced, we identified the predominance of HIV-1 subtype CRF02_AG (11/16, 68%). Subtypes G (2/16, 13%), dual CRF02_AG/G (2/16, 13%), and CRF01_AE (1/16, 6%) were also observed. Major nucleoside reverse transcriptase inhibitor (NRTI) resistance mutations, M184V/I, D67N, T215F, and K70R/E were found. Non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance mutations, K103N, Y181C, V90I, F227L, and V106A were also prevalent. Additionally, and at a lower level, protease inhibitor (PI)-resistance mutations, M46I, I54V, V82A, L90M, and I71V, were also present in the sequences from antiretroviral therapy (ART)-experienced individuals. Two NRTI-associated drug resistance mutations (DRMs) (D67N and T69N) were present in sequences from 1 ART-naive individual.

HIV-1 subtype CRF02_AG was most frequently detected in this study thus confirming earlier reports of dominance of this subtype in the West-African sub-region and Ghana in particular. The detection of these drug resistance mutations in individuals on first-line regimen composed of NRTI and NNRTI is an indication of prolonged drug exposure without viral load monitoring. Routine viral load monitoring is necessary for early detection of virologic failure and drug resistance testing will inform appropriate choice of regimens for such patients.

Abbreviations: 3TC = Lamivudine, AIDS = acquired immune deficiency syndrome, ART = antiretroviral therapy, AZT = Zidovudine, CRF02_AG = circulating recombinant form 02_AG, DRM = drug resistance mutation, EDTA = ethylene diamine tetraacetic acid, EFV = Efavirenz, HAART = highly active antiretroviral therapy, HIV-1 = human immunodeficiency virus type 1, HIV-2 = human immunodeficiency virus type 2, IRB = Institutional Review Board, NNRTI = non-nucleoside reverse transcriptase inhibitor, NRTI = nucleoside reverse transcriptase inhibitor, NVP = Nevirapine, PBMC = peripheral blood mononuclear cells, PI = protease

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1. Introduction

Human immunodeficiency virus (HIV) and its associated acquired immunodeficiency syndrome (AIDS) remain global health challenges, especially in Sub-Saharan Africa, the region with about 70% of the global disease burden. The joint United Nations programme on HIV/AIDS (UNAIDS), estimates the number of people living with HIV worldwide in 2015 to be 36.7 million, with 9 million of them in Eastern and Southern Africa, and 6.5 million in Western and Central Africa. In Ghana, the national HIV/AIDS prevalence in 2015 was estimated at 1.47%, with the Eastern Region having the highest prevalence of 3.7% followed by the Greater Accra region with 3.1%. To effectively tackle this epidemic, it is necessary to continually monitor factors that influence the epidemiology of the disease.

HIV-1 is genetically diverse, with variations of 25% to 35% and 15% to 20% between and within subtypes, respectively. This diversity arises from many factors, including the rapid replication of HIV-1 in vivo and the error prone nature of the HIV reverse transcriptase. The genetic diversity of HIV has very important clinical implications, with the infecting HIV-1 subtype linked to differing rates of disease progression. In a study with a prospective cohort of 615 seroconvertors in Sub-Saharan Africa, it was found that patients with HIV-1 subtype C infection progressed faster to a selected Cluster of Differentiation 4 + (CD4 +), viral load, and clinical AIDS endpoints than subtype A infected patients. Another study with South African women who were infected with HIV-1 found faster disease progression associated with subtype C infections, and subtype D infections also progressed faster to viral load endpoint and twice as fast to clinical AIDS than subtype A. Infecting subtypes have also been linked to the rate of CD4 + decline, diagnosis, transmission, and even response to treatment. In Western Africa, CFR02_AG and A are the most common circulating subtypes, with the recombinant subtype (CFR02_AG) dominating in Ghana.

A major problem with HIV-1 management has been drug resistance. Since the upscale of Highly Active Anti-Retroviral Therapy (HAART), there has been a documented gradual increase in antiretroviral drug resistance in Sub-Saharan Africa, and these findings and patterns have been reviewed several times. Previous research in Ghana has shown low prevalence (5%) of drug resistance, with some studies reporting no transmitted drug resistance mutations, while others observed only minor mutations L10I, L10V, V11I, and E35G in 4 patients and V179E in another. Two major drug resistance mutations (M184V and Y181C) in 1 patient and M46L another were observed in the threshold survey. An increased coverage of antiretroviral therapy in Ghana, from 29% in 2012 to an estimated 50% in 2016, is likely that the prevalence of antiretroviral drug resistance has also seen an increase.

