การศึกษาความผิดปกติในระดับชีวเคมีและโมเลกุลของผู้ป่วยไทยโรคเกาเชอร์

นายกำพล พิพัฒนานันท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์การแพทย์
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2548
ISBN 974-53-2948-7

BIOCHEMICAL AND MOLECULAR ANALYSIS OF THAI PATIENTS WITH GAUCHER DISEASE

Mr. Kampon Phipatthanananti

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Science
Faculty of Medicine
Chulalongkorn University
Academic Year 2005

ISBN 974-53-2948-7

Thesis Title	BIOCHEMICAL AND MOLECULAR ANALYSIS OF THAI PATIENTS WITH GAUCHER DISEASE
By	Mr. Kampon Phipatthanananti
Field of Study	Medical Science
Thesis advisor	Associate Professor Vorasuk Shotelersuk, M.D.

Accepted by the Faculty of Medicine, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

P. Kamolratanakul .....Dean of the Faculty of Medicine
(Professor Pirom Kamolratanakul, M.D.)

THESIS COMMITTEE

Apiwat Mutirangura .....Chairman
(Professor Apiwat Mutirangura, M.D. Ph.D.)

Vorasuk Shotelersuk .....Thesis Advisor
(Associate Professor Vorasuk Shotelersuk, M.D.)

Issarang Nuchprayoon .....Member
(Associate Professor Issarang Nuchprayoon, M.D.)

Kanya Suphapeetiporn .....Member
(Kanya Suphapeetiporn M.D.)

กำพล พิพัฒนานันท์ : การศึกษาความผิดปกติในระดับชีวเคมีและโมเลกุลของผู้ป่วยไทยโรคเกาเชอร์ (BIOCHEMICAL AND MOLECULAR ANALYSIS OF THAI PATIENTS WITH GAUCHER DISEASE) อ. ที่ปรึกษา : รศ.นพ.วรศักดิ์ โชติเลอศักดิ์, 56 หน้า. ISBN 974-53-2948-7

โรคเกาเชอร์เป็นโรคพันธุกรรมเมแทบอลิซึมที่มีการถ่ายทอดแบบลักษณะด้อยบนออโตโซม เกิดจากความผิดปกติของเอนไซม์ glucocerebrosidase ซึ่งสร้างโดยยีน *GBA* ในประเทศไทยยังไม่มีรายงานการศึกษาทางชีวเคมีและการกลายพันธุ์ของโรคเกาเชอร์ ในการศึกษาครั้งนี้ได้รวบรวมผู้ป่วยโรคเกาเชอร์ 5 ราย ซึ่งได้รับการวินิจฉัยโดยลักษณะทางคลินิกและการพบ Gaucher cell จากการตรวจไขกระดูก ผู้วิจัยได้พัฒนาวิธีการตรวจวัดระดับการทำงานของเอนไซม์ glucocerebrosidase โดยในคนไทยปกติจำนวน 6 คนพบว่า ระดับค่าเฉลี่ยเป็น 11.63±4.97 nmol/mg protein/h ส่วนในผู้ป่วยทั้ง 5 รายพบว่าผู้ป่วยทุกรายมีระดับการทำงานของเอนไซม์ลดลง (น้อยกว่า 5 เปอร์เซ็นต์เมื่อเปรียบเทียบกับคนปกติ) การศึกษาหาการกลายพันธุ์ในยีน *GBA* ซึ่งมี 11 exons และ cDNA มีขนาด 2,276 bp โดยวิธี long-template PCR, conventional PCR และ sequencing บริเวณ coding region ทั้งหมด แล้วยืนยันผลโดย restriction enzyme digestion พบการกลายพันธุ์ L444P บ่อยที่สุด (4 ใน 5 คนหรือ 6 ใน 10 อัลลีล) และพบการกลายพันธุ์ใหม่ 3 ชนิดคือ Y363H, IVS6(-1)G⟶C และ X498A โดย IVS6(-1)G⟶C น่าจะทำให้เกิดโรคจากการที่ mRNA ไม่เสถียรและถูกทำลายไป โดยสรุปคณะผู้วิจัยได้พัฒนาวิธีการวินิจฉัยโรคเกาเชอร์ด้วยวิธีการตรวจระดับการทำงานของเอ็นไซม์ glucocerebrosidase โดย L444P เป็นการกลายพันธุ์ชนิดที่พบบ่อยที่สุดและพบการกลายพันธุ์ที่ไม่เคยมีการรายงานมาก่อน 3 ชนิด การวินิจฉัยทั้งทางชีวเคมีและการกลายพันธุ์จะทำให้การวินิจฉัยโรคถูกต้องรวดเร็วและส่งผลถึงการให้คำปรึกษาทางพันธุศาสตร์ที่แม่นยำขึ้น

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต กำพล พิพัฒนานันท์
ปีการศึกษา 2548 ลายมือชื่ออาจารย์ที่ปรึกษา [illegible]
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม -

477 47093 30 : MAJOR MEDICAL SCIENCE
KEYWORD: GAUCHER DISEASE / GBA / GLUCOCEREBROSIDASE
KAMPON PHIPATTHANANANTI : BIOCHEMICAL AND MOLECULAR ANALYSIS OF THAI PATIENTS WITH GAUCHER DISEASE.
THESIS ADVISOR: ASSOC. PROF. VORASUK SHOTELERSUK, M.D.,
56 pp, ISBN 974-53-2948-7

Gaucher disease (GD) is an autosomal recessive disorder caused by the deficiency of lysosomal β-glucosidase, or glucocerebrosidase, an enzyme that participates in the degradation of glycosphingolipids. Deficiency of this enzyme results in the storage of glucocerebrosides in lysosomes of macrophage. Gaucher disease has three phenotypes based on the absence (type 1) or presence and severity (types 2 and 3) of primary CNS involvement. Although many clinical diagnoses of Thai patients with Gaucher disease have been reported, their biochemical and molecular characteristics were not included. We established a glucocerebrosidase activity assayed by fluorimetric method and studied 6 controls, 5 patients with GD and a carrier. We have found a remarkably decreased activity (< 5% of normal) of the glucocerebrosidase in all patients, whereas the mean glucocerebrosidase activity was 11.63±4.97 nmol/mg protein/h in control group. Mutation analysis, performed by long-template PCR, PCR using specific primers, direct sequencing in all coding regions of the *GBA* gene and restriction enzyme digestion, showed that L444P is the most common mutant allele found in 4 patients (6 of 10 alleles). Three novel mutations were found including a missense mutation (Y363H), a termination codon mutation (X498A), and a frameshift mutation (IVS6(-1)G→C). mRNA of the IVS6(-1)G→C is expected to be unstable and degraded. In conclusion, we established an enzyme assay to diagnose GD. L444P allele is the most frequent mutation identified in Thai patients and 3 novel mutations were identified. These biochemical and molecular tests will facilitate definite diagnosis of GD and have implications on genetic counseling.

Field of study Medical Science Student's signature Kampon Phipatthananti

Academic year 2005 Advisor's signature Vorasuk Shotelersuk

ACKNOWLEDGEMENTS

First, I really would like to express my gratitude and sincere appreciation to all those who participated in the success of this work. First of all, I am deeply indepted to my advisor, Assoc. Prof. Dr. Vorasuk Shotelersuk for his continuous support, suggestions and encouragement during the M.Sc. program. Without his encouragement and constant guidance, I could not have finished my thesis. He was always there to meet with me to talk about my thesis, to proofread and mark up my thesis, and to ask me good questions to help me think through my problems.

Besides my advisors, I would like to greatly express my heartfelt thanks to the rest of my thesis committee, Prof. Dr. Apiwat Mutirangura, Assoc. Prof. Dr. Issarang Nuchprayoon and Dr. Kanya Suphapeetiporn for their helpful suggestions and corrections during my study.

I am also grateful to Miss Siraprapa Thongkorbpetch, Miss Thivaratana Sinthuwiwat, Miss Petcharat Leoyklang and all the members in our laboratory for their help and support from the beginning of my laboratory practice. Moreover, they asked me good questions, rescued me from various crises, encouraged me to do my thesis, gave insightful comments and reviewed my work all the time.

A special thanks goes to many teachers in the past: Assist. Prof. Dr. Jessada Denduangboripant for teaching me, introducing me to, and getting me interested in Molecular Biology. He taught me how to write academic papers, made me a better scientist, gave many chances to me and had confidence in me when I doubted myself.

Let me also say ‘thank you’ to the following people: P’Jib, Arm, Neng, another OMG friend, Big, Phung, Bitoey, Bifern, Gigg who heard my complaints about any problem and frustration. They gave me willpower and cheerfulness and were always on my side.

Last, but not least, I thank my family: my parents for giving me life in the first place, for educating me with aspects from both arts and sciences, and for unconditional support and encouragement to pursue my interests.

This work was supported by Chulalongkorn University.

TABLE OF CONTENTS

Abstract(Thai).....iv

Abstract(English).....v

Acknowledgments.....vi

Table of contents.....vii

List of Tables.....ix

List of Figures.....x

List of Abbreviation.....xi

Chapter

I. Introduction.....1

Background and rationale.....1

Research Questions.....4

Objectives.....4

Hypothesis.....4

Key Words.....5

Expected Benefit.....5

Conceptual Framework.....6

Research Methodology.....7

II. Review of Related Literatures.....8

Gaucher Disease.....8

Clinical Features.....8

Classification.....8

Morphological Diagnosis.....9

Enzymatic Diagnosis.....9

Molecular Diagnosis.....11

Prenatal Diagnosis.....14

III. Materials and Methods.....15

Subjects and Sample Collection.....15

Enzymatic Diagnosis.....15

Molecular Diagnosis (DNA analysis).....18

IV. Results.....25

Glucocerebrosidase Activity Assay.....25

Identification of the Mutant Alleles.....26

Confirmation of Mutant Alleles.....34

V. Discussion and Conclusion.....39

References.....42

Appendices.....48

Biography.....56

LIST OF TABLE

Table Page

1. Phenotypes of Gaucher Disease—Clinical Types…….2
2. Enzyme activity of glucocerebrosidase in leukocytes from controls, GD patients and obligated GD heterozygotes. ……10
3. Allele distribution in Gaucher disease type 1 patients. ……...13
4. PCR primers used to amplify *Functional GBA* and *Pseudo GBA*……..20
5. Components of LT-PCR reaction……21
6. PCR primers used to amplify *functional GBA*..... 22
7. Component of PCR reaction for mutation detection in each exon……..22
8. PCR cycle conditions……...23
9. Glucocerebrocidase activity in patients and normal adults…….25
10. Mutations in glucocerebrosidase gene encountered in GD patients ……..26
11. Primers, restriction enzymes and expected size of PCR product ……..34
12. PCR primers used to amplify cDNA of the patient 5…….38

LIST OF FIGURES

Figure Page

1. Substrates and products of the glucocerebrosidase reaction……...1
2. Schematic of glucocerebrocidase gene and pseudogene……..3
3. Gaucher cells (left) and pseudo-Gaucher cells (right)……..3
4. Structure of glucocerebrosidase substrate and their reaction products……...17
5. Long-template PCR amplification of the entire glucocerebrosidase gene…..27
6. Electropherogram of the normal sequence and the T1448C (L444P) mutation in the patient 3 and their parents. ……..…..….28
7. Electropherogram of the normal sequence and the T1448C (L444P) mutation in the patient 1,2,3 and 4…….29
8. Electropherogram of the normal sequence in the patient 2 and the T681G (N188K) mutation in the patient 1…….……30
9. Electropherogram of the normal sequence in the patient 2 and the T1204C (Y363H) mutation in the patient 4……..…….31
10. Electropherogram of the normal sequence in the patient 2 and the splice site mutation in the patient 5……32
11. Electropherogram of the normal sequence in the patient 2 and the mutation in stop codon (X) at position 498 in the patient 5……...……33
12. Restriction enzyme digestion with *Nci* I of gDNA for detection L444P mutation…….35
13. Restriction enzyme digestion with *Rsa* I of gDNA for detection Y363H mutation……36
14. Restriction enzyme digestion with *Cac* 8 I of gDNA for detection X498A mutation……37
15. Electropherograme of the cDNA amplified from mRNA of patient 5……..38

LIST OF ABBREVIATIONS

GD	=	Gaucher disease
GBA	=	Acid beta Glucocerebrosidase gene
PCR	=	Polymerase Chain Reaction
LT-PCR	=	Long-Template Polymerase Chain Reaction
4-MU	=	4-Methly-Umbelliferone
CNS	=	Central Nervous System
AJ	=	Ashkenazi Jewish

CHAPTER I

INTRODUCTION

Background and rationale

Gaucher disease (GD)(OMIM 230800), the most prevalent lysosomal storage disorder, is caused by a deficiency of lysosomal enzyme glucocerebrosidase, or acid β-glucosidase, which normally hydrolyzes glucocerebroside, or glucosylceramide to glucose and ceramide (Fig.1), leading to the accumulation of glucocerebroside in macrophages and resulting in multiorgan involvement. The disease is an autosomal recessive disorder resulting from mutations in the gene for the lysosomal hydrolase, acid β-glucosidase or glucocerebrosidase (EC.3.2.1.45), which has been localized to the chromosomal region 1q21 (1,2).

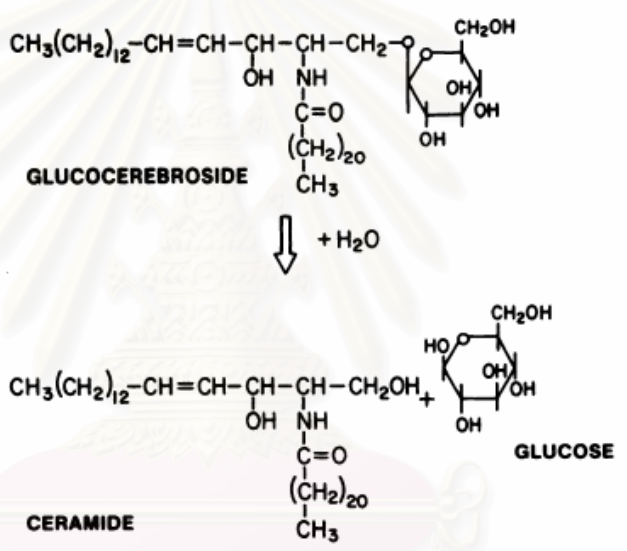


Figure 1. Substrates and products of the glucocerebrosidase reaction.

The human *GBA* gene (GenBank accession number J03059), located on chromosome 1q21, has a pseudogene (GenBank accession number J03060) that shares 96% exonic sequence homology, located 16 kb downstream of the functional gene. The 1q21 region is gene rich, with seven genes and two pseudogenes located in close proximity (Fig.2) (3,4).

There are three clinical subtypes of Gaucher disease, which are distinguished by the absence or presence and severity of neurologic complications (Table 1). Type 1 disease (MIM 230800), or nonneuronopathic, which does not involve the nervous system, occurs with high prevalence among Ashkenazi Jews. Type 2 disease (MIM 230900), or acute-neuronopathic, is a fatal neurodegenerative disorder of infancy, similar to Tay-Sachs disease. Type 3 disease (MIM 231000), or chronic-neuronopathic, is a slowly progressive neurologic disease with survival into

adulthood. The occurrence of type 2 or 3 Gaucher disease among Ashkenazi Jews is extremely rare, and the incidence of these subtypes is not increased beyond what is observed in other populations (5,6).

Table 1. Phenotypes of Gaucher Disease—Clinical Types.

