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PRACTICAL TECHNIQUES OF MUTATION DETECTION FOR PARTICULAR GENETIC DISEASES IN THAI PATIENTS

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	Practical techniques of mutation detection for particular genetic diseases in Thai patients
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ประมุข อัมรินทร์นุเคราะห์ : วิธีการตรวจหาการกลายพันธุ์ของโรคทางพันธุกรรมบางโรคในประชากรไทย (Pratical techniques of mutation detections for particular genetic diseases in Thai patients) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ศ.นพ.วรศักดิ์ โชติเลอศักดิ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.พญ.ดร.กัญญา ศุภปีติพร , 73 หน้า

โรคทางพันธุกรรมคือโรคที่มีสาเหตุจากการกลายพันธุ์ในยีนหรือความผิดปกติในระดับโครโมโซม ซึ่งความผิดปกตินี้สามารถเป็นได้ตั้งแต่ การเปลี่ยนแปลงเพียงเบสเดียวในสายดีเอ็นเอจนถึงลักษณะของโครโมโซมที่ผิดไป โดยวิธีการตรวจสอบความผิดปกตินี้มีหลายวิธีด้วยกัน ซึ่งหนึ่งในวิธีตรวจสอบที่มีประสิทธิภาพ คือ การตรวจสอบในระดับพันธุกรรม ในการศึกษาครั้งนี้จะนำเสนอวิธีการตรวจสอบในระดับพันธุกรรมที่เหมาะสมกับโรคทางพันธุกรรมบางโรคที่พบได้บ่อยในคลินิกพันธุศาสตร์ของโรงพยาบาลจุฬาลงกรณ์ โดยเริ่มจากการวิเคราะห์โรค X-linked adrenoleukodystrophy (X-ALD) ในยีน *ABCD1* ด้วยวิธี direct sequencing ซึ่งพบมีการกลายพันธุ์แบบที่เคยมีรายงานก่อนหน้านี้ 3 แบบ ได้แก่ A646P, E609K, R401W และพบยังไม่เคยมีรายงานมาก่อนอีก 2 แบบ ได้แก่ L392P และ A247D โรคที่สองที่ทำการศึกษาวิเคราะห์ คือ Pompe disease ในยีน *GAA* ด้วยวิธี PCR-RFLP บนตำแหน่งการกลายพันธุ์ที่พบได้บ่อยของโรคนี้ (hotspot) และวิธี direct sequencing ใน cDNA ซึ่งพบการกลายพันธุ์ที่พบได้บ่อยในโรคนี้คือ D645E ในผู้ป่วยทุกราย และพบครอบครัวหนึ่งมีการกลายพันธุ์อื่นร่วมด้วย คือ G576S โรคที่สามที่ได้วิเคราะห์คือ hyper-IgE syndrome (HIE) ในยีน *STAT3* โดยวิธี direct sequencing ครอบคลุมเฉพาะบริเวณ DNA binding และ SH2 ของยีนดังกล่าว ซึ่งผลการวิเคราะห์พบการกลายพันธุ์ R382W ที่เคยมีรายงานมาแล้วบนบริเวณ DNA binding domain โรคต่อมาที่ได้ทำการวิเคราะห์ คือ Holt-Oram syndrome (HOS) ในยีน *TBX5* และ *SALL4* ในผู้ป่วยทั้ง 4 คน ซึ่งไม่พบการกลายพันธุ์ในยีนทั้งสอง นอกจากนี้ ได้ทำการวิเคราะห์ยีนซึ่งอาจจะมีความเกี่ยวข้องกับโรค Systemic lupus erythematosus (SLE) คือ *DcR3* โดยทำการวิเคราะห์โดยวิธี PCR-sequencing และวัดระดับ DcR3 ในซีรั่ม โดยวิธี ELISA พบว่า ผู้ป่วย SLE ที่มีอาการรุนแรง จะมีระดับซีรั่ม DcR3 โดยเฉลี่ย 436.35 pg/µl (±433.71) ผู้ป่วย SLE ที่มีอาการไม่รุนแรง จะมีระดับซีรั่ม DcR3 โดยเฉลี่ย 68.04 pg/µl (±158.52) และ คนที่ไม่เป็นโรค SLE จะมีระดับซีรั่ม DcR3 โดยเฉลี่ย 222.9141pg/µl (±194.8946) จากผลดังกล่าวจะเห็นได้ว่าระดับ DcR3 ไม่มีความแตกต่างในผู้ป่วย SLE กลุ่มรุนแรงกับกลุ่มตัวอย่างที่ไม่เป็นโรค SLE แต่ระดับ DcR3 ในผู้ป่วย SLE ที่มีอาการรุนแรง มีระดับที่สูงกว่าในผู้ป่วย SLE ที่มีอาการไม่รุนแรงอย่างมีนัยสำคัญทางสถิติ การตรวจการกลายพันธุ์โดย PCR-sequencing พบมีการกลายพันธุ์ใหม่ที่ยังไม่เคยมีรายงาน คือ H122Y อยู่บนบริเวณ Fas binding domain ในผู้ป่วย SLE หนึ่งราย ซึ่งการกลายพันธุ์ดังกล่าวอาจเป็นปัจจัยหนึ่งที่ทำให้ผู้ป่วยเกิดเป็น SLE ขึ้นได้ การตรวจการกลายพันธุ์มีหลายวิธี การเลือกใช้ให้เหมาะสมเพื่อได้วิธีที่เชื่อถือได้ ถูกต้อง รวดเร็วขึ้นอยู่กับลักษณะของยีนที่เกี่ยวข้องกับการเกิดโรคในแต่ละโรค เช่น ขนาด การแสดงออกในเม็ดเลือดขาว ลักษณะการกลายพันธุ์ที่พบมาก่อนหน้านี้ ช่วยให้ได้การทดสอบทางพันธุกรรมที่เหมาะสมในแต่ละโรคและแต่ละประชากร

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....ประมุข อัมรินทร์นุเคราะห์.....

ปีการศึกษา 2552 ลายมือชื่อ อ .ที่ปรึกษาวิทยานิพนธ์หลัก .....

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PRAMUK AMARINTHNUKROWH: PRATICAL TECHNIQUES OF MUTATION DETECTIONS FOR PARTICULAR GENETIC DISEASES IN THAI PATIENTS. THESIS ADVISOR: PROF. VORASUK SHOTELERSUK, M.D. THESIS CO-ADVISOR: ASST. PROF. KANYA SUPHAPEETIPORN, M.D., Ph.D., 73 pp

Genetic disorders are caused by mutations in genes or abnormalities of chromosomes. The abnormality can range from a discrete mutation in a single base in the DNA to a gross chromosome abnormality. There are several methods for mutation detection. In this study, we demonstrated some of methods to properly characterize genetic diseases that were identified at Pediatric Clinic of the King Chulalongkorn Memorial Hospital. We have collected 9 patients with X-Adrenoleukodystrophy (X-ALD) and performed PCR-sequencing of entire coding region of the *ABCD1* gene. Three known mutations, A646P, E609K, R401W and two novel mutations, L392P and A247D were detected. We also analyzed the *GAA* gene causing Pompe disease by PCR-RFLP or direct sequencing on the previously reported mutations in the Chinese population. The possible founder effect mutation, D645E was found in all families and G576S known mutation in one family. A recently identified gene responsible for hyper-IgE syndrome (HIE) was also studied in one patient. Direct sequencing covering the region coding for the DNA binding and SH2 domains in the *STAT3* gene was performed and revealed the known mutation, R382W on the DNA binding domain. We also found 4 patients with clinical features consistent with Holt-Oram syndrome. However, PCR-direct sequencing could not identify disease-causing mutations in the *TBX5* and *SALL4* genes. Finally, a possible candidate gene, *DcR3*, for SLE was characterized. ELISA was used to detect the level of DcR3 in serum of 52 SLE patients and 25 controls who were unaffected with SLE. The DcR3 levels were significantly higher in the active group compared to the inactive SLE patients. However, there was no difference between the SLE patients and the unaffected controls. Interesting, a novel mutation, H122Y, was detected in the *DcR3* gene in one SLE patient. The mutation was on the Fas binding domain. Further studies are required to elucidate its functional significance.

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Student's Signature Pramuk Amarinthnukrowh

Advisor's Signature Vorasuk Shotelersuk

Co- Advisor's Signature Kanya Suphapeetiporn

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LIST OF ABBREVIATIONS

X-ALD	=	X- Linked Adrenoleukodystrophy
HIES	=	Hyper-IgE syndrome
HOS	=	Holt-Oram syndrome
SLE	=	Systemic Lupus Erythematosus
Ig	=	Immunoglobilin
ELISA	=	Enzyme-linked immunosorbent assay
SLEDAI	=	Systemic Lupus Erythematosus Disease Activity Index

CHAPTER I
INTRODUCTION

Background and rationale

Genetic disorders are caused by abnormalities in an individual's genetic material (genome). The abnormality can range from a discrete mutation in a single base in the DNA of a single gene to a gross chromosome abnormality involving the addition or subtraction of an entire chromosome or set of chromosomes.

Mutations are changes to the nucleotide sequence of the genetic material of an organism. It can be caused by copying errors in the genetic material during cell division. Mutations can affect the function of genes leading to genetic disorders. Some examples include X-linked adrenoleukodystrophy, Glycogen storage disease, Hyper-IgE syndrome, Holt-Oram syndrome, systemic lupus erythematosus.

X-linked adrenoleukodystrophy (X-ALD) is a neurodegenerative disorder, with a minimum incidence of 1:21,000 males. X-ALD is cause by alterations in the *ABCD1* (ATP-binding cassette, sub-family D [ALD]) gene. Mutations in this gene cause a defect in peroxisomal β-oxidation leading to an accumulation of saturated very long chain fatty acids (VLCFA) in all tissues of patients, which results in demyelination. Patients with X-ALD can be diagnosed by an increase in long chain fatty acid levels in plasma and confirmed by mutation analysis in the *ABCD1* gene.

Glycogen storage disease type II (Pompe disease) is an autosomal recessive progressive muscular disorder caused by mutations in the acid α-glucosidase (*GAA*) gene. Patients with Pompe disease have impaired lysosomal degradation causing an accumulation of glycogen in multiple tissues, which results in severe muscle weakness. The diagnosis of Pompe disease can be confirmed by mutation analysis in the *GAA* gene.

Hyper-IgE syndrome (HIES), a rare primary immune deficiency, is caused by mutations in the *STAT3* (SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 3) gene. The clinical features of HIES patients include dermatitis, and

recurrent skin and lung infections. HIES can be diagnosed by a finding of increased IgE levels in plasma or mutation analysis of the *STAT3* gene

Holt-Oram syndrome (HOS) is an autosomal dominant disorder, which has an estimated frequency of 1 in 100,000 live births. The HOS patients were found to harbor mutations in the *TBX5* gene. The clinical features of HOS patients are a variety of upper limb skeleton malformations and congenital heart defects. HOS can be diagnosed by clinical features showing upper limb skeleton malformations with congenital heart defects or mutation analysis by PCR-sequencing of the *TBX5* gene.

Systemic lupus erythematosus (SLE) is an autoimmune disease inflicting damage to multiple organs. The disease prevalence is 0.05% in general population, with 80-90% of patients being female. The exact etiology of SLE has not been clear, but genetics, gender, and environment are involved in its pathogenesis. A previous report in 2007 showed that overexpression of DcR3 in mice resulted in a SLE–like syndrome. In addition, another report in 2008 revealed that SLE patients had elevated serum DcR3 levels when compared with DcR3 levels of normal controls. DcR3 might be a new diagnostic parameter and risk factor for SLE. The *DcR3* gene becomes an interesting new candidate for SLE.

Development in genetic testing has led to significant benefits in improving patient management including more accurate diagnosis, genetic counseling as well as prenatal diagnosis.

In this study, we demonstrated various approaches for mutation analysis in several genetic disorders. These methods were developed to help improve diagnosis of particular genetic disorders.

Research questions

1. Are Thai patients with clinically diagnosed ALD, Pompe disease, HIE, HOS, and SLE caused by mutations in the *ABCD1*, *GAA*, *STAT3*, *TBX5*, *SALL4*, *and DcR3* respectively?
2. Are these techniques practical to detect mutations in *ABCD1*, *GAA*, *STAT3*, *TBX5*, *SALL4*, *and DcR3*?

Objective

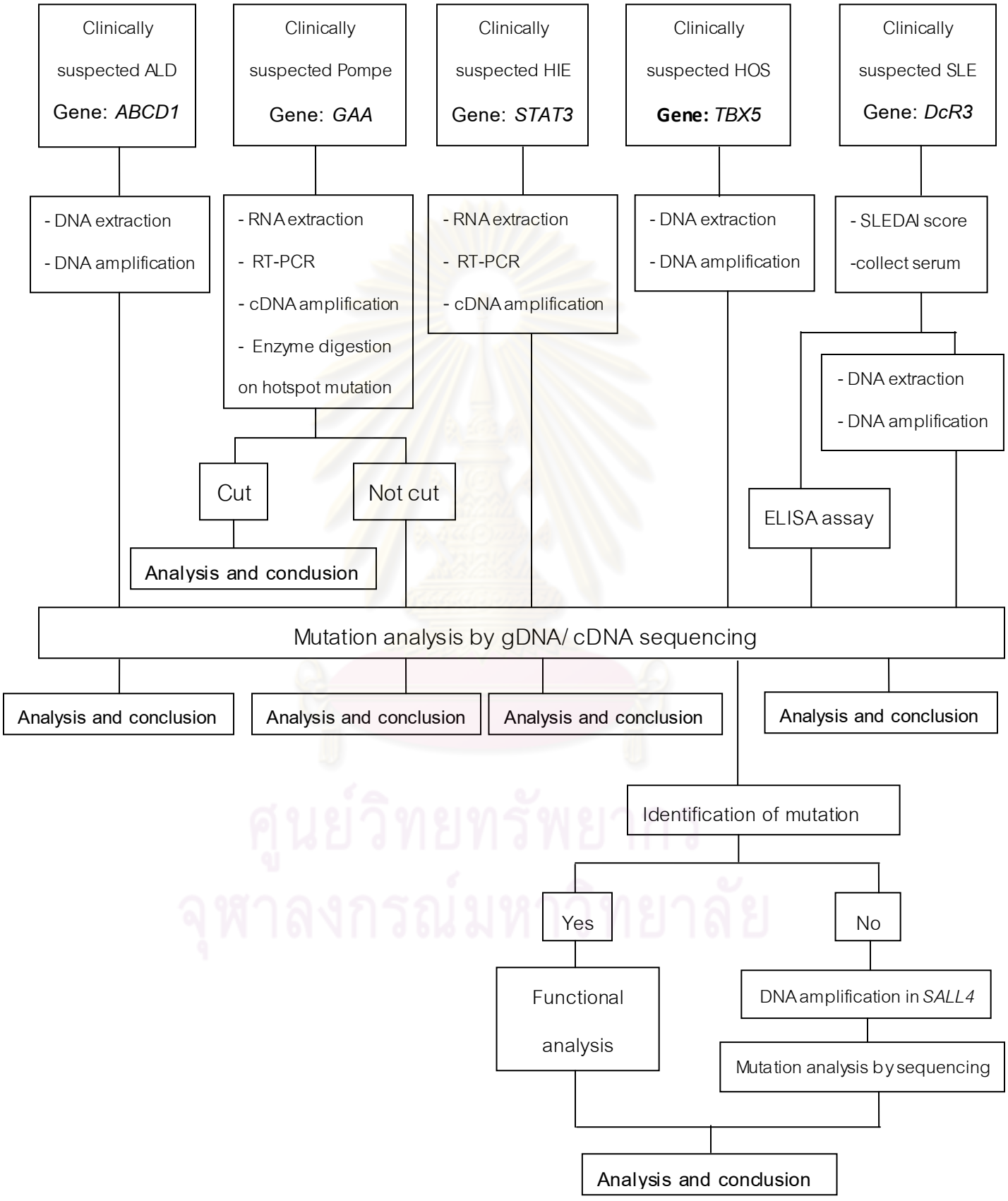
1. To develop techniques of mutation detection in the *ABCD1*, *GAA*, *STAT3*, *TBX5*, *SALL4*, *and DcR3* genes in patients with clinical features consistent with ALD, Pompe disease, HIE, HOS, and SLE, respectively.
2. To investigate efficiency of techniques for detection mutation in the *ABCD1*, *GAA*, *STAT3*, *TBX5*, *SALL4*, *and DcR3* genes.

