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BIOCHEMICAL AND MOLECULAR ANALYSIS OF THAI PATIENTS WITH MULTIPLE CARBOXYLASE DEFICIENCY

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โรคเอนไซม์คาร์บอกซิเลสบกพร่องเป็นโรคพันธุกรรมเมแทบอลิซึมที่มีการถ่ายทอดแบบลักษณะด้อยบนออโตโซม อาจเกิดจากความผิดปกติของเอนไซม์ holocarboxylase synthetase ซึ่งสร้างโดยยีน *HLCS* หรือเอนไซม์ biotinidase ซึ่งสร้างโดยยีน *BTD* ในประเทศไทยยังไม่มีรายงานการศึกษาทางชีวเคมีและการกลายพันธุ์ของโรคเอนไซม์คาร์บอกซิเลสบกพร่อง ในการศึกษาครั้งนี้ได้รวบรวมผู้ป่วยโรคเอนไซม์คาร์บอกซิเลสบกพร่อง 4 รายซึ่งได้รับการวินิจฉัยโดยตรวจวิเคราะห์กรดอินทรีย์ในปัสสาวะ และได้พัฒนาวิธีการตรวจวัดระดับการทำงานของ biotinidase และตรวจวัดในคนไทยปกติจำนวน 245 คน พบว่า ระดับค่าเฉลี่ยเป็น 5.63±1.25 nmol/min/ml จากนั้นทำการตรวจวัดระดับการทำงานของ biotinidase ในผู้ป่วยทั้ง 4 ราย พบว่าผู้ป่วยทุกรายมีระดับการทำงานของ biotinidase ปกติ จึงทำการศึกษาหาการกลายพันธุ์เฉพาะในยีน *HLCS* ซึ่งยีน *HLCS* นี้มี 11 exons และ cDNA มีขนาด 2178 bp ศึกษาโดยใช้วิธี PCR cDNA ที่ได้จาก RNA ของเม็ดเลือดขาวและ sequencing บริเวณ coding region ทั้งหมด แล้วยืนยันผลโดย restriction enzyme digestion ผลการศึกษาพบว่ามีการกลายพันธุ์แบบ homozygous R508W ในผู้ป่วย 2 ราย อีก 2 รายเป็น R508W/G505R และ R508W/ตรวจไม่พบ นั่นคือตรวจพบ R508W 6 ใน 8 alleles ของผู้ป่วย 4 ราย จากนั้นจึงทำการศึกษา haplotype analysis เพื่อดูว่าการกลายพันธุ์นี้เป็นผลมาจาก founder mutation หรือไม่ พบว่า R508W อยู่บน 3 haplotype ซึ่งอาจเป็นเพราะการกลายพันธุ์นี้เป็น hot spot หรือเป็นผลจาก founder effect ที่อยู่บนจุดที่มี crossing over บ่อย โดยสรุปผลการศึกษานี้เป็นการแสดงค่า biotinidase activity ในคนไทยเป็นครั้งแรกและพบการกลายพันธุ์ G505R ซึ่งเป็นการกลายพันธุ์ใหม่ที่ไม่มีรายงานมาก่อนและไม่พบในคนไทยปกติ 50 ราย นอกจากนี้การตรวจหาการกลายพันธุ์ R508W อาจสามารถให้การวินิจฉัยผู้ป่วยไทยได้ทั้งหมด ซึ่งจะทำให้การวินิจฉัยโรคถูกต้องรวดเร็วและส่งผลถึงการให้คำปรึกษาทางพันธุศาสตร์ที่แม่นยำขึ้น

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Multiple carboxylase deficiency (MCD) is a rare autosomal recessive disorder of biotin-responsive carboxylases. There are two genetically distinct causes, mutations in *HLCS* or *BTD*. In Thailand, there have been no previous reports of biochemical and molecular characteristics of Thai patients. In this study, we have demonstrated biotinidase activity in the plasma using colorimetric assay of four patients and 245 normal controls. The mean control activity was 5.63±1.25 nmol/min/ml. All 4 patients had normal biotinidase activity. Mutation analysis is performed by direct sequencing all coding region of the *HLCS* having 11 exons and 2178 bp coding region and confirmation by restriction enzyme digestion. The results show that two patients were homozygous for the common R508W mutation and the other two were compoundly heterozygous for R508W/G505R and R508W/mutation-not-found. The G505R has not been previously reported. The R508W was present in 6 of 8 alleles found in four patients. To determine the origin of these mutations, we determined microsatellite markers in the *HLCS* and found that R508W was present in three haplotypes. In conclusion, we developed and determined biotinidase activity in Thais for the first time and found a novel mutation. In addition, our results suggest that the R508W could make diagnosis of MCD in all Thai patients, making diagnose of the disease faster and having genetic counseling implication.

Field of study Medical Science — Student's signature Suphawee Janklat

Academic year 2005 — Advisor's signature Vorasuk Shotelersuk

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LIST OF ABBREVIATIONS

MCD	=	Multiple Carboxylase Deficiency
PC	=	Pyruvate Carboxylase
PCC	=	Propionyl-CoA Carboxylase
MCC	=	Methylcrotonyl-CoA Carboxylase
ACC	=	Acetyl-CoA Carboxylase
BTD	=	Biotinidase
HLCS	=	Holocarboxylase Synthetase

CHAPTER I

INTRODUCTION

Background and rationale

The biotin-responsive multiple carboxylase deficiencies (MCDs) are autosomal recessively inherited disorders of metabolism in which biotin-dependent carboxylases. Patients with multiple carboxylase deficiency have reduced activity of all biotin-dependent carboxylases. Most patients present a characteristic pattern of organic acids in the urine which there are increased quantities of 3-hydroxyisovaleric acid, 3-methylcrotonylglycine, 3-hydroxypropionic acid, methylcitric acid and lactic acid (1, 2).

Biotin is a water-soluble B vitamin that functions as cofactor of enzymes known as biotin-dependent carboxylases(3). In human cells, there are four biotin dependent carboxylases: pyruvate carboxylase (PC), propionyl-CoA carboxylase (PCC), methylcrotonyl-CoA carboxylase (MCC) and acetyl-CoA carboxylase (ACC). These carboxylases are involved in branched-chain amino acid catabolism, fatty acid synthesis and gluconeogenesis(4)(Fig 1). The enzyme pyruvate carboxylase (PC) is located in mitochondria and catalyzes tranformation of pyruvate to oxaloacetate, an intermediary product in phosphoenolpyruvate synthesis, hence essential for glucose synthesis.The enzyme propionyl-CoA carboxylase (PCC) is involved in catabolism of branched chain amino acids and fatty acids of odd-numbered chain length by catalyzing transformation of propionyl-CoA to methylcrotonyl-CoA. Enzyme methylcrotonyl-CoA carboxylase (MCC) is involved in catabolism of amino acid leucine catalyzing carboxylation of 3 - methylcrotonyl-Co A to 3-methylglutaconyl-CoA. The enzyme ACC catalyzes carboxylation of acetyl-Co A to malonyl-Co A, an essential step in biosynthesis of fatty acids. Because these enzymes play such an important role in intermediary metabolism, biotin starvation or deficiencies of one enzyme involved in biotin utilization is potentially lethal. In humans, the balance between the utilization of exogenous and recycled biotin can be disrupted by genetic abnormalities with devastating consequences for metabolic homeostasis(5).

