CHAPTER IV

RESULTS

1. X-linked adrenoleukodystrophy (X-ALD)

1.1 Mutation analysis of the *ABCD1* gene

Sequence analysis of the *ABCD1* gene from the 9 unrelated patients with X-ALD identified 5 disease-causing mutations. There were 5 different sequence variants including 2 novel mutations.

Patient 1: A boy with clinical features consistent with X-ALD was found to have a known missense mutation on exon 9. He was hemizygous for a G to C mutation at nucleotide position 1936 (c.1936G>C) (Figure 10). The mutation is expected to result in an alanine to proline substitution at codon 646 (p. A646P).

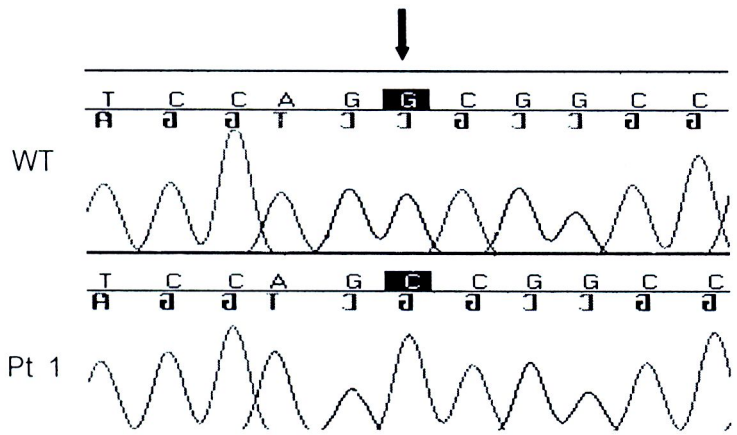

Figure 9. Electropherogram showing the c.1936G>C (p.A646P) mutation in the *ABCD1* gene.

Patient 2: A boy with X-ALD was found to have a known missense mutation on exon 8. He was hemizygous for a G to A mutation at nucleotide position 1825 (c.1825G>A) (Figure 11). The mutation is expected to result in a glutamic acid to lysine substitution at codon 609 (p.E609K).

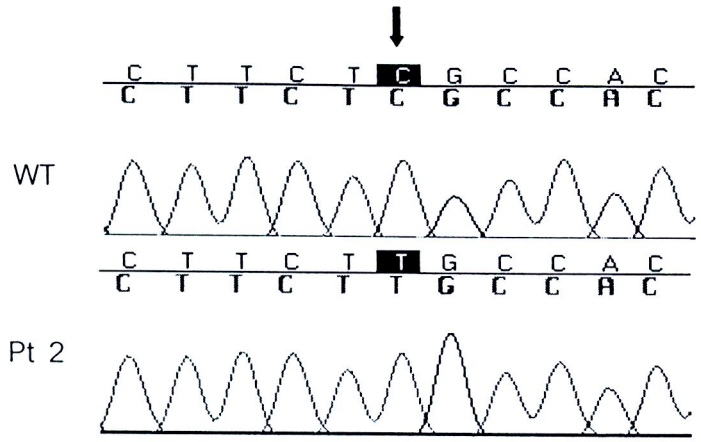

Figure 10. Electropherogram showing the c.1825G>A (p.E609K) mutation in the *ABCD1* gene.

Patient 3: A boy with X-ALD was found to have a known missense mutation on exon 3. He was hemizygous for a C to T mutation at nucleotide position 1201 (c.1201C>T) (Figure 12). The mutation is expected to result in an arginine to tryptophan substitution at codon 401 (p. R401W).