The purpose of this study was to determine the circulating HIV-1 subtypes and examine antiretroviral resistance in patients after a decade of introducing antiretroviral therapy in Ghana.

2. Methods

This study investigated HIV-1 subtypes and genotypic drug resistance as part of a larger study on the role of T cells in the persistence and progression of HIV-1 infection. The study protocol was approved by the Institutional Review Board (IRB) of Noguchi Memorial Institute for Medical Research, University of Ghana, Legon (IRB approval # CPN 089/15–16), and the study aim was explained to all study participants who provided informed consent for their enrollment.

2.1. Study population

Patients who were infected with HIV-1 were enrolled as study participants from Korle Bu Teaching Hospital, Accra in the Greater Accra Region and Koforidua Regional Hospital, Koforidua in the Eastern Region from 2014 to 2016. Both hospitals provide support and care, including antiretroviral therapy (ART), to HIV infected patients in Ghana. Clinical and demographic data were obtained from hospital records for all the patients who were enrolled in the study. Overall, 80 consenting HIV-1 infected patients were recruited for the study, and the participants were grouped according to ART exposure, ART regimen, and rate of disease progression as described previously. All patients were previously diagnosed as having HIV-1 using the national testing algorithm that included 2 rapid assays First Response HIV-1/2 (Premier Medical Corporation Limited, India) and OraQuick Rapid Antibody HIV1/2 Test (OraSure Technologies Inc., Pennsylvania, USA). Seventeen (17) participants who had undetectable or extremely low HIV-1 RNA copies/mL were therefore excluded from the study.

2.2. Sample collection and processing

In this cross-sectional study, venous blood (7mL) was collected, once from the patients, into tubes containing ethylene diamine tetraacetic acid (EDTA) (Becton-Dickinson, Mountain view, CA) and transported in cool boxes to the Virology Department of the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, where all molecular analyses were performed. The blood was processed to obtain plasma and peripheral blood mononuclear cells (PBMCs) using Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient separation. Plasma and PBMCs obtained were stored at −80°C until use.

2.3. CD4+ cell count and HIV-1 viral load determination

The CD4+ cell count and plasma HIV-1 viral load were determined at the clinical laboratory immediately after sample collection. The CD4+ cell counts were determined using FACSScalibur flow cytometer (BD Biosciences, San Jose, CA) and data acquisition was done using CellQuest software (New Jersey, USA). Plasma HIV-1 RNA was quantified by the COBAS Ampli-Prep/COBAS AMPLICOR HIV-1 Monitor Test, version 2.0, according to the manufacturer’s instructions, with limit of detection of 20 copies/mL to determine the viral load.
2.4. Viral RNA extraction, RT-PCR, and nested PCR

Viral RNA was extracted from plasma samples using the QIAamp Viral RNA Mini kit (QIAGEN, Maryland, USA) following the manufacturer’s protocol, and stored at −80°C until use. Reverse transcription polymerase chain reaction (RT-PCR) of the reverse transcriptase (RT) and protease (PR) genes was done using QIAGEN One Step RT-PCR Kit according to the manufacturer’s instructions. The RT-PCR products were further amplified through nested PCR using the OneTaq PCR Master Mix according to the manufacturer’s instructions using previously reported cycling conditions. Primers used have been previously described (Supplementary Digital Content 2). Phylogenetic analysis and HIV-1 subtype information and drug resistance mutations were done using MEGA software version 5.

2.5. HIV-1 subtypes and drug resistance mutations

HIV-1 subtype information and drug resistance mutations were obtained by submitting after analyzing the nucleotide sequences with the Stanford University HIVDB program. Phylogenetic trees were constructed using CLUSTAL-X and the neighbor-joining method in MEGA software version 5 (http://www.megasoftware.net).