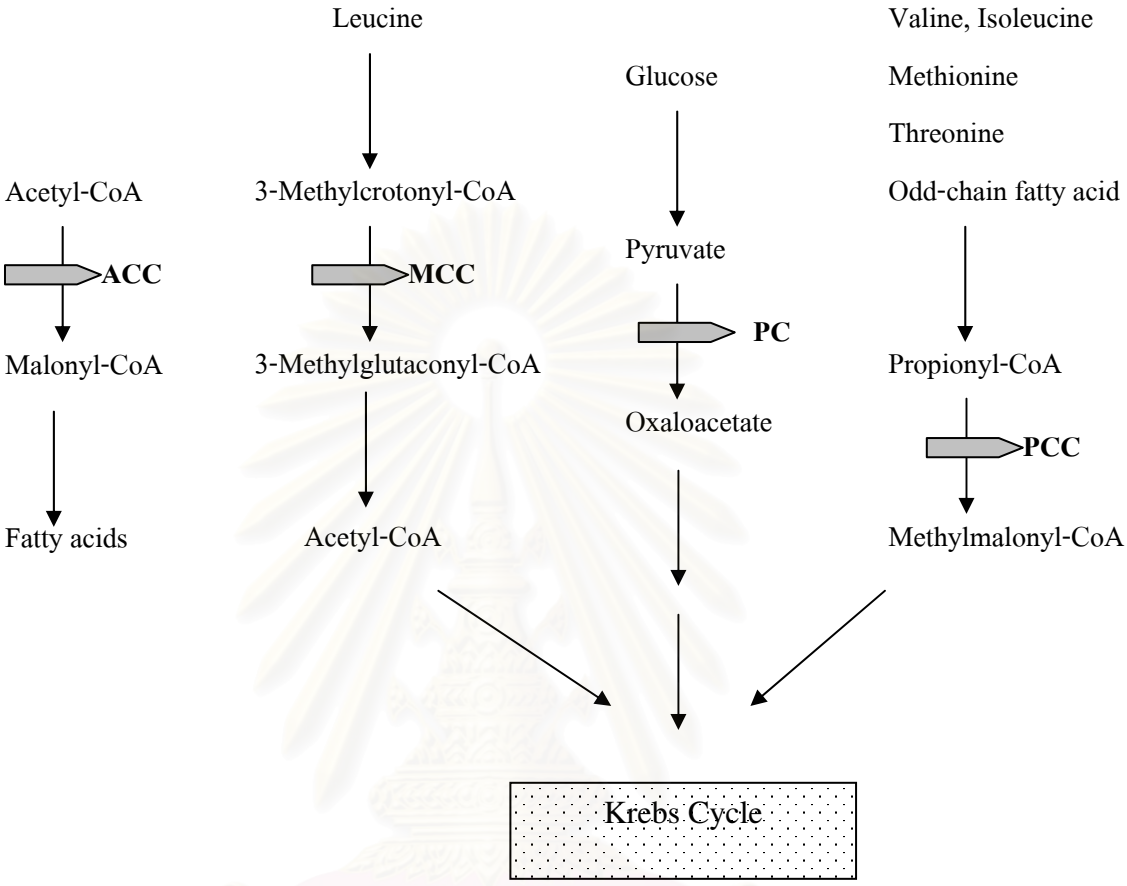


Figure 1. Role of biotin dependent carboxylases in human metabolism

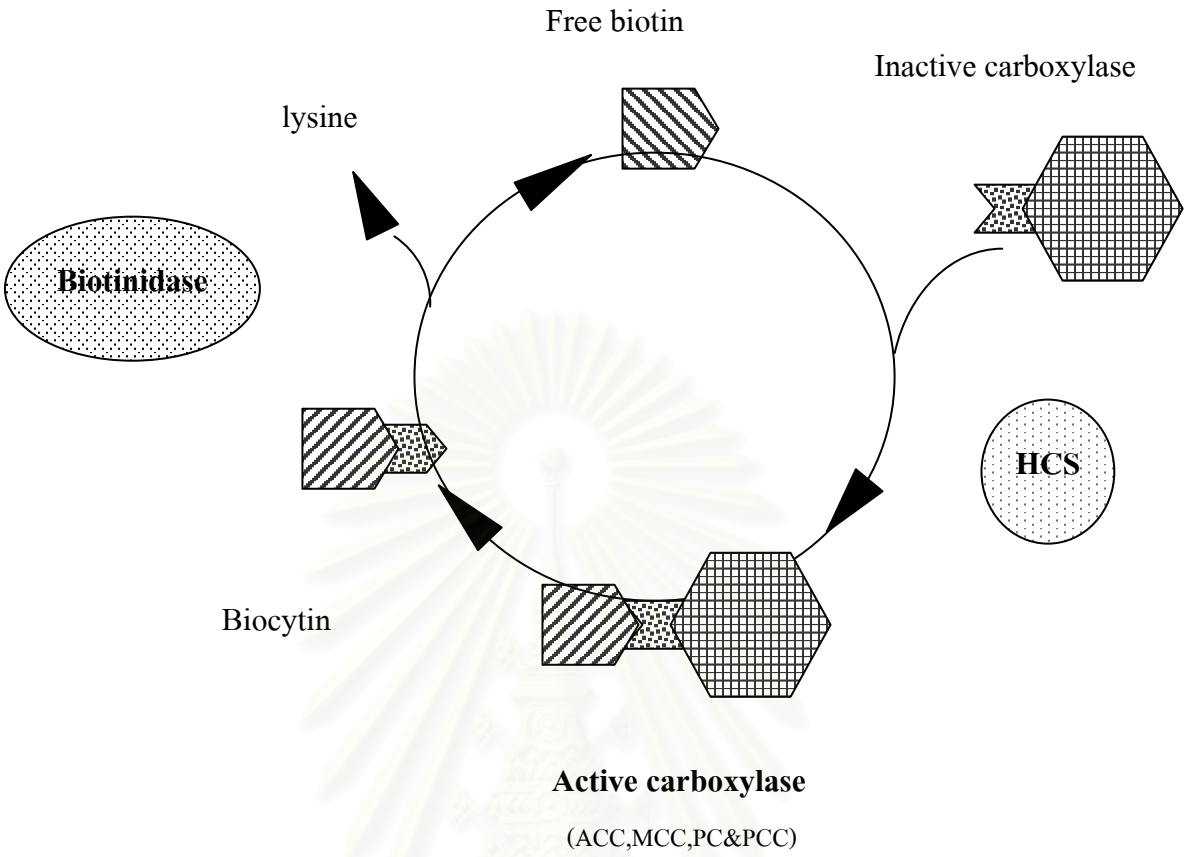


Figure 2. The biotin cycle

The vitamin biotin acts as a carboxyl carrier in carboxylation reactions. The carboxyl group of biotin is covalently attached though an amide bond to an E-amino group of a lysyl residue of the apocarboxylases. This attachment is catalyzed by holocarboxylase synthetase, thereby forming holocarboxylases. The holocarboxylase are degraded proteolytically to biocytin (biotin-E-lysine) which is then cleaved by biotinidase (biotin-amide amidohydrolase) releasing biotin for reutilization, thereby completing the biotin cycle (Fig.2). Most biotin present in foods is not readily available because it is protein-bound and must be released from the carboxylases to which it is attached before it can be used in carboxylation reactions.

In MCD, all carboxylase activities are simultaneously deficient owing to universally defective biotinylation. There are two genetically distinct cause mutations in *HLCS* or in biotinidase(*BTD*). In BTD deficiency, the defect lies in blocking reentry of biotin into the biotin cycle due to defective release of the proteolysis of carboxylases. HCS deficiency may be life threatening during infancy. Biotinidase deficiency tends to be of later onset and has milder symptoms. The lack of activity causes a characteristic pattern of accumulation of organic acids in the patient's urine. The nervous system, skin, respiratory system, digestive system and immune system are involved in MCD(6, 7). Two variants have been identified a neonatal or early onset form caused by a holocarboxylase synthetase deficiency and a juvenile or late-onset form caused by a deficiency of enzyme biotinidase(8,9). Both forms have a good prognosis if biotin therapy is introduced early(10,11). If left untreated, severe forms of MCD can lead to coma and death(12).

Holocarboxylase synthetase (HCS) is the enzyme responsible for attaching biotin to carboxylases. Most patients with holocarboxylase synthetase deficiency manifest symptoms that include tachypnea, seizures, difficulties in feeding and dermatitis in the early infantile period. Biochemical findings include metabolic ketoacidosis, hyperammonemia, and excretion of abnormal organic acid metabolites(13). Most defects in HCS result in a severe, neonatal disease characterized by metabolic ketoacidosis and organic acidemia accompanied by developmental delay, hypotonia, and erythematous rashes. Early diagnosis of HCS deficiency is crucial, because administration of biotin can prevent the occurrence of ketoacidosis. Assays for either multiple carboxylase or HCS have been the only means available to establish the diagnosis of HCS deficiency. DNA analysis will provide another tool for diagnosing the disease(14).

Biotinidase catalyze the cleavage of biotin from biocytin or biotinyl peptides. Biotinidase deficiency results in a similar disease though it tends to be of later onset and a milder clinical course(15,16). However, there is considerable variation in the age of onset and severity of symptom(17).

Because biotinidase deficiency meets the criteria for inclusion in newborn screening programs at least 25 countries have included the disorder in their programs(18). The incidence of disorder is estimate to be approximately 1:60,000. Biotinidase deficiency is detected by determining the activity of the biotinidase enzyme utilizing the newborn dried blood spot using a qualitative colorimetric assay in which biotinyl-p-aminobenzoate(B-p-ABA) is the substrate(19). Confirmation of biotinidase activity in serum was obtained for babies suspected of having the deficiency and for their parents by a qualitative colorimetric assay using biotinyl-p-aminobenzoate as described previously. Normal serum enzyme activity ranged from 4 to 10 umol p-aminobenzoate formed. The importance of early diagnosis is higlighted by the fact that treatment with biotin is highly effective while the untreated disease is fatal(20, 21). The first hour of life may be highly catabolic and death has occurred as early as 12 hours of life. Therefore in a family at risk, a molecular genetic approach is very helpful to establish the definitive diagnosis in these inherited metabolic diseases(22).

