CHAPTER IV

RESULTS

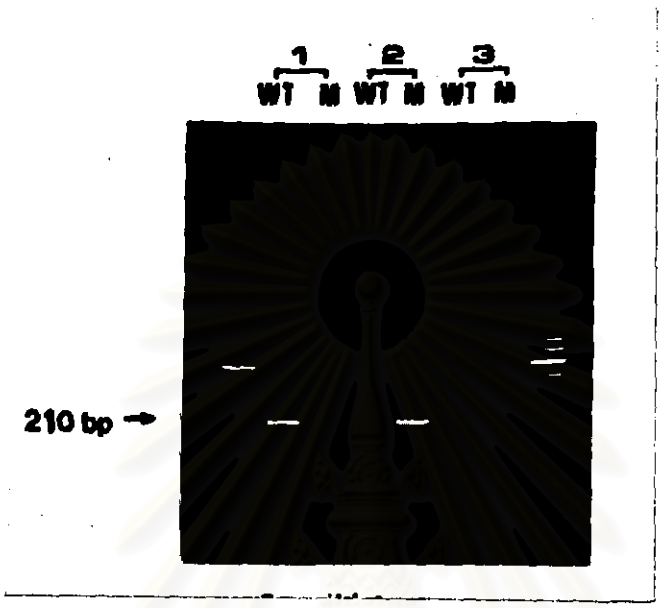
Part I : Develpoment of selective PCR for genotypic analysis of codon 215 AZT-resistant mutant.

1. Standardization of the previously described assay in plasmid DNA control

Plasmid DNAs (wild type 215 and mutant 215) were analysed for the presence or absence of mutation at codon 215. The plasmid wild type showed the 210 bp fragment product obtained by PCR with wild type primer only, whereas the mutant plasmid showed the 210 bp amplified product only when mutant primer was used. (Figure 2.)

2. Validation of RT-PCR for the detection of HIV-1 subtype B and E

The previousely described PCR assay failed to amplify HIV-1 subtype E but was not the case for subtype B. As shown in Figure 3, there was no 210 bp amplified product found in the sample (no. 2) which was subtype E as compared to the sample (no. 1) which was subtype B. When the PCR was modified by leaving out the "sense" outer primer (L1M) and by the use of seminested RT-PCR, i.e. ANMER B/AS62 as the first round primer, both HIV-1 subtype B and E virus were detectable for the codon 215 genotype (as shown in Figure 3, the sample no. 6 and 7, respectively).



Lane no. 1 2 3 4 5 6 7 8

sample 1 = wild type control plasmid (lane 2,3)

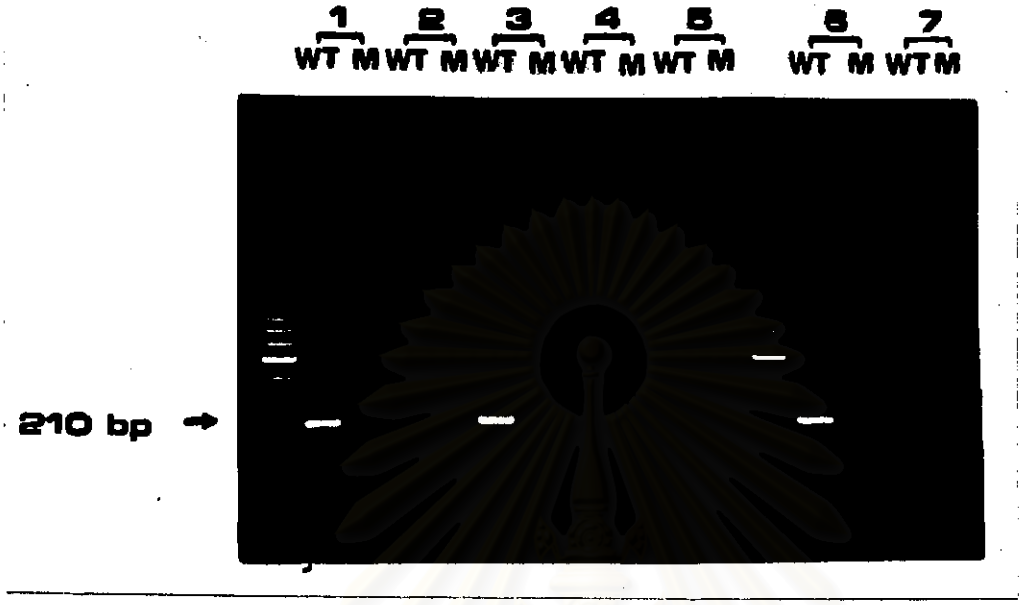
sample 2 = mutant control plasmid (lane 4,5)

sample 3 = negative control (lane 6,7)

100 bp DNA Ladder was used as DNA size marker in lane 1 and 8.