3. Results

3.1. Demographic and clinical characteristics of the study participants

Eighty (80) participants were enrolled in this study to determine HIV-1 diversity and drug resistance mutations in HIV-1 from a group of ART-exposed or -naïve HIV-1 infected patients in Ghana. Approximately 81% (51/63) of the participants were on ART, out of which 74.5% (38/51) were on first line ART and 25.5% (13/51) on second line ART (Table 1). All fast progressors, with a CD4 count below 200 cells/mm³ within 3 years of infection, were on first line therapy. Figure 1 showed the summaries of viral load and CD4 counts among the different categories of participants studied. Nineteen percent (19%) of the participants were long-term non-progressors, patients who remain symptom-free with a CD4 cell count above 500 cells/mm³ without therapy for at least 8 years after their infection and had no ART exposure. The mean age of the participants was 45 years (IQR 38–50 years), and two-thirds (42/63) were women. Examination of clinical records indicated the previous and current ART regimens of the patients enrolled into the study. The records (Supplementary Digital Content 2) showed that therapy was dominated by Lamivudine, Zidovudine, Nevirapine, and Efavirenz.

3.2. Subtype and drug-resistance mutations analyses

Viral genes for the PR and RT encoding regions were amplified in 29 and 21 patient samples respectively by nested PCR, and 8 PR and 13 RT amplified products were successfully sequenced.

Subtype analyses of PR and RT genes from the 16 patients’ samples revealed CRF02_AG (N=11), subtype G (N=2), dual CRF02_AG/G (N=2), and CRF01_AE (N=1). Estimated evolutionary divergence between sequences in study participants is shown in Figs. 2 and 3.

Majority of the drug resistance mutations were in the RT gene against NRTI and NNRTI drug classes. The predominant major NRTI mutation was M184I/V, while K103N was the predominant NNRTI mutation (Table 2). One therapy-naïve patient showed evidence of drug resistance with D67N and T69N NRTI mutations. In therapy exposed patients, 3 (25%) had triple-class resistance to PI, NRTI, and NNRTI, and 3 (25%) had dual-class resistance to NRTI and NNRTI, and 1 patient each had mutations against PI and NNRTI only.

4. Discussion

This study investigated HIV-1 subtypes and drug resistance mutations among ART exposed and naïve patients in 2 health facilities in Ghana. Similar to previous studies, this study identified a predominance of CRF02_AG (11/16, 68%) in the study population, with subtype G (2/16, 13%), dual CRF02_AG/G (2/16, 13%), and CRF01_AE (1/16, 6%) also present. The CRF02_AG samples clustered around the Nigerian CRF02_AG reference strain (Ref.02 AG.NG), which suggests a close evolutionary relationship between the 2 strains (Fig. 2). The CRF01_AE subtype was distant from the reference Chinese and Afghan CRF01_AE reference subtypes, suggesting that either this recombinant subtype did not originate from the 2 reference subtypes or that it is gradually evolving (Fig. 3).

Overall, these findings confirm earlier reports that CRF02_AG is the predominant HIV-1 subtype in West Africa, and also suggest that other subtypes originally associated with HIV-1 infection in...
other geographical areas\(^{30}\) may contribute to HIV-1 infection in Ghana. Detection of dual CRF02_AG/G HIV-1 infection from the study population confirms the genetic diversity of HIV and the existence of recombinant forms among infected persons.

One of the causes of immunologic and virologic failure in patients on ART is the presence of pre-existing drug resistance mutations.\(^{31}\) In our study, one ART-naive patient had major NRTI mutation and this could affect the effectiveness of the first-line regimen when ART is initiated. Majority of our study patients (90%) had high viral load despite therapy for a mean of 3 years, indicative of virologic failure (Supplementary Digital Content 1, http://links.lww.com/MD/D716). The therapy regimen remained unchanged for these patients because viral load test was not done routinely for patients during their periodic visit to the hospital thus the Doctors did not have the viral load information. Our study made provision to measure viral load so this information only became available as a result of our study. Consequently, there was a high level of NRTIs and NNRTIs