Research question

1. What are the mutations in Thai patients with multiple carboxylase deficiency ?
2. What is the normal range level of biotinidase activity in Thai population ?

Objective

1. To detect mutation in Thai patient with multiple carboxylase deficiency.
2. To determine biotinidase activity in plasma by using colorimetric assay of Thai patient with multiple carboxylase deficiency.

Hypothesis

1. Patients with multiple carboxylase deficiency who have decreased biotinidase activity, have mutations in *BTD*.
2. In patients with multiple carboxylase deficiency, who have normal biotinidase activity, have mutations in *HLCS*.

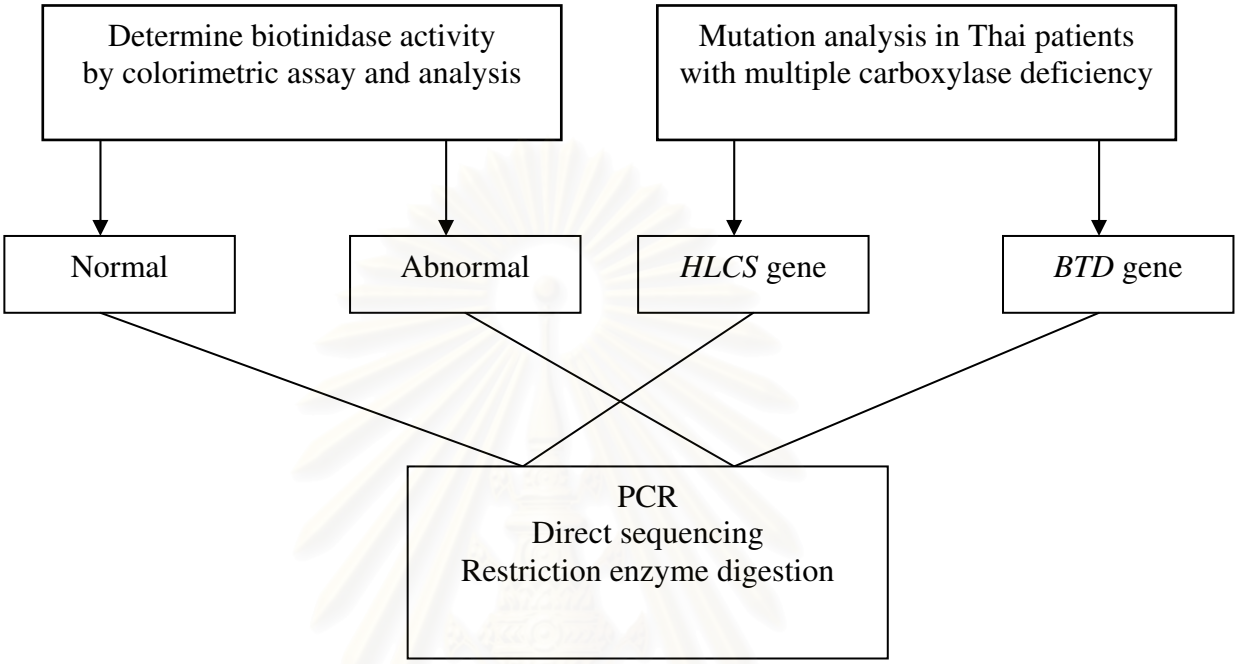
Key words

1. Biotin
2. Biotinidase
3. Holocarboxylase synthetase
4. Multiple carboxylase deficiency

Expected benefit

In this study, we developed and determined biotinidase activity in Thai for the first time. Mutation analysis in this research will help physicians to diagnosic patients with MCD faster and give genetic counseling more accurately.

Conceptual Framework



CHAPTER II

REVIEW OF RELATED LITERATURE

Multiple carboxylase deficiency

Multiple carboxylase deficiency is a rare autosomal recessive disorder of biotin utilization associated with deficiency of all four biotin-dependent carboxylases. MCD may be caused by a primary deficiency of holocarboxylase synthetase or biotinidase. The lack of activity causes a characteristic pattern of accumulation of organic acids in the patient's urine. The nervous system, skin, respiratory system, digestive system, and immune system are involved in MCD. Two variants have been identified, a neonatal or early onset form caused by a holocarboxylase synthetase deficiency, and a juvenile or late-onset form, caused by a deficiency of enzyme biotinidase. Both forms can be treated with pharmacological supplementation with biotin.

The role of biotin in carboxylases is to act as vector for carboxyl-group transfer between donor and acceptor molecules during carboxylation reaction. Covalent addition of biotin to these proteins is catalyzed by biotin ligases, which in prokaryotes are known as BirA protein and eukaryotes as holocarboxylase synthetase (HCS). Deficiencies in these enzymes document failure of the biotinylation of these carboxylase enzymes. Although biotin is required in only trace levels, biotin deficiency has occasionally been documented and shown to be associated with neurologic and developmental abnormalities and skin lesions.

The diagnosis of MCD can be made by urine organic acid analysis which shows increased excretion of several metabolites, including 3-methylcrotonylglycine, 3-hydroxyisovaleric and 3-hydroxypropionic acids, as a result of defective biotinylation of the carboxylase enzymes. Enzyme assays for the carboxylases can be performed on cultured skin fibroblasts and blood leukocyte. Further enzymatic assays in a biotin-deficient medium may help to differentiate between biotinidase deficiency and holocarboxylase deficiency. Biotinidase activity is easily measured in serum or cultured fibroblast so this assay should also serve to distinguish between these two causes of MCD.

Biotinidase deficiency

Biotinidase deficiency is an autosomal recessive trait. Patients can not recycle biotin. The age at onset of symptom ranges from several weeks to several years with median and mean ages of 3 and 5.5 months. Biotinidase activity is detected by determining the activity of the biotinidase enzyme in serum. Most symptomatic children have profound biotinidase activity (<10% of mean normal activity). Newborn screening also has identified children with partial biotinidase deficiency (10-30% of mean normal activity). Biotinidase activity can be determined in the dried blood spot using a qualitative colorimetric assay using biotinyl-p-aminobensoate(B-p-ABA) is the substrate and confirmation of biotinidase activity in serum(23). The most common assay for biotinidase activity screening measures the quantitative of p-aminobenzoate (P-ABA) producted from hydrolysis of biotinyl-p-aminobensoate. This method was adapted also to screening purposes. More than 25 states in the United States and 25 countries are screening for the enzyme defect in the newborn. The mean biotinidase activity is 7.1 nmol/min /ml serum in normal adults, range 3.5-12 nmol/min/ml. The enzyme activity may be determined by radiochemical assay that measures the liberation of (^{14}C)biocytin using separation by ion-exchange chromatography, fluorometric assay and by a microbiological bioassay(24). The enzyme also hydrolyzes some artificially synthesized biotin derivertives such as biotin-6-amidoquinoline using the fluorimetric assay, that was introduced by Wastell et al. (25, 26).

There are many factors that can result in falsely low enzyme activity(27). This problem is usually attributable to poor imprenation of blood on filter paper, increase humidity or heat exposure. The problem with discrepancies in quantitative enzyme determination is usually failure to rapidly freeze and ship the sample on dry ice or failure to store the samples in the laboratory at -80 C if not immediately assayed. In patient with liver disease found that biotinidase activity was decrease(28).

A molecular genetic approach is very helpful to establish the definitive diagnosis in most inherited metabolic diseases. There are many reports of mutations in *BTD* that cause profound biotinidase deficiency(29-31). The cDNA for human biotinidase has been sequenced. Pomponio R.J. et al. (1994) have isolated and characterized the cDNA for normal human serum biotinidase and localized the gene to chromosome 3p25. The cDNA consists of two possible ATG initiator codons and an open reading frame of 1929 bp (32). This cDNA encodes for a mature monomeric protein of 543 amino acids with a molecular mass of 56,771 Da. The gene consists of four exons and spans at least 23 kb. There are five common mutations among the over 40 that have been shown to cause reduced biotinidase activity. The first is G98:d7i3 in exon 2 which was originally identified in about 50% of the symptomatic children. The second is missense mutation (C1612>T; R538C) in exon4.The third mutation is a missense mutation (A1368>Q456H) in exon4. A fourth mutations is another missense mutation(G511>A;A171T)(33, 34).

