CHAPTER V

DISCUSSION AND CONCLUSIONS

5.1. Minority Y181C and M184V mutants in patients recently infected with HIV-1 and those with first-line failure to ARV in Thailand (paper 1)

In Thailand, 3TC and NVP are components in the most commonly prescribed, fixed-dose combination tablet GPO VIR® S. Both of these drugs have a low resistance genetic barrier that can lead to a rapid selection of specific mutations in the case of virological failure. Prevalence reported in patients failing first-line regimen for 20 months was 89% and 61% for M184V and Y181C/I mutations, respectively [31]. These mutations can be transmitted to patients recently infected with HIV and naïve to the drugs as already described in resource-rich countries where ARV treatment has been extensively used. Furthermore, some minority resistant variants can also be present in people infected with HIV but are not detected by the current routine genotypic tests, leading to early ARV failure in treatment naïve patients who receive HAART containing low genetic resistance barrier drugs [14, 17].

This study focused on patients recently infected and patients with first-line failure to ARV without the detection of DRAM by standard genotypic tests. The mutation M184V associated to resistance to 3TC/FTC and the mutation Y181C associated to resistance to NVP/EFV [32] were selected for the development of quantitative PSQ assays. The PSQ assay was successfully developed to detect and quantify the Y181C and M184V mutations. Currently, there have been a number of techniques which could be classified into three common approaches to detect minority variants of DRAMs (1) point mutations testing, (2) clonal sequencing, and (3) ultra-deep sequencing [179]. The PSQ is in group of point mutations assays which include heteroduplex tracking assay [157], line probe assay [158], oligonucleotide ligase-based assay [162], and allele-specific real-time PCR (AS-PCR) [14,

17, 134, 149, 151, 180]. The point mutations assays quantify the low-abundance of DRAMs which is below the detectable level of conventional drug resistance genotypic test (20% of the total viral population), especially AS-PCR can achieve sensitivity as low as 0.001% [163]. This PSQ assays have detection limits of 0.85% and 1.19% for Y181C and M184V mutations, respectively. The cutoff values of detection are in line with other PSQs used for quantifying point mutations of other diseases [168, 169], but are lower than PSQs used for quantitative analysis of influenza drug resistant quasispecies that are above 10% of the total viral population [175]. Unlike clonal sequencing and ultra-deep sequencing, the limitation of point mutation assays is that they do not allow the detection and quantification of several mutations at the same time, thus the genetic linkage and evolution of drug resistant viral quasispecies cannot be evaluated by using this assay [181].

This is the first report to study the prevalence of minority HIV-1 drug resistance in Thailand which HIV-1 subtype is predominantly CRF01_AE as in other parts of Southeast Asia [23]. The prevalence of minority M184V and Y181C variants is low. However, this prevalence does not represent the actual prevalence of DRAM in the drug naïve homosexual population due to the procedure of the sample collection. Conventional genotyping assays excluded samples with major DRAMs. From 104 samples of individuals recently infected with HIV-1 with no DRAM detected by standard genotypic tests, low abundance Y181C variants were found in only one sample (0.96%). In addition, minority M184V variants were detected in three (3%) from 101 samples which were successfully pyrosequenced. Globally our results are in accordance with the low prevalence of majority DRAMs found in persons recently infected with HIV and naïve to treatment in several resistance surveys using standard resistance testing. In two threshold surveys of HIV drug resistant transmission in 50 consecutive specimens collected during 2005-2006 from blood donors and clients from a voluntary counseling and testing centre in Bangkok, Thailand and in 56 samples of HIV-1 infected, drug naïve pregnant women in 2009 following the WHO protocol of HIV Drug

Resistance Threshold Survey, none of the majority DRAMs were detected [27]. Other two studies on the prevalence of DRAM in newly diagnosed, treatment naïve persons infected with HIV-1 with one study including 113 samples in 2007 and the other of 151 samples with unknown duration of HIV-1 infection from 12 provinces in central Thailand from 2005 to 2007 showed no Y181C species found and 3.5% containing M184V variants [182] and 0.7% harboring Y181C and 3.9% expressing M184V, respectively [30]. In contrast, both majority and minority DRAMs have been reported at higher frequencies (>5% for majority, and >10% for minority) in developed countries where HAART has been widely available and used for a long time [151, 164, 183-186].