	Type 1	**Type 2**	**Type 3**
Patients affected	Adults, children	Infants	Children, adolescents
Age at onset	Variable; late adolescence	Uniform; 4-5 months	Variable; preschool
Organs affected	Spleen, liver, bone	Brain, spleen, liver	Brain, spleen, liver, bone
Neurologic symptoms	None	Sever, multiple seizures; hypertonus; profound mental retardation; apnea	Myoclonus, seizures, dementia, ocular motor apraxia
Rate of progression	Slow, highly variable	Rapid, stereotypic	Intermediate, variable
Life span	Shortened to normal	Death before 2 years of age	20-30 years
Ethnicity/Demographic group	Panethnic, Ashkenazi Jewish	Panethnic	Panethnic, Norrbottnian
Frequency	1/60,000 1/450 (AJ)	1/100,000	1/100,000

The most common symptoms of Gaucher Disease are enlargement of the liver and spleen, anemia, reduced platelets (resulting in easy bruising and long clotting times), bone crises, bone infarctions often leading to damage to the shoulder or hip joints, and a generalized demineralization of the bones (osteoporosis). The weakening of the bones can then lead to spontaneous fractures. The course of the disease is quite variable, ranging from no overt symptoms to skeletal problems, liver or spleen damage, bleeding, or other problems (7).

Diagnosis of Gaucher disease, based initially on the symptoms described above, can be confirmed by morphologic, enzymatic, and molecular diagnoses. In the past, bone-marrow biopsy was a rapid and traditional procedure although it is a very invasive diagnosis. The presence of Gaucher cells (Fig.3) is indicative of disease. False negatives can occur because Gaucher cells are sparsely distributed. Other diseases sometimes present with engorged cells that resemble Gaucher cells, which can be easily misdiagnosed. Conditions for which these pseudo-Gaucher cells (Fig.3) have been detected include chronic granulocytic leukemia, multiple myeloma, Hodgkin's disease, thalassemia. Heterozygous carriers can not be identified by this test (8,9).

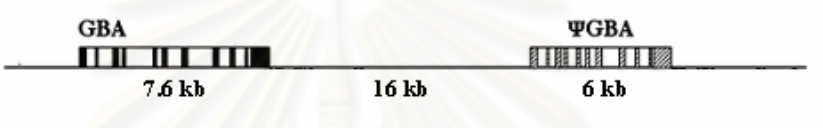


Figure 2. Schematic of glucocerebrocidase gene and pseudogene.

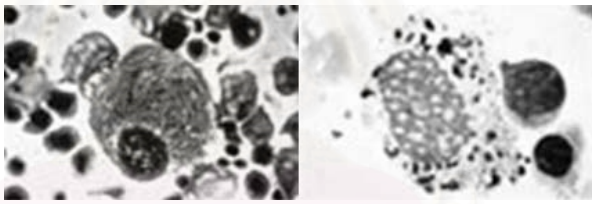


Figure 3. Gaucher cells (left) and pseudo-Gaucher cells (right).

Lipid-engorged cells with eccentric nuclei, known as Gaucher cells, accumulate and displace healthy normal cells in bone marrow and visceral organs, causing a host of signs, including skeletal deterioration, anemia, hepatosplenomegaly, and organ dysfunction. In rare cases Gaucher cells affect the brain and nervous system (6).

Today, enzyme assay can be used to measure glucocerebrosidase activity in cells obtained from the patient, providing definitive diagnosis of Gaucher disease: enzyme activity of 30% or less than normal definitively indicates Gaucher disease. Enzyme activity can be measured either in leukocytes, which are fairly easily obtained, or in skin fibroblasts (10). Enzyme assay is more precise and less invasive than bone marrow biopsy. Moreover, this assay is important because it can identify patient enzyme activity , which is necessary to introduce an appropriated therapy (to determinate the enzyme replacement dose). A disadvantage of this assay is that carrier detection is less precise and cannot be used to detect carriers reliably (11).

Molecular diagnosis has the advantages of being qualitative and using stable samples. The major disadvantages are the extreme specificity and dependence on allele frequencies. Although many Gaucher disease alleles have been characterized, others have not and the inability to detect a mutant allele does not exclude its presence. DNA testing can be used to aid diagnosis in Ashkenazi Jews, where four specific gene mutations, N370S, 84GG, L444P and IVS2(+1), occur in 89% to 96% of Gaucher disease patients. This method is considered less sensitive than enzyme analysis when used as a diagnostic tool in the general population. DNA testing, however, provides the most reliable means of identifying carriers. Carrier testing is recommended for all close relatives of a confirmed Gaucher disease patient (12).

Glucocerebrosidase activity is readily measured in amniotic fluid cells and chorionic villus samples and, thus, prenatal diagnosis is available for all types of Gaucher disease. Affected sibling histories provide a guide to counseling of at-risk families. Identification of the fetus' genotype when the disease alleles are known can also guide the counselor as to the disease variant and the disease severity in the absence of a sibling history. Diagnosis as to which of the three types of Gaucher disease an individual has is based on the symptoms, rather than on test results (13,14,15).

In Thailand, there are no reports in biochemical and molecular analysis in Thai patients. In these study, we have demonstrated glucocerebrosidase activities in the leukocytes using fluorimetric assay and performed molecular analyses of five patients and normal controls.

Research Questions

1. How much glucocerebrosidase activity in Thai normal population and Thai patients diagnosed Gaucher disease by clinical manifestations?
2. What are the genetic defects in Thai patients with Gaucher disease?

Objectives

1. To develop a non-invasive definitive diagnostic test for Gaucher disease by measuring glucocerebrosidase activity in WBC.
2. To analyse molecular defects of Thai patients with Gaucher disease.

Hypothesis

The patients with Gaucher disease have abnormal glucocerebrosidase activity and genetic defects in *GBA gene.*

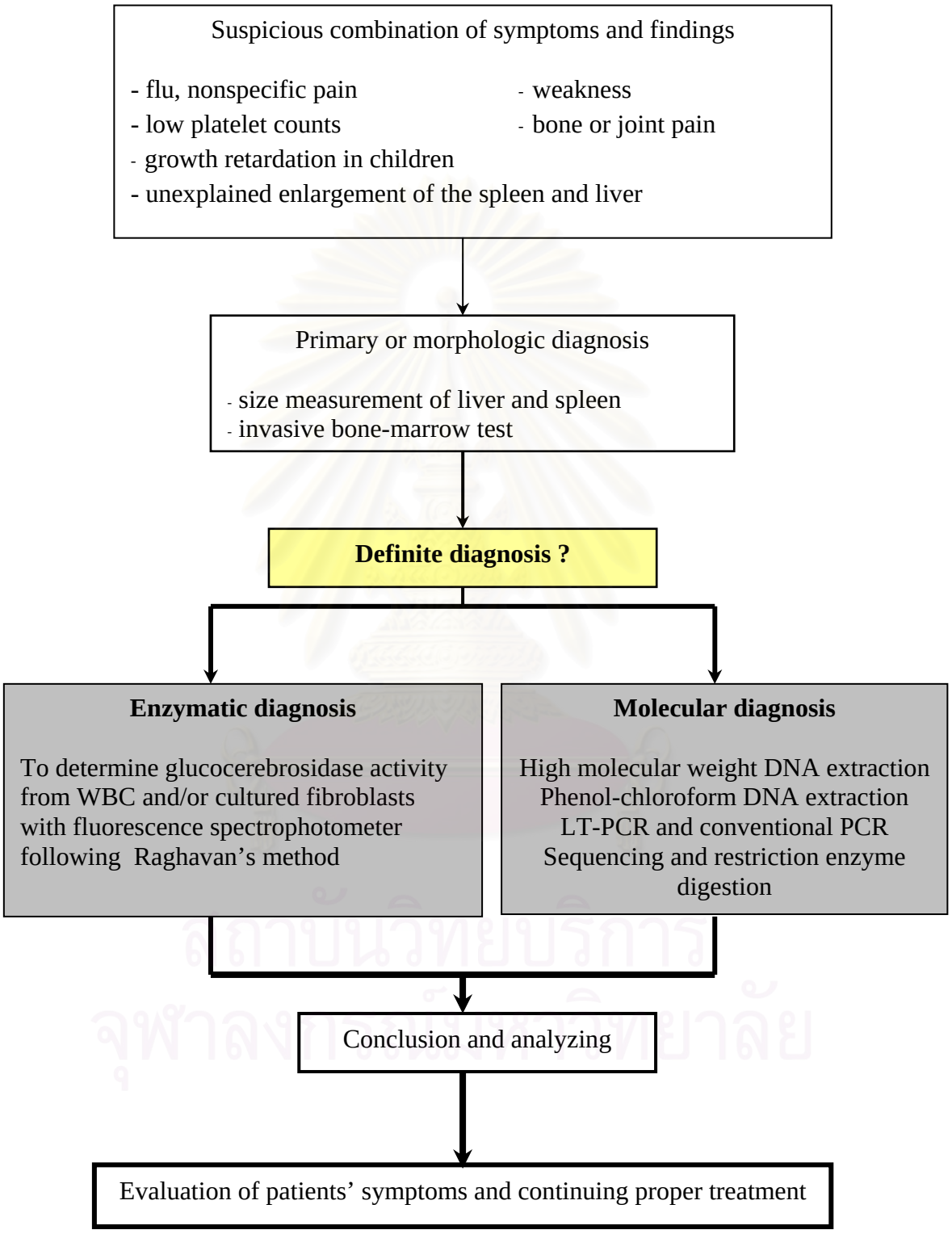
Key words

1. *GBA*
2. β-glucosidase
3. glucocerebrosidase
4. GD; Gaucher disease

Expected Benefit

In this study, we developed and determined glucocerebrosidase activity in Thais for the first time. Mutation analysis in this research will help physicians to diagnose patients with GD faster and give genetic counseling more accurately.

Conceptual framework



Research Methodology

1. Sample Collection

1.1 Thai patients with Gaucher disease who were diagnosed by doctor at King Chulalongkorn Memorial hospital and Ramathibodi hospital.

1.2 Thai healthy blood-donor volunteers from King Chulalongkorn Memorial Hospital.

2. Processes of study
   - 2.1 Blood collection
   - 2.2 WBC extraction
   - 2.3 Determining concentrations of protein by Lowry assay
   - 2.4 Determining glucocerebrocidase activity by fluorimetric assay
   - 2.5 DNA extraction
   - 2.6 DNA amplification
   - 2.7 Sequencing
   - 2.8 Restriction enzyme analysis
   - 2.9 Agarose gel electrophoresis

3. Data collection and analysis

CHAPTER II

REVIEW OF RELATED LITERATURE

Gaucher disease

Gaucher disease is an autosomal recessive inborn error of metabolism caused by the deficiency of the lysosomal enzyme glucocerebrosidase (EC 3.2.1.45), which is responsible for the cleavage of β-glycoside bonds resulting in glucose and ceramide residues. Deficiency of this enzyme leads to the accumulation of glucocerebrosides in the lysosomes of some cells, called Gaucher cells (1).

Clinical features

The symptoms associated with Gaucher disease result from the accumulation of a fatty substance, a lipid called glucocerebroside. This lipid is a byproduct of the normal recycling of red blood cells. When the gene with the instructions for producing an enzyme to break down this byproduct is defective, the lipid accumulates. Gaucher disease is, therefore, a lipid storage disease, and is the most common disease of this type. The lipid is found in many places in the body, but most commonly in the macrophages in the bone marrow. It interferes with normal bone marrow functions, such as production of platelets (leading to bleeding and bruising) and red blood cells (leading to anemia).

The presence of glucocerebroside seems to also trigger the loss of minerals in the bones, causing the bones to weaken, and can interfere with the bone's blood supply, causing areas of bone-death, or infarctions. The most immediate human cost of Type I Gaucher disease is related to the loss of function when a hip or shoulder becomes infarcted or long bone fractures, the great pain experienced during reduced blood flow to the bones (bone crises), abdominal problems related to massive enlargement of the liver and spleen, poor blood clotting, and anemia. Type 2 and Type 3 Gaucher disease result in severe neurological impairment or early demise (7).

Classification

The disease can be classified into three clinical types. Type 1, the most common, is the chronic non-neuropathic form of the disease, which shows highly variable signs and symptoms and a variable course, with visceral and skeletal involvement (splenomegaly, hepatomegaly and bone damage that might lead to fractures) and hematologic abnormalities (pancytopenia), among others. The clinical onset of the disease can be observed at any age, but typically occurs after childhood and in some cases only manifests during adult life. The neurological involvement can be observed in types 2 and 3 (Table 1)(16).

Morphological diagnosis

The diagnosis of Gaucher disease is frequently suspected by finding Gaucher cells in bone marrow during the workup for splenomegaly. Similar appearing cells, pseudo-Gaucher cells, occur in other disorders, including chronic granulocytic leukemia (17,18), thalassemia (19), multiple myeloma (20), Hodgkin disease (21), plasmacytoid lymphomas (22), and in AIDS patients with Mycobacterium avium infections (23). Furthermore, in some instances, it has been difficult to locate Gaucher cells in a bone-marrow aspirate, especially in individuals with mild symptomology. These pseudo-Gaucher cells do not contain the typical tubular inclusions of Gaucher cells (24). The invasiveness and non-specificity of histologic studies limit their use to monitoring therapeutic progress in selected patients.

Enzymatic diagnosis

Glucocerebrosidase is expressed in all tissues and is localized to the lysosomes (25,26). There are roughly three different levels of enzymatic activity expressed in tissues. The highest activity is in placenta and fibroblasts (27,28). These activities range from 100 to 300 nmol substrate cleaved/h/mg of tissue protein. The brain, liver, spleen and lung have about 50 to 75% of these activities or 40 to 90 nmol/h/mg protein. The lowest levels are in white blood cells, the most available source, with 50% lower levels than those observed in the liver or 10 to 20 nmol/h/mg protein. The enzyme is not present normally in serum or plasma and is in very small amounts in urine. These latter sources cannot be used for diagnosis. A tissue source containing nucleated cells is required for confirmatory and diagnostic enzymatic assays (29,30,31,32). These sources also include cultured amniotic cells, or direct or cultured chorionic villi samples (33). Tissue sources from a suspected patient used to establish the diagnosis have 5 to 20% of normal levels of enzymatic activity. Since glucocerebrosidas is relatively stable to freezing in intact tissue samples, such tissue samples or frozen isolated white blood cells are reliable sources for diagnosis. Since specific tissue or cell types can express larger or smaller amounts of glucocerebrosidase activity, any tissue source will be a composite activity from the variety of cells present in the specimen. This is usually not of importance, but in the peripheral blood, different levels of enzymatic activity are present in lymphocytes and neutrophils. As a result it is important to establish the normal ranges based upon the mixture of white blood cells in samples (34,35). This admixture of cells is one source of the unreliability of heterozygote testing for Gaucher disease in peripheral white blood cell sources (36). More homogeneous mononuclear cell preparations may provide a more reliable enzyme source. Establishing the diagnosis by enzyme assay is an essential component of the standard of care for affected patients.

The most specific method for detection of an increase in acid phosphatase activity in serum of patients with Gaucher disease is that described by Chambers et al.(37), in which 4-methylumbelliferyl phosphate is the substrate. The assay is performed at pH 6 in the presence of 2-mercaptoethanol, 3.0 mol/L. This method is significantly more selective and sensitive than procedures in which p-nitrophenyl

phosphate or thymolphthalein phosphate is the phosphatase substrate. However, the colorimetric procedures have their limitations, the principal one being that an unremarkable serum acid phosphatase value is not uncommon in someone with proven Gaucher disease. Although an increase in serum acid phosphatase activity has been seen in most patients with Gaucher disease (38) when assays are performed under optimum conditions, acid phosphatase activity can also be increased in several other disease states (39,40); an increase in type 5 acid phosphatase isoenzyme activity in serum is not specific for Gaucher disease.