Hypothesis

Thai patients with clinically-diagnosed ALD, Pompe disease, HIE, HOS, and SLE carry mutations in the *ABCD1*, *GAA*, *STAT3*, *TBX5*, *SALL4*, *and DcR3* genes, respectively.

Techniques of mutation detection have efficiency to detect mutations in Thai patients with these particular disorders.

Conceptual framework



Assumption

Cases are the patients with clinically-diagnosed ALD, Pompe, HIE, HOS, or SLE in which selection criteria were based on clinical presentation.

Controls are healthy volunteers who are unaffected with ALD, Pompe, HIE, HOS, SLE and have no family history of ALD, Pompe, HIE, HOS, SLE.

Key words

Mutation analysis, X-linked adrenoleukodystrophy, X-ALD, *ABCD1*, Glycogen Storage Disease Type II, Pompe disease, *GAA*, Hyper-IgE Syndrome, HIES, *STAT3*, Holt-Oram Syndrome, HOS, *TBX5*, *SALL4*, Systemic Lupus Erythematosus, SLE, *DcR3*

Operational Definition

Controls: Blood samples from the healthy volunteers who are unaffected with ALD, Pompe, HIE, HOS and SLE and have no family history of ALD, Pompe, HIE, HOS, and SLE.

Cases: Blood samples from the patients who are diagnosed with ALD, Pompe, HIE, HOS or SLE.

Sequencing is the process of determining the nucleotide order within DNA and RNA.

Enzyme-linked immunosorbent assay (ELISA) is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample.

Research Design

Descriptive and *in vitro* studies

Ethical Consideration

This study has been approved by the local Ethics Committee. Written informed consent was obtained from all patients or their parents who participated in the study.

Limitation

Some diseases have small sample size. Some genes do not express in leukocytes.

Expected Benefit and Application

This study will help identify Thai patients with ALD, Pompe, HIE, HOS and SLE as well as to expand the mutation spectrum of the *ABCD1*, *GAA*, *STAT3*, *TBX5*, *SALL4* and *DcR3* genes. In addition, testing for mutations will help physicians to correctly diagnose Thai patients leading to appropriate genetic counseling, therapy and prenatal diagnosis.

Research Methodology

1. Sample collection

1.1 Cases wereThai patients with ALD, Pompe, HIE, HOS or SLE who were diagnosed by clinical geneticists at King Chulalongkorn Memorial Hospital. Initial diagnosis was based on the clinical presentations.

1.2 Controls were unrelated healthy blood donors who were unaffected with ALD, Pompe, HIE, HOS and SLE and had no family history of ALD, Pompe, HIE, HOS and SLE.

2. Study process

2.1 Blood collection

2.2 Mutation analysis

- RNA and DNA extraction
- RNA and DNA amplification
- PCR-RFLP on particular regions
- Nucleotide sequencing

2.3 Agarose gel electrophoresis

2.4 Enzyme-linked immunosorbent assay

3. Data collection and analysis

CHAPTER II
BACKGROUND AND LITERATURE REVIEWS

1. Genetic disorder

A genetic disorder is an illness caused by abnormalities in genes or chromosomes from a discrete mutation in a single base in the DNA to a gross chromosome or set of chromosomes. Most disorders are quite rare and affect one person in every several thousands or millions. There are four different types of genetic disorders (1) single-gene, (2) multifactorial, (3) chromosomal, and (4) mitochondrial.

1.1 Single-gene (also called Mendelian or monogenic)

This type is caused by changes or mutations that occur in the DNA sequence of one gene. Genes code for the proteins for functions and even make up the majority of cellular structures. When a gene is mutated, its protein product can no longer carry out its normal function, causing a disorder. There are more than 6,000 known single-gene disorders, which occur in about 1 out of every 200 births. Single-gene disorders are inherited in recognizable patterns: autosomal dominant (AD), autosomal recessive (AR) and X-linked.

1.1.1 Autosomal dominant

Only one mutated copy of the inherited gene will be necessary for a person to develop a disorder. Each affected person usually has one affected parent. The autosomal dominant diseases have a 50-50 chance of passing the mutant gene and therefore the disorder onto each of their children. Examples of this type of disorder are Huntington's disease, Hyper IgE syndromes, Holt-Oram syndrome, etc.

1.1.2 Autosomal recessive

Two copies of the gene must be mutated for a person to be affected by an autosomal recessive disorder. An affected person usually has unaffected parents who each carry a single copy of the mutated gene (and are referred to as carriers). Two unaffected people who each carry one copy of the mutated gene have a 25% chance with each pregnancy of having a child affected by the disorder. Examples of this type of disorder are cystic fibrosis, sickle cell anemia, glycogen storage disease type II (Pompe disease)

1.1.3 X-linked

X-lined can be classified into 2 classes

1.1.3.1 X-linked dominant

X-linked dominant disorders are caused by mutations in genes on the X chromosome. Only a few disorders have this inheritance pattern. Males are more frequently affected than females, and the chance of passing on an X-linked dominant disorder differs between men and women. The sons of a man with an X-linked dominant disorder will not be affected, and his daughters will all inherit the condition. A woman with an X-linked dominant disorder has a 50% chance of having an affected daughter or son with each pregnancy. Some X-linked dominant conditions, such as Aicardi syndrome, are fatal to boys, therefore only girls have them (and boys with Klinefelter syndrome). Other examples of this type of disorder are X-linked hypophosphatemia (hypophosphatemic rickets, vitamin D-resistant rickets), Aicardi syndrome, and Rett's syndrome.

1.1.3.2 X-linked recessive

X-linked recessive disorders are also caused by mutations in genes on the X chromosome. Males are more frequently affected than females, and the chance of passing on the disorder differs between men and women. The sons of a man with an X-linked recessive disorder will not be affected, and his daughters will carry one copy of the mutated gene. With each pregnancy, a woman who has an X-linked recessive disorder (X^rX^r) has a 50% chance of having sons who are affected and a 50% chance of having daughters who carry one copy of the mutated gene. Examples of this type of disorder include hemophilia A, Duchenne muscular dystrophy, red-green color blindness, and X-linked adrenoleukodystrophy (X-ALD).

1.2 Multifactorial (polygenic or complex disorders)

Genetic disorders may also be complex, multifactorial or polygenic. This means that they are likely associated with the effects of multiple genes in combination with lifestyle and environmental factors. Although complex disorders often cluster in families, they do not have a clear pattern of inheritance. This makes it difficult to determine a person's risk of inheriting or passing on these disorders. Complex disorders are also

difficult to study and treat because the specific factors that cause most of these disorders have not yet been identified. Examples of disorders in this group include heart disease, systemic lupus Erythematosus (SLE), Alzheimer's disease, arthritis and diabetes.

1.3 Chromosomal

Chromosomes are distinct structures made up of DNA and protein, located in the nucleus of each cell. Because chromosomes are carriers of genetic material, such abnormalities in chromosome structure as missing or extra copies or gross breaks and rejoinings (translocations) can result in diseases. Some types of major chromosomal abnormalities can be detected by microscopic examination. Down syndrome is a common disorder that occurs when a person has three copies of chromosome 21.

1.4 Mitochondrial

Mitochondria are small round or rod-like organelles involved in cellular respiration and found in the cytoplasm of plant and animal cells. They can convert the energy of food molecules into the ATP that powers most cell functions. Mitochondrial diseases, rare type of genetic disorders, are caused by mutations in the nonchromosomal DNA of mitochondria. The effects of mitochondrial disease can be quite varied. The mutation that in one person may cause liver disease might in another person cause a brain disorder. Some minor defects cause only "exercise intolerance", with no serious illness or disability. Other defects can more severely affect the operation of the mitochondria and can cause severe body-wide impacts. These diseases that have neuromuscular symptoms are often referred to as a mitochondrial myopathy.

Most genetic disorders are cause by mutations in some genes. Mutation is a permanent change to the nucleotide sequence in the DNA sequence of a gene. It can be caused by copying errors in the genetic material during cell division. Mutations can be subdivided into germ line mutations, which can be passed on to descendants through the reproductive cells, and somatic mutations.

- **Heterozygous mutation** is a mutation of only one allele.
- **Homozygous mutation** is an identical mutation of both the paternal and maternal alleles.

- **Hemizygous mutation is** a mutation on only a single copy of a gene instead of the customary two copies. All of the genes on the single X chromosome in the male are in the hemizygous state
- **Compound heterozygous** mutations comprise two different mutations in the paternal and maternal alleles.

A mutation that is not inherited from either parent is called a *de novo* mutation.

2. **Effect of mutation**

Mutations can be classified based on their effect into 2 classes.

1. Effect on structure

The sequence of a gene can be altered in a number of ways. Gene mutations have varying effects on health depending on where they occur and whether they alter the function of essential proteins. Structurally, mutations can be classified as:

1.1 Small-scale mutations

It will affect one or a few nucleotides, including

1.1.1 **Point mutations**, often caused by chemicals or malfunction of DNA replication, exchange a single nucleotide for another. These changes are classified as transitions or transversions. Most common is the transition that exchanges a purine for a purine (A ↔ G) or a pyrimidine for a pyrimidine (C ↔ T). Less common is a transversion, which exchanges a purine for a pyrimidine or a pyrimidine for a purine (C/T ↔ A/G). Point mutations that occur within the protein coding region of a gene may be classified into three kinds, depending upon what the erroneous codon codes for :

- Silent mutations: which code the same amino acid.
- Missense mutations: which code a different amino acid.
- Nonsense mutations: which code a stop causing truncation of the protein

1.1.2 **Insertions**, add one or more extra nucleotides into the DNA. They are usually caused by transposable elements, or errors during replication of repeating elements. Insertions in the coding region of

a gene may alter splicing of the mRNA, or cause a shift in the reading frame (frameshift)

1.1.3 **Deletions**, remove one or more nucleotides from the DNA. Like insertions, these mutations can alter the reading frame of the gene.

1.2 Large-scale mutations in chromosomal structure, including

1.2.1 **Amplifications** (or gene duplications) leading to multiple copies of all chromosomal regions, increasing the dosage of the genes located within them.

1.2.2 **Deletions of large chromosomal regions**, leading to loss of the genes within those regions.

1.2.3 **Chromosomal translocations**: interchange of genetic parts from non homologous chromosomes.

1.2.4 **Chromosomal inversions**: reversing the orientation of a chromosomal segment.

1.2.5 **Loss of heterozygosity**: loss of one allele, either by a deletion or recombination event, in an organism that previously had two different alleles

2. Effect on function

Mutations are effect on functions of gene, can classifies as :

2.1 **Loss-of-function mutations** are the result of gene product having less or no function. When the allele has a complete loss of function (null allele), it is often called an amorphic mutation. Phenotypes associated with such mutations are most often recessive. Exceptions are when the organism is haploid, or when the reduced dosage of a normal gene product is not enough for a normal phenotype (this is called haploinsufficiency).

2.2 **Gain-of-function mutations**, change the gene product such that it gains a new and abnormal function. These mutations usually have dominant phenotypes (neomorphic mutation).

2.3 **Dominant negative mutations** (also called antimorphic mutations) have an altered gene product that acts antagonistically to the wild-type allele. These mutations usually result in an altered molecular function (often inactive) and

are characterized by a dominant or semi-dominant phenotype. In humans, Hyper IgE syndromes is an example of a dominant negative mutation occurring in an autosomal dominant disease.

Mutations on codon of DNA sequence of gene that can alter the amino acid sequence of the protein encoded. It can be classified as:

1. **frameshift mutation** is a mutation caused by insertion or deletion of a number of nucleotides that is not evenly divisible by three from a DNA sequence. Due to the triplet nature of gene expression by codons, the insertion or deletion can disrupt the reading frame, or the grouping of the codons, resulting in a completely different translation from the original.
2. **Missense mutations** or *nonsynonymous mutations* are types of point mutations where a single nucleotide is changed to cause substitution of a different amino acid. This in turn can render the resulting protein nonfunctional.
3. **nonsense mutation** is a point mutation in a sequence of DNA that results in a premature stop codon, or a *nonsense codon* in the transcribed mRNA, and possibly a truncated, and often nonfunctional protein product.
4. **Silent mutations** are mutations that do not result in a change to the amino acid sequence of a protein. They may occur in a region that does not code for a protein, or they may occur within a codon that does not alter the final amino acid sequence.

At present, with more than 3,000 genes identified, and over 4,000 diseases caused by genetic disorders, mutation detection has become an increasingly important to detect genetic disorders. Available types of testing can be divided into

Newborn screening: is used just after birth to identify genetic disorders that can be treated early in life of baby.

Diagnostic testing: is used to diagnose or rule out a specific genetic or chromosomal condition. In many cases, genetic testing is used to confirm a

diagnosis. The results of a diagnostic test can influence a person's choices about health care and the management of the disease.

Carrier testing: is used to identify people who carry one copy of a gene mutation. This type of testing is offered to individuals who have a family history of a genetic disorder and to people in some ethnic groups with an increased risk of specific genetic conditions. If both parents are tested, the test can provide information about a couple's risk of having a child with a genetic disorder.

Prenatal testing: is used to detect changes in a fetus's genes or chromosomes before birth. This type of testing is offered to couples with an increased risk of having a baby with a genetic or chromosomal disorder. It cannot identify all possible inherited disorders and birth defects.

Preimplantation genetic diagnosis: Genetic testing procedures that are performed on human embryos prior to the implantation as part of an in vitro fertilization procedure.

Predictive and presymptomatic testing: are used to detect gene mutations associated with disorders that appear after birth, often later in life. These tests can be helpful to people who have a family member with a genetic disorder. It can determine whether a person will develop a genetic disorder. The results of predictive and presymptomatic testing can provide information about a person's risk of developing a specific disorder and help with making decisions about medical care.

Most of the time, practical technique for testing is used to find changes that are associated with inherited disorders. It is most helpful in providing diagnosis, early treatment, or prenatal diagnosis that might help a person make good life decisions. In this time, several hundred genetic tests are currently in use, and more are being developed.

This research will evaluate some practical techniques for mutation detection in patients who were clinically diagnosed with genetic disorders at Pediatric Clinic of the

King Chulalongkorn Memorial Hospital. Selection criteria were based on clinical presentations.

3. X- Linked Adrenoleukodystrophy (X-ALD)

Adrenoleukodystrophy (ALD) is a rare inherited disorder that leads to progressive brain damage, failure of the adrenal glands and eventually death. The prevalence of X-linked adrenoleukodystrophy is approximately 1 in 21,000 males[1]. ALD is one disease in a group of inherited disorders called leukodystrophies. Adrenoleukodystrophy progressively damages the myelin, a complex fatty neural tissue that insulates many nerves of the central and peripheral nervous systems, eventually destroying it. Without myelin, nerves are unable to conduct an impulse, leading to increasing disability as myelin destruction increases and intensifies.

An essential protein, called a transporter protein, is missing in sufferers. This protein is needed to carry very long-chain fatty acids to break down in peroxisomes that found in the normal diet. The lack of this protein can give rise to accumulation of very long-chain fatty acids 24–30 carbon atoms (VLCFA) in the body, which can damage the brain and the adrenal gland. The elevation in VLCFA was originally described by Moser *et al.* in 1981.[2]

Patients with X-linked ALD are all male, but about one in five women carrying the disease develop a milder form in adult life, called *adrenomyeloneuropathy*. There are several different types of the disease which can be inherited, but the most common form is an X-linked condition.

The clinical presentation is largely dependent on the age of onset of the disease. Symptoms normally start between the ages of 4 and 10 and include loss of previously acquired neurologic abilities and die soon after. This severe form of the disease was first described by Ernst Siemerling and Hans Gerhard Creutzfeldt.

The diagnosis is established by clinical findings and the detection of serum very long-chain free fatty acid levels[2], MRI examination which reveals white matter abnormalities, neuro-imaging and mutation analysis in the *ABCD1* gene.