In the cohort of 22 patients experiencing first-line ARV failure with standard drug resistant genotyping shown no M184V and Y181C variants, only one patient was found harboring minority M184V variants. The result was confirmed by standard clonal analysis. This low prevalence of minority DRAM variants was possibly due to the fact that a fraction of patients had viral load lower than the minimum level required to detect minority variants at a frequency of 1% [155] leading to a higher limit of PSQ sensitivity in these patients. Thus, an underestimation of the true prevalence of minority viruses in the studied population could occur. In addition, because the detection thresholds of our PSQ were higher than AS-PCR [14, 163], all extremely low abundant DRAM variants were not detected. This explanation is supported by the result of a study using AS-PCR for detecting minority DRAMs. They showed that all mutations identified were at the frequencies lower than 1% of the viral population [187]. The other possibility explanation is that during the time of treatment failure, DRAM strains outgrow and become dominant in the viral population under drug pressure. By excluding all samples with majority DRAMs detected by standard genotyping, our selected samples from the cohort of patients included only those who have other causes [188, 189], rather than minority DRAM variants, of drug failure. Among causes, low or no adherence might be a primary factor leading to the absence of minority DRAM despite treatment failure

since the process of DRAM selection could not be initiated under insufficiently selective pressure given by the drugs [190, 191]. Therefore, monitoring and boosting adherence in patients on HAART is of critical importance in treatment management.

The patient harboring minority M184V variants achieved suppression of viremia when she switched to a second line regimen with TDF, 3TC and LPV/RTV. This virological success was consistent with the current findings [186, 192]. Furthermore, the recent result of HIV STAR study showed that patients who failed NNRTI-based HAART with major DRAMs could achieve viral suppression if they switched to a second line regimen containing recycled TDF plus 3TC and LPV/RTV [193]. While the clinical relevance of minority DRAMs detected at baseline or before the initiation of HAART containing compounds with low genetic resistance barriers is still a controversial issue with supported [14, 17, 150, 164] and opposed [151] publications, the minority M184V variants in RT gene might not have a significant influence on virological outcome in patients who receive a regimen containing a compound with high drug resistance barrier as RTV/LVP.

Nonetheless, there were several limitations in our study. As stated above, the cutoff values of detection of the PSQ were higher than that of AS-PCR, leading to an underestimation of low abundant DRAM existing less than 1% of viral population. In addition, the PSQ sensitivity was identified using plasmid mixtures and this limit could be higher in clinical samples in the context of HIV's heterogeneity. The relatively small size of the cohort of patients in first-line ARV therapy failure may have been underpowered. Furthermore, samples prior to HAART initiation were not available; viral resistant dynamics and evolution thus could not be evaluated. Additionally, a selection bias could have been introduced in the study as mentioned above. Finally, since there were no data available on treatment adherence, therefore the potential contribution of adherence in virological failure could not be assessed. Indeed, as adherence above 95% is required to obtain therapeutic

success, tools to evaluate and reinforce adherence should be implemented to avoid switching to second or third lines in patients without virological failure associated with selected resistance.

In conclusion, the prevalence of minority M184V and Y181C variants in Thai homosexual men recently infected with HIV-1 and naïve to ARV treatment was still low. Together with the result of low frequency of majority transmitted HIV-1 drug resistance in Thailand, this result did not support using PSQ to routinely screen for DRAMs in persons infected with HIV-1 and naïve to treatment. Low abundant M184V mutation found at the time of failed regimen containing 3TC and NVP in patients whose plasma samples showed no DRAMs found by standard genotypic test might have no impact on virological outcomes in patients who subsequently receive RTV boosted LPV containing regimen as second-line therapy. Since there were relatively low prevalence of minority resistance variants and no correlation between minority resistance variants and the treatment outcome, it is not recommend using this assay in patients failing first-line therapy. In HIV-infected mothers pre-exposed to a single dose NVP to prevent mother-to-child transmission; this PSQ assay may be beneficial prior to commencing HAART. Systematic survey to monitor primary HIV-1 drug resistance among recently infected and newly diagnosed patients in countries such as Thailand where universal access to ART has been implemented for several years is warranted.

5.2. Minority N155H drug resistance has no association with different RAL resistant profiles (paper 2)

Our study described the dynamics of the N155H mutation present in minor and major variants circulating in plasma and archived in cells by a longitudinal analysis of samples from heavily treated patients failing RAL-containing regimens and harboring multiple drug-resistance mutations. No mutation N155H was identified as both minority and majority variants at baseline prior to RAL initiation by bulk sequencing and AS-PCR despite the fact

that viral load levels were sufficient (> 5 $\log_{10}$ copies/ml for all except patient 4). This finding is in line with other studies using similar [152] or different technologies [153, 194] to detect minority N155H as well as other primary mutations in INI-naïve patients. Thus, the N155H mutation that usually emerges first in patient failing RAL-based regimen is selected *de novo* as a result of continued drug pressure rather than a polymorphism conferring natural resistance to RAL or a growing mutated strain developed from a resistant transmitted isolate.