The deletion/insertion mutationG98-G104:ins TCC or G98:d7i3 occurred in half of the symptomatic children is the most common mutation in the biotinidase gene that causes profound biotinidase activity in symptomatic children. Currently, 55 mutations that cause biotinidase deficiency have been identified. The most common cause of profound biotinidase deficiency in children ascertained by newborn screening in the United States is the missense mutation Q456H in exon 4. The second most common mutation is a double mutation A171A and D444H. The D444H mutation in one allele in combination with a mutation for profound deficiency in the other allele is the common cause of partial biotinidase deficiency. Haplotype analysis of five common mutation reviews evidence for possible founder effects for four of the five common mutations(35).

Prenatal diagnosis in families with milder forms biotinidase deficiency has to be performed by enzyme assays in culture amniotic cells using the colorimetric assay. Biotin administered prenatally is effectively taken up by the fetus and prevents functional deficiency of the carboxylases in an affected newborn.

Holocarboxylase synthetase deficiency

Holocarboxylase synthetase deficiency is an autosomal recessive. Heterozygosity cannot be confirmed by measuring enzymatic activity but should be determined by mutation analysis. Holocarboxylase synthetase deficiency can be diagnosed prenatally by measuring the concentration of abnormal organic acids in the amniotic fluid. Prenatal diagnosis also should be possible by mutation analysis. Most patients have become symptomatic soon after birth. The age of onset of symptoms varied from a few hours after birth to 21 months of age. Decreased HCS activity was first demonstrated in fibroblasts from patients with this disease, using apo-PCC as a substrate(36). Decrease in HCS activity also has been demonstrated in lymphoblasts from a patient with HCS deficiency. This method was suitable for assay of multiple fractions on column chromatography, but was not sufficiently sensitive to detect lower HCS activity in cultured cells. HCS deficiency has been diagnosed indirectly by measuring activities of multiple carboxylases in cultured cells from patients(37).

Several new methods have been developed to assay holocarboxylase synthetase activity that use prepared acceptors of biotin. One method measures the incorporation of (^{3}H)biotin into apocarboxyl carrier protein.

The *HLCS* is located on chromosome 21q22.1 and consists of 14 exons and 13 introns in a span of 240 kb(38). The human *HLCS* cDNA was cloned in 1994 and shown to encode 726 amino acids (39). Three types of *HLCS* mRNA that start at different exons. The first methionine codon has been found to be located in exon 6 and stop codon was present in exon 14. Three types of the mRNA that differ at the 5' end are now known. Type 1 mRNA starts at exon 1. Type 2 and 3 mRNA start at exon 3 and 2 respectively. The *HLCS* probably has three transcription starting sites. Among 13 introns, intron 9 is the longest(130kb)(40, 41). It contains two polymorphic tetra-nucleotide repeats that were used for haplotyping of the *HLCS* (42).

By the analysis of mutations in patient cDNA. There are different mutations have been identified so far. About ten mutations in *HLCS* have been identified seven novel mutations were identified in the cDNA of the patients. This included missense mutations, two single–base deletions that resulted in a termination codon, a three-base in-frame deletion and a 68 bp deletion(43). These data suggest that a variety and of mutations is responsible for decreasing HCS activity. The most common Leu 237Pro and a 1-bp deletion (1067delG) have frequently been detected in Japanese patients. The results suggest that a variety of mutations is causative in patients from European or Middle Eastern countries, whereas Leu237Pro and 1067delG may be founder mutations in Japanese patients. Four of the mutations are within the putative biotin-binding domain, including two more common mutations R508W and V550M. The R508W mutation was found in a heterozygous from in three Japanese patients and in a homozygous from in one Taiwanese patient. This mutation has also been found in patients from many ethnic(44, 45). Another mutation found in many ethnic groups is the V550M change.

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 four childrens with multiple carboxylase deficiency at King Chulalongkorn Memorial Hospital. All patients were diagnosed by urine analysis for abnormal organic acids.

Plasma from 245 healthy blood-donor volunteer from Thai Red Cross Institute (Kamphaeng Phet Province) were determined biotinidase activity by colorimetric assay(46).

2. Determine biotinidase activity by colorimetric assay

The plasma from healthy blood-donor volunteer and the patients were determined biotinidase activity by colorimetric assay.

Principle

Biotinidase cleaves the amide bond of biotinyl-p-aminobenzoate (B-p-ABA) as a substrate to release free biotin and p-ABA. Then p-ABA will couple with naphthol reagent have formed purple product, which can detect by spectrophotometer.

Reagent

1. KPB (50 mM potassium phosphate buffer, pH 6.0, 0.05 mM EDTA
2. Substrate buffer (0.3 umol B-p-ABA in 50 mM KPB, pH 6.0
3. 1 M NaHCO3
4. 30%(v/v) Trichloroacetic acid (TCA)
5. 0.1%(w/v) Sodium nitrite
6. 0.5%(w/v) Ammonium sulfamate
7. 0.1%(w/v) N-1-naphthyl-ethylenediamine dihydrochloride (NEDD)
8. 0.2 mM p-ABA in 50 mM KPB (standard)

Method

1. Mix reagent in tube

Reagent	Control	Patient	Control background	Patient background	standard	blank
B-p-ABA buffer	1.9 ml	1.9 ml	-	-	-	-
KPB	-	-	1.9 ml	1.9 ml	1.9 ml	2 ml
p-ABA	-	-	-	-	0.1 ml	-
Incubate 37°C 10 min						
Patient's plasma	-	0.1 ml	-	0.1 ml	-	-
Control's plasm	0.1 ml	-	0.1 ml	-	-	-
Mix & Incubate 37°C 30 min						
30% TCA	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml
Centrifuge 700g 10 min						

2. Pipette 1.5 ml of the supernatant from each tube into a new clean tube, add 0.5 ml DW and mix.

3. Pipette the following color-developing reagents, add 0.5 ml of 0.1%(w/v) Sodium nitrite, 0.5 ml of 0.5%(w/v) Ammonium sulfamate and 0.5 ml of 0.1%(w/v) NEDD consecutively into each tube at 3 min intervals. Vortex the tubes after each addition.

4. Allow color development of the solution to proceed for 10 minutes and measure the Abs. in each tube at 546 nm within 30 min.

5. Calculate biotinidase activity (nmol/min/ml)

3. Identification of mutation

The total RNA was isolated from peripheral blood and were converted to cDNA by Reverse transcription-PCR. For PCR amplification of the cDNA, sense and anti-sense primers were designed to amplify DNA fragment. Then detect the PCR product by 1.5% agarose gel electrophoresis

To screen mutation in *HLCS* and *BTD*, primers were designed to amplify cover the entire encoding region (Table1).

Table 1. PCR primers used to amplify cDNA of *HLCS* and *BTD*

Gene	Primer sequence	Product size(bp)
HLCS/1	HLCS / F1- 5' CTG GGG ATC CTT ATC GGC TA 3' HLCS /R1- 5' CAT GTC ACA GCT GAG GCC AA 3'	1200
HLCS/2	HLCS /F2- 5' TCC CAG CTC CAA CAT AGT GC 3' HLCS /R2- 5' CAG ATG CAT GGG CAC GGA CA 3'	1160
BTD	BTD /F- 5' GGA GCG TTT TCG GGG CTG TA 3' BTD /R- 5' CTT GCT GAT CCA CAT CTG CT 3'	1753

3.1 PCR amplification cDNA

Table 2 Mixture of PCR reaction for mutation detection in cDNA

Component	HLCS 1	HLCS2	BTD
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 dNTP	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	14.9	14.9
8.50ng/μl Genomic DNA	1.0 (2.5 ng/μl)	1.0 (2.5ng/μl)	1.0 (2.5ng/μl)
Total volume (μl)	20	20	20

Table 3 PCR cycle condition for detection mutation in cDNA

Step	HLCS 1	HLCS 2	BTD
1. Initial denaturation	95°C/ 5 min	95°C/ 5 min	95°C/ 5 min
2. PCR cycle	30 cycles	35 cycles	35 cycles
Denature	94°C/ 1 min	94°C/ 1 min	94°C/ 1 min
Annealing	64°C/ 1 min	62°C/ 1 min	62°C/ 1 min
Extension	72°C/ 1 min 30sec	72°C/ 1 min 30sec	72°C/ 1 min 30sec
3. Final extension	72°C/ 10 min	72°C/ 10 min	72°C/ 10 min

3.2 Sequencing of cDNA

The PCR products of cDNA from patients were sequencing. Primer ; HLCS3/F;5' TTT CTC AGG GAG GGA AGG TG 3' and HLCS4/F; 5' TTC AGA CAC CGC AGG AAA TG 3' were design for direct sequencing.