The ALD gene (*ABCD1*), discovered in 1993, is located on chromosome Xq28. The gene consists of 10 exons spanning 20 kb of DNA and encodes a 4.2 kb of RNA.

It coded for a protein of 745 amino acid that was a member of a family of transporter proteins. This gene has 2 domains, as shown in figure 1.

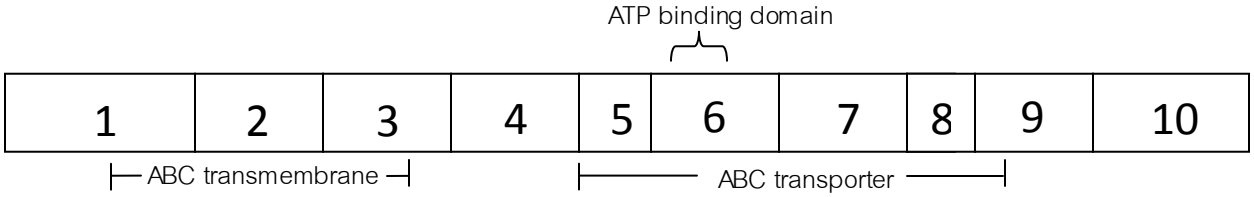


Figure 1. All exons and domains of *ABCD1*

ABCD1 or "ATP-binding cassette, subfamily D, member 1" codes for a protein that transfers fatty acids into peroxisomes, that is the cellular organelles where the fatty acids undergo β-oxidation.[3] A dysfunctional *ABCD1* gene, leads to the accumulation of very long chain fatty acids (VLCFA) because VLCFA cannot be transferred to peroxisomes, which can damage the myelin and neural tissues.

At present, more than 500 mutations in the *ABCD1* gene have been identified. Each mutation was found private and spreaded throughout the *ABCD1* gene.[4]

4. Glycogen Storage Disease Type II (Pompe disease)

Pompe disease is a neuromuscular autosomal recessive metabolic disorder in the family of lysosomal storage diseases caused by a deficiency of the enzyme Acid alpha-glucosidase (EC 3.2.1.20) in lysosomes, which is needed to break down glycogen (a long branched glucose polymer and stored form of sugar used for energy), leads to lysosomal accumulation of glycogen in many different cell types.[5] The disease is named after Johann Pompe, who characterized it in 1932.

The clinical features of Pompe disease have been divided into three forms defined by age of onset and progression of symptoms. Infantile, or early onset, is noticed shortly after birth. Symptoms include severe lack of muscle tone, weakness, and enlarged liver (hepatomegaly) and heart. Mental function is not affected. Development appears normal for the first weeks or months but slowly declines as the disease progresses. Most children die from respiratory or cardiac complications before 2 years of age.[6] Juvenile onset symptoms appear in early to late childhood and include progressive weakness of respiratory muscles in the trunk, diaphragm and lower limbs.

Intelligence is normal. Finally, adult onset symptoms also involve generalized muscle weakness and wasting of respiratory muscles in the trunk, lower limbs, and diaphragm.

Diagnosis and testing: Type II GSD can be diagnosed by determining the activity of the specific enzyme acid alpha glucosidase testing that can be performed on blood samples, muscle biopsy, cultured cells from a skin biopsy, test accumulation of glycogen in lysosomes, and mutation analysis in the *GAA* gene.

The disorder is estimated to occur in about 1 in 40,000-300,000 live births. It has an autosomal recessive inheritance pattern. Children have a 1 in 4 chance of inheriting the disorder when both parents carry one copy the defective gene.

GAA (acid alpha glucosidase) gene is mapped to human chromosome 17q25. This gene consists of 20 exons spanning 18.4 kb of DNA and encodes a 3.8 kb of RNA. It coded for a protein of 952 amino acids. This gene has lysosomal alpha-glucosidase domain, as shown in figure 2.

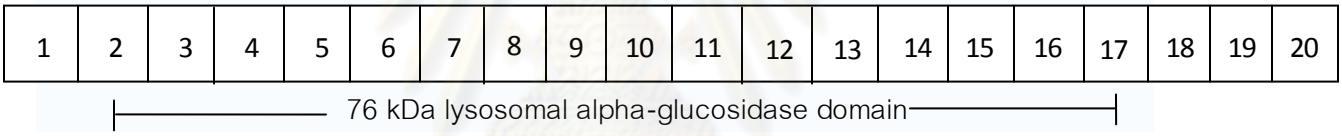


Figure2. All exons and domains of *GAA*

Dysfunctional *GAA* gene, leads to accumulation of glycogen in many different cell types because enzyme acid alpha-glucosidase(GAA) cannot break down glycogen. More than 200 mutations in the *GAA* gene have been identified.

5. Hyper-IgE syndrome (HIES)

HIE or Job's syndrome, is a complex primary immunodeficiency characterized by high levels of serum IgE and recurrent bacterial infection. HIES can be classified into 2 types.[7]

Type 1 HIES (Autosomal Dominant type)

This group of HIES represents the most common and typical form and includes sporadic and familial autosomal dominant type.[8] Its clinical presentations include recurrent skin and pulmonary infections, atopic dermatitis, elevated serum IgE levels. By age 16 years, all patients show distinctive facial appearance: skeletal and dental

abnormalities, asymmetric facial appearance, deep-set eyes, and parenchymal lung abnormality.[9] Recent studies have showed mutations in the signal transducer and activator of transcription-3 (*STAT3*) gene as major causes of AD and sporadic HIES.[10, 11]

Type 2 HIES (Autosomal Recessive type)

The patients showed no apparent abnormalities in their skeletal and dental systems but suffered from recurrent and severe infections with *S. aureus*, *S. pneumonia*, or *H. influenzae*, as observed in type 1 HIES.[12] Most of the type 2 HIES patients also suffered from severe viral infections without pneumatoceles, which were not observed in type 1 HIES. In 2006, Minegishi et al. have recently identified a homozygous mutation of the tyrosine kinease-2 gene (*TYK2*) in a patient with AR-HIES.[13] The classification of HIES can be summarized in Table 1

Table 1. A new classification of Hyper-IgE syndrome

Type	Inheritance	Distinguished clinical finding	Genes
Type 1 (multisystem)	Sporadic (most cases), familial with AD inheritance (rare)	skeletal and dental abnormalities, parenchymal lung abnormality	*STAT3*
Type 2 (nonskeletal)	Familial with AR inheritance	severe viral infections, possible CNS involvement	*TYK2* (JAK)

Diagnosis of HIES can be made clinically by eye examination which may reveal signs of dry eye syndrome. A physical exam may show signs of osteomyelitis, and recurrent sinus infections. A chest x-ray may reveal lung abscesses. Tests used to confirm a diagnosis include absolute eosinophil count, complete blood count, serum globulin electrophoresis to look for high blood IgE levels and genetic testing for a mutation in the *STAT3* or/and *TYK2* genes by direct sequencing.

The cytokine can regulate cells by JAK (TYK2) and STAT signaling pathways as shown in figure 3.

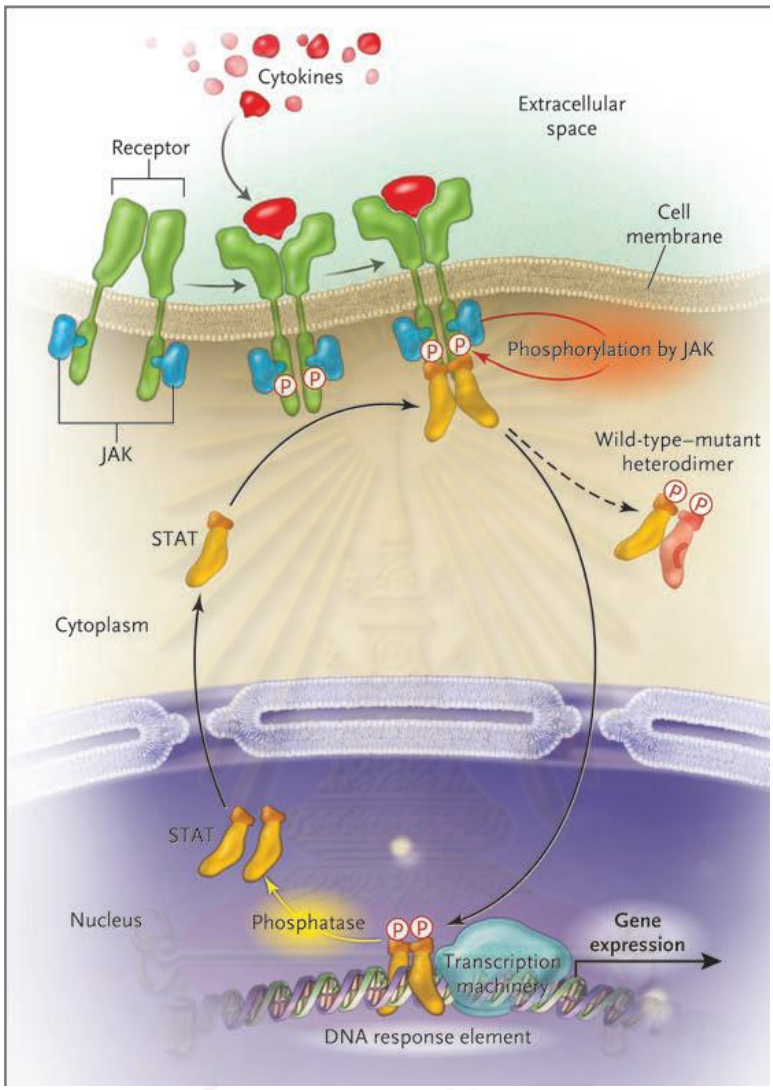


Figure 3. JAK-STAT signaling pathway[14]

From figure 3, when cytokines bind with receptors, receptors will sent the signal to JAK protein. The JAK protein will be phosphorylated and aggregate to the binding site for STAT. The STAT protein will be phosphorylated by JAK and form dimers and accumulate in the nucleus. The STAT dimers will bind to the promoters of the target genes in nucleus and activate transcription. If JAK or STAT does not function properly, it can have an affect on the regulation of cytokines causing HIES.[14]

In this study, the patient had clinical features consistent with type 1 HIES. Therefore, mutation analysis in the *STAT3* gene was performed. *STAT3* is mapped to human chromosome 17q21. This gene consists of 24 exons spanning 30 kb of DNA and encodes a 4.7 kb of RNA. Its protein contains 770 amino acids. This gene has 6 domains as shown in figure 4.

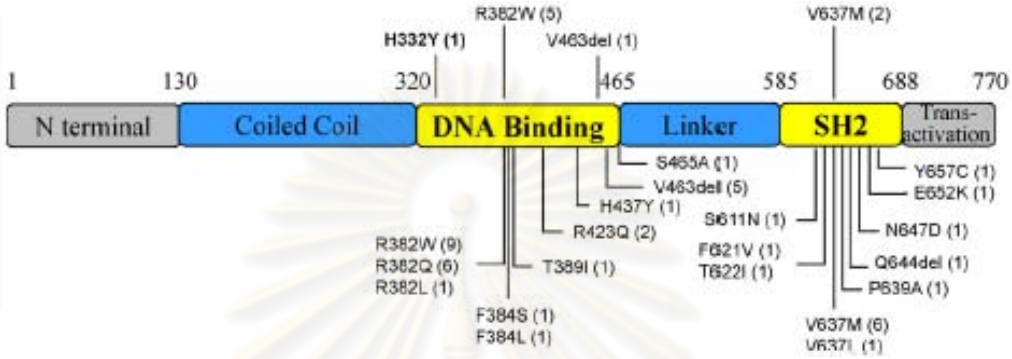


Figure4. Mutations detected in the *STAT3* gene.[15]

From the figure 4, all mutations described recently were localized to regions encoding the DNA binding domain and the SH2 domain.[10] We therefore could select the regions for primer design to detect mutations in the *STAT3* gene.

6. Holt-Oram syndrome (HOS)

Holt-Oram syndrome or heart-hand syndrome has an autosomal dominant inheritance pattern. The disorder is estimated to occur in about 1 in 100,000 live births.[16] HOS was first reported in 1960 by Holt, M. and Oram, S. They found patients with atrial septal defect and upper limb abnormality in 4 generations.[17] In 1997, Bosson et al. found the T-box transcription factor 5 (*TBX5*) gene on chromosome 12q24.1 responsible for this syndrome and 70%of HOS patients harbored mutations in this gene.[18] The *TBX5* gene, a member of T-box gene family, is important transcription factor of heart and upper limb development in embryogenesis. In 2001, Bruneau, B.G et al studied homozygous knockout mice ($Tbx5^{-/-}$) and found that they died early in embryogenesis while heterozygous knockout mice ($Tbx5^{-/+}$) appropriately mimicked Holt-Oram syndrome.[19]

Clinical features of HOS include at least one limb abnormality that affects bones in the wrist, a missing thumb or a thumb that looks like a finger, partial or complete

absence of bones in the forearm, and an underdeveloped bone of the upper arm. In some cases, only one arm and/or hand is affected.

About 75 percent of individuals with Holt-Oram syndrome have congenital heart defects such as atrial septal defects (ASD), or ventricular septal defects (VSD). The most common problem is a defect in the muscular wall, or septum. Sometimes people with Holt-Oram syndrome have cardiac conduction disease, which is caused by abnormalities in the electrical system that coordinates contractions of the heart chambers leading to problems such as a slow heart rate or a rapid and ineffective contraction of the heart muscles.

The diagnosis is established by clinical findings, radiographs of affected bones, echocardiograms to detect congenital heart defects and genetic testing for the analysis of the mutation in *TBX5*.

TBX5 is mapped to human chromosome12q24.1. This gene consists of 9 exons spanning 54 kb of DNA and encodes a 3.9 kb of RNA. It codes for a protein of 518 amino acids. This gene has 2 domains as shown in figure 5

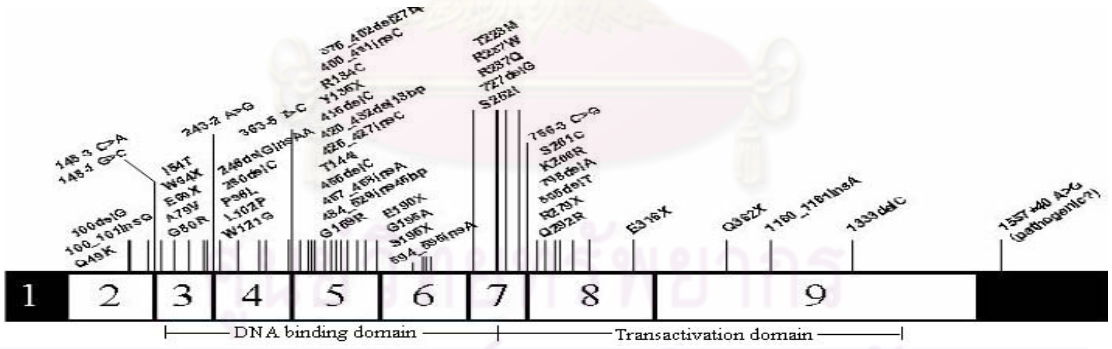


Figure 5. TBX5 Domains and mutations of *TBX5.*[16]

From figure 5, mutations in the *TBX5* gene can be found at exons 2-9 with the most commonly detected is a point mutation.[16] If mutations occur on the DNA binding domain. TBX5 transcription factor cannot bind to the promoter of the target genes. Mutations on transactivation domain lead to a decrease of efficiency of transcription.[20]

A previous cytogenetic report of a de novo pericentric inversion of chromosome 20q13.2, where *SALL4* was located, revealed that it was associated with a clinical presentation of bilateral absence of the thumbs and an atrial septal defect.[21] This phenotype was taken to represent Holt-Oram syndrome and the suggestion was made that there were likely to be other causes of apparent Holt-Oram syndrome but without *TBX5* mutation. From a report in 2003, they suggested that patients with clinical features consistent with Holt-Oram syndrome but without mutations in *TBX5*, *Sall4* might be another candidate gene for analysis.[22]

SALL4 is mapped to human chromosome 20q13.2. This gene consists of 4 exons spanning 18.4 kb of DNA and encodes a 3.4 kb of RNA. It codes for a protein of 1053 amino acids. This gene has a zinc finger domain as shown figure 6.