During RAL failure, the selection of the N155H mutation was detected early in 3 patients of our study. The frequent selection of N155H variants was also described in patients failing RAL-combined therapy in ARV-naïve patients [195] as well as ARV-experienced patients [39]. In contrast, in 2 patients of our study harboring viruses with the mutations Q148H + G140S detected throughout RAL failure by bulk sequencing, no minority N155H mutant was found at various time-points by AS-PCR.

The evolution of INI resistance observed in patient 3 has already been described [42] with the single N155H mutant firstly selected and then replaced by the double mutant Q148H + G140S. The mutant N155H decreased from major to minority levels when the double mutant appeared in mixtures of mutated and WT variants. This observation was consistent with findings *in vivo* and *in vitro* showing that: (i) N155H and Q148H mutations are independent and mutually exclusive [42] and (ii) the Q148H + G140S double mutants display a strongly selective advantage in reducing RAL susceptibility while maintaining viral replicative capacity compared to the single mutant N155H [38, 44, 46, 128].

Unexpectedly, in patients 1 and 2, the N155H mutation was maintained during the failure therapy with the selection of additional secondary mutations V151L and G163R. Unfortunately, this finding was not explained by the presence of the N155H mutation as minority variant at baseline in plasma or archived in cells in our study. Nevertheless, of note, the rapid disappearance of the N155H mutation as minor and major variants after RAL

withdrawal observed in all 3 patients displaying the N155H resistance pathway confirmed the impairment in viral replicative capacity of viruses carrying this resistance mutation compared with WT strain or to some extent, other mutated species. Ferns *et al* studied the dynamics of INI-resistant variants during and after RAL therapy and found that in patient with single N155H mutation the viral population rapidly disappeared only one month after stopping RAL and in those displaying N155H associated with other secondary mutations the time to disappear took longer (5 months) with the loss of N155H mutation usually being the first to occur [196]. *In vitro*, in the absence of RAL, recombinant isolates containing single N155H mutation were less fit than those bearing WT and some combinations of mutations particularly Q148H + G140S [38, 44, 197].

To date, although explained in part *in vitro*, the mechanism that could drive the different evolutions of RAL-resistance profiles has not been fully understood. Despite being the first mutation selected in RAL resistance evolution, in our study on patients failing RAL following 3 RAL-associated resistance profiles, the N155H mutation present at various levels from minority to majority showed no relation with these 3 profiles, suggesting this mutant might not play a role in determining different resistance profiles. However, due to the limited number of patients in the present study, this conclusion might need to be supported by further studies with a larger number of participants. Other factor able to impact resistance and fitness such as genetic background of IN in combination with multiple mutations in reverse transcriptase and protease in those patients involved in determining different resistance patterns could also be an interesting topic for further investigation.

5.3. Genetic barrier to the development of integrase inhibitor resistance is generally similar between HIV-1 subtype B and CRF01_AE (paper 3)

Genetic barrier is an important factor contributing to the development of drug resistance. Given the great genetic diversity of HIV, different subtypes with existing

variability at nucleotide level could have an effect on the genetic barrier of drug. In this study, 66 mutations at 41 amino acid positions associated with resistance to RAL, EVG, and DTG were evaluated in 144 sequences derived from stored samples collected from INI-naïve individuals (109 isolates from France, Cambodia, Thailand, and Vietnam infected with subtype CRF01_AE, 35 people from France infected with subtype B). Overall, the majority of amino acid positions (28/41) showed the high conservation of the predominant codon usage, indicating the same genetic barrier for the two subtypes. Of 28 these conserved amino acid positions, 6 consisted of 9 RAL and EVG primary mutations. Thus, RAL and EVG have a similar genetic barrier for the major mutations in the two subtypes CRF01_AE and B. Piralla *et al* evaluated genetic barrier for 28 DRAMs associated with RAL and EVG resistance at 27 amino acid positions in 41 ARV- naïve patients infected with HIV-1 subtype B, A, C, G, F, CRF02_AG, CRF01_AE, CRF12_BF and 54 NRTI/NNRTI/PI- experienced patients infected with HIV-1 subtype B, A, A/K, C, D, G, D/F, F, CRF02_AG, CRF01_AE, CRF19_CPX and found that the majority of the studied positions showed a high degree of conservation across all subtypes including CRF01_AE [198]. Maiga *et al* obtained the concordant finding in a study of 27 substitutions related to RAL and EVG resistance at 19 amino acid positions in IN sequences isolated from samples of 73 HIV-1 subtypes B and 77 HIV-1 subtype CRF02_AG, ARV-naïve individuals [53]. The absence of 9 primary mutations associated with RAL and EVG (Q148H/K/R, N155H, Y143R/C, T66I, E92Q, and S147G) in two HIV-1 subtype groups was observed in our study. This is also correspondent with other reports indicating that major RAL and EVG associated mutations were very infrequent in INI-naïve persons [60, 61, 198].