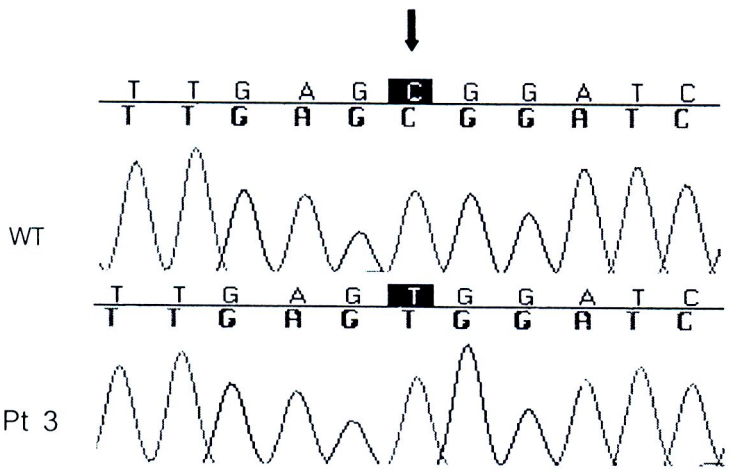

Figure 11. Electropherogram showing the c.1201C>T (p.R401W) mutation in the *ABCD1* gene.

Patient 4: A boy with X-ALD was found to have a novel missense mutation on exon 3. He was hemizygous for a T to C mutation at nucleotide position 1175 (c. 1175T>C) (Figure 13). The mutation is expected to result in a leucine to proline substitution at codon 392 (p. L392P).

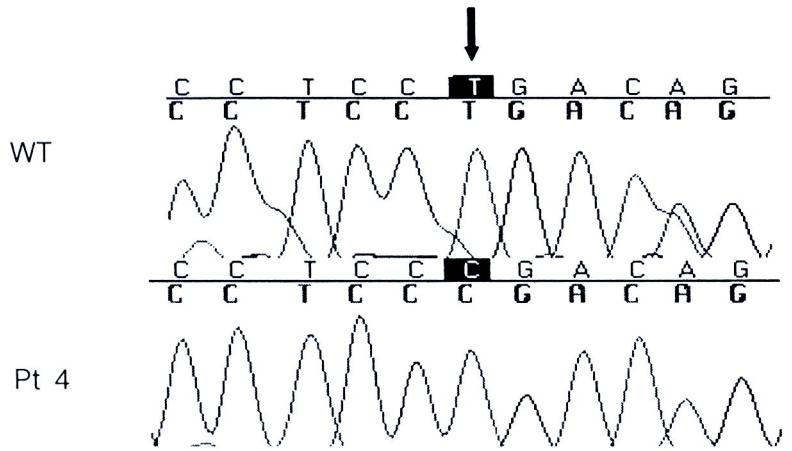

Figure12. Electropherogram showing the c. 1175T>C (p.L392P) mutation in the *ABCD1* gene.

Patient 5: A boy with X-ALD was found to have a novel missense mutation on exon 1. He was hemizygous for a C to A mutation at nucleotide position 740 (c. 740C>A) (Figure 14). The mutation is expected to result in an alanine to aspartic acid substitution at codon 247 (p. A247D).

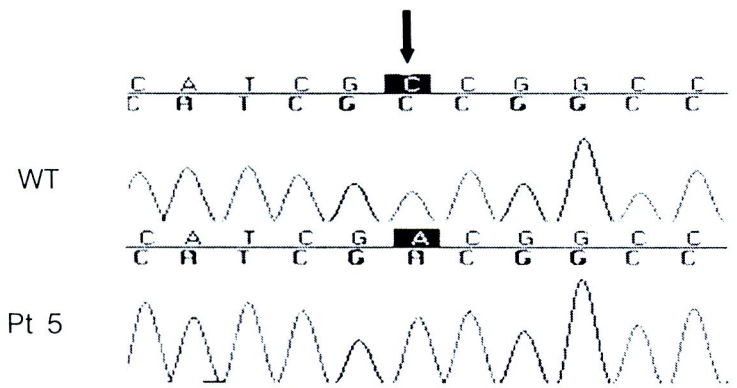

Figure.13Electropherogram showing the c. 740C>A (p.A247D) mutation in the *ABCD1* gene .