The definitive biochemical test for the diagnosis of Gaucher disease is the demonstration of the deficiency of tissue glucocerebrosidase. Although any one of a variety of tissues could be used to demonstrate the glucocerebrosidase deficiency, leukocytes or fibroblasts are the most readily available tissues and therefore the most commonly used source of enzyme. With the methods currently available, glucocerebrocidase activity cannot be measured in serum or urine.

Glucocerebrosidase activity can be measured by using the authentic glycolipid substrate (Fig. 1), radiolabeled in either the glucose (34) or the long-chain fatty acid moiety (41,42). However, methods that involve radiolabeled authentic substrate are relatively time-consuming, require expensive equipment, and are not readily applicable to the usual clinical laboratory setting. Consequently, various procedures in which nonspecific artificial glucosides are substrates have been developed to avoid some of the operational problems involved in using the authentic substrate.

In 2004 Michelin et al. reported that the levels of enzyme activity in leokocytes ranges from 7.0 to 25.3 nmol/h/mg protein in normal individuals and from 0 to 3.9 nmol/h/mg protein in individuals homozygous for GD. In fibroblasts, enzyme activity ranges from 130 to 872.4 nmol/h/mg protein in normal individuals and from 0 to 67.2 nmol/h/mg protein in patients with GD (43). One year later, Michelin et al. measured levels of glucocerebrosidase in leukocytes as shown in Table 2 (44).

Table 2. Enzyme activity of glucocerebrosidase in leukocytes from controls, GD patients and obligated GD heterozygotes.

Groups	Activity (43) (nmol/h/mg protein)	Range (43) (nmol/h/mg protein)	Range (44) (nmol/h/mg protein)
Controls	14.5±5.54 (n=8)	6.0-27.1	7.0-25.3 (n=35)
GD patients	0.917±0.572 (n=13)	0.41-2.6	0-3.9 (n=33)
Obligate heterozygotes	7.768±3.083 (n=27)	3.8-10.8	-

In heterozygotes peripheral blood leukocytes and cultured skin fibroblasts have 50 to 60% of normal glucocerebrosidase activity. Using peripheral blood leukocytes, considerable overlap (up to 20%) is found between the normal and heterozygote ranges. This results in large numbers of false positive and false negative results. The 95% confidence ranges for obligate heterozygotes contains about 25 to 30% of normal controls. Consequently, enzyme assays for heterozygote detection are not suitable for mass screening and should be cautiously interpreted in at-risk families.

Molecular Diagnosis

The glucocerebrosidase gene is located on chromosome 1q21 and organized into 11 exons coding for 498 amino acids, and, to date, more than 200 different mutations have been identified (45,46,47,48). There are several methods of identifying the mutations; most use modifications of the polymerase chain reaction (PCR) technique. Most mutations resulting in Gaucher disease are point mutation that are distributed throughout the gene, with a majority clustered between exons 8 and 11. Several missense mutations, particularly N370S and L444P, are encountered with an increased frequency. Other mutations include frameshifts, deletions (with or without insertions), and splice-site mutations. Another important group of mutation results from homologous recombination between the *GBA* gene and pseudogene.

All the mutations result in a glucocerebrosidase enzyme that is either ineffective in catalysis or unstable. The frequency of the various mutations is somewhat different in Jewish and non-Jewish populations. In the Ashkenazi Jewish population, four mutations termed N370S, 84GG, L444P and IVS2(+1) account for >95% of all mutations (Table 3). The most common mutation is a point mutation in which an adenine at nucleotide 5841 is changed to a guanine, which leads to the substitution of the serine for an asparagine at amino acid 370 (N370S). The second most common mutation in the Jewish population is the insertion of an extra guanine at nucleotide 84, which results in a shift of the protein translation frame and a premature termination of the resulting protein. Another common mutation in the Jewish population is the point mutation at amino acid 444 (leucine→proline; L444P). This mutation is found at high frequencies in Type 2 and Type 3 Gaucher disease. However, it may also be found in Type 1. In contrast, the N370S mutation is almost never found in Type 2 or Type 3 disease and almost excludes neurologic complications. Finally, a guanine to adenine transition of the first nucleotide in the second intron results in a defect of the 5'-donor splice site of exon 2 of the pre-mRNA glucocerebrosidase IVS2(+1). In the Ashkenazi Jewish population, therefore, it is relatively easy to use genotyping for population screening because there are only four mutations that account for >95% of all mutations.

The N370S mutation is the most common mutation in patients with GD, and this mutation is associated with Type 1 disease. Approximately 90% of individuals who are homoallelic for N370S have mild Type 1 disease (49). Disease type and severity is usually consistent within families; however, clinical heterogeneity even among siblings with Type 1 disease occurs (50). The L444P mutation is associated with all types of GD but is most commonly identified in neuronopathic forms of the

disease. The L444P mutation occurs more frequently in the non-Jewish population. Individuals who are homoallelic for L444P usually have neurologic involvement. This mutation may be present in homoallelic or heteroallelic states in all types of the disease (51,52,53). For patients with Type 1 disease in which the 84GG mutation occurs in combination with N370S, disease is usually moderate to severe (54). No patient homozygous for 84GG has been identified and, because of the nature of the mutation, it is predicted that 84GG in the homoallelic state is incompatible with survival. The IVS2(+1) mutation has been reported in patients with Types 1 and 3 GD. In Type 1 patients, IVS2(+1) occurs most frequently in combination with N370S and disease is moderate to severe. Individuals with GD who are IVS2(+1) homozygotes have not been reported, and it is predicted that an individual with this genotype would have severe, rapidly fatal GD (55,56). Patients with a recombinant allele containing three single point mutations—two that introduce the amino acid substitutions L444P and A456P, and a silent mutation, V460V, in exon 10—were first reported by Eyal et al. (57). Homologous recombination resulting in a fusion that includes the 5′ segment of the GBA functional gene extending from exon 1 to intron 9, with the remainder of the sequence corresponding to the pseudogene, was shown by Zimran et al. (58). Strasberg et al. (59) first reported homozygosity for a recombinant allele in a patient with lethal type 2 Gaucher disease. Another type of mutant allele, a 55-bp deletion in exon 9 resulting from gene conversion from the pseudogene sequence, has also been reported (60,61). Koprivica et al. (62) reported that four common mutations accounted for only 49% of mutant alleles in the non-Jewish type 1 patients. Homozygosity or heterozygosity for N370S resulted in type 1 Gaucher disease, whereas homozygosity for L444P was associated with type 3. Genotype L444P/recombinant allele resulted in type 2 Gaucher disease, and homozygosity for a recombinant allele was associated with perinatal lethal disease. Stone et al. (63) have performed molecular analyses of a cohort of 31 patients with type 2 Gaucher disease. Mutation L444P was found on 25 patient alleles. Southern blots and direct sequencing demonstrated that mutation L444P occurred alone on 9 alleles, with E326K on one allele and as part of a recombinant allele on 15 alleles. There were no homozygotes for point mutation L444P. The recombinant alleles that included L444P resulted from either reciprocal recombination or gene conversion with the nearby glucocerebrosidase pseudogene. Homozygosity for a recombinant allele was associated with early lethality.

Genotyping by various techniques has also been shown to be useful in identifying heterozygotes and in prenatal diagnosis in Ashkenazi Jewish populations, in which the gene frequency may be as high as 1 in 10. Among non-Jewish patients, mutations at L444P and N370S account for 60 to 75% of the disease-producing alleles (Table 3). The existence of many rare alleles in the non-Jewish population limits DNA testing to families in which the disease alleles are known (52,64,65).

Table 3. Allele distribution in Gaucher disease type 1 patients.

Allele	No. of alleles (%) in Jewish patients	No. of alleles (%) in Non-Jewish type1 patients
N370S	71.82	43.60
84GG	11.20	0.20
L444P	2.84	25.60
IVS2	1.72	0.70
Rec	1.42	3.50
Alleles detected	89	73.69
Alleles undetected	11	26.31
Total alleles	1160	419

DNA-based technology has major advantages over enzymatic diagnosis of Gaucher disease in that the results are qualitative rather than quantitative and in that the samples are extremely stable. However, although many Gaucher disease alleles have been identified, others have not. Thus, even when DNA is examined for many different known mutations, a normal result does not ensure the absence of a Gaucher disease allele.

Genetic DNA analysis was performed for initial diagnosis in none of the patients in the present series. Even in patients who were diagnosed because a sibling had already been identified as having Gaucher disease, genetic analysis was done only after and complementary to enzyme assay or pathologic assessment of tissue biopsy specimens. Clearly abnormal glucocerebrosidase measurements are usually sufficient to make a definite diagnosis of homozygotes and may make genetic analysis unnecessary, at least for clinical reasons. However, in family members with borderline abnormal enzyme assays, genetic diagnosis may help to identify heterozygous subjects. For the routine diagnostic workup in non-Jewish populations, genotype identification has to be considered confirmatory, provided that the enzyme assay has been done properly.

Prenatal diagnosis

Glucocerebrosidase activity is readily measured in amniotic fluid cells and chorionic villus samples and, thus, prenatal diagnosis is available for all types of Gaucher disease. Affected sibling histories provide a guide to counseling of at-risk families. Identification of the fetus' genotype when the disease alleles are known can also guide the counselor as to the disease variant and the disease severity in the absence of a sibling history (66,67).

CHAPTER III

MATERIALS AND METHODS

Procedure

1. Subjects and sample collection

After clinical diagnosis and informed consents were received, 3 ml of peripheral blood for mutation analysis was collected from 4 Thai patients with Gaucher disease at King Chulalongkorn Memorial Hospital and 1 Thai patient with Gaucher disease at Ramathibodi Hospital. All patients were diagnosed by morphological diagnosis.

5 ml of peripheral blood from 3 Thai patients with Gaucher disease and 6 Thai healthy blood-donor volunteers from King Chulalongkorn Memorial Hospital were determined glucocerebrocidase activity by fluorimetric assay(46).

2. Enzymatic Diagnosis

2.1 White Blood Cell extraction

WBC were extracted from 5 ml of peripheral blood from 3 patients with Gaucher disease and 6 healthy blood-donor volunteers**.** The method was performed as follow:

1. 5 ml. of heparinized whole blood is centrifuged for 10 minutes at 2,000 rpm.

2. Remove supernatant and collect buffy coat to a new polypropylene tube. Then add 0.85% NaCl until total volume is 5 ml.

3. Add 10 ml. of 3% dextran in 0.85% NaCl, then mix thoroughly by invert and let the tube stand for 30 minutes – 2 hours until separating phase.

4. Transfer the supernatant to a new 15 ml conical tube, then centrifuge at 1,200 g for 7 minutes.

5. Remove supernatant and place the tube on ice.

6. Add 2 ml of cold distilled water followed by vortexing to suspend all of the pelleted cells.

7. After 45 seconds, 2 ml of 1.7% NaCl is added to each tube, and after mixing they are centrifuged at 1,200 g for 2 minutes.

8. The supernate is removed, and the yellow-white pellets are resuspended in 1 ml of 0.85% NaCl.

9. The cell suspensions from each person are pooled into individual 1.5 ml tube .

10. After centrifugation at 1,200 g for 2 minutes, the supernate is removed. To this pellet is added 0.3-0.8 ml of distilled water , depending on the estimated size of the pellet, which is then stored at -20°C until the day of assay.

2.2 Determining concentrations of protein by Lowry assay

Peripheral blood from 3 patients with Gaucher disease and 6 healthy blood-donor volunteers were determined the concentration of protein.

Principle

Under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue.

Reagent

1. reagent A (2% sodium carbonate in 0.1 N NaOH)
2. reagent B (0.5% $CuSO_4$. $5H_2O$ in 1% sodium potassium tartrate)
3. reagent C ; mix 5.0 ml of reagent A with 0.1 ml of reagent B
4. folin-ciocalteu reagent
5. protein standard (bovine serum albumin; BSA)
   - 5.1 stock solution 0.25 mg/ml
   - 5.2 dilution ; 1:1 dilution of stock solution – 0.125 mg/ml
     ; 1:4 dilution of stock solution – 0.0625 mg/ml

Assay

1. Set up the following tubes in duplicate except for the blank

	unknown (ml)	standard (ml)	blank (ml)
cell extract	0.05	-	-
H_2O	0.15	-	0.2
Standard	-	0.2 ml of stock and 1:1, 1:4 dilution	-

2. Add 1 ml of reagent C to each tube, then mix well and stand at room temperature for 10 minutes.
3. Add 0.1 ml of diluted folin-ciocalteu reagent to each tube, then mix and stand at room temperature for 30 minutes.
4. At the end of 30 minutes, read absorbance of each sample at 500 nm on spectrophotometer.

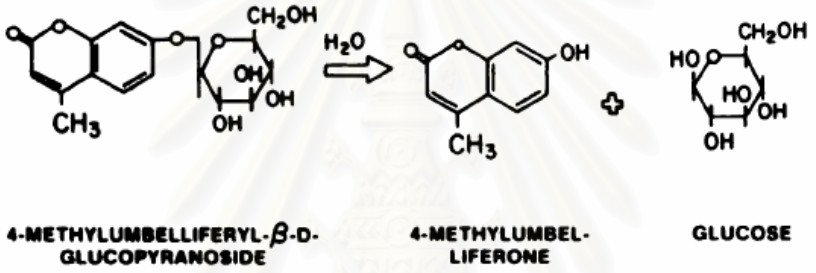
2.3 Determining glucocerebrocidase activity by fluorimetric assay

Peripheral blood from 3 patients with Gaucher disease and 6 healthy blood-donor volunteers were determined glucocerebrocidase activity.

Principle

4-methyl-umbelliferone-β-D-glucopyranoside (4-MU-glucopyranoside) does not fluoresce unless cleaved to release the fluorophore (4-MU). Fluorometric enzyme assays are based on the hydrolysis of 4-MU-containing substrates such as 4-MU-glucopyranoside by glucocerebrosidase. Cleavage of 4-methyl-umbelliferone-ß-D- glucopyranoside by glucocerebrosidase yields the fluorescent molecule 4-MU that emits light at 450 nm when excited by 365 nm light. (Fig.4)

Figure 4. Structure of glucocerebrosidase substrate and their reaction products.



Reagent

1. citric acid, 1.0 M
2. dibasic sodium phosphate, 2.0 M
3. phosphate-citrate buffer, pH 5
4. 4-MU- β-D-glucopyranoside, 20 mM
5. 2 % sodium taurodeoxycholate
6. AMP buffer, 0.025 M
7. fluorescent standard solution (4-MU in AMP buffer)

Assay

1. Set up the following tubes and run duplicate samples on the unknown.

	Unkno wn (ml)	cell blank (ml)	standard (ml)
4-MU-β-D-glucopyranoside	0.05	-	0.05
H_2O	-	0.05	0.03
buffer	0.01	0.01	0.01
2 % Na taurodeoxycholate	0.01	0.01	0.01
WBC sonicate	0.08	0.03	-

2. Immediately mix the above solutions, and incubate for 2 hours at 37°C in water bath.

3. At the end of 2 hours incubation, stop reaction with 3.9 ml of 0.025 M AMP buffer.

4. Measure absorbance of each sample at 365 nm on fluorescence spectrophotometer.

5. Calculate glucocerebrosidase activity (nmol/hour/mg protein).

3. Molecular diagnosis (DNA analysis)

3.1 Phenol-chloroform extraction of DNA

The extraction of DNA from peripheral blood leukocyte was performed as follow:

1. 3 ml. of whole blood is centrifuged for 10 minutes at 3,000 rpm.

2. Remove supernatant and collect buffy coat to a new polypropylene tube. Then add 10 volumes of cold lysis buffer1 (or 10 ml.), mix thoroughly and incubate at –20°C for 5 minutes.