#### Table 2

| Sample ID | PR subtype | PI mutation | PI resistance | RT subtype | NRTI mutation | NRTI resistance | NNRTI mutation | NNRTI resistance |
|-----------|------------|-------------|---------------|------------|---------------|----------------|----------------|-----------------|
| AR-14-01\(^a\) | CRF02_AG | L47I | LPV/r, NFV | CRF02_AG | M184I | 3TC, ABC, DDI | V90I, K103N, Y181C, H221Y | EFV, NVP |
| AR-14-03\(^a\) | CRF02_AG | K20I | None | None | N/A | None | None | None |
| AR-14-09\(^a\) | CRF02_AG | K20I | None | None | N/A | None | None | None |
| AR-14-13\(^a\) | CRF02_AG | M46I | LPV/r, NFV | G | M184V, T215Y | 3TC, ABC, AZT, D4T, DDI | Y181C | EFV, NVP |
| AR-15-06\(^a\) | CRF02_AG | K20I | None | None | N/A | None | None | None |
| AR-15-09\(^a\) | CRF02_AG | V32I | LPV/r, NFV | CRF02_AG | M184I | 3TC, ABC, AZT, D4T, DDI | V90I, K103N | EFV, NVP |
| EL-14-01\(^b\) | CRF02_AG | None | None | CRF01_AE | None | None | None | None |
| EL-14-05\(^b\) | CRF02_AG | None | None | CRF02_AG | None | None | None | None |
| EL-14-09\(^b\) | CRF02_AG | None | None | CRF02_AG | None | None | None | None |
| EL-14-12\(^b\) | CRF02_AG | None | None | CRF02_AG | None | None | None | None |
| FG-15-02\(^b\) | CRF02_AG | None | None | CRF02_AG | None | None | None | None |
| FG-15-04\(^b\) | CRF02_AG | None | None | G | D67N, K70E, M184V | 3TC, ABC, AZT, D4T | V106A, F227L | EFV, NVP |
| FG-15-05\(^b\) | CRF02_AG | None | None | G | D67N, K70E, M184V | 3TC, ABC, AZT, D4T | V106A, F227L | EFV, NVP |
| FG-15-14\(^b\) | CRF02_AG | None | None | G | None | None | None | None |
| AK-15-15\(^b\) | CRF02_AG | None | None | CRF02_AG | None | None | None | None |
| AK-15-16\(^b\) | CRF02_AG | None | None | CRF02_AG | None | None | None | None |

3TC=Lamivudine, ABC=Abacavir, AZT=Zidovudine, D4T=Stavudine, DDI=Didanosine, EFV=Efavirenz, LPVr=Lopinavir/Ritonavir, N/A=not applicable, NNRTI=non-nucleoside reverse transcriptase inhibitor, NRTI=nucleoside reverse transcriptase inhibitor, NVP=Niverapine, PI=Protease Inhibitor, PR=Protease, RT=Reverse transcriptase, TDF=Tenofovir. \(^{30}\)Low level resistance to underlined drug; \(^{31}\)intermediate level resistance to underlined drug; \(^{32}\)high level resistance to underlined drug.

\(^a\)K20I though a drug resistance mutation in reference HXB2 genome, I at amino acid position 20 is the consensus for CRF02_AG subtype.

\(^b\)Therapy naive patient.

\(^c\)Therapy experienced patient.

\(^d\)Patient on first line antiretroviral therapy.

\(^e\)Patient on second line antiretroviral therapy.
resistance mutations (dual-class) in patients on first-line ART, and triple-class resistance in patients on second-line ART. This high level drug resistance may be due to the drug pressure experienced by the patients who have been on therapy for a long period without viral load measurements. Our study provides points to the importance of routine viral load measurements for early detection of virologic failure. While third-line regimens are more expensive and not yet available in Ghana, the main solution for these patients would be to use another boosted PI and a second-generation NNRTI as previously suggested. It is suggested that routine CD4+ count and viral load estimations should be implemented to enhance early detection of treatment failure in Ghana to avoid a situation where individuals are maintained on failing drug regimens. It has been reported previously that, at sites with viral load monitoring, patients are switched to second-line drugs earlier than at sites without viral load monitoring. In the 2 hospitals, routine viral load monitoring of these HIV patients had been unavailable for 2 years preceding the study due to financial and logistical constraints, and so patients were inadvertently maintained on same “failing” drug regimens. To avert the situation, there is the need for the development of cheaper but effective assays for viral load monitoring and drug resistance testing, which will be a helpful tool for HIV patient management in resource-limited settings.

Genetic diversity of HIV has major implications on disease pathogenesis including infectivity, transmissibility, and development of drug resistance mutations. Mutations associated with NRTI, NNRTI, and PI resistance have previously been reported in CRF02_AG strains from Ghana’s neighboring countries, Ivory Coast and Togo. In the present study, CRF02_AG had mutations associated with resistance to NRTIs, NNRTIs, and PIs, subtype G had mutations associated with NRTIs and NNRTIs, while CRF01_AE had no resistance mutation. This suggests that the rate of antiretroviral drug resistance mutation selection may be influenced by viral genetic diversity, as previously observed.