4. Confirm Mutation analysis

4.1 The extraction of DNA from peripheral blood leukocyte

Genomic DNA was extracted from 3 ml. of whole blood were added to a new polypropylene tube and mix with 10 volumes of cold lysis buffer I (or 10 ml.). The mixture was centrifuge for 8 minutes at 3300 rpm for 8 minutes, then remove supernatant. Add 3 ml. cold lysis buffer I, mix thoroughly and centrifuge for 8 minute at 3300 rpm. After discard supernatant, add 900µl lysis buffer2, 10µl 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 50µl. Mix vigorously for 15 seconds. The mixture was incubated in 37°C shaking water bath overnight for complete digestion. Add 1 ml. Phenol-chloroform-isoamyl alcohol shake vigorously for 15 seconds and centrifuge at 6,000 rpm for 5 minutes. The supernatant was transfered to a new microcentrifuge tube then add 0.5 volumes of 7.5 M CH_3COONH_4 and 1 volume of 100% ethanol 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 and rinse the pellet with 70% ethanol. Decant the ethanol and air-dried the pellet. (It is important to rinse well to remove any residual salt and phenol). Resuspend the digested DNA in 20-300 µl of the double distilled water at 37°C until dissolved.

4.2 PCR amplification genomic DNA

Since DNA extracted from whole blood was obtained, genomic DNA were amplified using the PCR primers, components and PCR condition showed in table 4 and table 5 respectively.

Table 4 PCR primers used to amplify gDNA of *HLCS*

Primer name	Primer sequence	Product size(bp)
HLCS exon-8	Forward 5' GAG TGT GTG GCC CTG GCA TA 3' Reverse 5' GCT GAG GTT CTA CAG CCA CC 3'	350
HLCS exon-7	Forward 5' CTC ATG GCT CCA CAT TCC TG 3' Reverse 5' CTC CAT TCC AGG CGG TTA TG 3'	373

Table 5 Mixture of PCR reaction for detection mutation in *HLCS*

Component	HLCS exon-8	HLCS exon-7
1.10X PCR buffer	2.0 (1X)	2.0 (1X)
2.25mM $MgCl_2$	1.2 (1.5mM)	1.2 (1.5mM)
3.10mM dNTP	0.4 (0.2mM)	0.4 (0.2mM)
4.10μM Forward primer	0.2 (0.1μM)	0.2 (0.1μM)
5.10μM Reverse primer	0.2 (0.1μM)	0.2 (0.1μM)
6 5U/μI Taq polymerase	0.1 (0.025U)	0.1 (0.025U)
7.Distilled water	15.1	13.9
8.50ng/μl Genomic DNA	0.8 (2.0 ng/μl)	2 (5.0 ng/μl)
Total volume (μl)	20	20

Table 6 PCR cycle condition for detection mutation in *HLCS*

Step	HLCS exon-8	HLCS exon-7
1. Initial denaturation	95°C/ 5 min	95°C/ 5 min
2. PCR cycle	35 cycles	35 cycles
Denature	94°C/ 30sec	94°C/ 30sec
Annealing	60°C/ 30sec	58°C/ 30sec
Extension	72°C/ 30sec	72°C/ 30sec
3. Final extension	72°C/ 10 min	72°C/ 10 min

4.3 Restriction enzymes digestion

10 μl of PCR products were digested with 5 units of restriction enzyme *Fox*I and *Hha*I. The digestion was incubated at 37 ^{0}C for 16 hours in order to completely digest the DNA. The PCR products were separated by electrophoresis on a 3% agarose gel containing ethidium bromide.

4.4 Agarose gel eletrophoresis

The PCR products were separated on 1.5-3.0% of agarose gel electrophoresis containing ethidium bromide.

5. Haplotype Analysis

5.1 Analysis of polymorphic marker

To determine the origin of the mutations, the polymorphic microsatellite markers in the HLCS gene were identified and anaIyzed the haplotypes of the patients and normal control. Tetra-nucleotide repeats, which was previously described by Yang X et al (2000), were amplified. One was located in intron 8 and the other in intron 9 of the *HLCS* gene. These were named the CAAA repeat and the ATTC repeat, respectively. The primer pairs used to amplify each region show in Table 7. The PCR reaction mixture of 20 ul, including 10 uM each of primers and 0.025U of Tag polymerase proceeded through 35 cycles of 94 ^{o}C for 30s, 58 ^{o}C for 30s, and 72 ^{o}C for 30s. The size of the PCR products was detected by 10% polyacrylamine gel electrophoresis and direct sequencing.

Table 7 The primer pairs used to amplify CAAA repeat and the ATTC repeat

Primer name	Primer sequence	Product size(bp)
CAAA	Forward 5' CTG TAG TCC CAG CTA GTT GA 3'	250 bp
	Reverse 5' CAT TTT CCA CCA CAG CTG AG 3'	
ATTC	Forward 5' CTC TGG TGA ATG GAA GAA CC 3'	402 bp
	Reverse 5' CAG CAG GAG ACC AGT ATA GG 3'	

5.2 Estimate of Haplotype frequency

Haplotypes determined by a combination of the CAAA and the ATTC genotype, EH program which was previously described by Zhao et al (2000), was employed to estimate haplotype frequencies in the normal controls.

CHAPTER IV

RESULT

1. Biotinidase Activity Assay

Biotinidase activity was measured in plasma by the colorimetric assay using biotinyl-p-aminobenzoate as a substrate. Biotinidase activity in four patients and normal control adults was shown in table 8. The mean value with S.D. was 5.63±1.25 nmol/min/ml. All patients have normal biotinidase activity. These result shows that patients do not have mutation in *BTD*.

Table 8 Biotinidase activity in patients and normal adults

Subject	Biotinidase activity (nmol/min/ml)
Normal control (n= 245) Mean ± SD (Range)	 5.63±1.25 (2.50-6.88)
Patient 1	7.64
Patient 2	8.48
Patient 3	6.60
Patient 4	4.88

2. Mutation in *HLCS*

Mutation analysis of cDNA is performed by direct sequencing and confirm mutation in gDNA by restriction enzyme digestion.

Patient 1 was heterozygous for the known R508W mutation. This mutation is due to C to T substitution at nucleotide position 1522 in codon 508 substitutes the wild type amino acid arginine(R) by tryptophan(W). Sequence from father confirmed the presence of the mutation in heterozygous(Fig.3).

Patient 2 was heterozygous for R508W mutation. In the other allele carried a novel missense mutation at codon 505 (G505R). It was a substitution of G to C at nucleotide position 1513. Amino acid changes from glycine to arginine. Both mutations were confirmed in genomic DNA (Fig.4).

Patient 3 and 4 were found to be homozygoue for R508W mutation. Parents of these cases were also confirmed to be heterozygote carriers(Fig.5).

The result shows the common mutation R508W accounted for six of eight disease alleles in four patients.