Figure 2. Analysis of plasmid DNA (wild type and mutant 215) with the selective 215 RT-PCR

(WT indicates 215 wild type primer and MT indicates 215 mutant primer that used for each analysis)



Lane no. 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

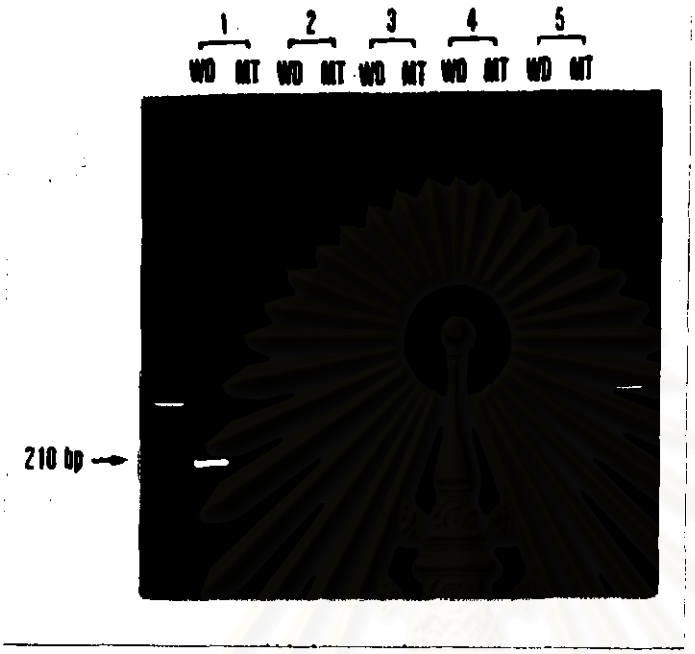
1. known HIV-1 subtype B isolate
2. known HIV-1 subtype E isolate
3. wild type control plasmid
4. mutant control plasmid
5. negative control
6. known HIV-1 subtype B
7. known HIV-1 subtype E

100 bp DNA Ladder were used as DNA size marker in lane 1 and 12.

Figure 3. The previousely described selective 215 RT-PCR needed modification for detecting HIV-1 subtype E.

(Sample no. 1 and 2 were analysed using the original selective 215 RT-PCR, whereas sample no. 6 and 7 were done by the use of the modified semi-nested 215 RT-PCR)

Part II Modified seminested RT-PCR to detect both HIV-1 subtype B and E



Lane no. 1 2 3 4 5 6 7 8 9 10 11 12

1. wild type control plasmid (lane 2,3)
2. patient no. 1, group I (lane 4,5)
3. patient no. 4, group II (lane 6,7)
4. mutant control plasmid (lane 8,9)
5. negative control (lane 10,11)

There were a non specific amplification in lane 9 and 11.

100 bp DNA Ladder were used as DNA size marker in lane 1 and 12.

Figure 4. Analysis of RT codon 215 in HIV-1 infected patients with modified selective PCR

(results from patients no.1 of group I, and no.4 of group II)

Part III. The Cross-sectional study for prevalence of AZT-resistant 215 mutant genotype

1. Demographic characteristics (Table IV,V)

There were no differences in sex, age and heterosexual risk factor among the 3 groups. However the patients in the group II and III were more advanced in disease progression than in the group I ($p < 0.01$).

The detection of AZT-R mutant at codon 215 after AZT therapy were 1 to 6 months (median =3.71) in the group II and 6.1 to 39 months (median = 15.7) in the group III.

2. The prevalence of AZT-resistant 215 mutant genotype (Table V)

All of the HIV-naive patients (group I) showed solely wild type at codon 215 (0% mutant). There were evidence of mutant variants in 11 of the 50 (22%) of patients from the group II (AZT-experienced < 6 months), and 21 of the 50 (42%) patients from the group III (AZT-experienced > 6 months). The prevalences of AZT-resistant mutant in the group II and III were significantly higher than that of the group I ($p < 0.01$ and $p = 0.001$, respectively).

