CHAPTER IV

RESULTS

PART I : DEVELOPMENT OF DUPLEX SELECTIVE PCR FOR GENOTYPIC ANALYSIS

To develop a successful duplex selective PCR for genotypic resistance analysis in patients, the most important is to ensure that the mutant specific primers allow efficient amplification of mutant template while minimizing amplification of nontarget (wild type) template. From this reason, all of the mutant (MT) specific primers were tested with WT template (WT plasmid and WT HIV-1_{IIIB} virions (WT virus stocks)). The results are summarized in Table V. However, all of the wild type (WT) specific primers were also tested with 151 (M) plasmid and with known mutant isolates that have been proven by sequencing analysis for each codon.

First generation primer design

Based on the ARMS technique, all of the first generation primers were designed by introducing an additional mismatch close to the 3'-terminal nucleotide in either WT and MT specific primers. The specificity was high for only some primers (codon 151, 181, and 215). However, an additional mismatch in the MT specific primer for codon 215 failed to detect 215Y mutation in known MT sequence isolates.

Second generation primer design

The second generation primer design for codon 103 was modified from MS-PCR primer as described previously by Frater et al.[106] Additional mismatches were introduced into the third and forth position from the 3' end of WT and MT primers, respectively. This MT specific primer showed good specificity with WT template and the WT specific primer could also distinguish known WT sequence isolates.

The primers for codon 215 were designed with some modification from the one previously described by Larder et al[20]. Although this MT specific primer was designed base on T215F mutation, it could recognized both 215Y and F mutation.

However, we found that it could detect only some 215Y mutation (4/12) of the known 215Y mutant samples in this study.

Table V : Evaluation of specificity of mutant specific primer of each codons with WT template (WT plasmid and WT virus stock)

Mutant specific primers	Mispriming[a] (WT template)	Resolved by dilution [b]
Codon 103		
1st generation primer		
5'-C ATC TCC CAC ATC TAG TAC TGT TAC TGA TT**G**-3'	Yes	No
2nd generation primer [c]		
5'-TCC CCC ACA TCT AGT ACT GTT ACT GA**C** TT**G**-3'	No	ND
Codon 181		
5'-A TCC TAC ATA CGA GTC ATC CTT GTA TTG **T**C-3'	No	ND
Codon 151		
5'-AT ATT GCC GGT GAT CCT TTC CAT CC**G** **AT**-3'	No	ND
Codon 215		
1st generation primer [d]		
5'-C CTT CTG ATG CTT TTT GTC TGG TGT **T**AA-3'	No	ND
2nd generation primer [d,e]		
5'-TT CTG ATG TTT TTT GTC TGG TGT **C**AA-3'	No	ND
3rd generation primer		
5'-TG ATG CTT TTT GTC TGG TGT **A**AA-3'	No	ND

[a] Misprimimg : amplification of WT template (WT plasmid, WT virus stock) with MT primer

[b] Resolved the mispriming by serial template dilutions.
(WT plasmid dilution (10^6 to 500), WT virus stock dilution (500,000 to 500)).

[c] This primer was modified from Frater et al.

[d] This primer was failed to detect the T215Y mutation.

[e] This primer was modified from Larder et al.

Base pairs comprising the codons of interest are in boldface type.

The sites of additional mismatches are in shading. ND : not determined.

Third generation primer design

The obstacle to development of the primer of codon 215 is the difference of nucleotide specific sequence between HIV-1 subtype B and subtype A/E. The comparison of the nucleotide specific sequence are shown in Table VI. This observation explains why previously described MT specific primers by Larder et al. [20] failed to detect 215Y mutation in this study. For this reason, we use nucleotides that are complementary to the HIV-1 subtype A/E at the third base form 3' end for both WT and MT primers. This MT specific primer could detect most (11/12) of the known 215Y mutants and all (8/8) of the known 215F mutants. The results of the primer development are shown in Figure 2.