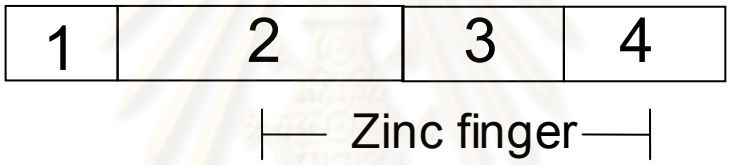


Figure 6. Structure of SALL4

7. Systemic Lupus Erythematosus (SLE)

Systemic lupus erythematosus is a potentially severe autoimmune disease. The abnormalities are caused by defects in apoptosis leading to the accumulation of autoreactive T and B cells resulting in damage to multiple organs.

Clinical features of SLE can be characterized by arthritis, proximal muscle aching or weakness, recurrence of inflammatory rashes, patchy or diffuse loss of hair, oral or nasal ulcerations, pericardial pain, evidence of kidney disease.

A number of tests are required before SLE can be diagnosed definitively. The diagnosis is established by tests for autoantibodies for measuring ANA, antiphospholipid antibodies, antibodies to SR proteins. Other tests include blood tests to measure C3, C4, C1q, and CH50 levels and protein in urine

The exact etiology of SLE has not been elucidated, but it is obvious that genetic factors, gender, and environment are involved in its pathogenesis.[23] It is a polygenic

disease, and as many as 30 susceptibility loci with possible links to its pathogenesis have been identified in mice.[24] In mice, mutations in the prototype proapoptotic molecules *Fas* or *Fas ligand* (*FasL*) lead to the occurrence of an SLE-like syndrome[25], but human SLE patients rarely have mutations in *Fas* or *FasL*.[26] However, it is possible that some molecules in the Fas/FasL pathway are SLE risk factors.

Decoy receptor 3 (DcR3)/TR6 is a secreted protein belonging to the tumor necrosis factor (TNF) receptor family. It binds to Fas ligand (FasL), LIGHT, and TL1A that are all TNF family members. It was noted that soluble or solid phase DcR3-Fc co-stimulated proliferation, lymphokine production and cytotoxicity of mouse and human T cells upon T-cell receptor (TCR) ligation. Recently, the investigators found that the serum level of soluble DcR3 was higher in SLE patients than in healthy control subjects. Taken together, the investigators propose that in autoimmune diseases, SLE activated T cells secrete more DcR3 than non-autoimmune controls, which may in turn costimulate T cells further and cause dysregulated lymphocyte activation. With the aim to establish the possible correlation between DcR3 and autoimmune phenotypes in children, we analyzed the serum DcR3 level and performed mutation analysis in the *DcR3* gene in children with SLE. The genetic analysis on the *DcR3/TR6* gene and circulating DcR3 level will be compared between SLE and non-autoimmune control subjects.

The disease prevalence is ~0.05% in the general population, with 80-90% of patients being women.[27] DcR3 is mapped to human chromosome at 20q13. This gene consists of 3 exons spanning 3.7 kb of DNA and encodes a 1.1 kb of RNA. It codes for a protein of 300 amino acids. This gene has 3 domains as shown in figure 7.

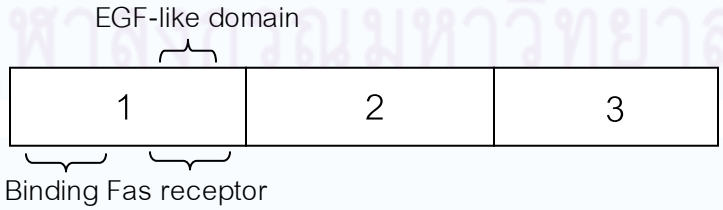


Figure 7. Structure of DcR3

Practical techniques of genetic testing are very helpful in providing additional information for patients with genetic diseases and family members leading to more

accurate diagnosis, effective therapy and prevention. This study presented practical techniques for mutation detection in the *ABCD1*, *GAA, STAT3*, *TBX5*, *SALL4*, *DcR3* genes responsible for ALD, Pompe disease, HIE, HOS, and SLE, respectively.

CHAPTER III

METHODOLOGY

Research Instruments

1. Automatic adjustable micropipette : P2(0.1-2 µl), P10(0.5-10 µl), P20(5-20 µl), P100(20-100 µl), P200(50-200 µl), P1,000 (0.1-1ml) (Gilson, France)
2. Pipette tip : 10µl, 100µl, 200µl, 1,000µl (Elkay, USA)
3. Microcentrifuge tube : 0.2ml, 0.5ml, 1.5ml (Bio-RAD, Elkay, USA)
4. Polypropylene conical tube : 16 ml (Elkay, USA)
5. Beaker : 50ml, 100ml, 200ml, 500ml, 1,000ml (Pyrex)
6. Reagent bottle : 100ml, 250ml, 500ml, 1,000ml (Duran, USA)
7. Flask : 250ml, 500ml, 1,000ml (Pyrex)
8. Cylinder : 25ml, 50ml, 100ml, 250ml, 500ml, 1,000ml (Witeg, Germany)
9. Pipette rack (Autopack, USA)
10. Thermometer (Precision, Germany)
11. Parafilm (American National Can, USA)
12. Stirring magnetic bar
13. Plastic wrap
14. Comb
15. Vortex (Scientific Industry, USA)
16. pH meter (Eutech Cybernatics)
17. Stirring hot plate (Bamstead/Thermolyne, USA)
18. Balance (Precisa, Switzerland)
19. Centrifuge (J.P.Selecta, Spain)
20. Microcentrifuge (Eppendorf, Germany)
21. Mastercycler personal (Eppendorf, Germany)
22. Thermal cycler (Touch Down, Hybraid USA)
23. Power supply model 250 (Gibco BRL, Scotland)
24. Power poc 3000 (Bio-RAD)
25. Horizon 11-14(Gibco BRL, Scotland)

26. Sequi-gen sequencing cell (Bio-RAD)
27. Heat block (Bockel)
28. Incubator (Memmert)
29. Thermostat shking-water bath (Heto, Denmark)
30. Spectronic spectrophotometers (Genesys5, Milon Roy, USA)
31. UV Trasilluminator (Fotodyne, USA)
32. UV absorbing face shield (Spectronic, USA)
33. Gel doc 1000 (Bio-RAD)
34. Refrigerator 4°C (Misubishi, Japan)
35. Deep freeze -20 and -80°C (Revco)
36. Water purification equipment (Water pro Ps, Labconco, USA)
37. Water bath (J.P.Selecta, Spain)
38. Sequencher software 4.2 Demo (Gene Codes Corporation, MI)
39. Oligo software
40. Autoclave
41. Automatic adjustable multichanal micropipette : P100(20-100 µl) (Eppendorf, Germany)
42. Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)

Reagent

1. General reagents

1.1 Absolute ethanol (Merck)

1.2 Agarose, molecular grade (Promega)

1.3 Ammonium acetate (Merck)

1.4 Boric acid (Merck)

1.5 Tri-base (USB)

1.6 Disodium ethylenediamine tetraacetic acid : EDTA(Merck)

1.7 Bromphenol blue (Pharmacia)

1.8 Ethidium bromide (Gibco BRL)

1.9 Hydrochloric acid (Merck)

1.10 Phenol (Sigma)

1.11 Chloroform (Merck)

1.12 Sodium chloride (Merck)

1.13 Sodium hydroxide (Merck)

1.14 Triton X-100(Pharmacia)

1.15 100 base pair DNA ladder (Biolabs)

1.16 1 Kb pair DNA ladder (Biolabs)

1.17 Water Distillation

2. PCR reagents

2.1 10X PCR buffer with KCl (500mM KCl, 100 mM Tris-HCl pH 8.8, 0.8% Nonidet P40) (Fermentas)

2.2 10X PCR buffer with $(NH_4)SO_4$ (200mM $(NH_4)SO_4$, 750 mM Tris-HCl pH 8.8, 0.1%Tween20) (Fermentas)

2.3 Magnesium chloride (Fermentas)

2.4 Deoxynucleotide triphosphates (dNTPs) (Fermentas)

2.5 Oligonucleotide primer (Operon)

2.6 Oligonucleotide primer (BioDesign)

2.7 *Taq* DNA polymerase (Fermentas)

2.8 100% DMSO

2.9 Genomic DNA sample

2.10 cDNA sample

3. Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) (Promega)

3.1 0.5µg/µl Oligo dT Primer

3.2 0.5ng/µl RNA template sample

3.3 5X ImProm II™ bufferbuffer

3.4 25mM Magnesium chloride for RNA

3.5 10mM Deoxynucleotide triphosphates (dNTPs) for RNA

3.6 40U/µl RNAsin RNase Inhibitor

3.7 ImProm II Reverse Transcriptase 40U/ml

4. Restriction enzymes

4.1 *Tag*I

4.3 *BsaH*I

4.4 *Rsa*I

5. ELISA reagents and materials

5.1. Biotin-Conjugate anti-human DcR3 monoclonal antibody

5.2. Streptavidin-HRP

5.3. Sample Diluent

5.4. Assay Buffer Concentrate (1%PBS, Tween 20, 10% BSA)

5.5. Wash Buffer Concentrate (1%PBS, Tween 20)

5.6. Substrate Solution (tetramethyl-benzidine)

5.7. Stop Solution (1M Phosphoric acid)

5.8. Microwell Plate coated with monoclonal antibody to human DcR3

6. Commercial Kits

6.1 QIAamp® RNA Blood Mini Kit (QIAGEN)

6.2 QIAamp® DNA Blood Mini Kit (QIAGEN)

6.3 QIAamp® Gel Extraction Kit (QIAGEN)

6.4 ELISA Kit (Bender Medsystem, Austria)

Experimental Procedure

1. Subjects and sample collection

1.1 Blood collection

After informed consent was received, approximate 5 ml of peripheral blood from each individual was collected in a polypropylene tube with EDTA for RNA and DNA extraction.

Approximate 5 ml of peripheral blood was obtained in a polypropylene tube for serum collection.

1.2 Subjects

Patients from unrelated families were clinically diagnosed with ALD, Pompe, HIE, HOS and SLE at Pediatric Clinic of the King Chulalongkorn Memorial Hospital and were included in the study. Selection criteria were based on clinical presentation.

1.3 Controls

Controls were healthy volunteers unaffected with ALD, Pompe, HIE, HOS and SLE, had no family history of ALD, Pompe, HIE, HOS and SLE. DNA and RNA from controls was used for mutation screening in *ABCD1* (ALD), *GAA* (Pompe), *DcR3* (SLE). Serum from controls was used for ELISA in *DcR3* (SLE). DNA from the patients' family members who were at risk was also investigated.

2. Genetic analysis

2.1 DNA extraction and collected plasma

After informed consent, genomic DNA was isolated from peripheral blood leukocytes. This procedure was performed as:

1. 3 ml. of whole blood was centrifuged for 10 minutes at 3,300 rpm.
2. Remove plasma (supernatant) to microcentrifuge tube 1.5 ml for ELISA and transfer buffy coat to a new polypropylene tube. Then add 10 volumes of cold lysis buffer 1 (or 10ml), mix with vortex and incubate at 20°C for 5 minutes.
3. Centrifuge for 8 minutes at 13,400 rpm, and remove supernatant.
4. Add 3 ml of cold lysis buffer 1,mix thoroughly and centrifuge for 8 minutes at 13,400 rpm.
5. Discard supernatant and add 900 µl of lysis buffer 2, 10 µl of proteinase K solution (20 mg of proteinase K in 1.0 ml of 1% SDS-2 mM EDTA, and 50 µl of 10% SDS). Mix vigorously for 15 seconds.
6. Incubate the tube(S) in a 37°C shaking waterbath overnight for complete digestion.
7. Add 1 ml of phenol-chloroform-isoamyl alcohol and shake vigorously for 15 seconds and centrifuge at 6,000 rpm for 5 minutes
8. Transfer the supernatant from each tube (containing DNA) to a new microcentrifuge tube.
9. Add 0.5 volumes of 7.5 M CH_3COONH_4 and 1 volume of 100% ethanol and mix by inversion. The DNA should immediately form a stringy precipitate. Recover the DNA by centrifugation at 13,400 rpm for 15 minutes. Then remove supernatant.

10. Rinse the pellet with 70% ethanol. Decant the ethanol and air-dry the pellet. (It is important to rinse well to remove any residual salt and phenol.)
11. Resuspend the DNA in 20-300 µl of the double distilled water at 37°C until dissolved.

Calculation of DNA concentration

The reading at 260 nm is used for calculating the DNA concentration. An OD of 1 concentration to approximately 50 µg/ml for double-strand DNA. So, DNA concentration can be calculated from the following

DNA concentration = OD X 50 X dilution ration (µg/ml)

2.2 RNA extraction

Total RNA was isolated from white blood cells using QIAamp® RNA Blood Mini Kit (Qiagen, Valencia, CA). Reverse transcription was perform using Improm-II™ reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions.

1. Mix 1 volume of human whole blood with 5 volumes of Buffer EL in an appropriately sized tube.
2. Incubate for 10-15 minutes on ice. Mix by vortexing briefly 2 times during incubation.
3. Centrifuge at 4,000 rpm for 10 minutes at 4°C, and completely remove and discard supernatant.
4. Add Buffer EL to the cell pellet (use 2 volumes of Buffer EL per volume of whole blood used in step 1). Resuspend cells by vortexing briefly.
5. Centrifuge at 4,000 rpm for 10 minutes at 4°C, and completely remove and discard supernatant.
6. Add Buffer RLT to the pellet and vortex or pipet to mix.

7. Pipet lysate directly into QIAshredder spin column in a 2 ml collection tube and centrifuge for 2 minutes at maximum speed to homogenize. Discard QIAshredder spin column and save homogenized lysate.
8. Add 1 volumn (350 µl or 600 µl) of 70% ethanol to the homogenized lysate and mix by pipetting. Do not centrifuge.
9. Carefully pipet sample, including any precipitate which may have formed, into a new QIAamp spin column in a 2 ml collection tube without moistening the rim. Centrifuge for 15 seconds at 13,400 rpm. Maximum loading aliquots onto the QIAamp spin column and centrifuge as above.
10. Transfer the QIAamp spin column into a new 2 ml collection tube. Apply 700 µl of Buffer RW1 to the QIAamp spin column and centrifuge for 15 seconds at 13,400 rpm to wash.
11. Place the QIAamp spin column in a new 2 ml collection tube (provided). Pipet 500 µl of Buffer RPE into the QIAamp spin column and centrifuge for 15 seconds at 13,400 rpm.
12. Carefully open the QIAamp spin column and add 500 µl of Buffer RPE and centrifuge at 13,400 rpm for 3 minutes.
13. Recommended: place QIAamp spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate. Centrifuge at 13,400 rpm for 1 minute.
14. Transfer the QIAamp spin column into a 1.5 ml microcentrifuge tube (provided) and pipet 30-50 µl of RNase-free water (provided) directly onto the QIAamp membrane. Centrifuge for 1 min at 13,400 rpm to elute. Repeat if >0.5 ml whole blood (or >2x10^6 leukocytes) has been processed.

2.3 DNA amplification by Polymerase Chain Reaction (PCR)

Primer design guidelines:

- Primer length between 18-25 bp.
- Keep G-C content in the 30-80% range.

- The T_m should be 55-60°C.
- G or C at the 3'-end of primers will increase priming efficiency.
- Avoid runs of an identical nucleotide, especially guanine.
- Avoid secondary structure (hairpin, self-complementary and primer dimer)
- The five nucleotides at the 3'-end should have no more than two G and/or C bases.
- Primer sequence should be searched using BLAST and checked for cross-homology
- Primers should be specific with the target gene and not anneal with other genes

2.3.1 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For mutation screening, RT-PCR was initially performed.

RT-PCR Protocol

1. Mixture for cDNA preparation(reverse transcription)

Components	Amount
0.5 µg/µl Oligo dT Primer	1.0 µl (0.5 µg)
0.5 ng/µl RNA template	10.1 µl (5.05 ng)
Conditions	70°C, 5 minutes
	4°C, 5 minutes

2. Add the following components to the product of step 1

Component	Amount
5X Buffer	4.0 µl (1X)
25mM $MgCl_2$	2.4 µl (3.0 mM)
10mM dNTPs	2.0 µl (0.5 mM)
40U/µl RNasin® RNase Inhibitor	0.5 µl (20.0 U)
Reverse transcriptase	1.0 µl
Total Volume	20.0 µl

Condition 25°C, 5 minutes

40°C, 60 minutes

70°C, 15 minutes

3. cDNA can be frozen for later use or used immediately for PCR.