Because DTG is a new drug, 8 mutations so far described as conferring resistance to this compound (E92Q, L101I, A124T, Q148H/R, S153F/Y, G193E) at 6 amino acid positions were assessed. Up to our knowledge, this is the first report on the genetic barrier for DTG resistance. Of six amino acid positions with different predominant codon usage leading to a

different genetic barrier between HIV-1 subtypes B and CRF01_AE, two were previously described to be associated with DTG resistance (L101I and A124T (T124A in some papers due to different reference sequence use)). The genetic barriers for the development of these two mutations related to DTG resistance as well as the other 4 RAL and EVG secondary mutations V72I, T125K, and G140C/S are higher in subtype CRF01_AE than in subtype B (Table 88, Figure 18). This higher genetic barrier was also found in subtype CRF02_AG in acquiring mutations G140S, G140C, and V151I [53] and in non- B subtypes in acquiring some PI, NRTI and NNRTI mutations [54] in comparison with subtype B. The mutations L101I and T124A were emerged by serial virus passages in the presence of DTG *in vitro* [199, 200]. They were highly prevalent and more frequent in subtype CRF01_AE than subtype B in our study (14% vs 0% for L101I and 87% vs 20% for T124A) (Table 1) and in non-B subtypes B than in B subtype (66.9% vs 45.7% for L101I and 61.7% vs 25.9% for T124A) in Garrido *et al*'s study [59]. The substitutions L101I and T124A are considered as polymorphic mutations [200, 201] and demonstrate a small fold change (<3) reduction in the susceptibility to DTG [200]. However, due to the fact that these two mutations are highly prevalent in ARV-naïve individuals and even more frequent in RAL-treated patients [51, 201] and the T124A mutation seems to be involved in the initial step of DTG resistance development [201], their presence may affect the genetic barrier for DTG resistance by favoring the selection of other DTG resistant mutations. If indeed present, this influence is different between subtypes B and CRF01_AE. A further study should be carried out in patients failing RAL and EVG with different mutation profiles subsequently treated with DTG to evaluate the impact of natural polymorphic mutations including L101I and T124A in the evolution of other mutations when DTG failure occurs.

The mutation G140S shown to recover the fitness of resistant viruses is usually associated with the mutations at position 148 with > 130 fold reduced susceptibility to RAL and EVG as well as 3-8 fold decreased sensitivity to DTG [47, 52, 178, 202]. Our finding that

calculated genetic barrier to develop mutation G140S is higher in HIV-1 subtype CRF01_AE than subtype B could display a possibility that it is more difficult for subtype CRF01_AE to select RAL, EVG, and DTG resistance following the Q148 pathway.

In our study, there were a number of patients with sequences containing natural codons encoding for INIs mutations, especially some mutated amino acids showed a high prevalence such as V72I, L101I, A124T, K156N, V165I , V201I, I203M , T206S, N232D, suggesting that the natural resistance to INIs already presents and/or the development of INIs resistance could be faster in those patients.

A number of natural polymorphisms with unknown significance (Q95H/S/A, L101V/P/F, S119P/G, A124N/S, T125A/V, V151E, M154T/L, K160R/T/Q/E, G163E, G193D, S230N, and N232E) were observed in our study, showing the diversity of HIV. These polymorphic substitutions should be studied further to know their roles in the evolution of INI resistance.

In summary, most studied amino acid positions including all corresponding to RAL and EVG primary mutations show a high degree of conservation, indicating a similar genetic barrier between subtypes CRF01_AE and B. The genetic barrier to acquire mutations V72I, L101I, A124T, T125K, and G140C/S is higher in subtype CRF01_AE whereas that to acquire mutation V201I is lower in subtype CRF01_AE in comparison with subtype B. These different genetic barriers might play a role in the development of resistance to RAL, EVG, and DTG.

5.4. General discussion and conclusion

5.4.1. Technical issues of assays for minority species detection

With the rapid development of technology, more techniques have been applied in the field of minority drug resistance investigation. However, as minority quasispecies are rare events following Poisson distribution, all assays designed for detection and quantification of these variants must take into account this factor. The most important point is the sample need to have sufficient volume to ensure the representative presence of minority strains in viral population. Mathematically, the result of an assay to detect minority variants (<20%) using an extract derived from 18 µl of plasma with viral load of 3,000 copies/ml is not reliable and one cannot find variants existing at a level of 0.1% in a sample with HIV-1 RNA 900 copies/ml given 1ml plasma was used for extraction. Therefore, the volume of plasma and viral load of samples are critical factors to be considered before performing any assay to detect minority variants.