Figure 2 : Comparison of the three generation mutant specific primers of codon 215 for T215Y/F mutation detection in known mutant isolates as proven by sequencing analysis. (N=20)

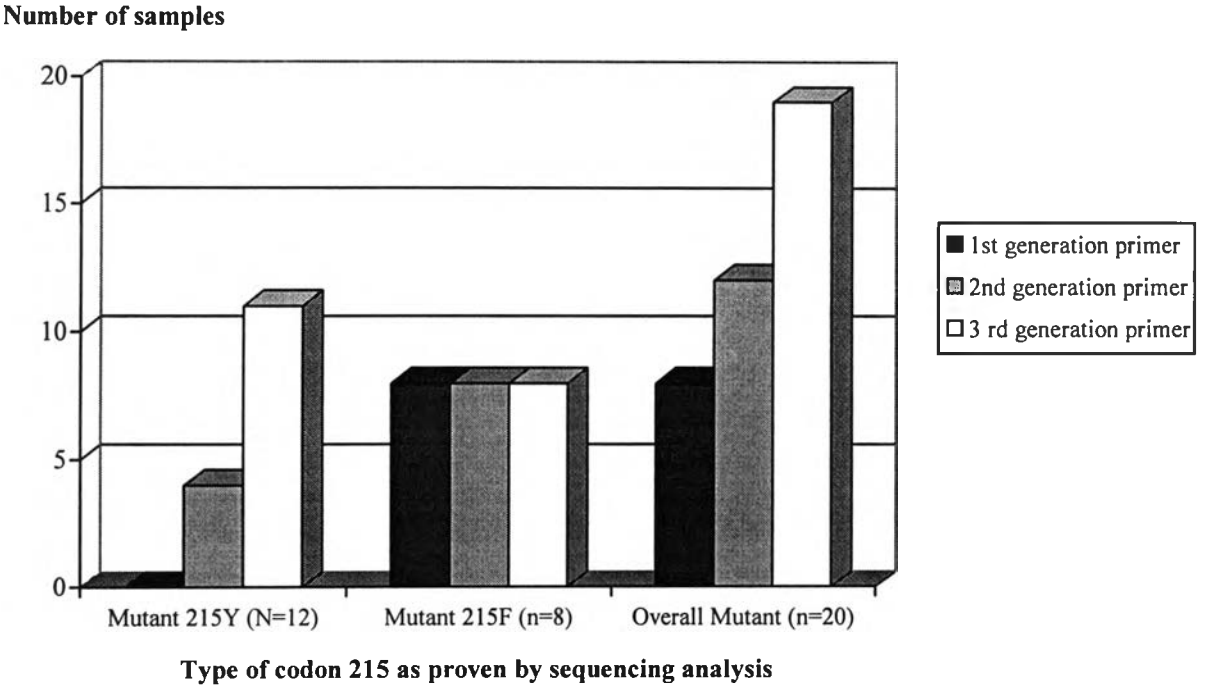


Table VI : Details of the primers for codon 215 (2nd and 3rd generation) with nucleotide specific sequence of subtype B and A/E

Amino acid Change	Subtype B specific sequence with 215 primers specific for subtype B [a]	Subtype A/E specific sequence with 215 primers specific for subtype B[a]	Subtype A/E specific sequence with 215 primers specific for subtype E
Wide type **Thr (T)**	← 3' TGG ACA CCA GAC 5' wt primer B CTT ACC ACA CCA GAC --3' wt DNA B G ACA CCA GAC 5' mt primer B ✗ 3' AA	← 3' TGG ACA CCA GAC 5' wt primer B CTT ACT ACA CCA GAC --3' wt DNA A/E ACA CCA GAC 5' mt primer B ✗ 3' AAG	← 3' TGA ACA CCA GAC 5' wt primer A/E CTT ACT ACA CCA GAC --3' wt DNA A/E ACA CCA GAC 5' mt primer A/E ✗ 3' AAA
Mutant Phe (F)	✗ 3' TG G ACA CCA GAC 5' wt primer B CTT TTC ACA CCA GAC --3' mt DNA B ← 3' AAG ACA CCA GAC 5' mt primer B	✗ 3' TGG ACA CCA GAC 5' wt primer B CTT TTT ACA CCA GAC --3' mt DNA A/E ← 3' AAG ACA CCA GAC 5' mt primer B	✗ 3' TGA ACA CCA GAC 5' wt primer A/E CTT TTT ACA CCA GAC --3' mt DNA A/E ← 3' AAA ACA CCA GAC 5' mt primer A/E
Mutant Tyr (Y)	✗ 3' TG G ACA CCA GAC 5' wt primer B CTT TAC ACA CCA GAC --3' mt DNA B ← 3' AAG ACA CCA GAC 5' mt primer B	✗ 3' TGG ACA CCA GAC 5' wt primer B CTT TAT ACA CCA GAC --3' mt DNA A/E ⇠ 3' AAG ACA CCA GAC 5' mt primer B	✗ 3' TGA ACA CCA GAC 5' wt primer A/E CTT TAT ACA CCA GAC --3' mt DNA A/E ← 3' AAA ACA CCA GAC 5' mt primer A/E

[a] the modified primers as previously described by Larder et al.[20]

The nucleotide specific sequence for each subtype are in border.