3. Centrifuge for 8 minutes at 1,000 g, then remove supernatant.

4. Add 3 ml. cold lysis buffer1, mix thoroughly and centrifuge for 8 minutes at 3,000 rpm.

5. Discard supernatant afterward add 900 µl lysis buffer2, 10 µl Proteinase K solution (20 mg Proteinase K in 1.0 ml. of 1% SDS-2mM EDTA, should be prepare 30 min before use.), and 10% SDS 50 µl. Mix vigorously for 15 seconds.

6. Incubate the tube(s) in 37°C shaking waterbath overnight for complete digestion.

7. Add 1 ml. phenol-chloroform-isoamyl alcohol, then shake vigorously for 15 seconds and centrifuge at 6,000 rpm for 5 minutes.

8. Transfer the supernatant from each tube (contains DNA) to a new microcentrifuge tube.

9. Add 0.5 volumes of 7.5 M CH_3COONH_4 and 1 volume of 100% ethanol, then mix by inversion. The DNA should immediately form a stringy precipitate. Recover the DNA by centrifugation at 14,000 rpm for 15 minutes. Then remove supernatant.

10. Rinse the pellet with 70% ethanol and air-dried the pellet until ethanol disappers. (It is important to rinse well to remove any residual salt and phenol.)

11. Resuspend the digested DNA in 20-300 μl of the double distilled water at 37°C until dissolved.

3.2 High molecular weight DNA extraction

High molecular weight DNA extraction method was performed as follow:

1. Add 9 ml. of cold lysis buffer1 in 3 ml. of whole blood, then mix thoroughly by vortex.

2. Centrifuge for 8 minutes at 1,000 g.

3. Remove supernatant, WBC sedimented at the bottom of a tube is washed by 5 ml. cold lysis buffer1 and then mix by gently vortex.

4. Centrifuge for 5 minutes at 1,000 g.

5. Discard supernatant and add 1 ml. lysis buffer2 according to WBC pellet volume

6. Add 10% SDS 50 μl : 1 ml. of lysis buffer2 and Proteinase K solution 20 μl : 1 ml. of lysis buffer2, then mix by gently vortex.

7. Incubate the tube(s) in 37°C waterbath overnight for complete digestion.

8. Remove mixture into 2 tubes of 1.5 ml. microtube. and add phenol-chloroform-isoamyl alcohol 1:1 of mixture, then mix by gently vortex.

9. Centrifuge for 5 minutes at maximum speed and collect buffy coat to a new polypropylene tube.

10. Add 0.3 volume of 7.5 NH_2OAc and 2 volume of 100% ethanol, then mix by gently vortex.

11. Remove DNA pellet into a new tube and centrifuge for 10 minutes at maximum speed, then discard supernatant and rinse the pellet with 70% ethanol 500 μl, gently vortex

12. Centrifuge for 5 minutes at maximum speed and air-dried the pellet until ethanol disappers.

13. Resuspend the digested DNA in 400-500μl of the distilled water at 37°C until dissolved.

3.2 Long Template PCR

Long template PCR amplification of the entire glucocerebrosidase gene was performed on high molecular weight DNA by use of the ELONGase™ enzyme mix to look for large deletions or insertions using the primers (53) and components of LT-PCR reaction listed in Table 4-5.

Table 4. PCR primers used to amplify *Functional GBA* and *Pseudo GBA*

Gene	Primer sequence	Product size (bp)
Functional GBA	GBA-F1 ; 5'- CCT AAA GTT GTC ACC CAT AC -3' GBA-R11 ; 5'- ACC ACC TAG AGG GGA AAG TG – 3'	~6.5 kb
Pseudo GBA	GBA-F1 ; 5'- CCT AAA GTT GTC ACC CAT AC -3' GBA-R11 ; 5'- ACC ACC TAG AGG GGA AAG TG – 3'	~5.2 kb

Table 5. Components of LT-PCR reaction

Mixture A	
1. 10mM dNTPs	1.0 μl
2. 10μM Forward Primer	1.0 μl
3. 10μM Reverse primer	1.0 μl
4. HW-DNA (100-200ng)	Varied by concentration of HW-DNA
5. Distilled water	Varied by volume of HW-DNA
Total volume (μl)	20
Mixture B	
1. 5X Buffer A	-
2. 5X Buffer B	10 μl
3. ELONGase™	2 μl
4. Distilled water	18 μl
Total volume (μl)	30

The DNA was denatured for 30 s at 94 ^{o}C, followed by 35 cycles of denaturation at 94 ^{o}C for 30 s, annealing at 55 ^{o}C for 30 s, and elongation at 68^{o}C for 7 min. The DNA amplified by long template PCR was gel purified with QIAquick gel extraction kit (Qiagen) and sequenced with internal primers for each exon.

3.3 Conventional PCR

Individual exons were amplified and sequenced with primers specific for the glucocerebrosidase gene (but not pseudogene) (53), using primers, component and conditions of PCR reaction listed in Table 6-8.

Table 6. PCR primers used to amplify *functional GBA*.

exon	Primer sequence	PCR product size (bp)
1-2	GBA-F1 ; 5'- CCT AAA GTT GTC ACC CAT AC -3' GBA-R2 ; 5'- CCA CCG AGC TGT AGC C -3'	1382
3-4	GBA-F34 ; 5'-ATG TGT CCA TTC TCC ATG TCT TCA -3' GBA-R34 ; 5'- ACG AAA AGT TTC CGT GGC TCT -3'	523
5-6	GBA-F56 ; 5'-GAC CTC AAA TGA TAT ACC TG-3' GBA-R56 ; 5'-GAA AGG TCA TGA ATG A-3'	1690
7	GBA-F7 ; 5'-TCA AGA CCA ATG GAG CGG TG -3' GBA-R7 ; 5'-AGT TTG GGA GCC AGT CAT TT -3'	953
8	GBA-F8 ; 5'-GTT GCA TTC TTC CCG TCA CC -3' GBA-R8 ; 5'-CTG GAC AGG AAG GGC TTC TG -3'	367
9-11	GBA-F911 ; 5'-AAC CAT GAT TCC CTA TCT TC -3' GBA-R911 ; 5'-ACC ACC TAG AGG GGA AAG TG -3'	1249

Table 7. Component of PCR reaction for mutation detection in each exon.

Component	Exon 1-2	Exon 3-4	Exon 5-6
1.10X PCR buffer	2.0 (1X)	2.0 (1X)	2.0 (1X)
2.25mM $MgCl_2$	1.2 (1.5mM)	1.2 (1.5mM)	1.2 (1.5mM)
3.10mM dNTPs	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)
4.10μM Forward primer	0.4 (0.2μM)	0.4 (0.1μM)	0.4 (0.1μM)
5.10μM Reverse primer	0.4 (0.2μM)	0.4 (0.1μM)	0.4 (0.1μM)
6 5U/μI *Taq* polymerase	0.1 (0.025U)	0.1 (0.025U)	0.1 (0.025U)
7.Distilled water	13.5	13.5	12.5
8.50ng/μl Genomic DNA	2.0	2.0	2.0
9. DMSO	-	-	1.0
Total volume (μl)	20	20	20

Table 7(continued). Component of PCR reaction for mutation detection in each exon.

Component	Exon 7	Exon 8	Exon 9-11
1.10X PCR buffer	2.0 (1X)	2.0 (1X)	2.0 (1X)
2.25mM $MgCl_2$	1.2 (1.5mM)	1.2 (1.5mM)	1.2 (1.5mM)
3.10mM dNTPs	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)
4.10μM Forward primer	0.2 (0.1μM)	0.2 (0.1μM)	0.2 (0.1μM)
5.10μM Reverse primer	0.2 (0.1μM)	0.2 (0.1μM)	0.2 (0.1μM)
6 5U/μI *Taq* polymerase	0.1 (0.025U)	0.1 (0.025U)	0.1 (0.025U)
7.Distilled water	14.9	13.5	13.5
8.50ng/μl Genomic DNA	1.0	1.0	1.0
9. DMSO	-	-	-
Total volume (μl)	20	20	20

Table 8. PCR cycle conditions.

Step	Exon 1-2	Exon 3-4	Exon 5-6
1. Initial denaturation	95°C/ 5 min	95°C/ 5 min	95°C/ 5 min
2. PCR cycle	35 cycles	35 cycles	35 cycles
Denature	94°C/ 1 min	94°C/ 1 min	94°C/ 1 min
Annealing	60°C/ 2 min	58°C/ 45sec	61°C/ 45sec
Extension	72°C/ 2 min	72°C/ 1 min	72°C/ 1 min
3. Final extension	72°C/ 10 min	72°C/ 10 min	72°C/ 10 min
Step	Exon 7	Exon 8	Exon 9-11
1. Initial denaturation	95°C/ 5 min	95°C/ 5 min	95°C/ 5 min
2. PCR cycle	35 cycles	35 cycles	35 cycles
Denature	94°C/ 1 min	94°C/ 30sec	94°C/ 45sec
Annealing	62°C/ 1 min	58°C/ 30sec	55°C/ 45sec
Extension	72°C/ 2 min	72°C/ 45sec	72°C/ 1min30sec
3. Final extension	72°C/ 10 min	72°C/ 10 min	72°C/ 10 min

3.4 Sequencing

The HW-DNA amplified by long-template PCR was gel purified with QIAquick gel extraction kit and sequenced with internal primers for each exon. Individual exons were also amplified, treated with ExoSAP-IT enzyme and sequenced with primer specific for glucocerebrosidase gene (but not pseudogene). Sequencing was performed with 3730xl DNA analyzer from Macrogen.

3.5 Restriction enzymes digestion

Restriction enzyme digestion of PCR products was performed with *Nci* I, *Rsa* I and *Cac* 8 I. Digested and undigested PCR products was analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide along with DNA markers under ultraviolet light.

3.6 Agarose gel eletrophoresis

The PCR products were separated on 1.5-2.0% of agarose gel electrophoresis stained ethidium bromide along with DNA markers under ultraviolet light.

CHAPTER IV

RESULTS

1. Glucocerebrocidase Activity Assay

Initially, the 3 groups were characterized by measuring levels of glucocerebrocidase activity in leukocytes using 4-MU-glucopyranoside as a substrate. Glucocerebrocidase activity in three patients, obligate heterozygote and 6 normal control adults was shown in table 9. The mean value of glucocerebrosidase activity in normal controls with S.D. was 11.63±4.97 nmol/mg protein/h.

Table 9. Glucocerebrocidase activity in patients and normal adults.

Subject	Glucocerebrocidase activity (nmol/mg protein/h)
Normal controls (n= 6) Mean ± SD (Range)	11.63±4.97 (5.97-19.58)
Patient 2	1.07
Patient 3	0.62
Patient 4	0
Obligate heterozygote (Mother of Patient 2)	6.84

2. Identification of the Mutant Alleles

Mutation analysis of DNA is performed by Long-template PCR amplification, conventional PCR amplification, direct sequencing and restriction enzyme digestion to confirm mutation. The mutant alleles identified in the DNA of the 5 GD patients studied are summarized in Table 10.

Table 10. Mutations in glucocerebrosidase gene encountered in GD patients.

Patient No.	Type of GD	Ethnic background	Genotype
1	2	Thai	L444P / N188K
2	1	Thai	L444P / L444P
3	1	Thai	L444P / L444P
4	1	Thai	L444P / Y363H
5	1	Thai	IVS6(-1)G⟶C / X498A

Patient 1 was heterozygous for the known L444P mutation(Fig.7). This mutation is due to T to C substitution at nucleotide position 1448 in codon 444 substitutes the wild type amino acid leucine (L) by praline (P). The other allele carried the known N188K mutation(Fig.8). This mutation is due to T to G substitution at nucleotide position 681 in codon 188 substitutes the wild type amino acid asparagine (N) by lysine (K).

Patient 2 and Patient 3 were found to be homozygous for L444P mutation (Fig.6,7). This mutation is due to T to C substitution at nucleotide position 1448 in codon 444 substitutes the wild type amino acid leucine (L) by proline (P).

Patient 4 was heterozygous for the known L444P mutation(Fig.7). The other allele carried a novel missense mutation at codon 363 (Y363H) (Fig.9). It was a substitution of T to C at nucleotide position 1204. Amino acid changes from tyrosine to histidine.

Patient 5 was heterozygous for the novel IVS6(-1)G⟶C mutation which replace G by C at the last base of intron 6 (Fig.10). The other allele were found to be the novel termination change which the stop codon (X) at position 498 is changed to a codon for arginine (A) (Fig.11). The stop codon is moved to the codon 513.

Long-template PCR amplification of the entire glucocerebrosidase gene produced the expected 6.6-kb PCR product (*functional GBA*) and a second smaller 4-kb PCR product (*pseudo GBA*) (Fig.5).

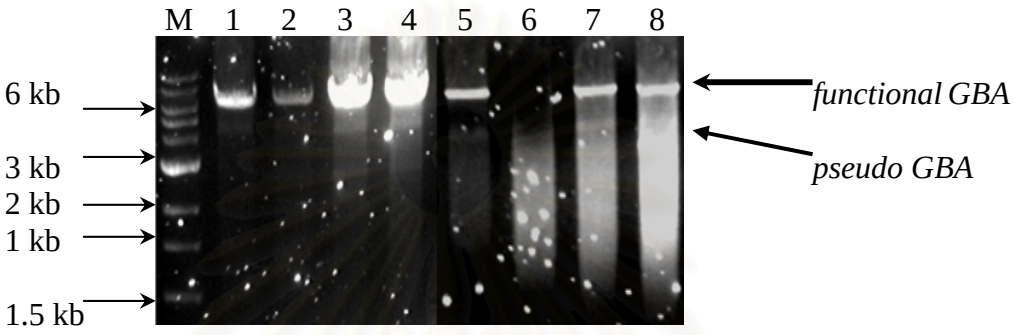


Figure 5. Long-template PCR amplification of the entire glucocerebrosidase gene. M: DNA marker; Lane 1: normal control DNA; Lane 2,5: patient 4: Lane 3-4: patient 3; Lane 6-8: patient 5

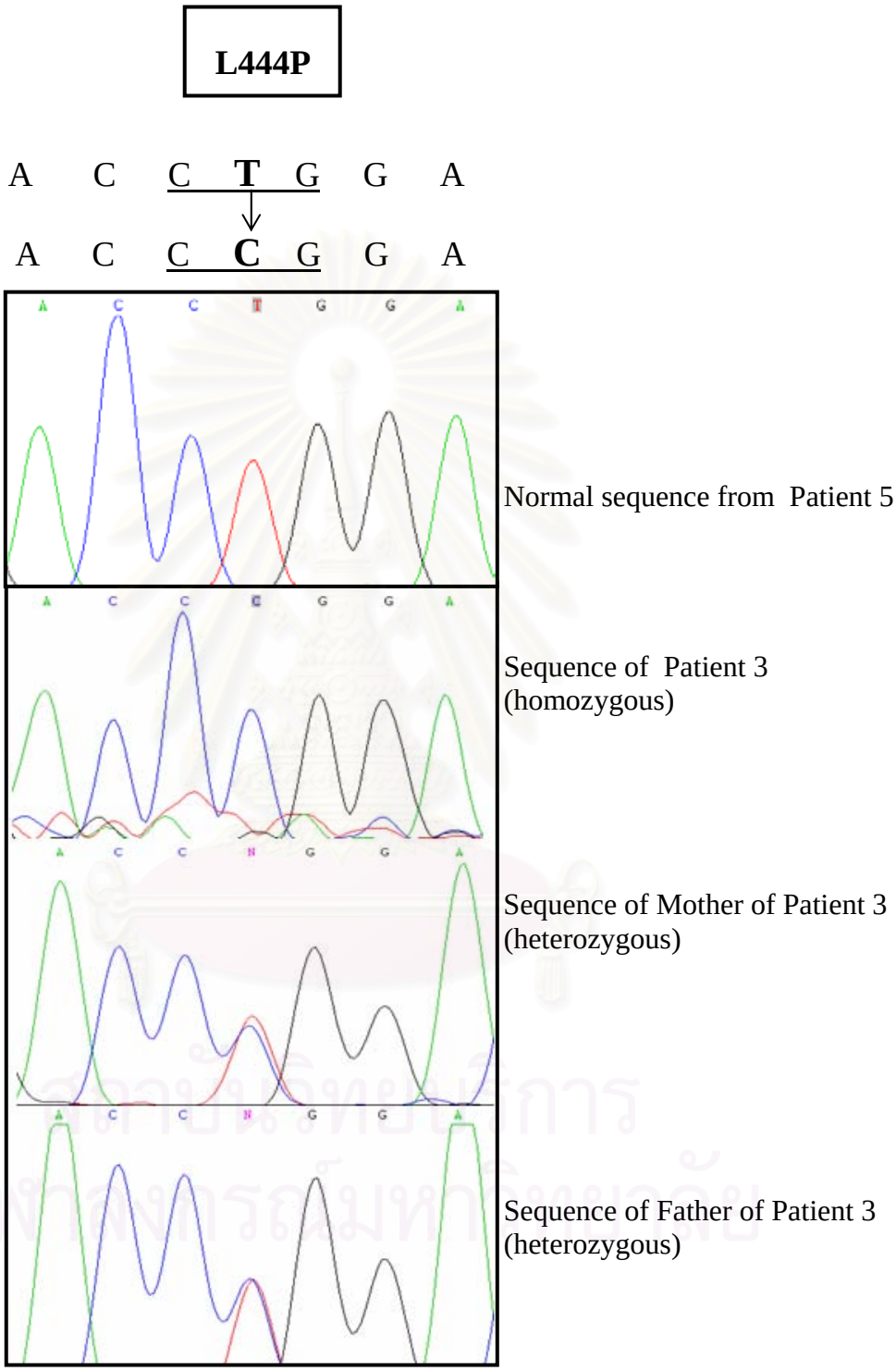


Figure 6. Electropherogram of the normal sequence and the T1448C (L444P) mutation in the patient 3 and their parents.
The arrow indicates a T to C substitution at nucleotide position 1448 in codon 444.