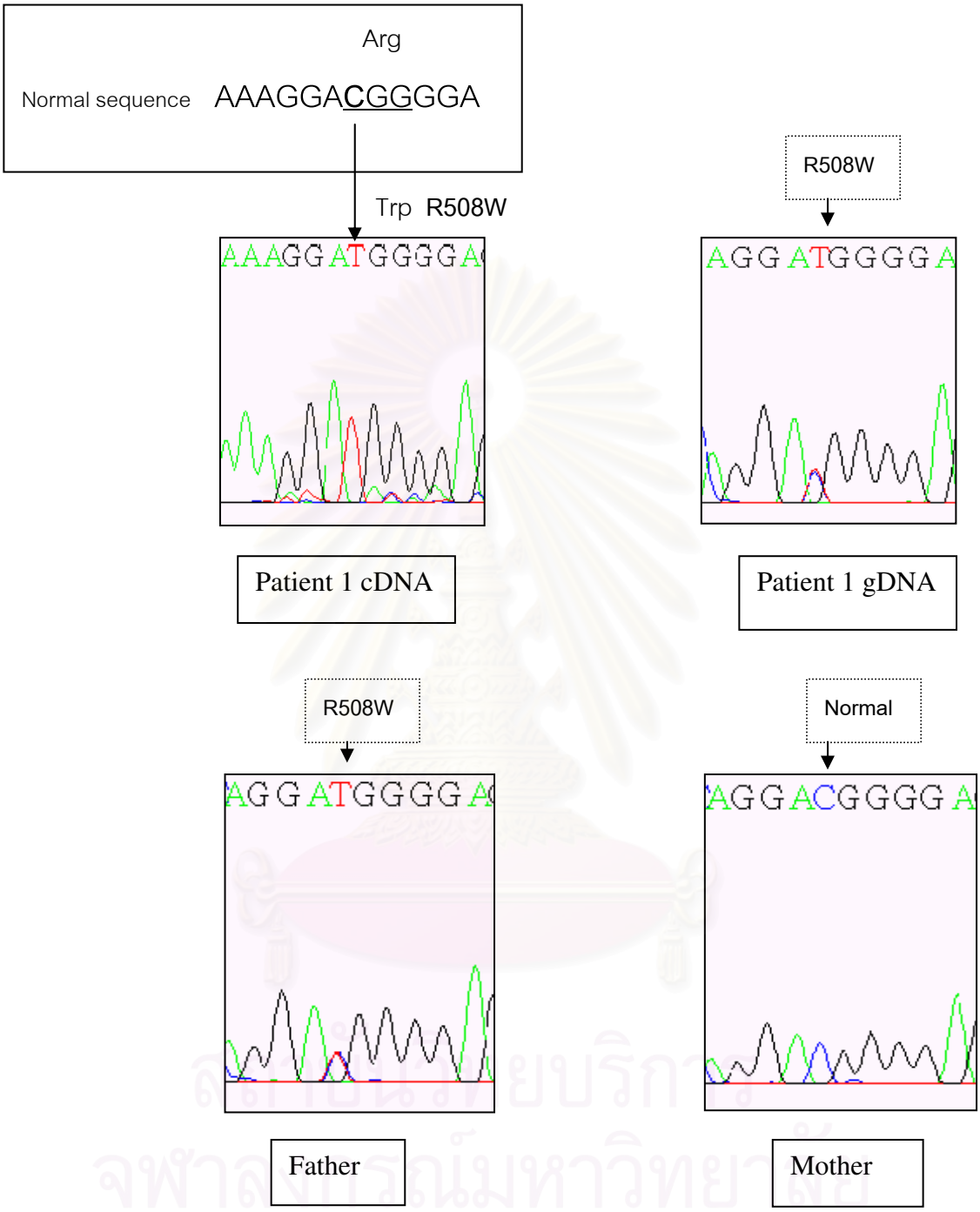


Figure3 Direct sequencing of patient 1 and parents.
The arrow indicates a C to T substitution at nucleotide position 1522 in codon 508.

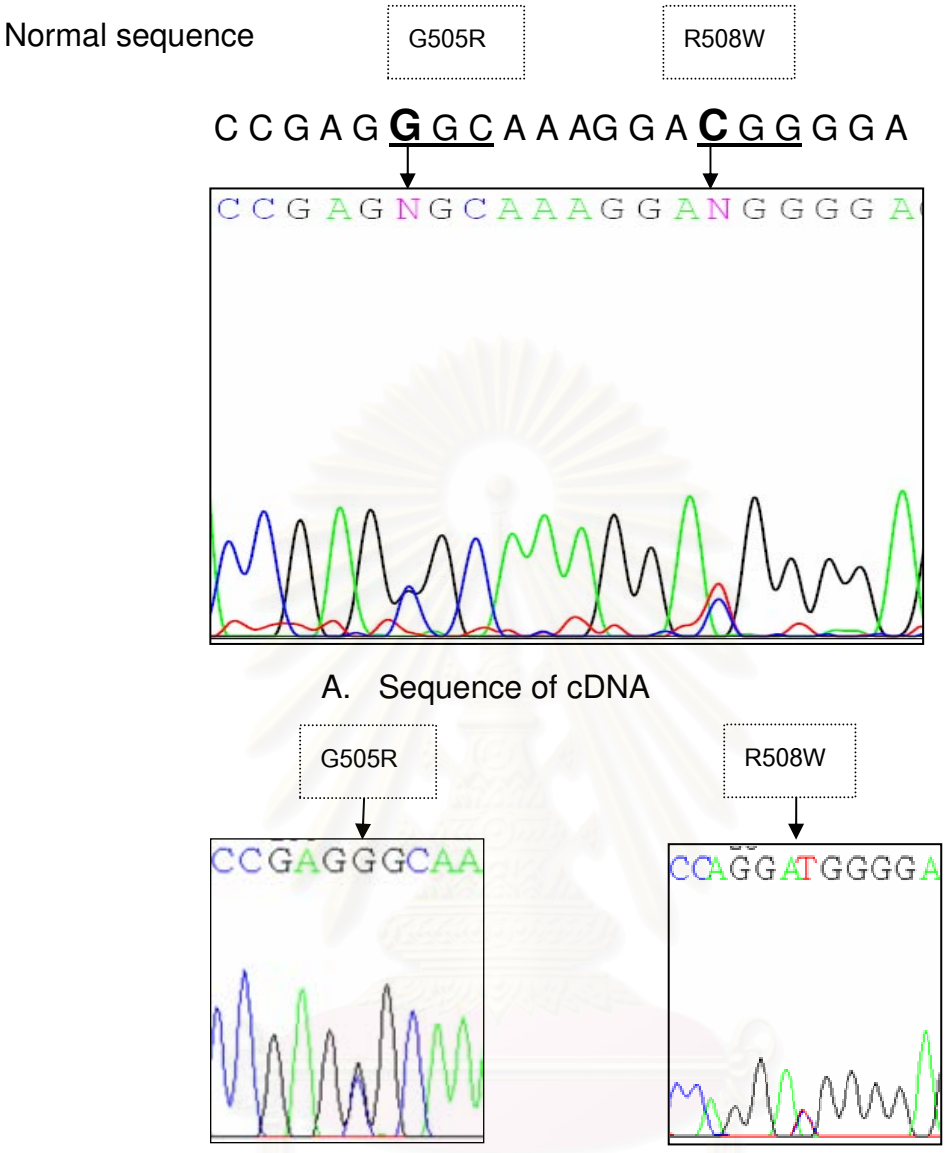


Figure 4 Direct sequencing of cDNA from patient who is heterozygous for the R508W

A. Sequence of cDNA from patient 2
B. Sequence of gDNA from patient 2

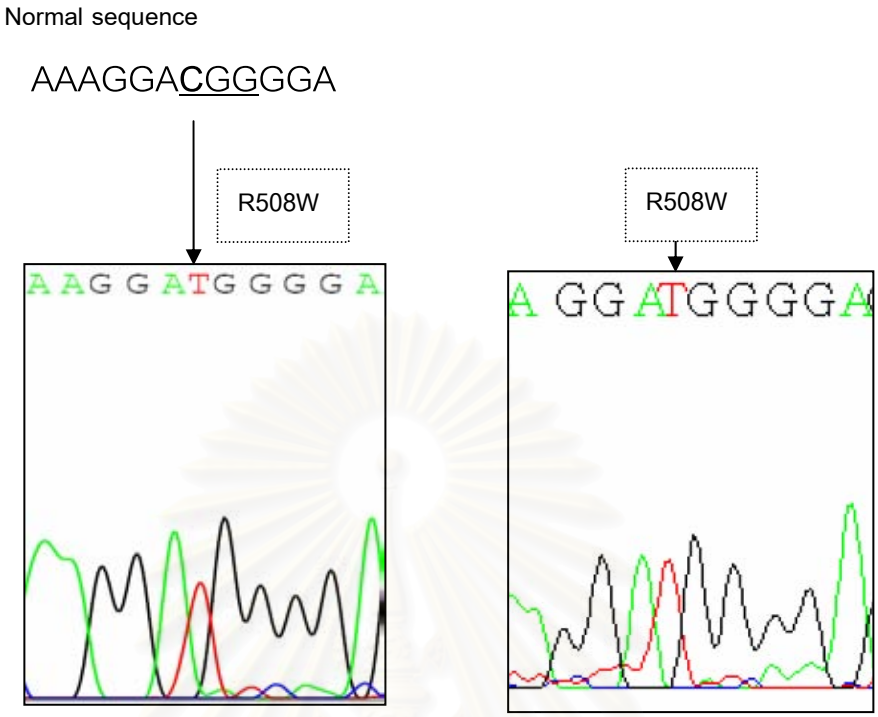


Figure 5 Direct sequencing of cDNA from patient 3 and patient 4.

Patient 3 and 4 are homozygous for the R508W mutation.

Table 9 Summary of mutations in four patients with multiple carboxylase deficiency.