The nucleotide complementary to the specific sequence in the primers for each subtype are in underline.

← Taq DNA polymerase **can** initiate DNA synthesis from the 3' end of the primer.

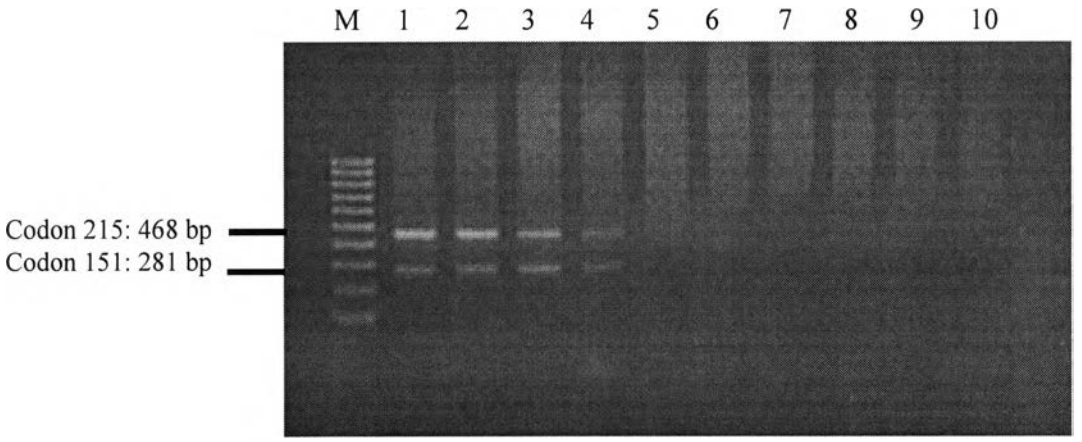
⇠ Taq DNA polymerase **can partially** initiate DNA synthesis from the 3'end of the primer.

✗ Taq DNA polymerase **cannot** initiate DNA synthesis from the 3'end of the primer.

In this study, primers were successfully developed (as shown in Table VII) and could lead to nice combination of two groups of duplex selective PCR (The failure of primer development are illustrated in Table VIII.)

1. K103N / Y181C duplex selective PCR
2. Q151M / T215Y/F duplex selective PCR

The sensitivity of both duplex selective PCR were evaluated with serial dilution of both WT plasmid and virus stocks. Both duplex selective PCR had the same detection limit of 1,000 copies of both plasmid and virus stocks template as shown in Figure 3.



Lane	Type of primers	WT (III_B) virus stocks (copies/mL)
1	151WT+215WT	500,000
2	151WT+215WT	50,000
3	151WT+215WT	5,000
4	151WT+215WT	1,000
5	151WT+215WT	500
6	151MT+215MT	500,000
7	151MT+215MT	50,000
8	151MT+215MT	5,000
9	151MT+215MT	1,000
10	151MT+215MT	500

Figure 3 : Determination of sensitivity and specificity of Q151M / T215Y/F duplex selective PCR with serial dilutions of wild type virus stocks.

Table VII : Summary of the successful primers design and development.

Type of mutation		Primer sequence	Primer design		No. of initial mismatches	No. of additional mismatches	Type of additional mismatches [a]
			Newly	Previous Report			
K103N	WT	5'-TCC CCC ACA TCT AGT ACT GTT ACT GAT ▓TT-3'		✓ [b]	1	1	G : A
	MT	5'-TCC CCC ACA TCT AGT ACT GTT ACT GA▓ TTG-3'					
Y181C	WT	5'-A TCC TAC ATA CGA GTC ATC CTT GTA TTG ▓T-3'	✓		1	1	C : T / T : T
	MT	5'-A TCC TAC ATA CGA GTC ATC CTT GTA TTG ▓C-3'					
Q151M	WT	5'-AT ATT GCC GGT GAT CCT TTC CAT CC▓ TG-3'	✓		2	1	G : T / G : G
	MT	5'-AT ATT GCC GGT GAT CCT TTC CAT CC▓ AT-3'					
T215Y/F	WT	5'-ATG ATG CTT TTT GTC TGG TGT AGT-3'	✓		2	0	None
	MT	5'-ATG ATG CTT TTT GTC TGG TGT AAA-3'					

[a] mismatches between the primer and template

[b] the modified primers as described previously by Frater et al.[106]

Base pairs comprising the codons of interest are in boldface type.