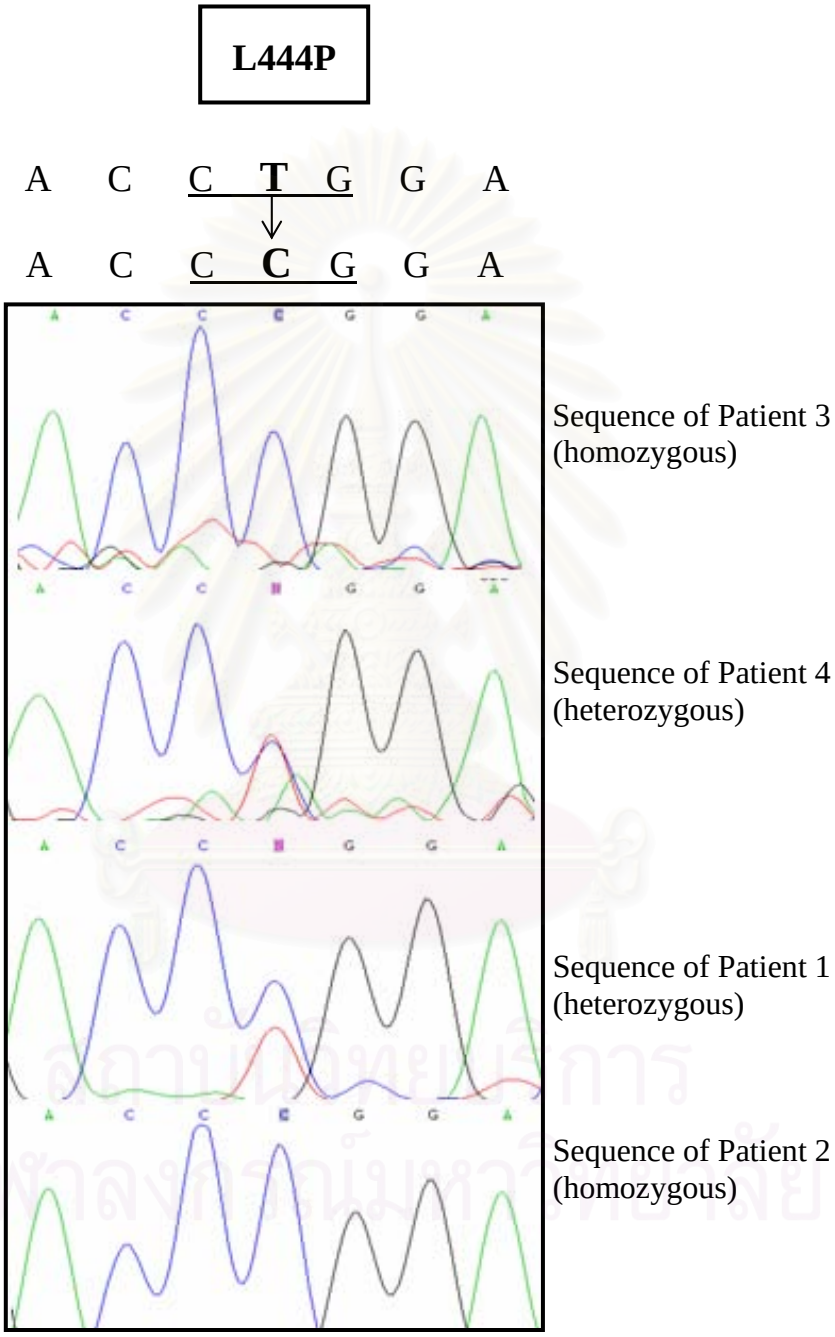


Figure 7. Electropherogram of the normal sequence and the T1448C (L444P) mutation in the patient 1,2,3 and 4.
The arrow indicates a T to C substitution at nucleotide position 1448 in codon 444.

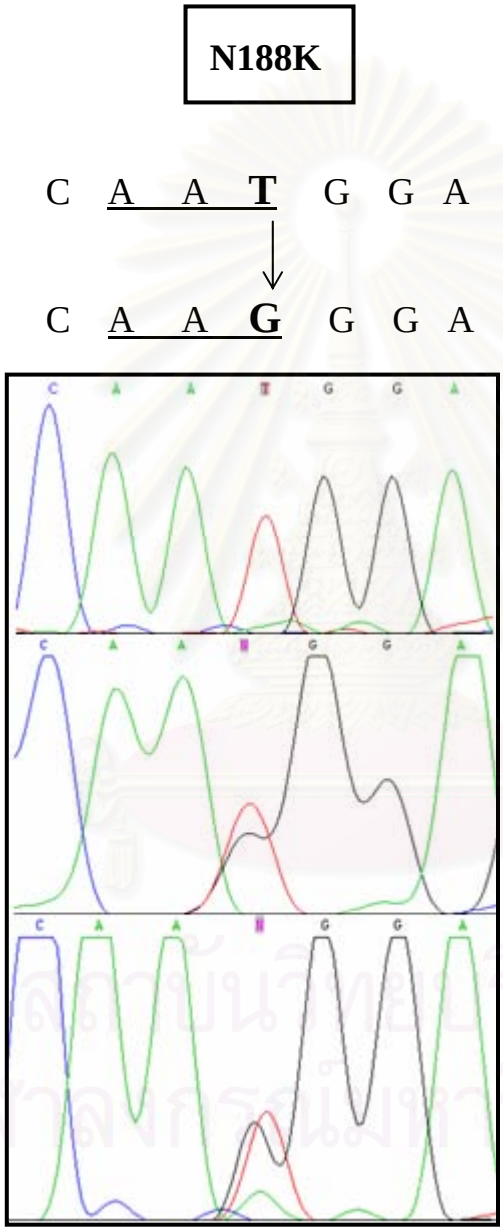
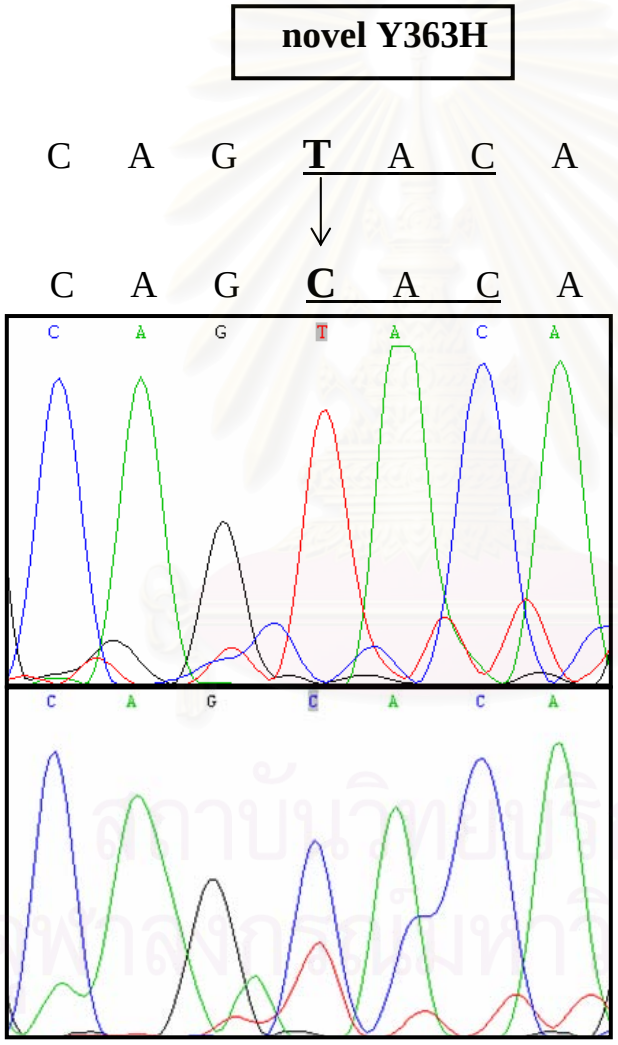


Figure 8. Electropherogram of the normal sequence in the patient 5 and the T681G (N188K) mutation in the patient 1.
The arrow indicates a T to G substitution at nucleotide position 681 in codon 188.



Normal sequence from Patient 2

Sequence of Patient 4 (heterozygous)

Figure 9. Electropherogram of the normal sequence in the patient 2 and the T1204C (Y363H) mutation in the patient 4.
The arrow indicates a T to C at nucleotide position 1204 in codon 363.

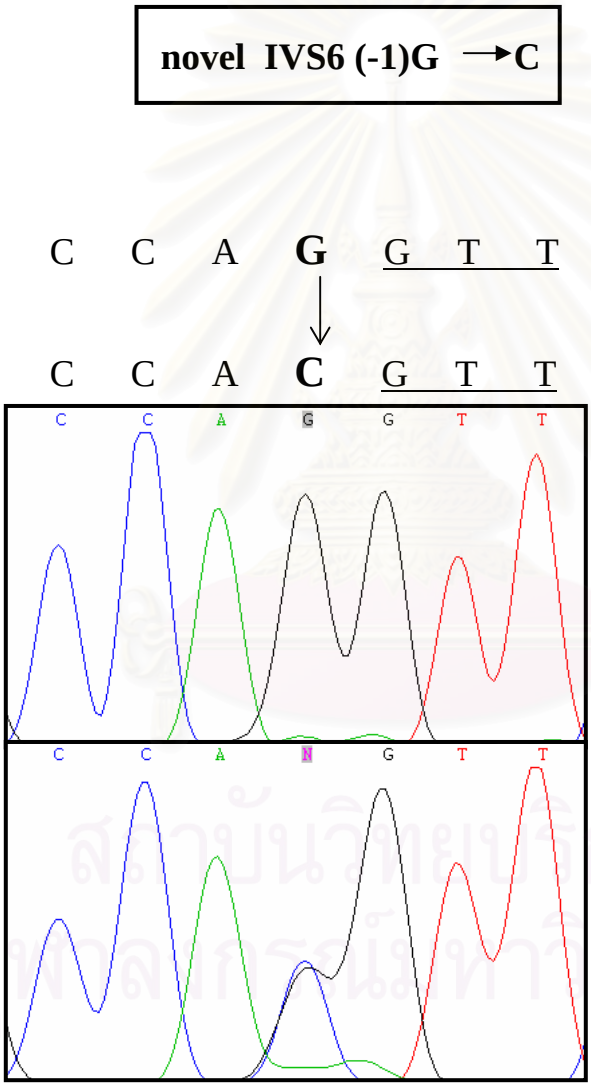


Figure 10. Electropherogram of the normal sequence in the patient 2 and the splice site mutation in the patient 5.
The arrow indicates a G to C at the last base of intron 6.

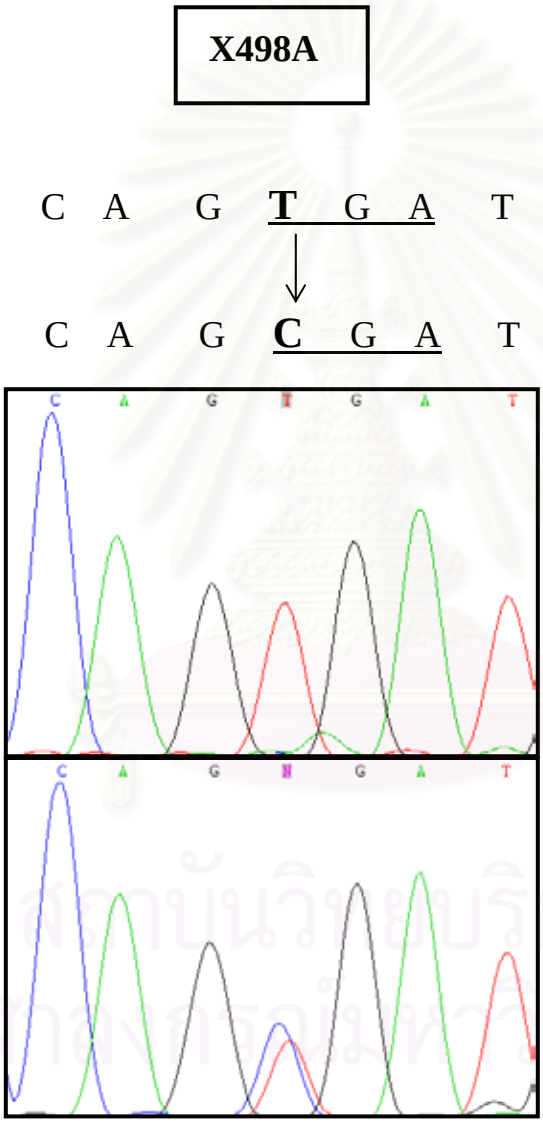


Figure 11. Electropherogram of the normal sequence in the patient 2 and the mutation in stop codon (X) at position 498 in the patient 5.
The arrow indicates a T to C at nucleotide position 1609 in codon 498.

3. Confirmation of the Mutant Alleles

Restriction enzyme digestion

Restriction enzyme digestion of PCR products was carried out to confirm mutations and for evaluation of carrier status in family members. To confirm the known mutation L444P and The novel mutation Y363H and X498A, DNA was screened by using primers, restriction enzymes and expected sizes of PCR product show in table 11.

Table 11. Primers, restriction enzymes and expected size of PCR product

Mutation	Primer Name	Expected size before digestion (bp)	Restriction enzyme	Expected sizes after digestion (bp)		
				normal	Homo zygous mutant	Hetero zygous mutant
L444P	GBAL444P-F GBAL444P-R	638	*Nci* I	638	102 536	102, 536, 638
Y363H	GBA-F8 GBA-R9	970	*Rsa* I	430	580	430, 580
X498A	GBAL444P-F GBA-R11	961	*Cac* 8I	121, 840	121, 172, 667	121, 172, 667, 840

Restriction enzyme digestion with *Nci* I

Restriction enzyme digestion with *Nci* I was performed by using GBAL444P-F and GBAL444P-R primers. Then digested and undigested PCR products were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide. The 638 bp PCR product is obtained after PCR amplification from genomic DNA. In normal control, the PCR product remains undigested whereas heterozygous L444P reveals 536 and 638 bp bands of PCR product. Homozygous L444P reveals a 536 bp band of PCR product.