Paredes R *et al* proposed a minimal requirement of the number of RNA molecules must be provided for cDNA synthesis and amplification and the viral load according to different minority variant proportions present in population given RNA is extracted from 1ml of plasma (Table 9). If the minority variants accounting for 1% of total population, at least 500 RNA molecules extracted from 1ml plasma of a sample with viral load more than 3,000 copies/ml must be tested to give a reliable result. However, a sample with low viral load is still able to perform if a larger volume of plasma is used. This aspect was discussed for studied population in paper 1 of the thesis.

Another issue for all methods based on hybridization PCR is the polymorsphims in primer binding sites especially those located near 3' end of primers. These polymorphisms can affect the amplification efficiency of primers and probes, resulting in a bias proportion.

The common approach to deal with this issue is to design primers and probes in the conserved regions of interested genes. This strategy was used in primer design for conventional genotyping of RT and IN genes used in the current thesis. Another approach is designing degenerate primers based on the consensus sequences which are used for PSQ assays in paper 1 of the thesis.

Table 9. Minimum HIV-1 RNA load required to detect minority variants at frequencies of 0.1%, 1%, and 10%, assuming that the RNA is extracted from 1ml of plasma

Variant frequency, λ	Number of RNA molecules to be tested, according to the Poisson distribution (P>.99)	Fraction of elution volume used for cDNA synthesis, f_e	Minimum HIV1 RNA load (copies/ ml)
0.1%	5000	0.25	29,762
		0.5	14,881
		1	7,440
1%	500	0.25	2,976
		0.5	1,488
		1	744
10%	50	0.25	298
		0.5	148
		1	74

HIV-1 RNA values were estimated using the following assumptions: V = 1 mL, E_{RNAX} = 0.96 and E_{cDNA}=0.7, based on http://www1.qiagen.com/literature/qiagennews/0398/983hiv1.pdf and http://omrf.ouhsc.edu/~frank/CDNA.html. Importantly, E_{RNAX} and particularly E_{cDNA} are subject to significant variation in different conditions, and may need to be determined empirically. Therefore, HIV-1 RNA values would need to be adjusted if different plasma volumes were used for the RNA extraction or if E_{RNAX} or E_{cDNA} were different. Note that the volume of plasma required for RNA extraction in order to detect a variant with frequency λ can be easily calculated as $V = N_{RNA(\lambda)}/(pVL\ f_e E_{RNAX} E_{cDNA})$.

[a] Calculations were derived from the formula: $pVL = N_{RNA(\lambda)/(}Vf_eE_{RNAX}E_{cDNA})$, where pVL is the plasma HIV-1 RNA copy number, $N_{RNA(\lambda)}$ the number of RNA copies that need to be tested according to the Poisson distribution to detect at least one variant with a probability >99% if this variant is present at a frequency λ, V the volume of plasma used for the RNA extraction in millilitres, f_e the fraction of the RNA elution volume used for cDNA synthesis, E_{RNAX} the efficiency of the RNA extraction process and E_{cDNA} is the efficiency of the cDNA synthesis.

Source: Paredes R, Marconi VC, Campbell TB, Kuritzkes DR. 2007. Systematic evaluation of allele-specific real-time PCR for the detection of minority HIV-1 variants with pol and env resistance mutations. J Virol Methods 146(1-2):136-146.

5.4.2. Prevalence and clinical relevance of minority variants

By using minority variant assays applied from different technologies, an undetectable viral quasispecies population of HIV resistance species by conventional genotyping was unveiled and in fact, it has been existed from the time of ARV introduction and present in all patients infected with HIV [181]. Despite different techniques performed to detect mutations at low levels, all studies including ours detected additional mutations to those found by bulk sequencing. In paper 1, minority mutations Y181C and M184V were found in population of drug naïve as well as treatment failures in Thailand in whom no DRAMs were found by conventional genotyping. Additional minority N155H mutants were also detected in longitudinal samples from 5 heavily treated patients failing RAL containing regimen in paper 2. However, despite some limitations, our findings suggested that the significant role of these found minority variants in clinical perspective is limited in the context of high genetic drug included in the regimen (paper 1), and in different INI resistance profiles (paper 2). Further studies with a larger sample size should be needed to confirm our preliminary results.