The sites of additional mismatches are in shading.

The nucleotide complementary to the specific sequence A/E in the primers are in underline.

Table VIII : Summary of the failure primers design and development .

Type of mutation		Primer sequence	Primer design: Newly	Primer design: Previous Report	No. of initial mismatches	No. of additional mismatches	Type of additional mismatches [a]
1st K103N	WT	5'-C ATC TCC CAC ATC TAG TAC TGT TAC TGA TT**T**-3'	✓		1	1	C : A
	MT	5'-C ATC TCC CAC ATC TAG TAC TGT TAC TGA TT**G**-3'					
1st T215Y/F	WT	5'-C CTT CTG ATG CTT TTT GTC TGG TGT **GT**-3'	✓		2	1	T : T
	MT	5'-C CTT CTG ATG CTT TTT GTC TGG TGT **AA**-3'					
2nd T215Y/F	WT	5'-TT CTG ATG TTT TTT GTC TGG TGT GGT-3'		✓ [b]	2	1 [c]	G : T
	MT	5'-TT CTG ATG TTT TTT GTC TGG TGT GAA-3'					

[a] mismatches between the primer and template.

[b] the modified primers as previously described by Larder et al.[20]

[c] mismatch nucleotide specific for subtype A/E (match for subtype B)

Base pairs comprising the codons of interest are in boldface type.

The sites of additional mismatches are in shading.

The nucleotide complementary to the specific sequence B in the primers are in underline.

PART II : GENOTYPIC RESISTANCE STUDY IN HIV-1 INFECTED PATIENTS

1. Demographic characteristic (Table IX)

There was no difference in plasma viral load between the two groups. However, the ART-experienced patients (group II) were more advance in term of disease progression than in the ART-naïve patients (group I) because all of them were randomly selected from patients attending the Immune Clinic at King Chulalongkorn Memorial Hospital.

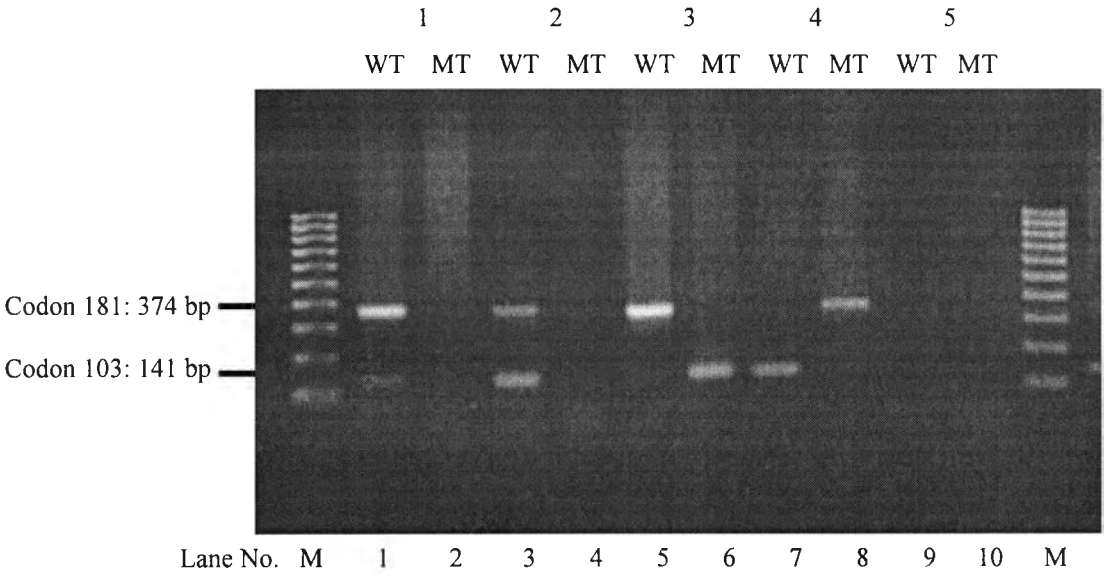
Table IX : Demographic characteristic of patients