The different band patterns generated after digestion with *Nci* I are shown in Figure 12.

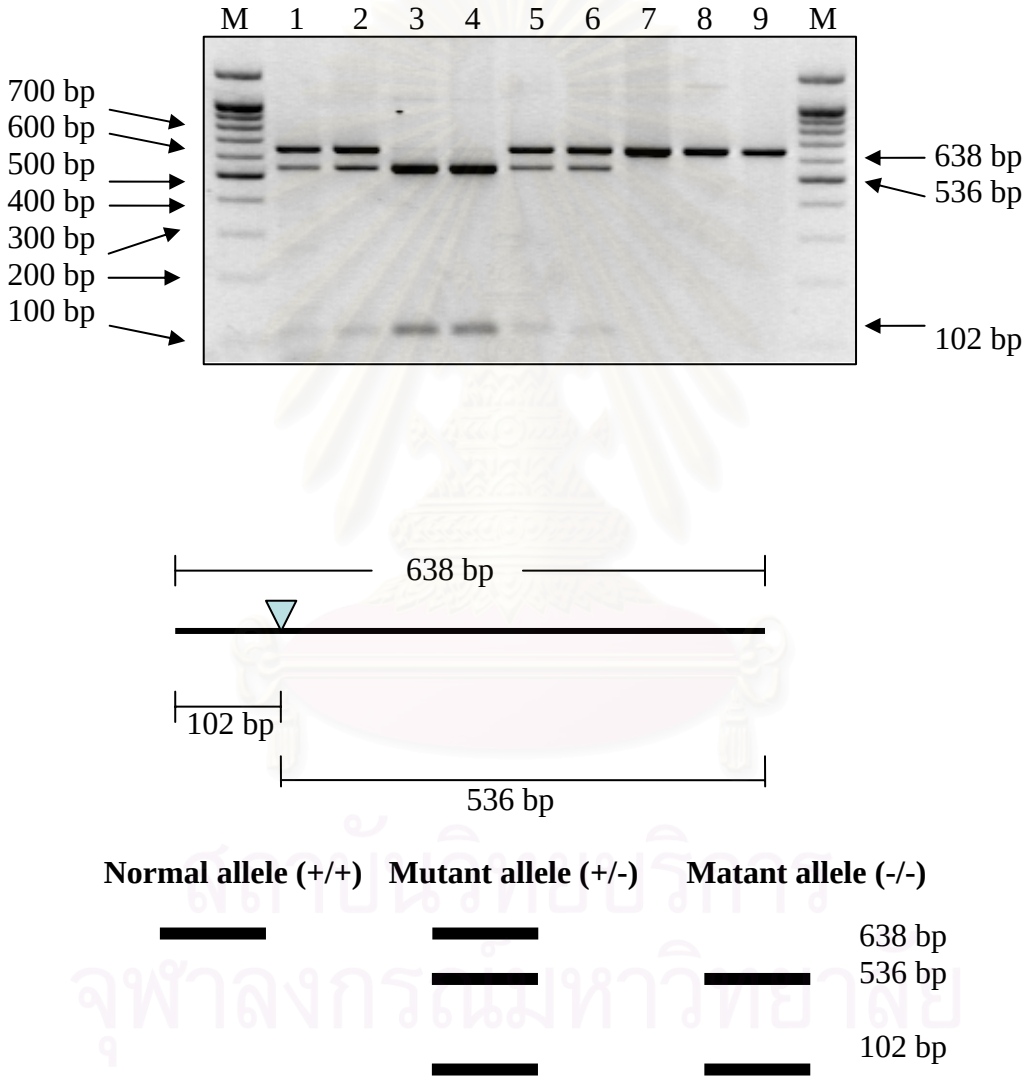


Figure 12. Restriction enzyme digestion with *Nci* I of gDNA for L444P mutation detection. M: DNA marker; Lane 1: father of patient 3; Lane 2: mother of patient 3; Lane 3: patient 3; Lane 4: patient 2; Lane 5: patient 1; Lane 6: patient 4; Lane 7: patient 5; Lane 8-9: normal controls. (+ : normal allele; - : mutant allele)

Restriction enzyme digestion with *Rsa* I

Restriction enzyme digestion with *Rsa* I was performed by using GBA-F8 and GBA-R9 primers. Then digested PCR products were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide. The 970 bp PCR product is obtained after PCR amplification from genomic DNA which was digested both in normal control and in patient. In normal control, the PCR product shows a 430 bp band whereas heterozygous Y363H reveals 430 and 580 bp bands of PCR product. Homozygous Y363H reveals a 580 bp band of PCR product.

The different band patterns generated after digestion with *Rsa* I are shown in Figure 13.

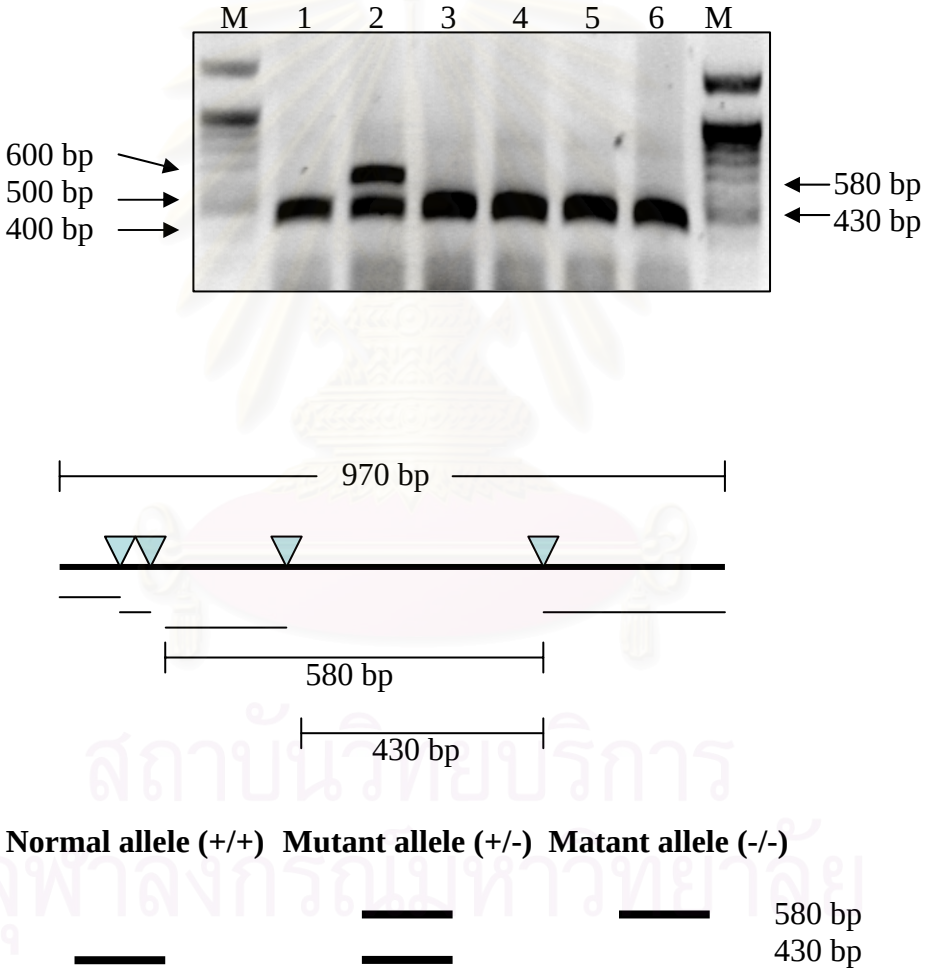


Figure 13. Restriction enzyme digestion with *Rsa* I of gDNA for Y363H mutation detection. M: DNA marker; Lane 1: normal control; Lane 2: patient 4; Lane 3: patient 5; Lane 4: patient 2; Lane 5: patient 3; Lane 6: patient 1.(+ : normal allele; - : mutant allele)

Restriction enzyme digestion with *Cac* 8 I

Restriction enzyme digestion with *Cac* 8 I was performed by using GBAL444P-F and GBA-R11 primers. Then digested PCR products were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide. The 961 bp PCR product is obtained after PCR amplification from genomic DNA which was digested both in normal control and in patient. In normal control, the PCR product shows 121 and 840 bp bands whereas heterozygous X498A reveals 121, 173, 667 and 840 bp bands of PCR product. Homozygous X498A reveals 121, 173 and 667 bp bands of PCR product.

The different band patterns generated after digestion with *Cac* 8 I are shown in Figure 14.

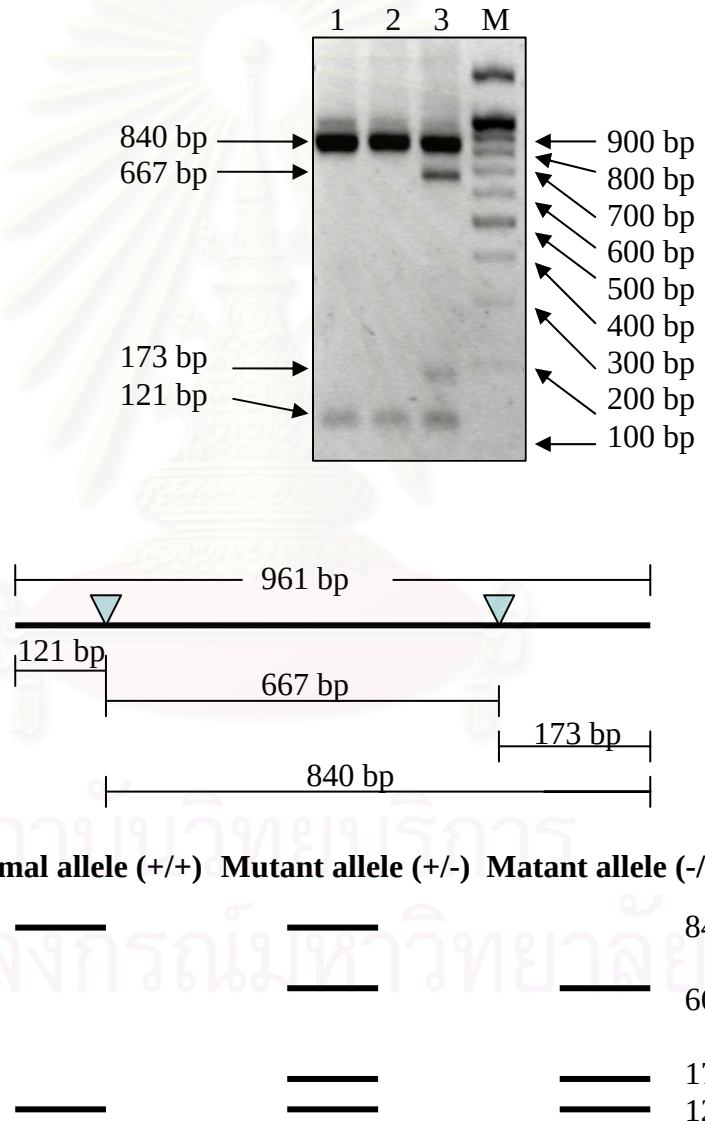


Figure 14. Restriction enzyme digestion with *Cac* 8 I of gDNA for X498A mutation detection. M: DNA marker; Lane 1: normal control; Lane 2: patient 3; Lane 3: patient 5. (+ : normal allele; - : mutant allele)

Analysis of mRNA

Total cellular RNA was obtained from patient 5, in whom a splice site mutation was identified. Double stranded cDNA was amplified with polymerase chain reaction for 35 cycles (94C, 45 sec; 57C, 30 sec, 72C, 1 min) using 2 specific primers as shown in Table 12.

Table 12. PCR primers used to amplify cDNA of the patient 5.

exon	Primer sequence	PCR product size (bp)
4-9	cGBA-F4 ; 5'- TGA CAG ATG CTG CTG CTC TC -3' cGBA-R9 ; 5'- GTT ACG CAC CCA ATT GGG TC -3'	935

The sequence of cDNA amplified from mRNA of patient 5 showed the sequence of each exon contiguous to each other (Fig.15).

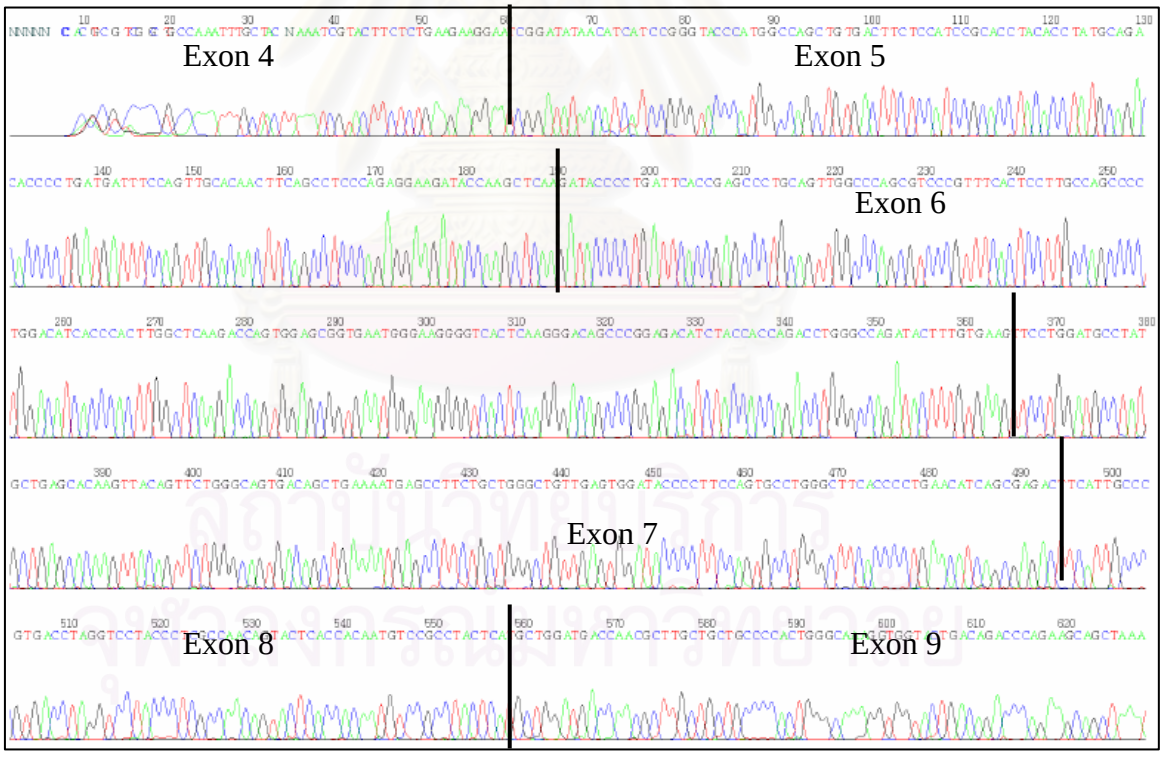


Figure 15. Electropherogram of the cDNA amplified from mRNA of patient 5.