The mother of patient 5: The mother of patient 5 was unaffected and was found to be carry the novel missense mutation on exon 1. She was heterozygous for a C to A mutation at nucleotide position 740 (c. 740C>A) as shown by restriction enzyme digestion with *Tag*I (Figure 15).

Table 10. The restriction enzyme digestion to screening for the c. 740C>A mutation

Mutation	Primer	Expected size before digestion	Restriction enzyme	Cut site 5' to 3'	Expected size after digestion		
					Normal	Homozygous mutant alleles	Heterozygous mutant alleles
c.740C>A (p. A247D)	ALDe1C-F ALDe1C-R	514	*Tag*I	T/CGA	512 - -	- 280 234	512 280 234

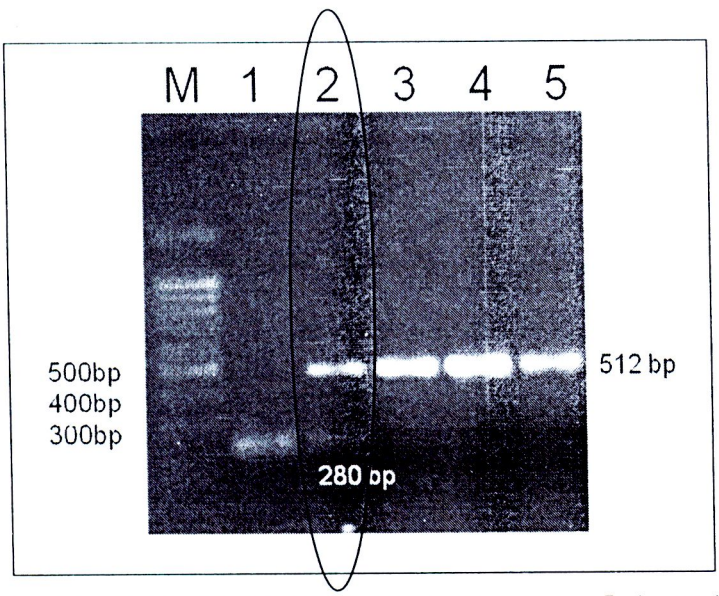

Figure14. Restriction enzyme digestion analysis of patient 5, the mother of patient 5 and 50 unaffected controls. M = 100 bp DNA marker; Lane1 = patient 5; Lane 2 = patient's mother; Lanes 3-5 = unaffected controls

1.2 Confirmation of two novel mutant alleles by restriction enzyme digestion and sequencing

Restriction enzyme digestion of PCR products of the patient and 50 normal controls was carried out to confirm the presence of the identified A247D novel mutation as shown in Table 11 and Figure 15.

PCR and direct sequencing of the patient and 50 normal controls was carried out to confirm the presence of the identified L392P novel mutation that as shown in figure16.

Table11. PCR-RFLP for confirmation of the novel mutation, c. 740C>A

Mutation	Primer	Expected size before digestion	Restriction enzyme	Cut site 5' to 3'	Expected size after digestion		
					Normal	Homozygous mutant alleles	Heterozygous mutant alleles
c.740C>A (p. A247D)	ALDe1C-F ALDe1C-R	514	*TagI*	T/CGA	512 - -	- 280 234	512 280 234