	ART-naive	ART-experienced
Patients (n)	20	25
Age (years) (Mean±SD, range)	36.8 ± 8.3	UD
	(25 – 53)	
Female : male ratio	10 : 10	5 : 20
Median CD4 cell count ($x10^6$ cells/l)	225	43 (n=5)
(range)	(24 - 693)	(10-410)
Median pVL (copies/mL)	23,528	34,232 (n=9)
(range)	(1,073 - 242,752)	(4,419 – 137,670)

ART : Antiretroviral therapy
pVL : plasma viral load
UD : unknown data

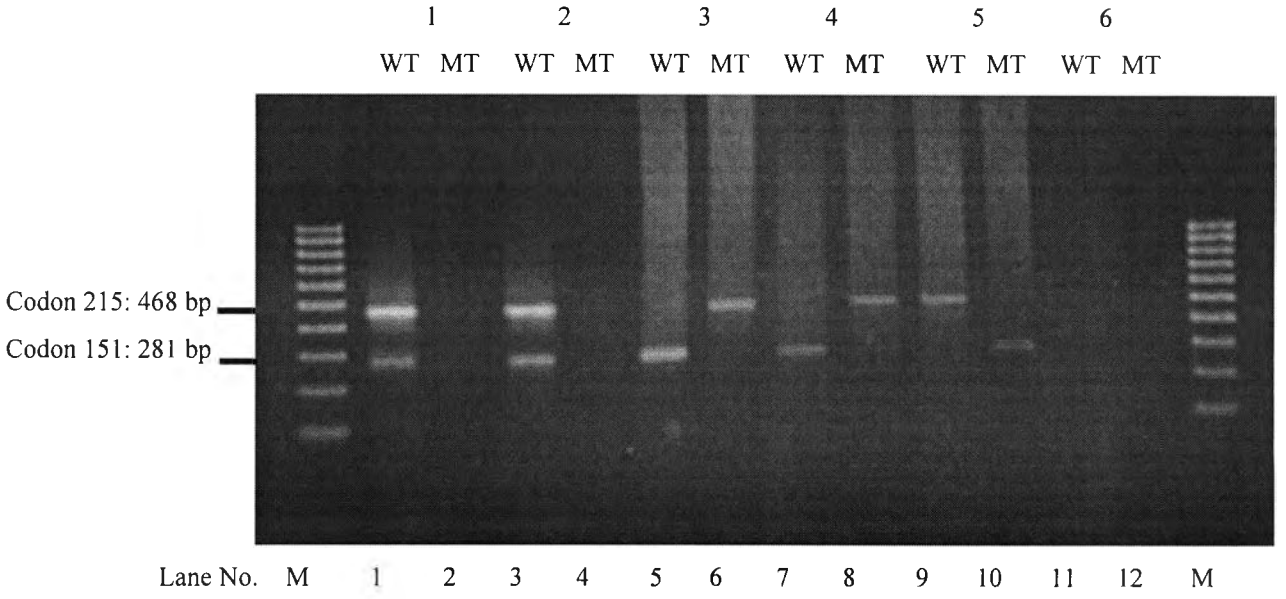
2. Genotypic resistance in HIV-1 infected patients by duplex selective PCR

The duplex selective PCR were used to evaluate genotypic resistance. Sequencing analysis was performed in 10 randomly selected ART-naïve patients and all of the ART-experienced patients. The results of duplex selective PCR assay were compared to those generated by sequencing analysis as shown in Figure 4 (for K103N/Y181C) and Figure 5 (for Q151M/T215Y/F).