The predominance of M184V and D67N among mutations associated with resistance to NRTIs in subtype G and CRF02_AG observed in this study. Supplementary Digital Content 3, http://links.lww.com/MD/D718) has been shown previously. M184V mutation causes high-level resistance to Lamivudine, and therefore the high frequency of the mutation observed in this study is not unusual since the drug is the most common NRTI in use in Ghana. M184V also increases susceptibility to Zidovudine, Stavudine, and Tenofovir, and is associated with a clinically significant reduction in HIV-1 replication in vivo. Due to the decrease in susceptibility, patients infected with HIV-1 with the M184V mutation continue treatment with Lamivudine, resulting in continuous exposure, and consequently, higher frequency of the mutation. The extended duration of treatment failure as a result of unavailability of viral load monitoring to inform change in therapy also accounts partly for the development of more/multiple mutations. Other mutations detected in this study, which have been shown to confer resistance to NRTIs, were M184I, T69N, L74I, M41L, K70R/E, T215Y/F, and K219E. These other mutations are thymidine analogue mutations (TAMs) which occur in patients on thymidine based drugs: Zidovudine and Stavudine. T215Y/F and K219E are major TAMs which give rise to high-level resistance to Zidovudine and Stavudine, especially when the
mutations occur in concert with accessory TAMs like M41L, D67N, and K70R.[27] The presence of the L74I mutation in combination with the M184V causes high-level resistance to both Abacavir and Didanosine.[37]

NNRTI mutations which confer intermediate to high level resistance to Nevirapine and Efavirenz were found in almost all the samples that had mutations. NNRTI associated mutations detected included V90I, K103N, Y181C, H221Y, K101H, G190A, V106A, F227L, and K238T. K103N and V106A individually can reduce susceptibility of HIV-1 to Nevirapine and Efavirenz by as much as 50-fold, and together with other mutations found in this study, high-level resistance can be acquired.[27] The present situation of resistance mutations suggest a looming crisis in HIV/AIDS management in Ghana, since the mutations which were identified confer resistance to most of the drugs recommended for use in the first-line regimen by the Ghana Health Service.[38]

In contrast to the high rate of mutations associated with resistance to NRTIs and NNRTIs, there were only a few mutations associated with PI resistance. The PI mutations from this study (Supplementary Digital Content 3, http://links.lww.com/MD/D718) are common ones which select for Lopinavir and Nelfinavir,[27,39]; the only PIs used in Ghana. As expected, only samples from patients who were on second line drug therapy had PI resistance conferring mutations.

For effective HIV management in the study population, boosted PI-based antiretroviral regimens might be a better alternative to NNRTI-based regimens when drug options are limited. Patient compliance is imperfect and viral load monitoring is infrequent in many HIV programs in resource-limited settings. Despite the low number of genotyped samples, this study shows high occurrence of RTI mutations in the study population and emphasizes the need to improve monitoring of ART resistance in Ghana. Monitoring the extent and significance of HIV-1 drug-resistance mutations in treatment-naive and exposed individuals is and will be key for an informed choice of optimal ART, and contribute to preventing the accumulation and spread of the resistance HIV-1 strains.

This study had some limitations: the sample size was small and the rate of amplification and sequencing of the 2 genes further reduced the numbers used in the final analysis thus the data presented cannot be generalized for the entire population of HIV infected persons in Ghana. Additionally, the cross-sectional nature of the study made it impossible to determine how the mutations observed emerged and whether the sequence of drug use could reduce the level of drug resistance observed in the study.
Finally, recombinant analysis was not performed and so unique recombinant forms were not detected in the study. Despite these limitations, the study has provided useful data on resistance mutations among patients on ART in Ghana and made recommendations that could be useful in reviewing treatment monitoring policies in the country since the same treatment guidelines are in use throughout the country.

5. Conclusion

This study confirms the dominance of CRF02 AG in HIV-1 infections in Ghana. It also points to the presence of other HIV-1 subtypes, notably CRF01 AE, which dominates in Asia. The study further found high NRTIs and NNRTIs resistance mutations and underscores the need for routine viral load and CD4+ cell count estimations to monitor treatment outcomes. This monitoring will enable early detection of treatment failure, since selection for antiretroviral drug-resistance mutation has been shown to be influenced by drug pressure and the continual exposure of patients to the same failing drugs for long periods of time. Despite the challenges and limitations of this research, we think it still provides relevant information about the subtype dynamics and drug resistance patterns in different groups of HIV-1 patients in Ghana. We highlight the need for continuous HIV-1 subtype and drug resistance pattern monitoring to mitigate the emergence of drug-resistant strains of the virus.

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