Patient	Description of the mutation in *HLCS*	Nucleic acid changes
1	R508W (1522C→T) / Not found	Arg508Trp / Not found
2	R508W (1522C→T) / G505R (1513G→C)	Arg508Trp / Gly505Arg
3	R508W (1522C→T) / R508W (1522C→T)	Arg508Trp / Arg508Trp
4	R508W (1522C→T) / R508W (1522C→T)	Arg508Trp / Arg508Trp

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 R508W and The novel mutation G505R was screened by using primers, restriction enzymes and expected size product show in table 10.

Table 10 Primers, restriction enzymes and expected size product

Mutation	Primer Name	Expected size (bp)	Restriction enzyme	Expected size after digestion (bp)
R508W	HLCS exon 8	350	*Fox*I	162,120,87,68 and 33
G505R	HLCS exon 7	373	*Hha*I	373,222 and 151

Restriction enzyme digestion with *Hha*I

Restriction enzyme digestion with *Hha*I was performed and electrophoresis on 3% agarose gel contain with ethidium bromide. The PCR product 373 bp is obtained after PCR amplification from genomic DNA. In normal, the PCR product remained undigested where as heterozygous G505R reveals 373, 222 and 151 bp.

The different band patterns generated after digested with *Hha*I (Figure 6).

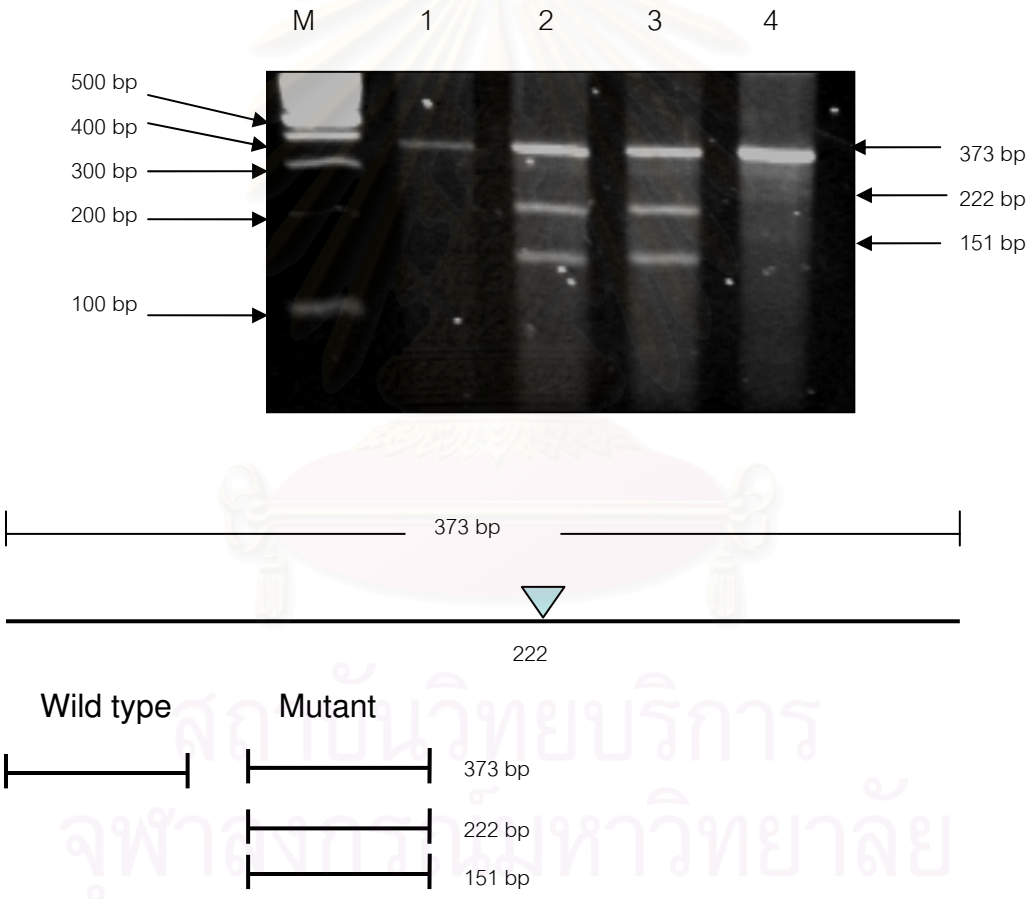


Figure 6 Restriction enzyme digestion with *Hha*I of gDNA for detection G505R.
Lane M, 100 bp DNA ladder, lane 1 father (373bp), lane 2 mother (373,222,151bp), lane3 patient (373,222,151bp), lane 4 control (373bp)

Restriction enzyme digestion with *Fox*I

Restriction enzyme digestion with Fox I was performed and electrophoresis on 3% agarose gel contain with ethidium bromide. The PCR product 350 bp is obtained after PCR amplification. In normal, the PCR product reveals 162,120, 68 and 33 bp where as heterozygous R508W reveals 162,120,87,68 and 33 bp.

In the homozygous R508W mutation, after digestion the PCR product reveals 162,87, 68 and 33 bp. The different band patterns generated after digested with Fox I show in Figure7.

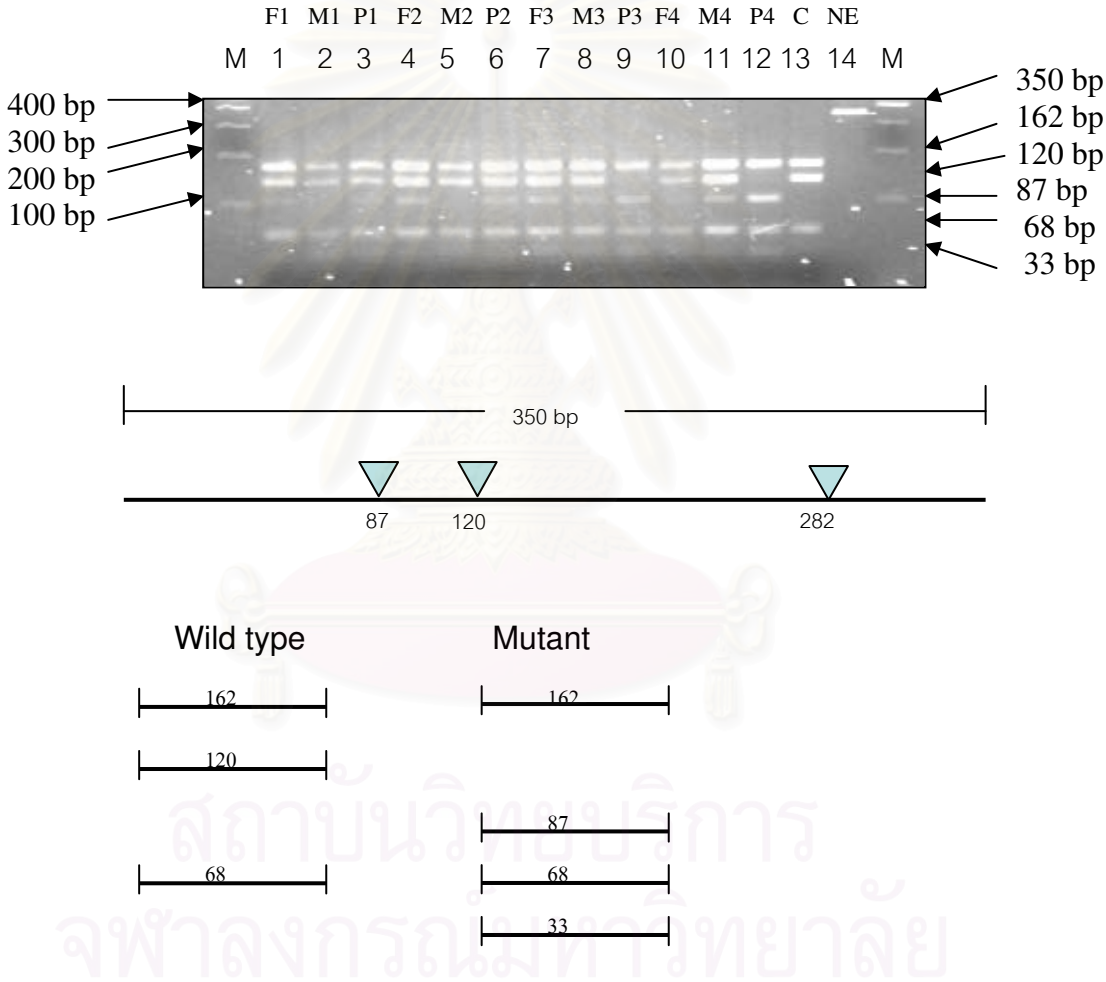


Figure7 Restriction enzyme digestion with Fox I
Lane M, 100 bp DNA ladder
Lane 1-3 are family of patient1 (the heterozygous R508W mutation)
Lane 4-6 are family of patient 2 (the heterozygous R508W mutation)
Lane 7-9 are family of patient 3 (the homozygous R508W mutation)
Lane 10-12 are family of patient 4 (the homozygous R508W mutation)
Lane 13 is control and lane 14 is non-enzyme digestion.