Lane No.	Type of primer	Type of sample (By sequencing analysis)	Expected Product size
1	103WT+181WT	1. Wild type control	141+374 bp
2	103MT+181MT	plasmid	-
3	103WT+181WT	2. Patient no.1, group I	141+374 bp
4	103MT+181MT	Wild type	-
5	103WT+181WT	3. Patient no. 4, group II	374 bp
6	103MT+181MT	K103N	141 bp
7	103WT+181WT	4. Patient no. 6, group II	141 bp
8	103MT+181MT	Y181C	374 bp
9	103WT+181WT	5. negative control	-
10	103MT+181MT		-

Figure 4 : Analysis of RT codon 103 and 181 in selected HIV-1 infected patients by duplex selective PCR. (M, Marker)
(results from patients no.1, group I, no.4, group II, and no.6, group II)



Lane No.	Type of primer	Type of sample (By sequencing analysis)	Expected Product size
1	151WT+215WT	1. Wild type control	281+468 bp
2	151MT+215MT	plasmid	-
3	151WT+215WT	2. Patient no.3, group I	281+468 bp
4	151MT+215MT	Wild type	-
5	151WT+215WT	3. Patient no.7, group II	281 bp
6	151MT+215MT	T215Y	468 bp
7	151WT+215WT	4. Patient no.8, group II	281 bp
8	151MT+215MT	T215F	468 bp
9	151WT+215WT	5. Patient no.12, group II	468 bp
10	151MT+215MT	Q151M	281 bp
11	151WT+215WT	6. negative control	-
12	151MT+215MT		-

Figure 5 : Analysis of RT codon 151 and 215 in selected HIV-1 infected patients by duplex selective PCR (M, Marker)
(results from patients no.3, group I, no.7, group II, no.8, group II, and no.12, group II)

3. Comparison of genotypic resistance in HIV-1 infected patients by duplex selective PCR (Figure 6a)

There was significant difference of genotypic resistance between ART-naïve (group I) and ART-experienced of more than 6 months patients (group II) ($p< 0.01$, Chi-square test).

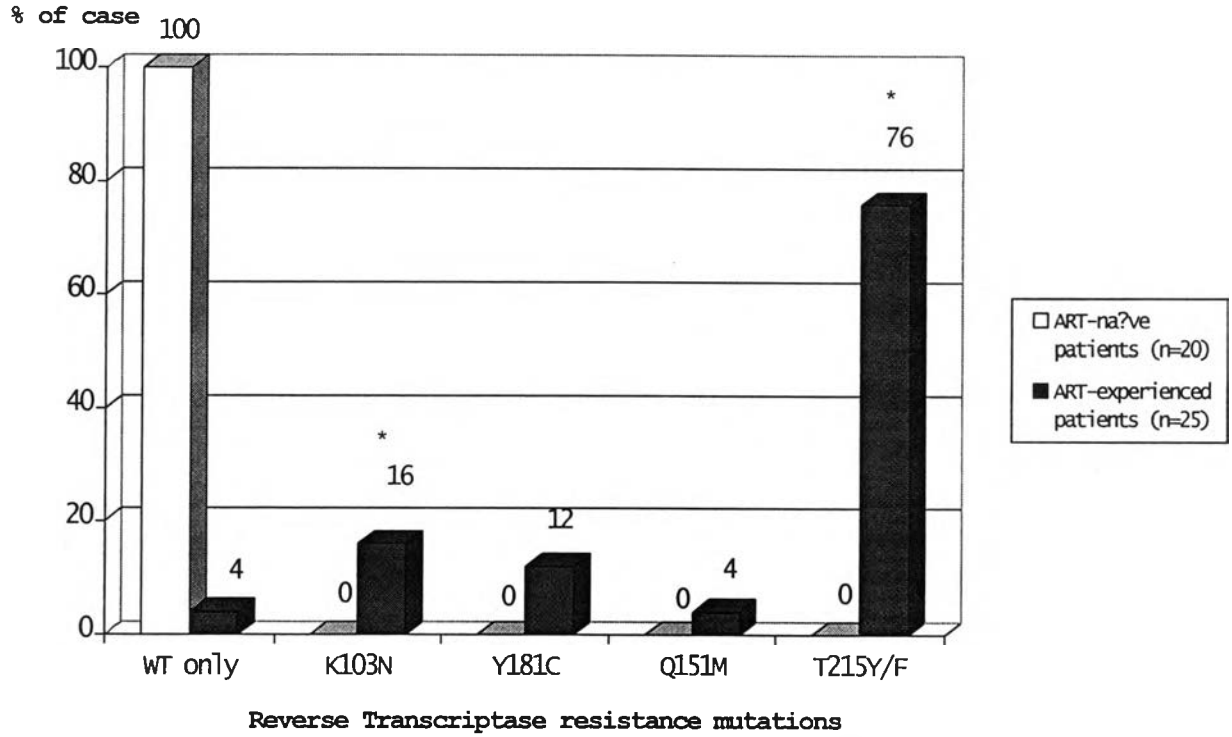
All of the ART-naïve patients showed solely wild type in all of four codons, whereas 23 (92%) of the 25 ART-experienced patients showed mutations. Mutations at codon 215 were found in 19 (76%) patients (while 20 patients were detected by sequencing analysis), whereas mutations at codon 151, 103, and 181 were found in 1 (4%), 4 (16%) (3 were detected by sequencing analysis) and 3 (12%), respectively. Four patients carried viruses with two resistance mutations. (Table X)