4. cDNA can amplify by PCR with forward and reverse primers

3. Mutation analysis

3.1. Mutation analysis with *ABCD1*

3.1.1 Polymerase Chain Reaction (PCR)

The first, primer were designed within introns to allow genomic amplification and sequencing of exons 1-10 including exon-intron boundaries as shown in Table2, 2.1, 2.2.

Table2. Primer sequences for *ABCD1* mutation analysis

Name	Primer sequence 5' to 3'	Product size (bp)
ALDe1A-F	ACA ACA GGC CCA GGG TCA GA	458
ALDe1A-R	AGG AAG GTG CGG CTC ACC A	
ALDe1B-F	AAC CGG GTA TTC CTG CAG CG	421
ALDe1B-R	ACT GGT CAG GGT TGC GAA GC	
ALDe1C-F	CCA CGC CTA CCG CCT CTA CTT	512
ALDe1C-R	AGA CTG TCC CCA CCG CTC	
ALDe2-F	GGC ACT GGG AGA CCC TG	368
ALDe2-R	TCA GCA CCC AGV GGT ATG G	
ALDe3-F	TTG CAG AAG AGC CTC GCC TT	304
ALDe3-R	TTG CAG GGA GAG AAG CAT GG	
ALDe4-F	GTC GTC GTA CAA GGA GGT AC	385
ALDe4-R	ACA GGA CAC TGC CCA GAG GC	
ALDe5-F	CTG CCA GGG ATG GGA ATG AG	373
ALDe5-R	TCT CAC CTT GAC CTT GGC CC	
ALDe6-F	GCC ATA GGG TAC GGG AAG GG	312
ALDe6-R	GCC TCT GCA GGA AGC CAT GT	
ALDe-7F	CGA TCC ACT GCC CTG TTT TGG	497
ALDe-7R	CTT CCC TAG AGC ACC TGG	

ALDE8/9-F	CTG AGC CAA GAC CAT TGC CCC CG	471
ALDe8/9-R	TGC TGC TGC CGG GCC CGC	
ALDe10-F	GAG GGG AGG AGG TGG CCT GGC	463
ALDe10-R	GCG GGG TGC GTG CAT GGG TGG	

Table2.1. PCR reaction of *ABCD1*

Component	Exon1A	Exon1B	Exon1C	Exon2	Exon3	Exon4
Distilled water (µl)	13.52 (1X)	13.52 (1X)	13.52 (1X)	13.52 (1X)	12.52 (1X)	13.52 (1X)
10XPCR buffer	2.0	2.0	2.0	2.0	2.0	2.0
25mM $MgCl_2$	1.2 (1.2mM)	1.2 (1.2mM)	1.2 (1.2mM)	1.2 (1.2mM)	1.2 (1.2mM)	1.2 (1.2mM)
10 mM dNTP	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)
10µM ALD-F	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)
10µM ALD-R	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)
5U/µl Taq polymerase	0.08 (0.02U)	0.08 (0.02U)	0.08 (0.02U)	0.08 (0.02U)	0.08 (0.02U)	0.08 (0.02U)
50ng/µl Genomic DNA	2	2	2	2	3	2
Total volume (µl)	20	20	20	20	20	20

Component	Exon5	Exon6	Exon7	Exon8/9	Exon10
Distilled water (µl)	13.52 (1X)	13.52 (1X)	13.2 (1X)	13.32 (1X)	13.5 (1X)
10XPCR buffer	2.0	2.0	2.0	2.0	2.0
25mM $MgCl_2$	1.2 (1.2mM)	1.2 (1.2mM)	1.5 (1.5mM)	1.2 (1.2mM)	1.2 (1.2mM)
10 mM dNTP	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)
10µM ALD-F	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)	0.5 (0.16µM)	0.4 (0.13µM)
10µM ALD-R	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)	0.5 (0.16µM)	0.4 (0.13µM)
5U/µl Taq polymerase	0.08 (0.02U)	0.08 (0.02U)	0.1 (0.025U)	0.08 (0.02U)	0.1 (0.025U)
50ng/µl Genomic DNA	2	2	2	2	2
Total volume (µl)	20	20	20	20	20

Table2.2. PCR cycle and condition of *ABCD1*

Step	Exon1A	Exon1B	Exon1C	Exon2	Exon3	Exon4
Initial	94°C/ 5	94°C/ 5	94°C/ 5	94°C/ 5	94°C/ 5	94°C/ 5
PCR cycle	35 cycles	35 cycles	35 cycles	35 cycles	35 cycles	35 cycles
Denature	94°C/	94°C/	94°C/ 1min	94°C/	94°C/ 1min	94°C/ 1min
Annealing	56°C/	60°C/	66°C/ 1min	62°C/	64°C/ 1min	64°C/ 1min
Extension	72°C/	72°C/	72°C/	72°C/	72°C/	72°C/
Final extension	72°C/ 5min	72°C/ 5min	72°C/ 5min	72°C/ 5min	72°C/ 5min	72°C/ 5min

Step	Exon5	Exon6	Exon7	Exon8/9	Exon10
Initial denaturation	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min
PCR cycle	35 cycles	35 cycles	35 cycles	35 cycles	35 cycles
Denature	94°C/ 30sec	94°C/ 30sec	94°C/ 1min	94°C/ 1min	94°C/ 30sec
Annealing	60°C/ 30sec	62°C/ 30sec	60°C/ 1min	64°C/ 45sec	62°C/ 30sec
Extension	72°C/ 45sec	72°C/ 45sec	72°C/ 45sec	72°C/ 45sec	72°C/ 45sec
Final extension	72°C/ 5min	72°C/ 5min	72°C/ 5min	72°C/ 5min	72°C/ 5min

3.1.2 Agarose gel electrophoresis and DNA sequencing

The PCR product were verified for correct size on an ethidium bromide-stained 1.5-2% agarose gel. The PCR product were then treated with ExoSAP-IT (USP Corporation, Cleveland, OH) according to the manufacturer's recommendation, and sent for direct sequencing at Macrogen Inc., Seoul, Korea. The sequence was analyzed by sequencher.

3.2 Mutation analysis with *GAA*

3.2.1. Polymerase Chain Reaction (PCR)

The primers were designed within cDNA. The forward primers were designed before ATG and the reverse primers were designed after TAA, TAG or TGA for allow amplification and sequencing as shown in Table 3, 3.1, 3.2.

Table3. Primer sequences for *GAA* mutation analysis

Name	Primer sequence 5' to 3'	Product size (bp)
GAA-F1	CAC CTC TAG GTT CTC CTC GT	1,331
GAA-R2	TCG TTC CAT TGG ACG TCC AG	
GAA-F2	ACC TGG ACG TTG TGG GAT AC	1909
GAA-R1	TCC AGG TGA CAC ATG CAA CC	
GAA-F3	TCT CTC CAC ACA CTA CAA CC	For sequencing

Table3.1. PCR reaction of *GAA*

Component	GAA-F1/R1	Nest GAA F1/R2	Nest GAA F2/R1
Distilled water (µl)	13.82 (1X)	15.02 (1X)	14.82 (1X)
10XPCR buffer	2.0	2.0	2.0
25mM $MgCl_2$	1.2 (1.2mM)	1.2 (1.2mM)	1.2 (1.2mM)
10 mM dNTP	0.3 (0.15mM)	0.3 (0.15mM)	0.3 (0.15mM)
10µM GAA-F	0.3 (0.1µM)	0.2 (0.067µM)	0.3 (0.1µM)
10µM GAA-R	0.3 (0.1µM)	0.2 (0.067µM)	0.3 (0.1µM)
5U/µl Taq polymerase	0.08 (0.02U)	0.08 (0.02U)	0.08 (0.02U)
50ng/µl cDNA	2	1	1
Total volume (µl)	20	20	20

Table3.2. PCR cycle and condition of *GAA*

Step	GAA-F1/R1	Nest GAA F1/R2	Nest GAA F2/R1
Initial denaturation	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min
PCR cycle	35 cycles	35 cycles	35 cycles
Denature	94°C/ 30sec	94°C/ 45sec	94°C/ 45sec
Annealing	57°C/ 30sec	64°C/ 45sec	66°C/ 45sec
Extension	72°C/ 3min 30 sec	72°C/ 2min 10 sec	72°C/ 2min 10 sec
Final extension	72°C/ 5 min	72°C/ 5 min	72°C/ 5 min

3.2.2 PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

Add enzyme *BsaH I*, 10xNEB, and BSA buffer to the PCR product as shown in Table 4.

Table 4. PCR-RFLP reaction of *GAA*

Component	solution
Distilled water (µl)	4.8 (1X)
10XNEB buffer	2.0
BSA	0.2 (0.2mM)
BsaH I	1 (10U)
PCR product	12
Total volume (µl)	20

The enzyme cleaved at position (c.1935C>A) (p.D645E) on cDNA. This mutation is a common mutation in Chinese, Taiwan, Thai patients. After enzyme digestion, the expected result on gel electrophoresis was shown in figure 9

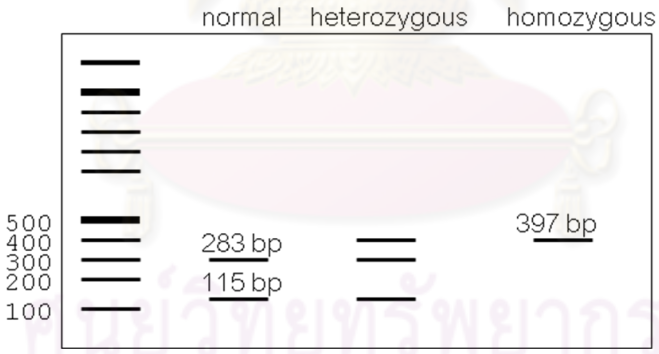


Figure 8. PCR-RFLP of *GAA* hotspot mutatoin

3.2.3 Agarose gel electrophoresis and DNA sequencing

The RFLP reaction was verified for correct size on an ethidium bromide-stained 1.5-2% agarose gel. The samples without p.D645E were sent for sequencing for mutation analysis.

3.3 Mutation analysis with *STAT3*

3.3.1 Polymerase Chain Reaction (PCR)

The primers were designed between DNA binding domain and SH2 domains in cDNA. The forward primer was designed before DNA binding domain and the reverse primer was designed after the SH2 domain domain for allow amplification and sequencing as shown in Table 5, 5.1, 5.2.

Table5. Primer sequences for *STAT3* mutation analysis

Name	Primer sequence 5' to 3'	Product size (bp)
STAT3-mRNA-F1	TCA CTG CGC TGG ACC AGA TG	1,780
STAT3-mRNA-R1.2	GAT GAT CTG GGG TTT GGC TG	
STAT3-mRNA-F2	AGA CTC TGG GGA CGT TGC AG	For sequencing
STAT3-mRNA-F3	CCT TCT GGG TCT GGC TGG AC	For sequencing

Table 5.1. PCR reaction of *STAT3*

Component	STAT3 F1/R1.2
Distilled water (µl)	14.5 (1X)
10XPCR buffer	2.0
25mM $MgCl_2$	1.2 (1.2mM)
10 mM dNTP	0.4 (0.2mM)
10µM STAT3-F	0.3 (0.1µM)
10µM STAT3-R	0.3 (0.1µM)
5U/µl Taq polymerase	0.1(0.025U)
50ng/µl cDNA	1
Total volume (µl)	20

Table5.2. PCR cycle and condition of *STAT3*

Step	STAT3 F1/R1.2
Initial denaturation	94°C/ 5 min
PCR cycle	35 cycles
Denature	94°C/ 45sec
Annealing	60°C/ 30sec
Extension	72°C/ 1min 50 sec
Final extension	72°C/ 5 min

3.3.2 Agarose gel electrophoresis and DNA sequencing

The PCR products were verified for correct size on an ethidium bromide-stained 1.5-2% agarose gel. The PCR products were then treated with ExoSAP-IT (USP Corporation, Cleveland, OH) according to the manufacturer's recommendation, and sent for direct sequencing at Macrogen Inc., Seoul, Korea. The sequence was analyzed by sequencher.

3.4 Mutation analysis with *TBX5*

3.4.1 Polymerase Chain Reaction (PCR)

The first primers was designed within introns to allow genomic amplification and sequencing of exons 2-9 including exon-intron boundaries as shown in Table 6, 6.1, 6.2.

Table 6. Primer sequences for *TBX5* mutation analysis

Name	Primer sequence 5' to 3'	Product size (bp)
TBX5-exon2-F	CCC TCC CTG TCA CTA GAA TTG	346
TBX5-exon2-R	AAG CCG AGC AGG AAA GCC AG	
TBX5-exon3-F	CTC TCT GAG ACC ACA GGC TC	387
TBX5-exon3-R	CCA GGA TCT ATC TTT CGC TC	
TBX5-exon4-F	GAT CTT GCG GAG AGC GGA AC	385
TBX5-exon4-R	CGC CTT TAG CAC ACA GTA GG	
TBX5-exon5-F	GGA GAG CCT CCA GAT TAT TC	452
TBX5-exon5-R	GGA AGT CCA GAT CAA GAA GG	
TBX5-exon6-F	CGA GAG CCG ATA TAA CAA GG	421

TBX5-exon6-R	ACT CTT AGG CTG CAG CTT TG	
TBX5-exon7-F	GAC GTG ACT GGC TTA ATT TG	352
TBX5-exon7-R	CCA TGT GCC TGG CAT TCT AC	
TBX5-exon8-F	TTC TGT GAC TTT TCT GGT GG	505
TBX5-exon8-R	GGA ACT TTT TGT TTT AGC TG	
TBX5-exon9-F	CGG TTA GGG CTA ACA GTC TC	840
TBX5-exon9-R	CGA CCT TGA GTG CAG ATG TG	

Table6.1. PCR reaction of *TBX5*

Component	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6
Distilled water (µl)	13.5 (1X)	13.5 (1X)	13.5 (1X)	13.5 (1X)	13.5 (1X)
10XPCR buffer	2.0	2.0	2.0	2.0	2.0
25mM $MgCl_2$	1.2 (1.2mM)	1.2 (1.2mM)	1.2 (1.2mM)	1.2 (1.2mM)	1.2 (1.2mM)
10 mM dNTP	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)
10µM TBX5-F	0.4	0.4	0.4	0.4	0.4
10µM TBX5-R	0.4	0.4	0.4	0.4	0.4
5U/µl Taq	0.1 (0.025U)	0.1 (0.025U)	0.1 (0.025U)	0.1 (0.025U)	0.1 (0.025U)
50ng/µl Genomic	2	2	2	2	2
Total volume (µl)	20	20	20	20	20

Component	Exon 7	Exon 8	Exon 9
Distilled water (µl)	13.5 (1X)	13.5 (1X)	13.5 (1X)
10XPCR buffer	2.0	2.0	2.0
25mM $MgCl_2$	1.2 (1.2mM)	1.2 (1.2mM)	1.2 (1.2mM)
10 mM dNTP	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)
10µM TBX5-F	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)
10µM TBX5-R	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)
5U/µl Taq polymerase	0.1 (0.025U)	0.1 (0.025U)	0.1 (0.025U)
50ng/µl Genomic DNA	2	2	2
Total volume (µl)	20	20	20

Table6.2. PCR cycle and condition of *TBX5*

Step	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6
Initial denaturation	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min
PCR cycle	35 cycles	35 cycles	35 cycles	35 cycles	35 cycles
Denature	94°C/ 45sec	94°C/ 45sec	94°C/ 45sec	94°C/ 45sec	94°C/ 45sec
Annealing	64°C/ 45sec	64°C/ 45sec	64°C/ 45sec	64°C/ 45sec	64°C/ 45sec
Extension	72°C/ 35sec	72°C/ 35sec	72°C/ 35sec	72°C/ 35sec	72°C/ 35sec
Final extension	72°C/ 10min	72°C/ 10min	72°C/ 10min	72°C/ 10min	72°C/ 10min

Step	Exon7	Exon8	Exon9
Initial denaturation	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min
PCR cycle	35 cycles	35 cycles	35 cycles
Denature	94°C/ 45sec	94°C/ 45sec	94°C/ 45sec
Annealing	64°C/ 45sec	58°C/ 45sec	64°C/ 45sec
Extension	72°C/ 35sec	72°C/ 35sec	72°C/ 1 min
Final extension	72°C/ 10min	72°C/ 10min	72°C/ 10min

3.4.2 Agarose gel electrophoresis and DNA sequencing

The PCR products were verified for correct size on an ethidium bromide-stained 1.5-2% agarose gel. The PCR products were then treated with ExoSAP-IT (USP Corporation, Cleveland, OH) according to the manufacturer's recommendation, and sent for direct sequencing at Macrogen Inc., Seoul, Korea. The sequence was analyzed by sequencher.