3. The correlation between the occurrences of AZT-215 resistant mutant and CD4 cell counts

In the groups II, the mean of the baseline CD4 cell counts of the patients who carried AZT-215 mutant was significantly lower than that of the patients who carried AZT-215 wild type virus, i.e. 63.6 ± 50.2 vs. 132.5 ± 107.6, but there no significant differences (225.6 ± 151.3 vs. 173.4 ± 141.3) in the group III. (Table V, Figure 5 and Figure 6)

Table IV. Characteristics of the study groups

Group	Sex		Age	Clinical stages				Risk factors		
	male	female	(years)	Asym	PGL	ARC	AIDS	Heterosexuals	Homo/Bisexual	IVDUs
I. Naive	35 (70%)	15 (30%)	18-56 ($\bar{X}$=32.1)	13 (26%)	5 (10%)	22 (44%)	10 (20%)	47 (94%)	3 (6%)	-
II. AZT-experienced < 6 months	39 (78%)	11 (22%)	22-57 ($\bar{X}$=34.4)	6 (12%)	-	19 (38%)	25 (50%)	48 (96%)	1 (2%)	1 (2%)
III. AZT-experienced > 6 months	40 (80%)	10 (20%)	21-67 ($\bar{X}$=35.9)	-	1 (2%)	28 (56%)	21 (42%)	46 (92%)	2 (4%)	2 (4%)

Table V. The prevalence of AZT resistance codon 215 genotype and the correlation with CD4 cell counts

Group	Month of AZT Treatment (mean)	Wild type		Mutant		Total
		Number of subjects	Mean of CD4 cell counts (±S.D.)	Number of subjects	Mean of CD4 cell counts (±S.D.)	
I : Naive	-	50 (100%)	237.6 ± 237.8	-	-	50
II : AZT-experienced < 6 months	3.7 ± 1.3	39 (78%) [a]	132.5 ± 107.6	11 (22%) [a]	63.6 ± 50.2 [c]	50
III : AZT-experience > 6 months	15.2 ± 8.6	29 (58%) [b]	225.6 ± 151.3	21 (42%) [b]	173.4 ± 141.3 [d]	50

[a] $p < 0.01$ (Chi-square test) as compared to that of group I

[b] $p = 0.001$ (Chi-square test) as compared to that of group I

[c] $p < 0.05$ (Mann-Whitney test) as compared to that of the subjects who carried wild type virus in group II

[d] $p > 0.1$ (Mann-Whitney test) as compared to that of the subjects who carried wild type virus in group III

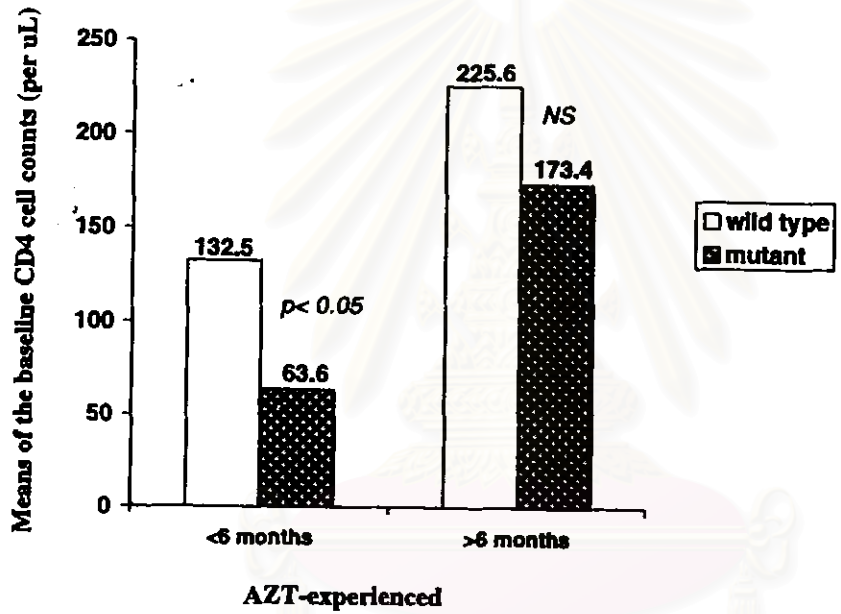


Figure 5. AZT-215 mutation and means of the baseline CD4 cell counts between the group of less than and more than 6 months of AZT treatment