CHAPTER V

DISCUSSION

Gaucher disease results from the inherited deficiency of the enzyme glucocerebrosidase. Patients usually present with splenomegaly, hepatomegaly, thrombocytopenia and often with bone disease. Patients can be divided into 3 clinical types on the basis of the progression of the disease and CNS involvement – type 1: nonneuronopathic, type 2: acute neuronopathic, type 3: subacute neuronopathic. Although an evaluation of signs and symptoms can help physicians arrive at a preliminary diagnosis, several tests are available for confirmation. In the past, invasive bone-marrow tests were most common, but this test is ineffective because there are both false negatives and false positives. So it has to be kept in mind that a large number of patients will be misdiagnosed before the disease is correctly identified. It is important to ask how to increase the number of patients who are correctly diagnosed by noninvasive biochemical or molecular tests, without undergoing invasive test.

There have been no reports of biochemical or molecular analysis of Thai patients. Glucocerebrosidase activity has not been routinely measured as only a few laboratories can perform this testing. In this study, glucocerebrosidase activity was determined by the fluorimetric assay using 4-MU-glucopyranoside as a substrate. The mean value of glucocerebrosidase activity in Thai normal controls with S.D. was 11.63$\pm$4.97 nmol/mg protein/h. The result is similar to the previous report of determining glucocerebrosidase activity. The mean value of glucocerebrosidase activity in 8 Brazil normal controls is 14.5$\pm$5.54 nmol/mg protein/h (range 6.0 to 27.1 nmol/mg protein/h)(43) and 7.0 to 25.3 nmol/mg protein/h determined in 35 Brazil healthy individuals (44). What's more, we found that the level of glucocerebrosidase activity remarkably decrease in 3 patients. We concluded that all patients had significant decrease of glucocerebrosidase activity and suggested that clearly abnormal glucocerebrosidase measurement is usually sufficient to make a definite diagnosis of patients and may make molecular analysis unnecessary. However, in family members with borderline abnormal enzyme assay, molecular diagnosis may help to identify heterozygous subjects. For the routine diagnostic workup in non-Jewish populations, genotype identification has to be considered confirmatory, provided that the enzyme assay has been done properly.

The molecular diagnosis of Gaucher disease has been difficult due to the existence of several different point mutations in the glucocerebrosidase gene and due to the presence of a tightly linked, highly homologous pseudogene (58). Many mutations encountered in Gaucher patients are identical to sequences ordinarily found only in the pseudogene, and some result from recombinant between the functional gene and pseudogene. Thus, for diagnostic purposes it is essential to differentiate between sequences from the functional gene and pseudogene. To avoid amplification of the pseudogene, oligonucleotide primers specific to the functional gene were chosen. In some case, large recombinant alleles or deletions (68) may remain undiagnosed by the currently used PCR techniques employing primers selectively amplifying the functional gene. The design of the primer used is problematic because of the close sequence homology to the pseudogene. As a result,

we have used long-template PCR to amplified simultaneously a 6.6 kb genomic fragment from the functional glucocerebrosidase gene and a corresponding 4 kb fragment from pseudogene, which can be directly sequenced or subsequently used as templates for further PCR reactions for mutation detection and screening methods.

Although >200 mutations in the gene for human glucocerebrosidase have been described, most genotype-phenotype studies have focused upon screening for a few common mutations. In this study, we used several approaches—including direct sequencing, long-template PCR, PCR using specific primer, restriction enzyme digestion to identify 5 Thai GD patients. All 10 mutant glucocerebrosidase alleles were identified. Five different mutant were found, including point mutations, splice junction mutations. Three novel mutations were identified in these patients: Y363H, IVS 6(-1)G→C and X498A. Mutation L444P was found on 6 patient alleles. Direct sequencing and restriction enzyme digestion demonstrated that L444P mutation occurred alone on one allele with N188K on one allele in type 2 GD patient 1 and occurred alone on one allele with Y363H on one allele in type 1 GD patient 4. In addition, L444P mutation was homozygous in two patients with Type 1 GD. Although 93% of the mutant alleles in our Ashkenazi Jewish type 1 patients were N370S, c.84-85insG, IVS2(+1)G→A or L444P, these four mutations accounted for only 49% of mutant alleles in the non-Jewish type 1 patients. Heterozygosity for the L444P mutation as either a single point mutation or in the context of a complex mutant allele can lead to a range of clinical subtypes depending on the severity of the second allele (69). The presence of type 2 disease in patient 1 with the genotype N188K in conjunction with L444P suggests that the substitution of the uncharged polar amino acid asparagines (N) by the polar positively charge lysine (K) at position 188 has a dramatic effect on glucocerebrosidase activity. Stone et al. (63) reported that point mutation L444P was detected on 25 patient alleles (41%) and was found frequently among the patients with type 2 GD. In addition, no patients were homozygous for this point mutation. The genotype L444P/L444P is often encountered in patients with type 3 GD, and has also been seen in young Gaucher patients without any evidence of neurologic involvement. It is not clear why humans with homozygous L444P seem to be protected frome type 2 GD, while those who have heterozygous L444P and a presumably more severe mutation develop type 2 GD.

The genotype of Swedish patients with type 3 GD is homozygous L444P mutation (70). However, no report has focused on Asian patients with neuropathic GD. One Japanese patient with type 2 had homozygous L444P mutation. None of the Japanese patients with type 3 had homozygous L444P mutation (71). In previous reports, all patients having homozygous L444P mutation showed the type 3 GD (53,72), the patients identified with the homozygous L444P genotype in Japanease patients were classified as type 1, though. In Asian, N370S, prominent in Ashkenazi Jews, has not been reported in Gaucher disease type 1 cases (73). We found that the L444P occurs in all populations and is the major allele in Swedish variant of Gaucher disease type 3 (74,75). As in other cohorts of Gaucher patients, the patients with the same genotype tended to have similar phenotypes, but they displayed a significant clinical variability. A role for unidentified modifier genes was postulated by different authors (76,77) to explain wider phenotypic range in the patients sharing the same genotype. Winfield and co-authors (78) speculated that a molecular lesion in genes clustered in the neighborhood of the glucocerebrosidase gene can contribute to the

phenotype of Gaucher patients, e.g., due to a contiguous gene syndrome. These findings suggest that a founder effect in each ethnic group is variable and the distribution of mutations was heterogeneous. These results indicate the potential need to design specific mutation panels for various populations.

In patient 5, we have found IVS 6(-1)G→C and X498A mutations so restriction enzyme digestion confirmed that X498A mutation is heterozygous. In case of IVS 6(-1)G→C mutation, firstly we expected that when double stranded cDNA was amplified with PCR in both this patient and normal control, we will see the same order of pseudogene sequence and the different order of functional sequence overlapping in both this patient and normal control. But the result does not exist in my expectation. So we thought that this allele was degraded by possibly nonsense mediated mRNA decay which would result from the premature termination. We continued to prove by amplifying cDNA having only X498A allele to show the sequence of each exon contiguous to each other. This suggests that these mutations are causative.

In conclusion, this study demonstrates the mean level of normal glucocerebrosidase activity in Thais for the first time and mutation analysis shows that the L444P is the most common mutation. Both enzymatic and molecular assays will help physician to definitely diagnose patients with Gaucher disease rapidly and give genetic counseling more accurately.

References

1. Charrow J, Andersson HC, Kaplan P, et al. The Gaucher registry: demographics and disease characteristics of 1698 patients with Gaucher disease. *Arch Intern Med* 160(2000): 2835-2843.

2. NIH Technology Assessment Panel on Gaucher Disease. Gaucher disease: current issues in diagnosis and treatment. *JAMA*. 275 (1996): 548-553.3. Tayebi N., Stubblefield B.K., Park J.K., Orvisky E., Walker J.M., LaMarca M.E., and Sidransky E. Reciprocal and nonreciprocal recombination at the glucocerebrosidase gene region: Implications for complexity in gaucher disease. *Am J Hum Genet* 72(2003): 519-534.

4. Winfield SL, Tayebi N, Martin BM, Ginns EI, Sidransky E. Identification of three additional genes contiguous to the glucocerebrosidase locus on chromosome 1q21: implications for Gaucher disease. *Genome Res* 7,10 (1997): 1020-1026.

5. Mignot C, Doummar D, Maire I, De Villemeur TB; French Type 2 Gaucher Disease Study Group. Type 2 Gaucher disease: 15 new cases and review of the literature. *Brain Dev* 28,1(2006): 39-48.

6. Brady RO, Barton NW, Grabowski GA. The role of neurogenetics in Gaucher disease. *Arch Neurol* 50(1993):1212-1224.

7. Grabowski GA. Lysosomal storage diseases. In: Braunwald E, Fauci AS, eds. *Harrison's Principles of Internal Medicine*. 15th ed. New York, NY: McGraw-Hill (2001): 2276-2281.

8. Beutler E, Grabowski G. Gaucher disease. In:Scriber CR, Beaudet AL, Sly WS, et al, eds. *The Metabolic and Molecular Bases of Inherited Disease*. 7th ed. New York, NY: McGraw-Hill 2(1995): 2641-2661.

9. Cox TM, Schofield JP. Gaucher's disease: clinical features and natural history. *Bailliere's Clinical Haematology* 10,4 (1997): 657-689.

10. Charrow J, Esplin JA, Gribble TJ, et al. Gaucher disease: recommendations on diagnosis, evaluation, and monitoring. *Ach Intern Med* 158(1998): 1754-1760.

11. Morales LE. Gaucher's disease: a review. *Ann Pharmacother* 30(1996): 381-388.

12. Grabowski G. Gaucher disease: enzymology, genetics, and treatment. In:Harris H, Hirshchorn K, eds. *Advances in Human Genetics*. New York, NY: Plenum Press (1993): 377-441.

13. Svennerholm L, Hakansson G, Lindsten J, Wahlstrom J, Dreborg S, Prenatal diagnosis of Gaucher disease. Assay of the beta-glucosidase activity in amniotic fluid cells cultivated in two laboratories with different cultivation conditions. *Clin Genet* 19(1981): 16-22.

14. Schneider EL, Ellis WG, Brady RO, McCulloch JR, Epstein CJ. Infantile (type II) Gaucher's disease: in utero diagnosis and fetal pathology. *J Pediatr* 81 (1972): 1134- 1139.

15. Heilbronner I-I, Wurster KG, Harzer K. Prenatal diagnosis of Gaucher's disease. *Dtsch Med Wochenschr* 106(1981): 652-654.

16. BarrangerJ A, Ginns EI. Glucosylceranlide lipidoses: Gaucher disease. In: Scriver CR, Beaudet AL, Sly WS, et al, eds. *The Metabolic Basis of Inherited Disease*. 6th ed. New York, NY: McGraw-Hill (1989): 1677-1698.

17. Keyserlingk DG, Boll I, Albrecht M. Electron microscopy and cytochemistry of Gaucher cells in chronic granulocytic leukaemia. *Klin Wochenschr* 50 (1972): 510-516.

18. Dosik H, Rosner F, Sawitsky A. Acquired lipidosis: Gaucherlike cells and 'blue cells' in chronic granulocytic leukemia. *Semin Hematol* 9(1972): 309-316.

19. Parkin JL, Brunning RD. Pathology of the Gaucher cell. *Prog Clin Biol Res* 95 (1982): 151-175.

20. Scullin DC Jr, Shelburne JD, Cohen HJ. Pseudo-Gaucher cells in multiple myeloma. *Am J Med* 67(1979): 347-352.

21. Zidar BL, Hartsock RJ, Lee RE, Glew RH, LaMarco KL, Pugh RP, Raju RN, Shackney SE. Pseudo-Gaucher cells in the bone marrow of a patient with Hodgkin's disease. *Am J Clin Patho* (1987): 533-536.

22. Parkin JL, Brunning RD. Pathology of the Gaucher cell. *Prog Clin Biol Res* 95 (1982) : 151-175.

23. Solis OG, Belmonte AH, Ramaswamy G, Tchertkoff V. Pseudogaucher cells in Mycobacterium avium intracellulare infections in acquired immune deficiency syndrome (AIDS). *Am J Clin Patho* 85(1982) :233-235.

24. Lee RE. The pathology of Gaucher disease. *Prog Clin Biol Res* 95(1982):177-217.

25. Desnick RJ, Gatt S, Grabowski GA. Gaucher Disease: A Century of Delineation and Research. New York: Alan R. Liss (1982).

26. Weinreb NJ, Brady RO, Tappel AL. The lysosomal localization of sphingolipid hydrolases. *Biochim Biophys Acta* 159(1968): 141-146.

27. Furbish FS, Blair HE, Shiloach J, Pentchev PG, Brady RO. Enzyme replacement therapy in Gaucher's disease: large-scale purification of glucocerbrosidase suitable for human administration. *Proc Nat Acad Sci* 74(1977): 3560-3563.

28. Pentchev PG, Brady RO, Hibbert SR, Gal AE, Shapiro D. Isolation and characterization of glucocerebrosidase from human placental tissue. *J Biol Chem* 24(1973): 5256-5261.

29. Beutler E, Kuhl W. The diagnosis of the adult type of Gaucher's disease and its carrier state by demonstration of deficiency of beta-glucosidase activity in peripheral blood leukocytes. *J Lab Clin Med* 76(1970):747-755.

30. Hakansson G, Dreborg S, Lindsten J, Svennerholm L. Assay of the beta-glucosidase activity with natural labelled and artificial substrates in cultivated skin fibroblasts from homozygotes and heterozygotes with the Norrbottnian type of Gaucher disease. *Clin Genet* 18(1980): 268-273.

31. Svennerholm L, Hlkansson G, Dreborg S. Assay of the betaglucosidase activity with natural labelled and artificial substrates in leukocytes from homozygotes and heterozygotes with the Norrbottnian type (Type 3) of Gaucher disease. *Clin Chim Acta* 106(1980): 183-193.

32. Daniels LB, Glew RH, Diven WF, Lee RE, Radin NS. An improved fluorometric leukocyte beta-glucosidase assay for Gaucher's disease. *Clin Chim Acta* 115(1981): 369-375.

33. Grabowski GA, Dinur T, Gatt S, Desnick RJ. Gaucher type I (Ashkenazi) disease: considerations for heterozygote detection and prenatal diagnosis. *Prog Clin Biol Res* 95(1982): 573-595.

34. Raghavan SS, Top01 J, Kolodny EH. Leukocyte beta-glucosidase in homozygotes and heterozygotes for Gaucher disease. *Am J Hum Genet* 32(1980): 158-173.

35. Svennerholm L, Hakansson G, Dreborg S. Assay of the betaglucosidase activity with natural labelled and artificial substrates in leukocytes from homozygotes and heterozygotes with the Norrbottnian type (Type 3) of Gaucher disease. *Clin Chim Acta* 106(1980): 183-193.

36. Grabowski GA, Dinur T, Gatt S, Desnick RJ. Gaucher type I (Ashkenazi) disease: a new method for heterozygote detection using a novel fluorescent natural substrate. *Clin Chim Acta* 124(1982): 123-135.

37. Chambers, J. P., Aquino, L., Glew, R. H., Lee, R. E., and McCafferty, L. R., Determination of serum acid phosphatase in Gaucher's disease using 4-methylumbelliferyl phosphate. *Clin Chim Acta* 80(1977): 67-77.

38. Daniels, L. B., Robinson, D. B., Glew, R. H., and Wylin, R. F., Normal serum acid phosphatase and angiotensin converting enzyme activities in serum of a patient with Gaucher's disease. *Clin Chem* 27(1981): 1782-1783 Letter.

39. Robinson, D. B., and Glew, R. H., Acid phosphatase in Gaucher's disease. *Clin. Chem* 26(1980): 371-382.

40. Yam, L. T., Li, C., and Finkel, H. E., Leukemic reticuloendotheliosis. The role of tartrate-resistant acid phosphatase in diagnosisand splenectomy in treatment. *Arch Intern Med* 130(1972): 248-256.

41. Snyder, R. A., and Brady, R. 0., The use of white cells as a source of diagnostic material for lipid storage diseases. *Clin Chim Acta* 25(1969): 331-338.