A previous report has recommended if *TBX5* mutation analysis fails to show a mutation, *SALL4* analysis should be considered.

3.5 Mutation analysis with *SALL4*

3.5.1 Polymerase Chain Reaction (PCR)

The primers were designed within introns to allow genomic amplification and sequencing of exons 1-4 including exon-intron boundaries as shown in Table 7, 7.1, 7.2.

Table 7. Primer sequences for *SALL4* mutation analysis

Name	Primer sequence 5' to 3'	Product size (bp)
Sall4-ex1-F1	CAG GAA TTT GTG GCG GAG AG	301
Sall4-ex1-R2	CTC CTG AAT TTG CGC TGG AC	
Sall4-ex2-F1	CGA GAG ACT TCC AGG CAT CA	2,937
Sall4-ex2-R1	GGC TGC TTC AAG TCA TAC TC	
Sall4-ex2-F2	TTC CCA ACC TCA GGT GAT CT	For sequencing
Sall4-ex2-F3	TGA ATC AGC GGA GCG CGG AT	For sequencing
Sall4-ex2-F4	GGA CTG ATA GCT CCT TGC AG	For sequencing
Sall4-ex3-F1	CCA GCT CCA GAC TCT CAA AC	624
Sall4-ex3-R1	GTG AGC TTG AGC TTG AGA TG	
Sall4-ex4-F1	CGC TGT AAG TCA AGG ATC ATC	693
Sall4-ex4-R1	GGT TGT GGT CAC AAC CAA CG	

Table7.1. PCR reaction of *SALL4*

Component	Exon1	Exon2	Exon3	Exon4
Distilled water (µl)	12.4 (1X)	13.4 (1X)	13.4 (1X)	13.4 (1X)
10XPCR buffer	2.0	2.0	2.0	2.0
25mM $MgCl_2$	1.5 (1.5mM)	1.5 (1.5mM)	1.5 (1.5mM)	1.5 (1.5mM)
10 mM dNTP	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)
10µM SALL4-F	0.3 (0.1µM)	0.3 (0.1µM)	0.3 (0.1µM)	0.3 (0.1µM)
10µM SALL4-R	0.3 (0.1µM)	0.3 (0.1µM)	0.3 (0.1µM)	0.3 (0.1µM)
100% DMSO	1.0 (5%)	-	-	-
5U/µl Taq polymerase	0.1 (0.025U)	0.1 (0.025U)	0.1 (0.025U)	0.1 (0.025U)
50ng/µl Genomic DNA	2	2	2	2
Total volume (µl)	20	20	20	20

Table 7.2. PCR cycle and condition of *SALL4*

Step	Exon1	Exon2	Exon3	Exon4
Initial denaturation	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min
PCR cycle	35 cycles	35 cycles	35 cycles	35 cycles
Denature	94°C/ 45sec	94°C/ 45sec	94°C/ 45sec	94°C/ 45sec
Annealing	64°C/ 45sec	64°C/ 45sec	68°C/ 45sec	60°C/ 45sec
Extension	72°C/ 20sec	72°C/ 3min	72°C/ 45sec	72°C/ 45sec
Final extension	72°C/ 5min	72°C/ 5min	72°C/ 5min	72°C/ 5min

3.5.2 Agarose gel electrophoresis and DNA sequencing

The PCR products were verified for correct size on an ethidium bromide-stained 1.5-2% agarose gel. The PCR products were then treated with ExoSAP-IT (USP Corporation, Cleveland, OH) according to the manufacturer's recommendation, and sent for direct sequencing at Macrogen Inc., Seoul, Korea. The sequence was analyzed by sequencher.

3.6 Mutation analysis with *DcR3*

3.6.1 Polymerase Chain Reaction (PCR)

The primers were designed within introns to allow genomic amplification and sequencing of all coding regions including exon-intron boundaries as shown in Table 8, 8.1, 8.2.

Table 8. Primer sequences for *DcR3* mutation analysis

Name	Primer sequence 5' to 3'	Product size (bp)
DcR3-gDNA-F1	CAC CCT TGG ACT GAG CTC TG	1,112
DcR3-gDNA-R1	GGC ATG CCT CAG GCT AGA TG	
DcR3-gDNA-F1.2	GAG TGG CAG AAA CAC CCA CC	For sequencing
DcR3-gDNA-R1.2	AAC TGG TGT CCT AGC TCA GG	
DcR3-gDNA-F2	AGC TCT CTG ACC GAA GGC TC	536
DcR3-gDNA-R2	CCT CTT TCA GTG CAA GTG GG	

Table 8.1. PCR reaction of *DcR3*

Component	DcR3-gDNA-F1/R1	DcR3-gDNA-F2/R2
Distilled water (µl)	12.4 (1X)	13.4 (1X)
10XPCR buffer	2.0	2.0
25mM $MgCl_2$	1.5 (1.5mM)	1.5 (1.5mM)
10 mM dNTP	0.4 (0.2mM)	0.4 (0.2mM)
10µM DcR3-gDNA-F	0.3 (0.1µM)	0.3 (0.1µM)
10µM DcR3-gDNA-R	0.3 (0.1µM)	0.3 (0.1µM)
100% DMSO	1.0 (5%)	1.0 (5%)
5U/µl Taq polymerase	0.1 (0.025U)	0.1 (0.025U)
50ng/µl Genomic DNA	2	1
Total volume (µl)	20	20

Table 8.2. PCR cycle and condition of *DcR3*

Step	DcR3-gDNA-F1/R1	DcR3-gDNA-F2/R2
Initial denaturation	94°C/ 5 min	94°C/ 5 min
PCR cycle	35 cycles	35 cycles
Denature	94°C/ 45sec	94°C/ 45sec
Annealing	64°C/ 45sec	66°C/ 45sec
Extension	72°C/ 1min 20 sec	72°C/ 45sec
Final extension	72°C/ 5 min	72°C/ 5 min

3.6.2 Agarose gel electrophoresis and DNA sequencing

The PCR products were verified for correct size on an ethidium bromide-stained 1.5-2% agarose gel. The PCR products were then treated with ExoSAP-IT (USP Corporation, Cleveland, OH) according to the manufacturer's recommendation, and sent for direct sequencing at Macrogen Inc., Seoul, Korea. The sequence was analyzed by sequencher.

3.6.3 SLE Disease Activity Index (SLEDAI)

This method reports disease activity, damage from disease, and health status in score form by filling clinical data into SLEDAI form (Table 21.) and

calculation of score. This method is useful for classification of severity as active and inactive SLE.

Table 9. SLEDAI form

Has the patient had the recent onset of seizures, unexplained by metabolic, infectious or drug causes?	◉ Yes ○ No
Has the patient shown psychotic behavior?	○ Yes ○ No
Does the patient show altered mental function, with impaired orientation, memory or other intellectual functions?	○ Yes ○ No
Does the patient have a visual disturbance, with retinal changes associated with lupus?	○ Yes ○ No
Does the patient have a new onset of sensory or motor neuropathy involving cranial nerves?	○ Yes ○ No
Does the patient have a severe, persistent headache, unrelieved by narcotic analgesics?	○ Yes ○ No
Has the patient had a recent cerebrovascular accident (CVA) or stroke, not due to arteriosclerosis?	○ Yes ○ No
Does the patient have skin ulcerations or areas of gangrene?	○ Yes ○ No
Does the patient have tender finger nodules, periungual infarctions, or splinter hemorrhages?	○ Yes ○ No
Does the patient have biopsy or angiographic evidence of vasculitis?	○ Yes ○ No
Number of joints with pain, tenderness, swelling and/or effusion	[ ] joints
Does the patient have proximal muscle aching or weakness?	○ Yes ○ No
Does the patient have elevated serum creatine phosphokinase or aldolase?	○ Yes ○ No
Does the patient have changes in an electromyogram or a biopsy consistent with myositis?	○ Yes ○ No
Does the patient have a new onset or recurrence of inflammatory type rash?	○ Yes ○ No
Has the patient had a new onset or recurrence of abnormal, patchy or diffuse loss of hair?	○ Yes ○ No

Has the patient had a new onset or recurrence of oral or nasal ulcerations?	Yes No
Has the patient had pleuritic chest pain with pleural rub or effusion, or pleural thickening?	Yes No
Does the patient have pericardial pain ?	Yes No
Does the patient have a pericardial rub or effusion?	Yes No
Does the patient have electrocardiogram or echocardiogram evidence of a pericardial effusion?	Yes No
Body temperature	°C °F
Platelet count	per µL 10^9/L
WBC count	per µL 10^9/L
Are the decrease in blood cell counts due to drugs or toxins?	Yes No
Current urine protein output	grams per day
Previous urine protein output	grams per day
Does the patient have heme-granular or red blood cell casts in the urine sediment?	Yes No
Number of red blood cells in the urine	RBCs per high power field
Number of white blood cells in the urine	WBCs per high power field
Can the urine findings be explained by stone, infection or other cause?	Yes No
Does the patient show a decrease in CH50, C3 or C4?	Yes No
Does the patient show evidence of increased DNA binding by the Farr or other assay?	Yes No

3.6.4 Enzyme-linked immunosorbent assay (ELISA)

1. Prepare solution and standard dilution for plotting standard curve

2. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control samples should be assayed in duplicate.

3. Wash the microwell strips twice with approximately 400 µl Wash Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about 10 – 15 seconds before aspiration. Take care not to scratch the surface of the microwells. After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer.

4. Add 100 µl of Sample Diluent in duplicate to all standard wells. Pipette 100 µl of prepared standard in duplicate into well. Mix the contents of wells by repeated aspiration and ejection and creating two rows of human DcR3 standard dilutions ranging from 5000 to 78 pg/ml

5. Add 100 µl of Sample Diluent in duplicate to the blank wells.

6. Add 50 µl of Sample Diluent to the sample wells.

7. Add 50 µl of each sample in duplicate to the sample wells.

8. Prepare Biotin-Conjugate

9. Add 50 µl of Biotin-Conjugate to all wells.

10. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours, if available on a microplate shaker set at 100 rpm.

11. Prepare Streptavidin-HRP

12. Remove adhesive film and empty wells. Wash microwell strips 6 times according to point b. of the test protocol. Proceed immediately to the next step.

13. Add 100 µl of diluted Streptavidin-HRP to all wells, including the blank wells.

14. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour, if available on a microplate shaker set at 100 rpm.

15. Remove adhesive film and empty wells. Wash microwell strips 6 times according to point b. of the test protocol. Proceed immediately to the next step.

16. Pipette 100 µl of TMB Substrate Solution to all wells.

17. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 min. Avoid direct exposure to intense light.

18. Stop the enzyme reaction when the highest standard has developed a dark blue colour by quickly pipetting 100 µl of Stop Solution into each well. It is important that the Stop Solution is spreaded quickly and uniformly throughout the microwells to completely inactivate the enzyme.

19. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

20. Plot standard curve and analyze concentration of DcR3 in each sample from standard curve.

3.7 Gel extraction

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.

2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.

4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).

5. Add 1 gel volume of isopropanol to the sample and mix. (This step increases the yield of DNA fragments <500 bp and >4 kb.)

6. Place a QIAquick spin column in a provided 2 ml collection tube.

7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.

8. Discard flow-through and place QIAquick column back in the same collection tube.

9. (Optional): Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.

10. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.

11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at >10,000 x *g* (~13,000 rpm).

12. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

13. To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or H_2O to the center of the QIAquick membrane and centrifuge the column for 1 min at maximum speed. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.

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).

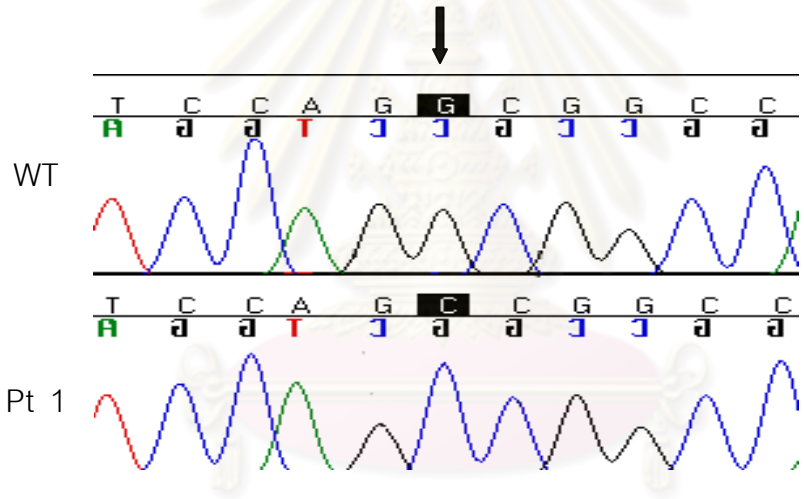


Figure 9. Electropherogram showing the c.1936G>C (p.A646P) mutation in the *ABCD1* gene.

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).

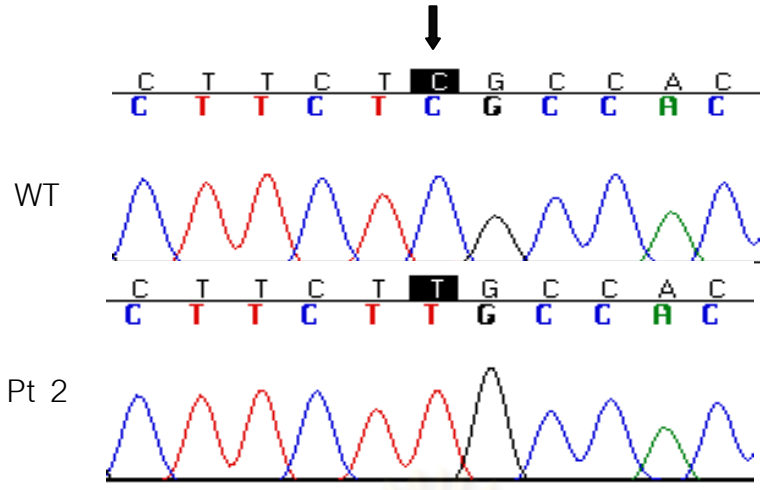


Figure 10. Electropherogram showing the c.1825G>A (p.E609K) mutation in the *ABCD1* gene.

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).

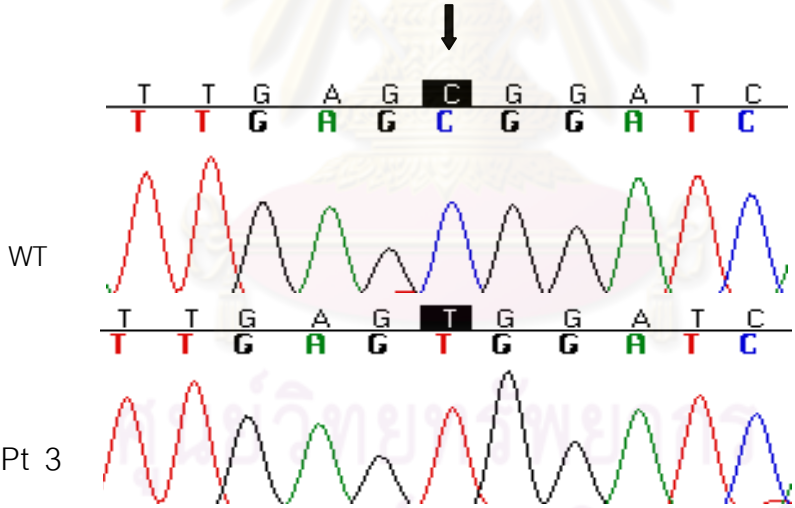


Figure 11. Electropherogram showing the c.1201C>T (p.R401W) mutation in the *ABCD1* gene.