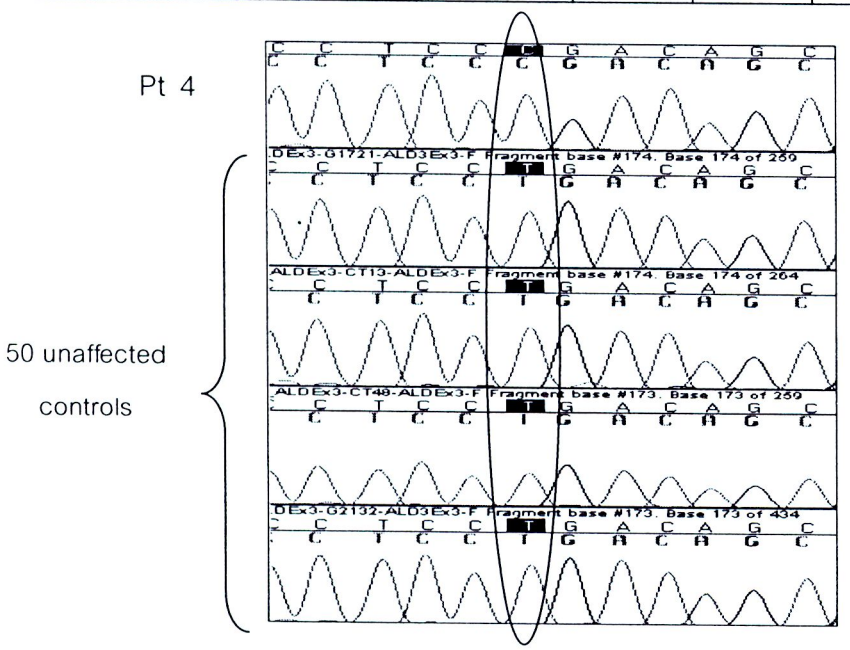

Figure 15 Electropherogram showing the p.L392P in patient 4 and 50 unaffected controls in the *ABCD1* gene.

2. Pompe disease

Mutation analysis of the *GAA* gene: 2 steps

We initially screened for a common mutation, p.D645E by PCR-RFLP. If the patient did not have the common mutation, pD645E, we performed direct sequencing in cDNA to screen for other mutations.

2.1 Restriction enzyme *BsaH I*

Restriction enzyme digestion is the simple and affordable method. We screened for the common mutation, D645E in Thai Pompe patients. There were totally 5 families. The results of PCR-RFPL studies were shown on table 12 and figure 17

Table12. PCR-RFLP for confirmation of the common mutation, c.1935C>A

Mutation	Primer	Expected size before digestion	Restriction enzyme	Cut site 5' to 3'	Expected size after digestion		
					Normal	Homozygous mutant alleles	Heterozygous mutant alleles
c.1935C>A (p. D645E)	GAA-ex15-F GAA-ex15-R	397	*BsaH I*	GR/CGYC	- 282 115	397 - -	397 282 115

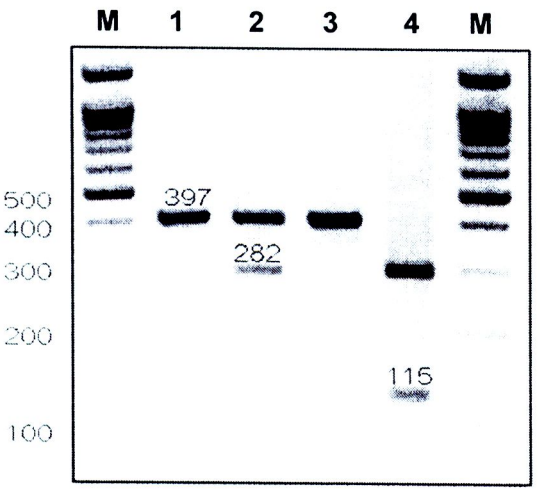

Figure16. Restriction enzyme digestion to detect the D645E mutation
M: 100 bp marker. Lane 1: A homozygous D645E control. Lane 2: A heterozygous D645E carrier. Lane 3: Patient 3. Lane 4: unaffected control.

2.2 Sequencing

For sequencing, RNA was changed to cDNA by reverse transcription.Then we used cDNA template for nested PCR amplify coding regions with the forward primer, GAA-F1 and reverse primer GAA-R1 produce 3,240 bp. Then amplified fragments from nested PCR by GAA-F1 and GAA-R2 produced the 1,330 bp and GAA-F2 and GAA-R1 produced the 1909 bp.