42. Harzer, K., Enzymic diagnosis in 27 caseswith Gaucher's disease. *Clin Chim Acta* 106(1980): 9-15.

43. Kristiane Michelin, Alessandro Wajner, Laureci da S. Goulart, Ângela A. Fachel, Maria Luiza S. Pereira, Alexandre S. de Mello, Fernanda T. S. Souza, Ricardo F. Pires, Roberto Giugliani and Janice C. Coelho. Biochemical study on Beta-glucosidase in individuals with Gaucher's disease and normal subjects. *Clin Chim Acta* 21(2004): 145-153.

44. Michelin K, Wajner A, Bock H, Fachel A, Rosenberg R, Pires RF, Pereira ML, Giugliani R, Coelho JC. Biochemical properties of beta-glucosidase in leukocytes from patients and obligated heterozygotes for Gaucher disease carriers. *Clin Chim Acta* 362(2005): 101-109.

45. Beutler E, Gelbart T Hematologically important mutations: Gaucher disease. *Blood Cells Mol Dis* 24(1998): 2–8.

46. Hodanova K, Hrebicek M, Cervenkova M, Mrazova L, Veprekova L, Zeman J Analysis of the beta-glucocerebrosidase gene in Czech and Slovak Gaucher patients: mutation profile and description of six novel mutant alleles. *Blood Cells Mol Dis* 25(1999): 287–298.

47. Koprivica V, Stone DL, Park JK, Callahan M, Frisch A, Cohen IJ, Tayebi N, Sidransky E. Analysis and classification of 304 mutant alleles in patients with type 1 and type 3 Gaucher disease. *Am J Hum Genet* 66(2000): 1777–1786

48. Orvisky E, Park JK, Parker A, Walker JM, Martin BM, Stubblefield BK, Uyama E, Tayebi N, Sidransky E. The identification of eight novel glucocerebrosidase (GBA) mutations in patients with Gaucher disease. *Hum Mutat* 19(2002): 458–459.

49. Zimran A, Sorge J, Gross E, et al. Prediction of severity of Gauchers disease by identification of mutations at DNA level. *Lancet*. 2(1989): 349-352.

50. Tsuji S, Martin BM, Barranger JA, et al. Genetic heterogeneity in type I Gaucher disease: multiple genotypes in Ashkenazi and non-Ashkenazi individuals. *Proc Natl Acad Sci* 85(1988): 2349-2352.

51. Tsuji S, Choudary PV, Martin BM, et al. A mutation in the human glucocerebrosidase gene in neuronopathic Gaucher's disease. *N Engl J Med* 316(1987): 570-575.

52. Theophilus BDN, Ladlam T, Grabowsh GA, et al. Gaucher disease: molecular heterogeneity and phenotype-genotype correlations. *Am J Hum Genet* 45(1989): 212-225.

53. Reissner K, Tayebi N, Stubblefield BK, Koprivica V, Blitzer M, Holleran W, Cowan T, Almashanu S, Maddalena A, Karson EM, Sidransky E. Type 2 Gaucher disease with hydrops fetalis in an Ashkenazi Jewish family resulting from a novel recombinant allele and a rare splice junction mutation in the glucocerebrosidase locus. *Mol Genet Metab* 63,4(1998): 281-288.

54. Beutler E, Gelbart T, Kuhl W, et al. Identification of the second common Jewish Gaucher disease mutation makes possible population-based screening for the heterozygous state. *Proc Natl Acad Sci* 88(1991): 10544-10547.

55. Sidransky E, Botder A, Stubblefield B, et al. DNA mutational analysis of type 1 and type 3 Gaucher patients: how well do mutations predict phenotype? *Hum Mutat* 3(1994): 25-28.

56. He G-S, Grabowski GA. Gaucher disease: a G(+1) to A(+l) IVS2 splice donor site mutation causing exon 2 skipping in the acid Beta-glucosidase mRNA. *Am J Hum Genet* 51(1992): 810-820.

57. Eyal N, Wilder S, Horowitz M. Prevalent and rare mutations among Gaucher patients. *Gene* 96(1990): 277–283.

58. Zimran A, Sorge J, Gross E, Kubitz M, West C, Beutler E. A glucocerebrosidase fusion gene in Gaucher disease: implications for the molecular anatomy, pathogenesis, and diagnosis of this disorder. *J Clin Invest* 85(1990): 219–222

59. Strasberg PM, Skomorowski MA, Warren IB, Hilson WL, Callahan JW, Clarke JT. Homozygous presence of the crossover (fusion gene) mutation identified in a type II Gaucher disease fetus: is this analogous to the Gaucher knock-out mouse model? *Bioche Med Metab Biol* 53(1994):16-21

60. Beutler E, Gelbart T, Demina A, Zimran A, LeCoutre P. Five new Gaucher disease mutations. *Blood Cells Mol* 21,2(1995): 20-24.

61. Tayebi N, Stern H, Dymarskaia I, Herman J, Sidransky E. 55-base pair deletion in certain patients with Gaucher disease complicates screening for common Gaucher alleles. *Am J Med Genet* 66(1996): 316–319.

62. Vuk Koprivica, Deborah L Stone,Joseph K. Park,Megan Callahan, Amos Frisch, Ian J. Cohen, Nahid Tayebi, and Ellen Sidransky. Analysis and Classification of 304 Mutant Alleles in Patients with Type 1 and Type 3 Gaucher Disease. *Am J Hum Genet* 66(2000):1777-1786.

63. Stone DL, Tayebi N, Orvisky E, Stubblefield B, Madike V, Sidransky E. Glucocerebrosidase gene mutations in patients with type 2 Gaucher disease. *Hum Mutat* 15,2(2000): 181-188.

64. Firon N, Eyal N. Kolodny EH, Horowitz M. Genotype assignment in Gaucher disease by selective amplification of the active glucocerebrosidase gene. *Am J Hum Genet* 46(1990): 527-532.

65. Mistry PK, Smith SJ, Ah M, Hatton CS, McIntyre N, Cox TM. Genetic diagnosis of Gaucher's disease. *Lancet* 339(1992): 889-892.

66. Svennerholm L, Hakansson G, Lindsten J, Wahlstrom J, Dreborg S, Prenatal diagnosis of Gaucher disease. Assay of the beta-glucosidase activity in amniotic fluid cells cultivated in two laboratories with different cultivation conditions. *Clin Genet* 19(1981): 16-22.

67. Heilbronner I-I, Wurster KG, Harzer K. Prenatal diagnosis of Gaucher's disease (author's transl). *Dtsch Med Wochenschr* 106(1981): 652-654.

68. Hatton CE, Cooper A, Whitehouse C, Wraith JE. Mutation analysis in 46 British and Irish patients with Gaucher's disease. *Arch Dis Child* 77(1997): 17–22.

69. Balicki D, Beutler E. Gaucher disease. *Medicine (Baltimore)* 71(1995): 305-323.

70. Dahl N, Lagerstrom M, Erikson A, Pettersson U Gaucher disease type III (Norrbottnian type) is caused by a single mutation in exon 10 of the glucocerebrosidase gene. *Am J Hum Genet* 47(1990): 275–278.

71. Ida H, Rennert OM, Kawame H, Ito T, Maekawa K, Eto Y. Mutation screening of 17 Japanese patients with neuropathic Gaucher disease. *Hum Genet* 98(1996): 167-71.

72. Horowitz M, Tzuri G, Eyal N, Berebi A, Kolodny EH, Brady RO, Barton NW, Abrahamov A, Zimran A Prevalence of nine mutations among Jewish and non-Jewish Gaucher disease patients. *Am J Hum Genet* 53(1993): 921–93.

73. Grabowski GA. Gaucher disease: gene frequencies and genotype/phenotype correlations. *Genet Test* 1,1(1997): 5-12.

74. Dahl N, Lagerstrom M, Erikson A, Pettersson U. Gaucher disease type III (Norrbottnian type) is caused by a single mutation in exon 10 of the glucocerebrosidase gene. *Am J Hum Genet* 47,2(1990): 275-278.

75. Latham T, Grabowski GA, Theophilus BD, Smith FI. Complex alleles of the acid beta-glucosidase gene in Gaucher disease. *Am J Hum Genet* 47,1(1990): 79-86.

76. Zimran A, Kay A, Gelbart T, et al. Gaucher disease. Clinical, laboratory, radiologic, and genetic features of 53 patients. *Medicine (Baltimore)* 71(1992): 337–353.

77. Grace ME, Desnick RJ, Pastores GM. Identification and expression of acid beta-glucosidase mutations causing severe type 1 and neurologic type 2 Gaucher disease in non- Jewish patients. *J Clin Invest* 99(1997): 2530–2537.

78. Winfield SL, Tayebi N, Martin BM, Ginns EI, Sidransky E. Identification of three additional genes contiguous to the glucocerebrosidase locus on chromosome 1q21: implications for Gaucher disease. *Genome Res* 7(1997): 1020–1026.

APPENDICES

APPENDIX A

Research Instruments

Pipette tip : 10 µl, 1,000 µl (Elkay, USA)

Microcentrifuge tube : 0.2 ml, 0.5 ml, 1.5 ml (Bio-RAD, Elkay, USA)

Polypropylene conical tube : 15 ml (Elkay, USA)

Beaker : 50 ml, 100 ml, 200ml, 500 ml, 1,000 ml (Pyrex)

Flask : 250 ml, 500 ml, 1,000 ml (Pyrex)

Reagent bottle : 100 ml, 250 ml, 500 ml, 1,000 ml (Duran, USA)

Cylinder : 25 ml, 50 ml, 100 ml, 250 ml, 500 ml, 1,000 ml (Witeg, Germany)

Glass pipette : 5 ml, 10 ml (Witeg, Germany)

Pipette rack (Autopack, USA)

Thermometer (Precision, Germany)

Parafilm (American National Can, USA)

Plastic wrap

Stirring-magnetic bar

Combs

Automatic adjustable micropipette : P2 (0.1-2 µl), P10 (0.5-10 µl),

P20 (5-20 µl), P100 (20-100 µl), P1000 (0.1-1 ml) (Gilson, France)

Pipette boy (Tecnomara, Switzerland)

Vortex (Scientific Industry, USA)

pH meter (Eutech Cybernatics)

tirring hot plate (Bamstead/Thermolyne, USA)

Balance (Precisa, Switzerland)

Centrifuge (J.P.Selecta, Span)

Microcentrifuge (Eppendorf, Germany)

Mastercycler personal (Eppendorf, Germany)

Thermal cycler (Touch Down, Hybraid USA)

Power supply model 250 (Gibco BRL, Scothland)

Power poc 3000 (Bio-RAD)

Horizon 11-14 (Gibco BRL, Scothland)

Sequi-gen sequencing cell (Bio-RAD)

Heat block (Bockel)

Incubator (Memmert)

Thermostat shaking-water bath (Heto, Denmark)

Spectronic spectrophotometers (Genesys5, Milon Roy USA)

UV Transilluminator (Fotodyne USA)

UV-absorbing face shield (Spectronic, USA)

Gel doc 1000 (Bio-RAD)

Refrigerator 4 ^{0}C (Misubishi, Japan)

Deep freeze -20 ^{0}C, -80 ^{0}C (Revco)

Water purification equipment (Water pro Ps, Labconco USA)

Water bath (J.P.Selecta, Span)

Storm 840 and ImageQuaNT solfware (Molecular dynamics)

General reagents

Absolute ethanol (Merck)

Agarose, molecular glade (Promega)

Ammonium acetate (Merck)

Boric acid (Merck)

Bromphenol blue (Pharmacia)

Disodium ethylenediamine tetracetic acid : EDTA (Merck)

Ethidium bromide (Gibco BRL)

Ficoll 400 (Pharmacia)

Hydrochloric acid (Merck)

Mineral oil (Sigma)

Phenol (Sigma)

Chloroform (Merck)

Isoamyl alcohol (Merck)

Sodium chloride (Merck)

Sodium dodecyl sulfate (Sigma)

Sodium hydroxide (Merck)

Sucrose (BDH)

Tris base (USB)

Triton X-100 (Pharmacia)

base pair DNA ladder (Biolabs)

40%acrylamide/bis solution 19:1 (Bio-RAD)

GelStar (Camberx)

Reagents of conventional PCR

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

Magnesium chloride (Fermentas)

Deoxynucleotide triphosphates (dNTPs) (Fermentas)

Oligonucleotide primers (BSU)

Oligonucleotide primers (Biogenomed)

Taq DNA polymerase (Fermentas)

100% DMSO

Genomic DNA sample

Reagents of long-template PCR

5X Buffer A (invitrogen)

5X Buffer B (invitrogen)

Magnesium chloride (Fermentas)

Deoxynucleotide triphosphates (dNTPs) (Fermentas)

Oligonucleotide primers (BSU)

Oligonucleotide primers (Biogenomed)

ELONGase^{TM} enzyme mix (invitrogen)

Genomic DNA sample

Restriction enzymes

Nci I (Biolabs)

Rsa I (Biolabs)

*Cac*8 I (Biolabs)

APPENDIX B

Buffers and Preparation

1. Lysis Buffer I

Sucrose	109.54	g
1.0 M Tris – HCl (pH 7.5)	10	ml
1.0 M $MgCl_2$	5	ml
Triton X – 100 (pure)	10	ml
Distilled water to	1,000	ml

Sterilize the solution by autoclaving and store in a refrigerator (at 4^0C).

2. Lysis Buffer II

5.0 M NaCl	15	ml
0.5 M EDTA (pH 8.0)	48	ml
Distilled water to	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

3. 10% SDS solution

Sodium dodecyl sulfate	10	g
Distilled water to	100	ml

Mix the solution and store at room temperature.

4. 20 mg/ml Proteinase K

Proteinase K	2	mg
Distilled water to	1	ml

Mix the solution and store in a refrigerator (at -20^0C).

5. 1.0 M Tris – HCl

   Tris base 12.11 g

   Dissolve in distilled water and adjusted pH to 7.5 with HCl

   Distilled water to 100 ml

   Sterilize the solution by autoclaving and store at room temperature.

6. 0.5 M EDTA (pH 8.0)

   Disodium ethylenediamine tetraacetate.$2H_2O$ 186.6 g

   Dissolve in distilled water and adjusted pH to 8.0 with NaOH

   Distilled water to 1,000 ml

   Sterilize the solution by autoclaving and store at room temperature.

7. 1.0 M $MgCl_2$ solution

   Magnesium chloride.$6H_2O$ 20.33 g

   Distilled water to 100 ml

   Dispense the solution into aliquots and sterilize by autoclaving.

8. 5 M NaCl solution

   Sodium chloride 29.25 g

   Distilled water to 100 ml

   Dispense the solution into aliquot and sterilize by autoclaving.

9. 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 store at room temperature.

10. 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

Mixed and stored at 4^0C

11. 7.5 M Ammonium acetate (CH_3COONH_4)

Ammonium acetate	57.81	g
Distilled water	80	ml

Adjust volume to 100 ml with distilled water and sterilize by autoclaving.

12. 25:24:1 (v/v) Phenol-chloroform-isoamyl alcohol

Phenol	25	volume
Chloroform	24	volume
Isoamyl alcohol	1	volume

Mix the reagent and store in a sterile bottle kept in a refrigerator.

13. 2% Agarose gel (w/v)

Agarose	1.6	g
1X TBE	80	ml

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

14. Ethidium bromide

Ethidium bromide	10	mg
Distilled water	1	ml

Mix the solution and store at 4^0C

BIOGRAPHY

Mr. Kampon Phipatthanananti was born in Bangkok, the capital city of Thailand, in August 25th,1982. In 2003, he received his bachelor degree in Biology from Faculty of Science, Chulalongkorn University. Consequently, with his interests in Human and Molecular Genetics, he had made one of his dicision to study in curriculum of Medical Science in Faculty of Medicine for his Master degree.