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).

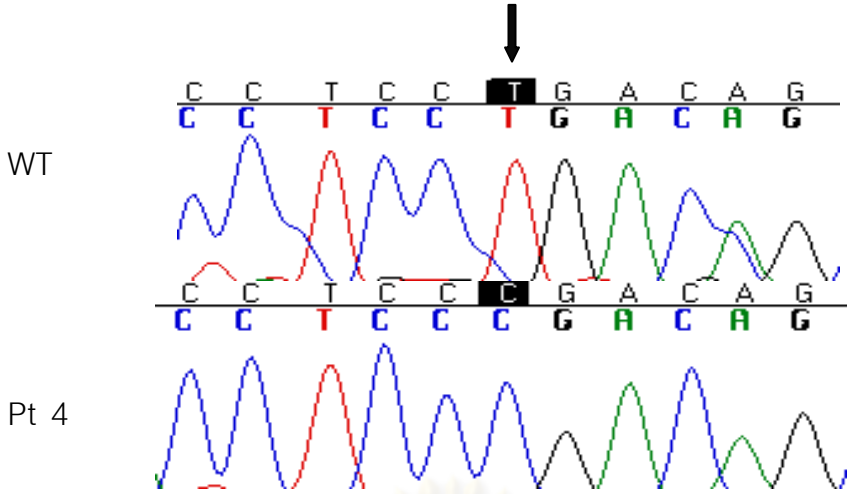


Figure12. Electropherogram showing the c. 1175T>C (p.L392P) mutation in the *ABCD1* gene.

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).

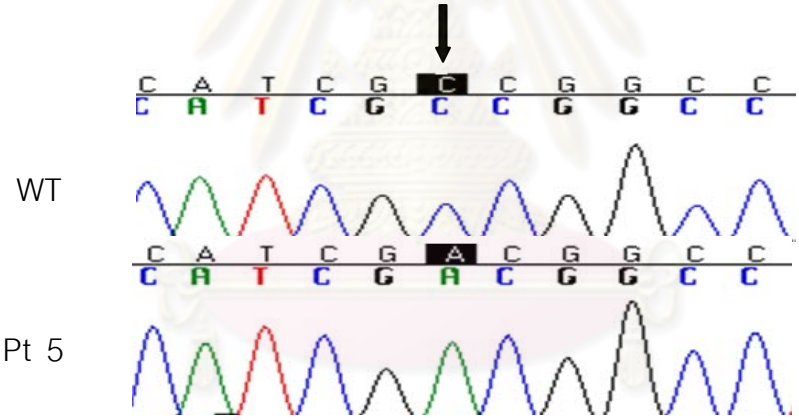


Figure.13Electropherogram showing the c. 740C>A (p.A247D) mutation in the *ABCD1* gene .

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

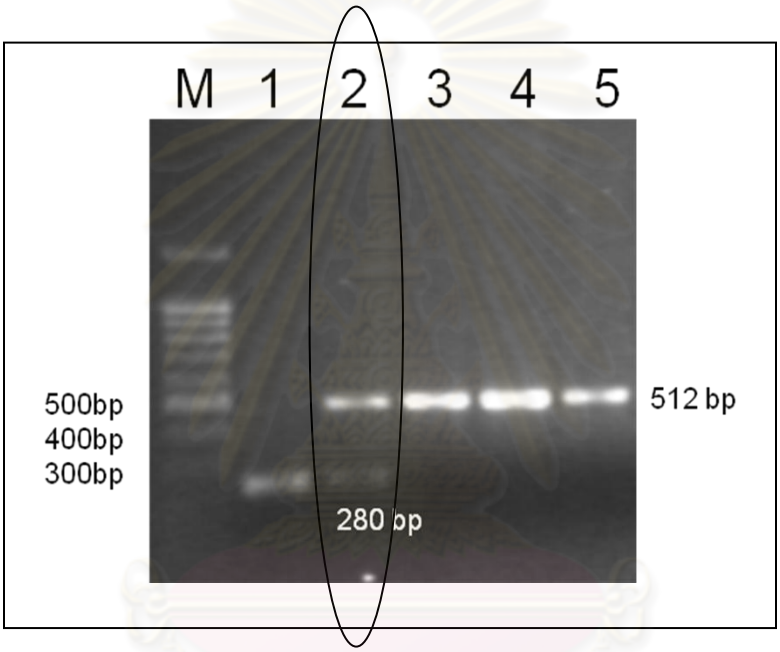


Figure14. Restriction enzyme digestion analysis of patient 5, the mother of patient 5 and 50 unaffected controls. M = 100 bp DNA marker;
Lane1 = patient 5; Lane 2 = patient's mother; Lanes 3-5 = unaffected controls

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

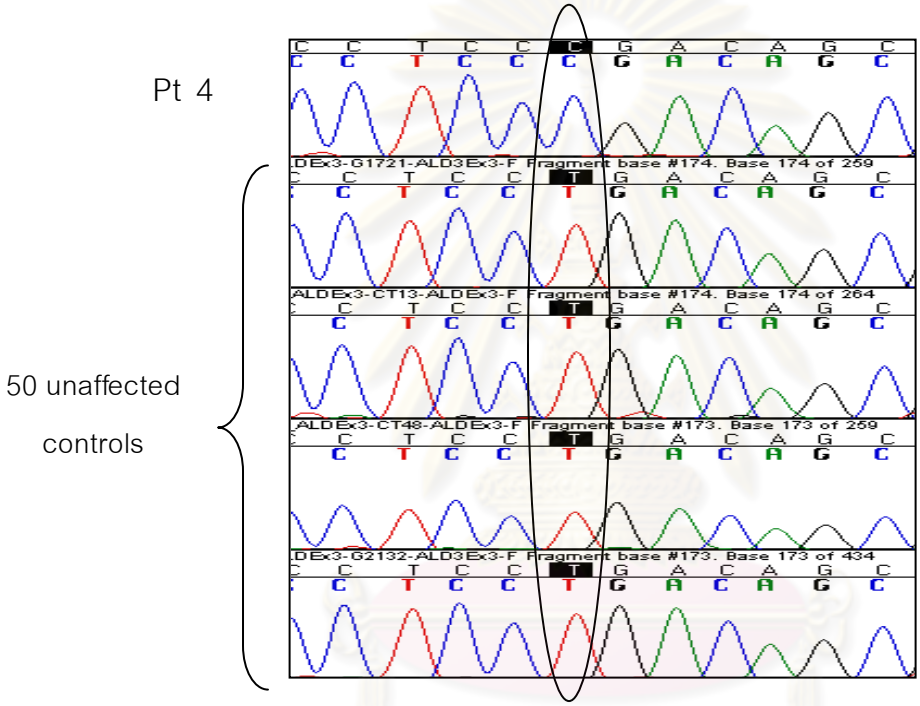


Figure 15 Electropherogram showing the p.L392P in patient 4 and 50 unaffected controls in the *ABCD1* gene.

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

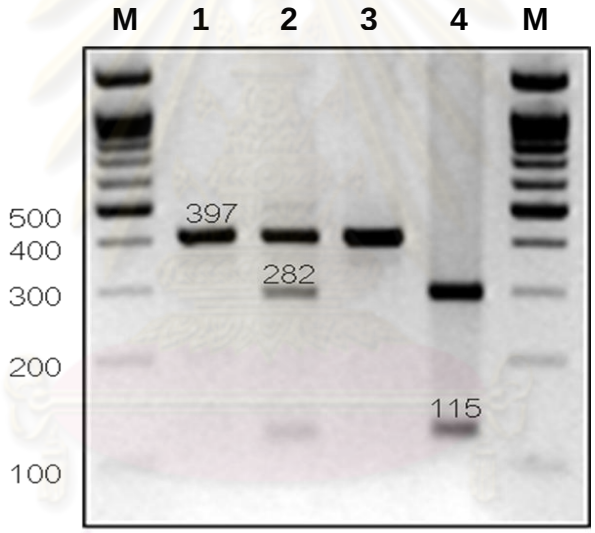


Figure16. Restriction enzyme digestion to detect the D645E mutation
M: 100 bp marker. Lane 1: A homozygous D645E control. Lane 2: A heterozygous D645E carrier. Lane 3: Patient 3. Lane 4 : unaffected control.

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.

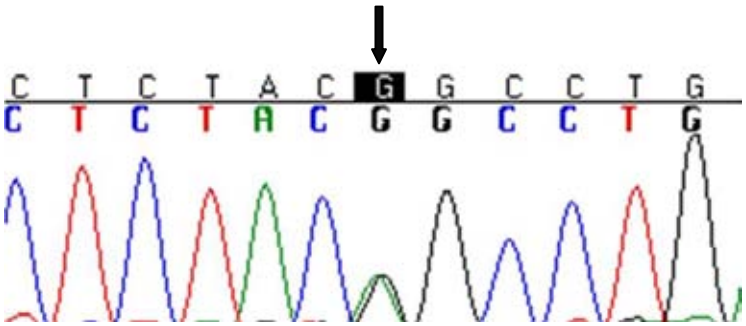


Figure 17. Electropherogram showing the c.1726G>A (p. G576S) mutation in the *GAA* gene.

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).

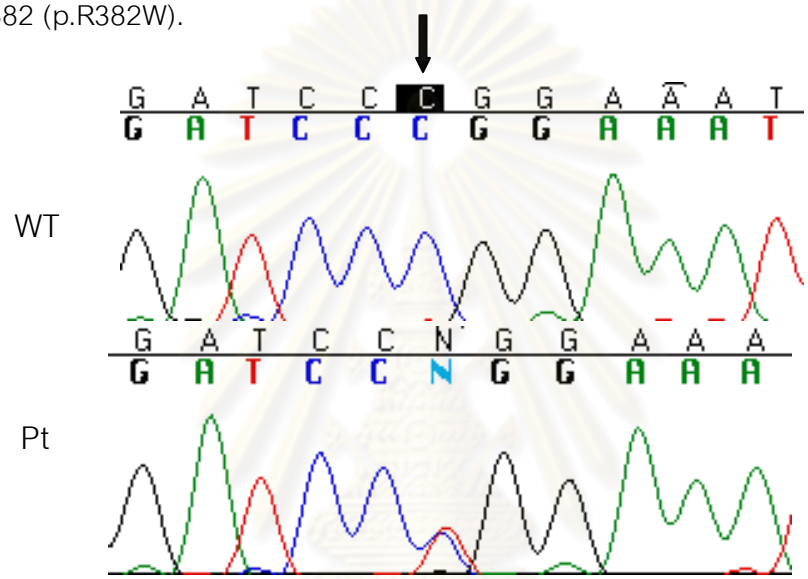


Figure18. Electropherogram showing the c.1144C>T (p.R382W) mutation in the *STAT3* gene.

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).

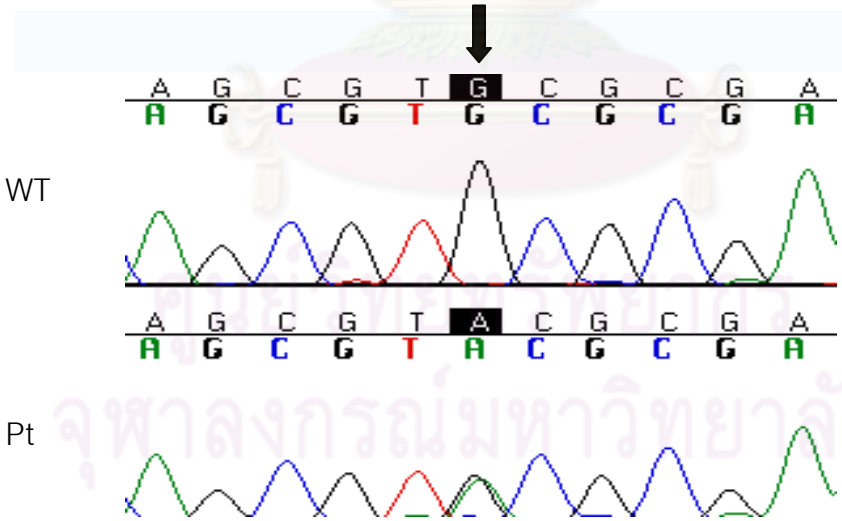


Figure19. Electropherogram showing the c.364C>T (p.H122Y) mutation in the *DcR3* gene.

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

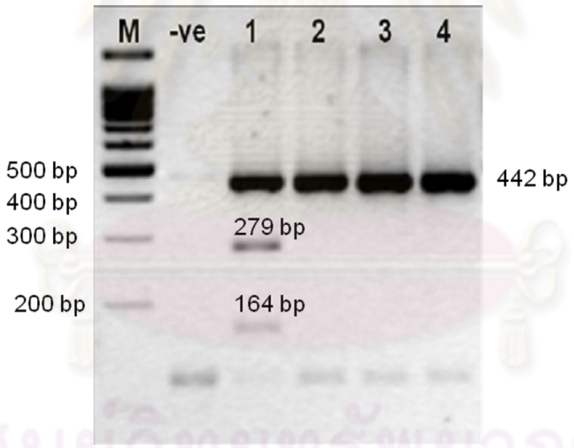


Figure 20 PCR-RFLP to confirm the presence of a novel mutation, c.364C>T in *DcR3*. M = 100 bp DNBA marker; -ve = no template; Lane 1 = patient; Lane 2-4 = unaffected controls

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.

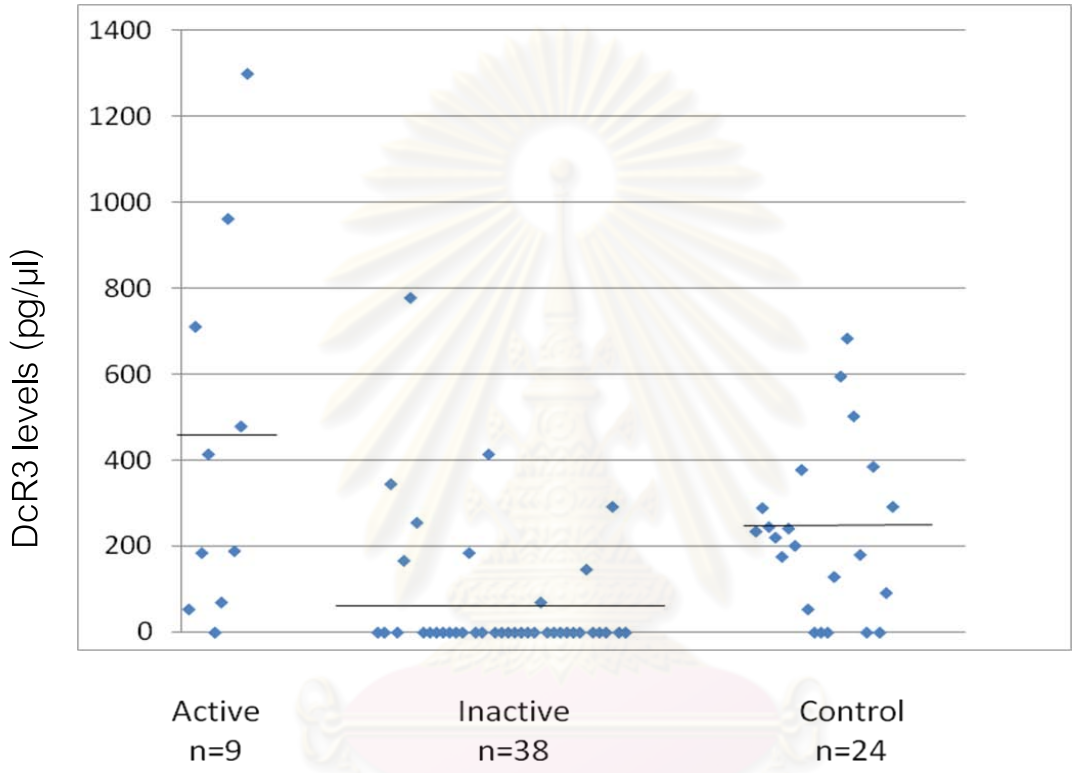


Figure 21 Serum DcR3 levels (pg/µl) in SLE patients and unaffected controls assayed by ELISA.