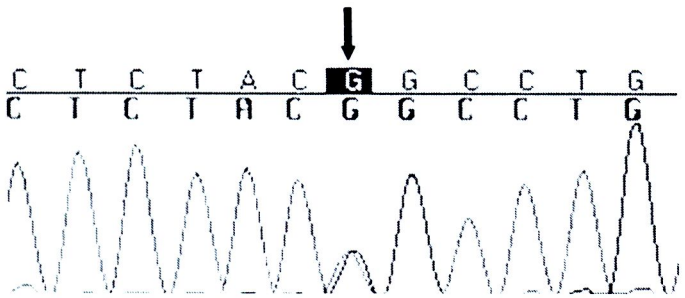

Figure 17. Electropherogram showing the c.1726G>A (p. G576S) mutation in the *GAA* gene.

We found the c.1935C>A (p.D645E) mutation in all families and found the c.1726G>A (p. G576S) mutation in one family summarized in table 13.

Table13. The result of *GAA* analysis in 6 unrelated Thai families

Family	Case	Patient	Genotype		
			Result	Methods	Diagnosis
Family 1	Prenatal	Patient 1	D645E / D645E	Sequence in Taiwan	Pompe
		Mother	D645E / Wild type	Restriction enzyme	Carrier
		Father	D645E / Wild type	Restriction enzyme	Carrier
		CVS from mother	D645E / Wild type	Restriction enzyme	Carrier
Family 2	Prenatal	Patient 2	D645E / D645E	Restriction enzyme	Pompe
		Mother	D645E / Wild type	Restriction enzyme	Carrier
		Father	D645E / Wild type	Restriction enzyme	Carrier
		AF from mother	D645E / D645E	Restriction enzyme	Pompe
Family 3	Prenatal	Mother	D645E / Wild type	Restriction enzyme	Carrier
		Father	G576S / Wild type	Sequencing	Carrier
		CVS from mother	D645E / G576S	Restriction enzyme & Sequencing	Pompe
Family 4	Confirm Diagnosis	Patient 3	D645E / D645E	Restriction enzyme	Pompe
		Mother	D645E / Wild type	Restriction enzyme	Carrier
		Father	D645E / Wild type	Restriction enzyme	Carrier
		Sister's Patient 3	Wild type / Wild type	Restriction enzyme	Normal
Family 5	Confirm Diagnosis	Patient 4	??? / D645E	Restriction enzyme & Sequencing	Pompe
		Father	Wild type / D645E	Restriction enzyme	Carrier
		Mother	??? / Wild type	Sequencing	Carrier

3. Hyper-IgE Syndrome (HIES)

For sequencing, RNA was changed to cDNA by reverse-transcription and the product was PCR-amplified. The product contained 1,780 bp

The patient : A boy with clinical features consistent with Hyper-IgE (AD) was found to have a known missense mutation on the DNA binding domain. He was heterozygous for a C to T mutation at nucleotide position 1144 (c.1144C>T) (Figure 19). The mutation was expected to result in an arginine to tryptophan substitution at codon 382 (p.R382W).