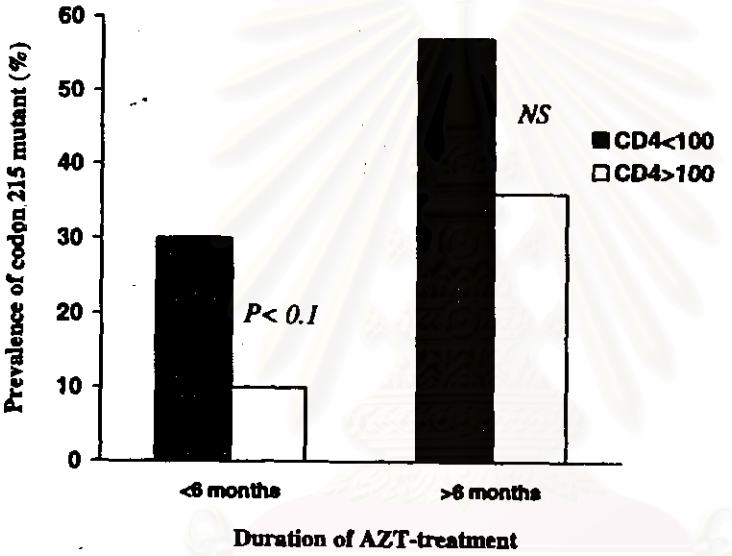


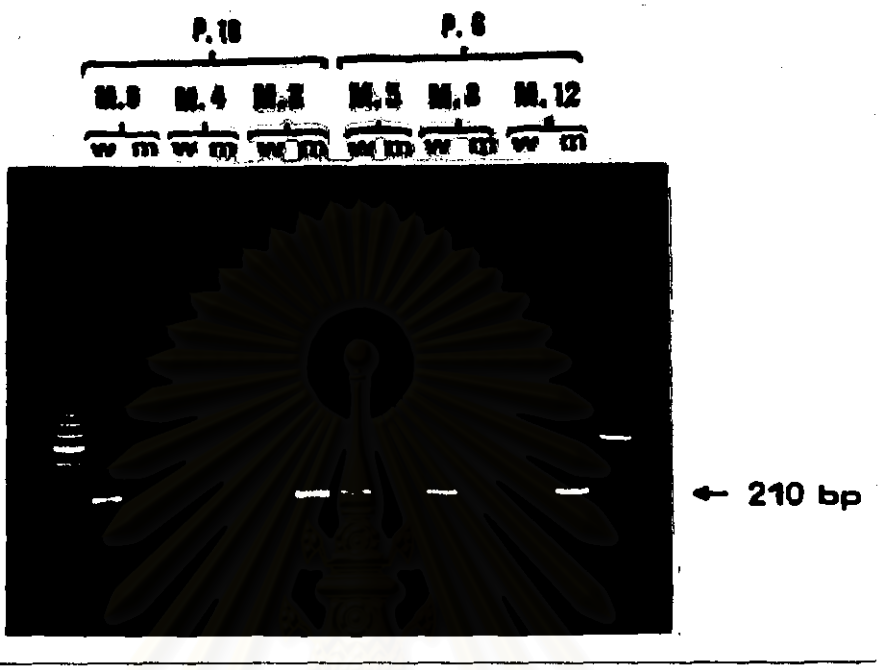
Figure 6. Comparison of the prevalences of codon 215 mutant between the patients with baseline CD4 less than and more than 100 /μL

4. Prospective study in ten subjects

In the prospective study, 9 out of the 10 were males, 7 were AIDS and 3 were ARC. The CD4 cell counts were 4-587 cells/μl. As summarized in Table VI, no resistant mutant was observed at the baseline. After 4-13 months of AZT monotherapy, 7 out of the 10 (70%) subjects showed existing of mutation at the codon 215. There were 4 subjects who had 3 time-points (8-12 months of follow-up after AZT treatment) of sample collection (P.3, P.4, P.6 and P.10), 2 out of the 4 showed a mixed genotype (both wild type and mutant) at the second time-point (P.4 at month 8^{th}, and P.10 at month 4^{th} after treatment. (Table VI and Figure 7)

Table VI. Clinical And Laboratory Details

Pateint number	Stage	Sex	Age	CD4 (per μL)	Month of AZT-treatment	RT 215
P.1	ARC	M	34	14	0	WD
				29	7	**MT**
P.2	AIDS	M	28	277	0	WD
				175	6	**MT**
P.3	AIDS	M	32	33	5.2	WD
				45	6	WD
				33	10	**MT**
P.4	AIDS	M	33	473	5	WD
				297	8	MIXED
				170	11	**MT**
P.5	AIDS	M	34	4	0	WD
				ND	6	**WD**
P.6	AIDS	M	26	120	5.2	WD
				72	8	WD
				ND	12	**MT**
P.7	AIDS	M	48	587	7	WD
				ND	13	**WD**
P.8	ARC	M	26	155	0	WD
				175	7	**MT**
P.9	ARC	F	30	323	0	WD
				342	5.2	**WD**
P.10	AIDS	M	30	93	0	WD
				18	4	MIXED
				ND	8	**MT**



Lane no. 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Lane 1, 14 = 100 bp. DNA Ladder were used as DNA size markers

Lane 2-3, 4-5, 6-7 = The samples of month 0, 4 and 8 of patient no. 10

Lane 8-9, 10-11, 12-13 = The samples of month 5, 8 and 12 of patient no.6

W = 215 wild type

m = 215 mutant

Figure 7. The prospective results of patients no. 6 and 10

(In patient no. 10, at month 4 of AZT monotherapy, there was a mixed codon 215 genotype, and at month 8, the only mutant genotype was observed. In patient no. 6, at the month 5 and 8 of therapy, the HIV -1 virus remaked wild type genotype, and was switched to mutant at month12)