CHAPTER V
DISCUSSION AND CONCLUSION

Genetic disorder is a disease caused by abnormalities in an individual's genetic material (genome). There are over 6000 genetic disorders that can be passed down through generations. In this study, we described some genetic disorders commonly found at Pediatric Clinic of the King Chulalongkorn Memorial Hospital.

X-ALD is a rare genetic disease with symptoms normally starting between the ages of 4 and 10 years. We identified 9 unrelated Thai patients clinically suspected of X-ALD. Mutation screening in the *ABCD1* gene by PCR-sequencing revealed 5 different mutant alleles including 2 novel ones. (A646P, E609K, R401W, L392P, A247D). Restriction enzyme analysis was also used to confirm and screen for the identified mutation in at-risk family members or suspected carriers. The results, revealing the mutations as either *de novo* or inherited, have proved to be helpful for more accurate genetic counseling as well as prenatal diagnosis.

In this study, a mutation in the *ABCD1* gene could not be identified in 4 X-ALD patients, it remains possible that mutations could be in the promoter or intron region which was not included in our method.

In Pompe disease, there are some populations in which particular mutations are more common due to founder effects. For example, the p.R854X mutation has been found in many African American and African cases; the p.D645E has been seen in many Chinese infantile cases; and the p.G925A mutation has been seen in many European cases.[28] In this study, we analyzed 5 unrelated Thai families with Pompe and found the p.D645E mutation in all families (100%). It might be the most commonly found mutation in Thai people caused by a founder effect similar to previous studies in the Chinese and Taiwan populations [28]. For a cheap, rapid and reliable test, genetic testing using restriction enzyme analysis for the D645E mutation can be performed in Thai patients with Pompe. If this mutation is not detected, further analysis by PCR-sequencing to identify other mutations in the *GAA* gene should be performed. Genetic analysis remains very useful for genetic counseling and prenatal diagnosis of this disease.

Mutations in *STAT3* have been recently identified in a disorder causing profound, multisystem inflammatory disorders.[11] STAT3 is an essential mediator of the immune suppressive action of interleukin-10, and the loss of responsiveness of this interleukin may underlie much of the proinflammatory nature of the hyper-IgE syndrome (HIE). The heterozygous missense mutations have been found only in the DNA-binding or SH2 domain. Mutations within the DNA binding domain resulted in a protein with impaired ability to target the promoter of the target genes and mutations in the SH domain caused reductions in target-gene expression.[10, 11] From these previous findings, we performed targeted mutation analysis by designing primers only around the DNA binding and SH domains for direct sequencing analysis. We identified the known mutation, c.1144C>T (p.R382W) in our Thai patient. This is the first molecularly-confirmed HIE Thai patient.

Holt-Oram syndrome (HOS) is a rare genetic disorder. It is characterized by malformations of upper limbs and variable cardiac defects. The diagnosis can be confirmed by mutation analysis of the *TBX5* gene. At present, mutations in *TBX5* can be found throughout in the coding region of the gene as shown in figure 6.[16] In this study, we analyzed all exons of the *TBX5* but could not identify the potential disease causing mutation. However, some previous studies found another gene responsible for HOS. It has been suggested that if the patients were diagnosed with Holt-Oram syndrome and *TBX5* analysis failed to show a mutation, *SALL4* analysis should be considered. [22] Therefore, we analyzed all coding regions of *SALL4* in our HOS patients without mutations in *TBX5* and could not identify the potential disease causing mutation. From this result, there could be other unidentified genes responsible for Holt-Oram syndrome in these Thai patients.

Systemic lupus erythematosus (SLE) is a severe autoimmune disease inflicting damage to multiple organs. The exact etiology of SLE has not been clear, but it is obvious that genetic factors, gender and environment are involved its pathogenesis. It has been shown that there are multiple genomic loci containing SLE susceptibility genes in humans and mice.[30] In 2007, studies in mice found that overexpression of human DcR3 in mice resulted in an SLE-like syndrome. In addition, some previous studies have shown elevated DcR3 levels in active SLE patients when compared with inactive SLE

patients and healthy controls, although the reasons of this association remain elusive. [31-33] Therefore, DcR3 is a potential new candidate gene for SLE.

Our study showed that DcR3 levels were not different between SLE patients and unaffected controls. Our results were not similar to the previous studies. The possibilities remain that the controls that we used in this study were individuals with other diseases but not autoimmune diseases, unlike the controls used in previous studies. Most of our samples were from inactive SLE patients. However, we found two severe SLE patients (with SLEDAI score 24 and 29) who had significantly increased levels of DcR3 (1,299 pg/µl and 961.7 pg/µl). There was one patient who we received two serum samples during inactive stage (score 0) with the DcR3 level of 255.3 pg/µl and during active stage (score 8) with up the DcR3 level to 711.01 pg/µl.

We identified one SLE patient with the c.364C>T mutation on exon1 of the *DcR3* gene. The mutation is expected to result in a histidine to tyrosine substitution at codon 122. The mutation is located on the Fas binding site. It is a region for binding with the Fas receptor to protect apoptosis. We tested whether this mutation was a potential disease-causing mutation by screening for its presence by PCR-RFLP in 500 unaffected controls. We found 1 in 500 unaffected controls carrying this mutation. We also measured the DcR3 level in this patient. The patient did not have an increased DcR3 levels. It remains possible that this mutation might result in gain of function leading to an increased ability of DcR3 in binding with the Fas receptor. The DcR3 level could be normal in the SLE patient with the mutation. Of note, although there have been many genome wide association studies for SLE, none have shown a positive association to a polymorphism nearby the *DCR3* locus. This does not absolutely contradict our hypothesis; *DCR3* could still be a gene underlying SLE. If the DNA change found in our patient is proved to be a *de novo* pathogenic mutation, then association studies are correctly unable to detect it.

The practical techniques to detect each mutation in this study can be concluded in Table 16.

Table16. Practical techniques to detect mutations in this study.

Mutation	Practical technique	Example disease
Distribute throughout the gene	Analysis of all exons or coding regions	X-linked ALD
Common /hotspot mutation	PCR-RFLP	Pompe disease
Some regions	Analysis of specific regions	HIES
Not found mutation	Maybe mutation in other genes	HOS

In conclusion, genetic testing can be developed for each genetic disease to provide the most accurate and affordable method for disease diagnosis. Developing such methods depend on characteristics of genes that associate with the diseases, for example, gene size, gene complexity, gene expression, previously reported mutations in the gene. These factors need to be taken into consideration for successfully developing practical genetic techniques.

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APPENDICES

APPENDIX A

BUFFERS AND REAGENT

1. **10X Tris borate buffer (10X TBE buffer)**

Tris – base	100	g
Boric acid	55	g
0.5 M EDTA (pH 8.0)	40	ml

Adjust volume to 1,000 ml with distilled water. The solution was mixed and stored at room temperature.

2. **6X loading dye**

Bromphenol blue	0.25	g
Xylene cyanol	0.25	g
Glycerol	50	ml
1M Tris (pH 8.0)	1	ml
Distilled water until	100	ml

Mix and store at 4^0C

3. **2% Agarose gel (w/v)**

Agarose	2.0	g
1X TBE	100	ml

Dissolve by heating in microwave oven and occasionally mix until no granules of agarose are visible.

4. **Ethidium bromide**

Ethidium bromide	10	mg
Distilled water	1	ml

Mix the solution and store at 4^0C

5. **Phosphate-Buffered Saline (PBS)**

Solution A

NaCl	8.0	g
KCl	0.2	g
$CaCl_2.2H_2O$	0.132	g
$MgCl_2.6H_2O$	0.1	g
Distilled water	800	ml

Solution B

Na_2HPO_4	1.15 g
KH2PO4	0.2 g
Distilled water	800 ml

Dissolve each solution in demineralized water. Autoclave solutions A and B separately at 15 pounds for 15 minutes. Mix A and B when cold: stir slowly; final pH 7.0 and store at 4^0C.

6. **100 bp ladder**

100 bp ladder stock	30 µl
TBE buffer	30 µl
1X loading dye	30 µl

Mix the solution and store at 4^0C

7. **1k bp ladder**

1k bp ladder stock	30 µl
TBE buffer	30 µl
1X loading dye	30 µl

Mix the solution and store at 4^0C

APPENDIX B

SAMPLE SIZE

Sample size (two independent groups) for ELIZA experiment

From previous report [31]

Patients (n_1) = 90	controls (n_2) = 123
mean ($\bar{X}_1$) = 32	mean ($\bar{X}_2$) = 5
SD (S_1) = $\pm$ 32	SD (S_2) = $\pm$ 12

Calculation

α = 0.05

β = 0.10

$Z_{\alpha/2} = Z_{0.05/2} = 1.96$ (two tails)

$Z_{\beta} = Z_{0.10} = 1.28$

$$\text{n/group} = 2(Z_{\alpha/2} + Z_{\beta})^2 \sigma^2 / (\bar{X}_1 - \bar{X}_2)^2$$

$\bar{X}_1$ = mean of patients

$\bar{X}_2$ = mean of controls

σ^2 = Pooled variance

$$= \frac{(n_1-1)S_1^2 + (n_2-1)S_2^2}{n_1+n_2-2}$$

$$= \frac{(90-1)32^2 + (123-1)12^2}{90+123-2}$$

$$= 515.185$$

$$\text{n/group} = \frac{2(1.96+1.28) \times 515.185}{(32-5)^2}$$

= 14.8

APPENDIX C

CRITERIA FOR SELECTION OF CONTROLS & RESULT OF ELISA

Controls of serum DcR3 levels using ELISA

In this study, serum from healthy children could not be obtained. Therefore, we used control serum from patients who met the following criteria.

Inclusion criteria for selection of the unaffected controls

1. Age

   ≤ 20 years old

2. Patients without the following disorders;

   2.1 Myeloma

   2.2 Nasopharyngeal carcinoma

   2.3 Pituitary adenoma

   2.4 Hepatocellular carcinoma

   2.5 Crohn's disease

   2.6 Pancreatic adenocarcinoma

   2.7 Gastric cancer

   2.8 Renal cancer

   2.9 Epithelial ovarian cancer

   2.10 B cell lymphoma

   2.11 Laryngeal carcinoma

   2.12 Ovarian cancer

   2.13 Glioma

   2.14 Colorectal cancer

   2.15 Gastrointestinal tract tumor

   2.16 Silicosis

   2.17 Leukemia

   2.18 Kidney transplant

3. The patients with mild illnesses

4. The patients/parents signed the consent form.

Table17. Evaluation of serum DcR3 (pg/µl) in SLE patients by ELISA

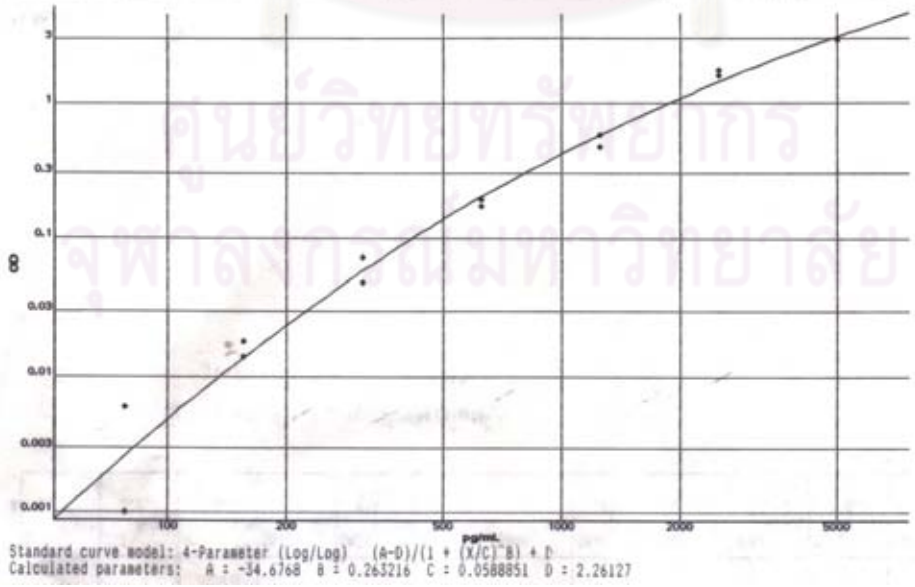
Plate ID: 7 **Biotrak II Reader** Results

Test : DCR3450/620
Measurement Date : 03.08.09 17:12
Measurement Filters : 450/620 nm

Legend: Layout / Absorbance / Concentration / Thresholds
Concentration range: 51.4526 pg/mL .. 7579.8 pg/mL
Thresholds not available.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BK 0.003	ST4 0.191 608.9	SM1 -0.015 1832	SM9 -0.005 1865	SM17 -0.007 1859	SM25 -0.004 1869	SM33 -0.005 1865	SM41 0.002 69.96	SM49 -0.010 1849	SM57 0.050 289.5	SM65 -0.008 1855	SM73 -0.013 1839
B	BK -0.003	ST4 0.170 568	SM2 -0.021 1813	SM10 -0.010 1849	SM18 0.020 185	SM26 -0.016 1829	SM34 -0.011 1845	SM42 -0.008 1855	SM50 0.393 961.7	SM58 0.036 245.4	SM66 -0.012 1842	SM74 0.086 385.6
C	ST1 2.900 4720	ST5 0.073 352.9	SM3 0.070 345.1	SM11 -0.020 1816	SM19 0.020 185	SM27 -0.021 1813	SM35 0.004 91.96	SM43 0.012 146.6	SM51 0.021 189.3	SM59 0.029 220.7	SM67 -0.004 1869	SM75 -0.004 1869
D	ST1 2.982 4849	ST5 0.048 283.5	SM4 0.001 53.95	SM12 -0.008 1855	SM20 -0.011 1845	SM28 -0.021 1813	SM36 -0.016 1829	SM44 -0.011 1845	SM52 0.127 479.3	SM60 0.018 176.2	SM68 0.009 129.2	SM76 0.004 91.96
E	ST2 1.593 2747	ST6 0.018 176.2	SM5 -0.019 1819	SM13 -0.019 1819	SM21 -0.028 1791	SM29 -0.018 1823	SM37 -0.005 1865	SM45 -0.026 1797	SM53 0.608 1299	SM61 0.035 241.9	SM69 0.184 595.4	SM77 1.940 3257
F	ST2 1.744 2968	ST6 0.014 157.06	SM6 0.016 166.9	SM14 -0.023 1806	SM22 -0.022 1810	SM30 -0.019 1819	SM38 -0.025 1800	SM46 -0.003 1872	SM54 -0.013 1839	SM62 0.024 201.6	SM70 0.231 683.8	SM78 0.051 292.4
G	ST3 0.472 1088	ST7 0.001 53.95	SM7 0.284 778.2	SM15 0.246 711.01	SM23 -0.011 1845	SM31 -0.018 1823	SM39 -0.021 1813	SM47 0.051 292.4	SM55 -0.025 1800	SM63 0.083 378.2	SM71 0.138 502.7	SM79 0.286 781.8
H	ST3 0.580 1256	ST7 0.006 108.7	SM8 0.039 255.3	SM16 -0.006 1862	SM24 0.098 414.3	SM32 0.002 69.96	SM40 -0.003 1872	SM48 -0.017 1826	SM56 0.033 235.05	SM64 0.001 53.95	SM72 0.019 180.6	SM80 0.131 487.9

Plate ID: 7 **Biotrak II Reader** Results



Standard curve model: 4-Parameter (Log/Log) (A-D)/(1 + (X/C) B) + D
Calculated parameters: A = -34.6768 B = 0.263216 C = 0.0588851 D = 2.26127

Figure22. Standard curve of DcR3 by ELISA

BIOGRAPHY

Mr. Pramuk Amarinthnukrowh was born in Bangkok, the capital city of Thailand, in November 8th, 1984. In 2007, I received my bachelor degree in Biochemistry from Faculty of Science, Chulalongkorn University. Consequently, with my interests in Human and Molecular Genetics, I had made a decision to study in curriculum of Medical Science in Faculty of Medicine for my Master degree.