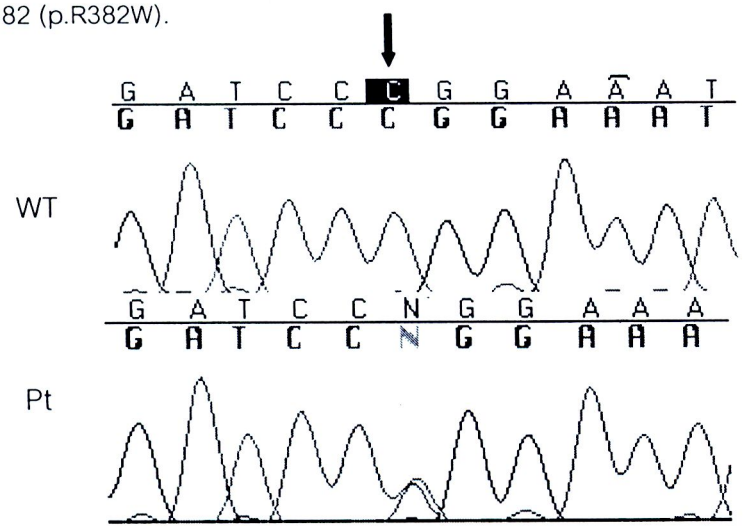

Figure18. Electropherogram showing the c.1144C>T (p.R382W) mutation in the *STAT3* gene.

4. Holt-Oram syndrome (HOS)

Sequence analysis of the *TBX5* gene in 4 unrelated patients with HOS could not identify disease-causing mutations in *TBX5*. However, a previous report identified a *de novo* pericentric inversion of chromosome 20q13.2, where *SALL4* was located, in a patient with clinical features consistent with HOS. We therefore performed mutation analysis of the *SALL4* gene in our patients with Holt-Oram syndrome but without mutations in *TBX5*. PCR-sequencing of the *SALL4* gene in 4 unrelated patients with HOS could not identify mutations in *SALL4*.

5. Systemic Lupus Erythematosus (SLE)

5.1 SLE Disease Activity Index (SLEDAI)

It is used to describe the disease activity. Using the SLEDAI score, there were 9 active patients and 38 inactive patients in our cohort.

Table14. SLEDAI score of 47 SLE patients

Inactive stage			Active stage							
Mild disease					Moderate to severe disease					Severe disease
0-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	>20
28	5	5	2	4	1	-	1	-	-	2

5.2 Mutation analysis of the *DcR3* gene

Sequence analysis of the *DcR3* gene from the 89 unrelated patients with SLE identified one potential disease-causing mutation which has never been described.

SLE Patients : Out of 89 unrelated SLE patients, a girl with SLE was found to have a novel missense mutation in the *DcR3* gene. She was heterozygous for a C to T mutation at nucleotide position 364 (c.364C>T) (Figure 20). The mutation is expected to result in a histidine to tyrosine substitution at codon 122 (p.H122Y).

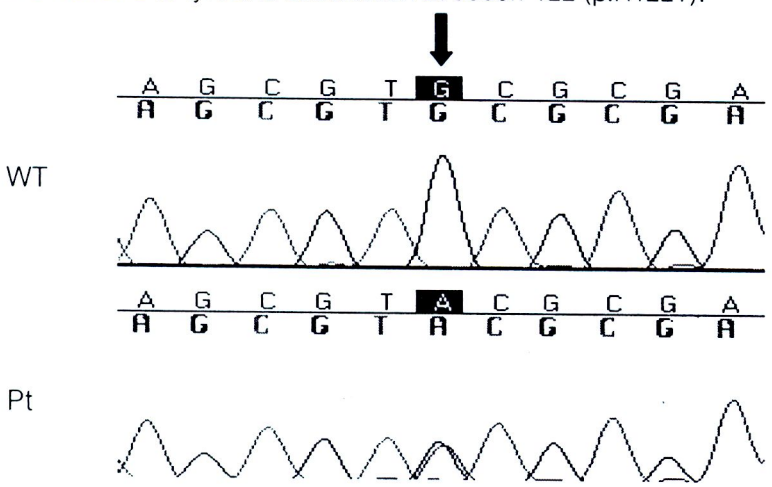

Figure19. Electropherogram showing the c.364C>T (p.H122Y) mutation in the *DcR3* gene.

5.3 Confirmation of the novel mutant allele by restriction enzyme digestion

Restriction enzyme digestion of PCR products of the patient and 500 unaffected controls was carried out to confirm the presence of the identified novel mutation as shown in Table 15 and Figure 21.

Table15. PCR-RFLP for confirmation of the novel mutation, c.364C>T.