In ART-experienced patients, patients who have been treated with 2 NRTIs showed 93% NRTI mutations (one of them is undetectable for 215Y mutation). Mutations at codon 215 were found in 14 (87%) patients (sequencing analysis showed 15 (93%)), while codon 151 was detected in one (6%) patient who had AZT/ddI failure. Patients who have been treated with 2 NRTIs plus NNRTI (triple combination) showed 89% mutations (one of them showed no mutations). The mutations found were 55% (5/9), 44% (4/9), and 33% (3/9) for codon 215, 103, and 181, respectively. (Figure 8)

Duplex selective PCR shows more than 94% (33/35) concordant results as compared to sequencing analysis. The results of sequencing analysis are shown in Figure 6b. Of the discordant results, one was 103N mutation by duplex selective PCR but 103K (WT) by sequencing, and one was 215Y mutation by sequencing but was unamplifiable by duplex selective PCR. The comparison of the discordant results is shown in Figure 7.

Figure 6 : Comparison of frequency of RT mutation codons between ART-naïve patients and ART-experienced of more than 6 months patients. (* discordant results)

6a : Duplex selective PCR analysis results



6b : Sequencing analysis results

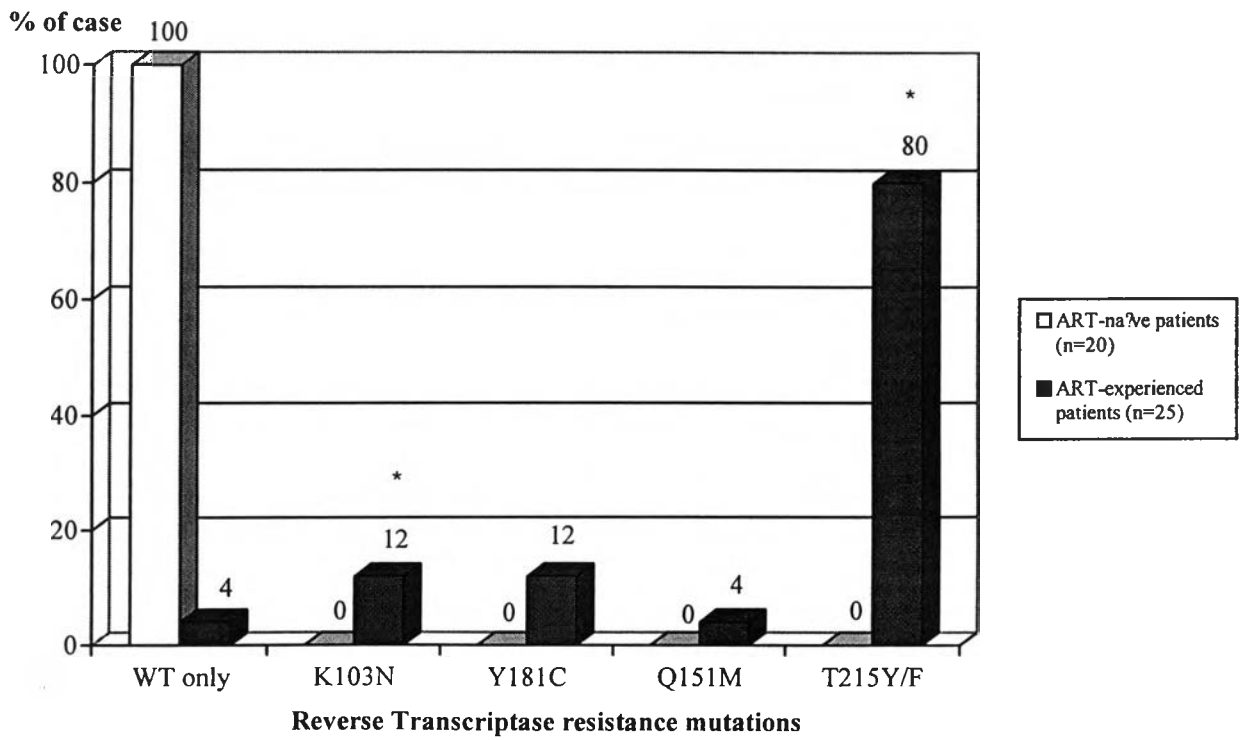


Table X : Frequency of mutations associated with resistance to antiretroviral drugs by duplex selective PCR in HIV-1 infected patients. (More than 94% (33/35) were concordant with sequencing analysis : the gold standard assay.)

		Codon Mutations						
Patients Classification	**N**	**103**	**181**	**151**	**215**	**103/215**	**181/215**	**Total patients**
ART-Naïve	20	0	0	0	0	0	0	0 (0%)
ART-experienced								
- 2 NRTIs	16	0	0	1[a]	14[b]	0	0	15[b] (94%)
- 2 NRTIs / NNRTIs	9	3	0	0	1	1[c]	3	8 (89%)

[a] AZT/ddI failure

[b] according to the gold standard (sequencing analysis), 215Y mutation was undetected in one patient.

[c] one patient showed discordant result for 103N mutation : it was 103N by duplex selective PCR but was 103K by sequencing analysis.

ART : Antiretroviral therapy

NRTIs : nucleoside reverse transcriptase inhibitors

NNRTIs : non nucleoside reverse transcriptase inhibitors

Figure 7 : Comparison of discordant results between duplex selective PCR and sequencing analysis. (N=25)

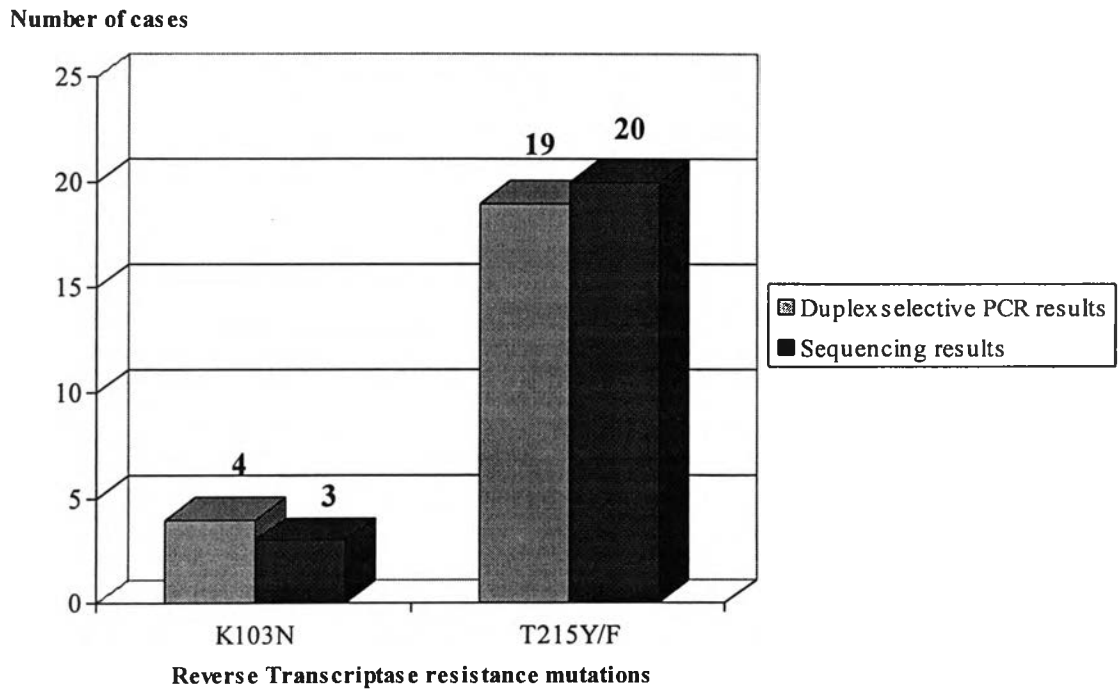


Figure 8 : Frequency of RT mutation at designated codons in ART-experienced patients (N=25) according to antiretroviral therapy using duplex selective PCR analysis.