Mutation	Primer	Expected size before digestion	Restriction enzyme	Cut site 5' to 3'	Expected size after digestion		
					Normal	Homozygous mutant alleles	Heterozygous mutant alleles
c.364C>T (p.H122Y)	DcR3-gDNA-F1.2 DcR3-gDNA-R1.2	442	*Rsa*I	GT/AC	442	- 279 164	442 279 164

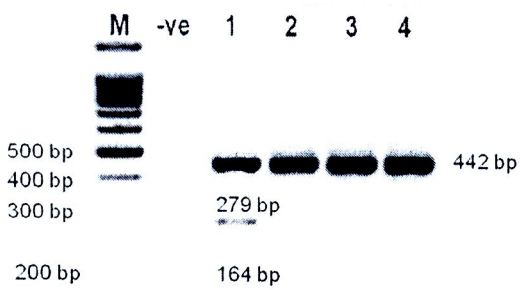

Figure 20 PCR-RFLP to confirm the presence of a novel mutation, c.364C>T in *DcR3*. M = 100 bp DNBA marker; -ve = no template; Lane 1 = patient; Lane 2-4 = unaffected controls

In 500 unaffected controls, we found one sample that can be cut by restriction enzyme digestion. The digestion pattern was similar to that of the patient with the c.364C>T mutation.

5.4 Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to detect the level of DcR3 in serum of 52 SLE patients and 25 controls who were unaffected with SLE. The serum DcR3 levels of active SLE patients, inactive SLE patients and unaffected controls were 436.35 ±433.71 pg/μl, 68.04 ±158.52 pg/μl and 222.9141±194.8946 pg/μl, respectively, as shown in figure 22.

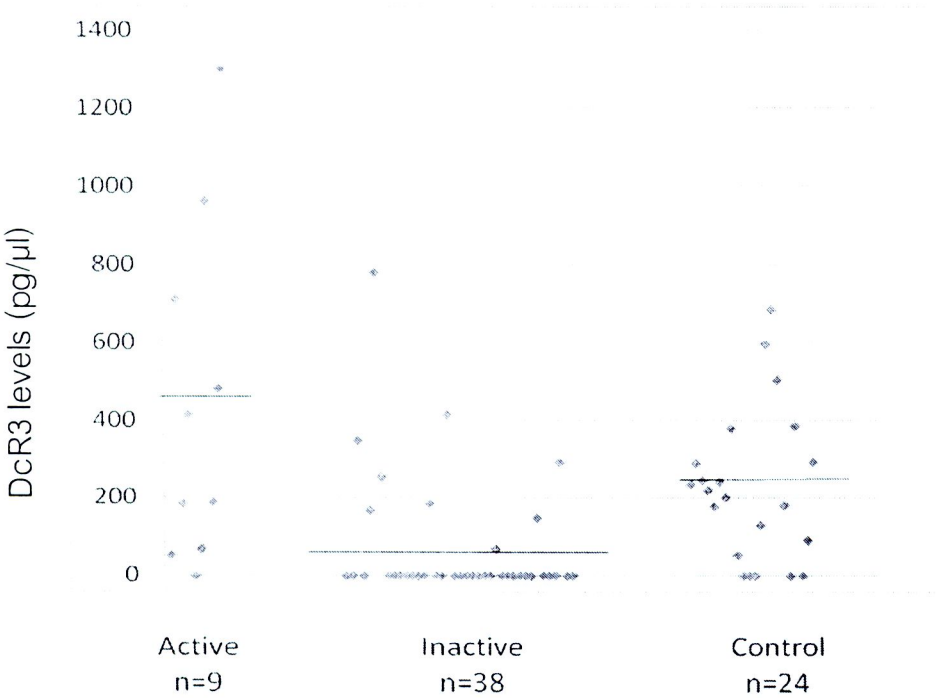

Figure 21 Serum DcR3 levels (pg/μl) in SLE patients and unaffected controls assayed by ELISA.