3. Haplotype analysis

To determine the origin of these R508W mutation, microsatellite markers in *HLCS* were identified and analysed haplotypes of the patients and normal. The result show that R508W were found in three haplotype. In patient 1 mutant allele associated with haplotype 2-1. The homozygous R508W mutation in patient 3 associated with haplotype 2-3 and patient 4 associated with haplotype 1-4(Table 12).

Data input looks as follows. It contains the numbers of alleles for each marker and the observations for each genotype.

```
3  4
0  0  0  0  1  0
0  0  0  0  0  0
0  2  1  1  2  0
0  0  0  0  0  0
0  0  0  1  6  0
1  1  2  3  4  0
0  1  1  0  1  1
1  4  2  6  1  2
0  1  0  0  0  0
0  0  0  3  1  0
```

Table11 Haplotype of the HLCS gene in 100 control alleles.

Haplotype CAAA-ATTC	Frequency(%)
1-1	0.1
1-2	0.1
1-3	0.1
2-1	0.7
2-2	16
2-3	13
3-1	11
3-2	25
3-3	20

Table12 Mutation and haplotypes of four patients with holocarboxylase synthetase deficiency

Patient	*HLCS* mutation	CAAA repeat	ATTC repeat	Haplotype
1	R508W (1522C>T) / Not found	2-2	1-4	2-1/2-4
2	R508W (1522C>T)/ G505R (1513G>C)	2-3	3-3	2-3/3-3
3	R508W (1522C>T)/ R508W (1522C>T)	2-2	3-3	2-3/2-3
4	R508W (1522C>T) R508W (1522C>T)	1-1	4-4	1-4/1-4

CHAPTER V

DISCUSSION

Multiple carboxylase deficiency (MCD) is caused by enzyme defects in either holocarboxylase synthetase or biotinidase that involves in biotinylation of the carboxylases. Early reports classified patients with MCD into neonatal and infantile forms and suggested that the neonatal form was due to holocarboxylase synthetase deficiency and late onset patients were deficient for biotinidase. Most patients with holocarboxylase synthetase deficiency have become symptomatic soon after birth, whereas most patients with biotinidase deficiency have not exhibited clinical manifestations prior to 3 months of age. Neverthelass, there is an overlap in the ranges of age of onset. Our four patients had onset at 4 months, 6 months, 2 years and 6 years showing that onset of disease can not indicate the defect enzyme. Biotinidase deficiency was found to be more common in French, Canadians than in other ethnic group. However, no evidence of regional clustering or founder effect was detected. The incidence of combined profound and partial biotinidase deficiency is about 1:60,000. About 25 states in the United States and 25 countries screen their newborns for biotinidase deficiency. True incidence of holocarboxylase synthetase in the newborn population cannot be cited for lack of data based on population screening. It is certainly true to say that holocarboxylase synthetase deficiency is among the most rare of inborn errors, with an estimated incidence of less than 1 case per 200,000 live births. In Thailand, there have no reports in biotinidase deficiency and holocarboxylase synthetase deficiency therefore the incidence is not known. Biotinidase activity has not been routinely measured as only a few laboratories can perform this testing. In this study, biotinidase activity was determined by the colorimetric assay using B-p-ABA as a substrate. The mean control biotinidase activity in the plasma of 245 normal Thai population was 5.63 ± 1.25 nmol/min/ml. The result is similar to the previous report of determining biotinidase activity. The mean normal activity in Brazil is 7.1 nmol/min/ml (range 4.5-10 nmol/min/ml)(27) and 5.29 ± 1.29 nmol/min/ml determined in 129 Japanese healtly adults(26). In the study of effect of neonatal jaundice on biotinidase activity shows the mean normal activity in full-term newborn is 4.99 ± 1.1 nmol/min/ml(28). In 2001, Broda, E. et al.(23) determine biotinidase activities of a healthy population of 651 newborns is 7.01 ± 1.92 nmol/min/ml (range 2.15-16.04 nmol/min/ml). Using our newly established method, four patients had biotinidase activity in the normal range. We concluded that all patients had normal activity. These findings suggest that all patients do not have mutations in *BTD* gene and should have mutations in *HLCS* gene These findings also suggest that biotinidase deficiency may be rare in Thai populations.

In mutation analysis, our patients were all confirmed to have holocarboxylase synthetase deficiency. The results show the most common R508W mutation was found in all patients. Patients 1 and 2 were heterozygous and patients 3 and 4 homozygous for the mutation. In patient1, the second mutant allele was not found in cDNA which may be due to unstability of its RNA, mutation in promoter region or a large deletion. Sequencing of his cDNA showed only the R508W but sequencing of his genomic DNA and restriction enzyme digestion show heterozygous R508W. Of the eight alleles analyzed, the R508W mutation present in six alleles. The R508W is a recurrent mutation that found in several ethnic group. It has been found in different races including Caucasians, Japanese and Chinese. It has been reported in three other patients (Chinese, American and Iranian) at the homozygous and five heterozygous(two Americans, two Japaneses and one Frech)(1). In all these cases, it has been associated with the late-onset holocarboxylase synthetase deficiency.

The structure-function relationship of the holocarboxylase synthetase is not understood. The only information available is that amino acid 448-701 have homology to *E. coli* BirA, yeast HCS and bacterial HCS-related enzymes.The region is probably important for affinity to biotin. The role of this site is probably important for affinity to biotin, affects the catalytic activity of holocarboxylase syntetase deficiency. The previous reports had found different types of mutations inside and outside the biotin-binding region. The most common R508W mutation is located in biotin binding domain. The changes 508Arg > Trp occurred at CpG dinucleotide which tends to be a hot spot for mutation and mutations at CpG dinucleotides have frequently been observed to result in human genetic disease.

There is the hypotheses to explain the origin of these mutations, the polymorphic microsatellite markers in the HLCS gene were identified and analyzed the haplotypes of the patients and 50 normal Thai populations. Tetra-nucleotide repeats, which was previously described by Yang X et al (2000)(42), were amplified. One was located in intron 8 and the other in intron 9 of the HLCS gene. These were named the CAAA repeat and the ATTC repeat, respectively. We found that, in patients R508W present in three haplotypes. These could be explained by the fact that, the nucleotide is proned to mutation. This finding is similar to those mutations are founder mutations in the Japanese population. Three Japanese 508Arg > Trp alleles were associated with several haplotypes. The haplotype of a Taiwanese patient homozygous for the 508Arg > Trp mutation was 2-3/2-3. The mutations were associated with at least two haplotypes and were found in several ethnic groups. Another explanation is it happened once but the mutation is in a region where crossing over happens frequently.

In previous reports the total 30 mutations was identified. We found a novel G505R mutation in patient 2. These mutation is due to G to C substitution at nucleotide position 1513 in codon 508 substitute the wild type amino acid from glycine to arginine. This was not found in 50 normal control supporting its pathogenic role.

In conclusion, this study show the mean normal activity in Thai for the first time and mutation analysis showing that the R508W is the most common mutation will help physician to diagnosis patients with multiple carboxylase deficiency rapidly and give genetic counseling more accurately. Prompt and correct diagnosis is important for these biotin-responsive disorders.

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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 PCR

10X PCR buffer (500 mM KCl, 200 mM Tris-HCl pH 8.4) (Promega)

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

Magnesium chloride (Promega)

Magnesium chloride (Fermentas)

Deoxynucleotide triphosphates (dNTPs) (Promega)

Deoxynucleotide triphosphates (dNTPs) (Fermentas)

Oligonucleotide primers (BSU)

Oligonucleotide primers (Biogenomed)

Taq DNA polymerase (Promega)

Taq DNA polymerase (Fermentas)

100% DMSO

Genomic DNA sample

Restriction enzyme

Fox I (Biolabs)

Hha I (Biolabs)

APPENDIX B

Bufer 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

APPENDIX C

Estimating Haplotype (EH)

File in this Window package.
1.EH.PAS : Source code of EH program.
2.EH.EXE : Executable code of EH program, which is compiled with a maximum of 30 alleles per locus. Loci, 1000 haplotypes, and 3600 genotypes (product of numbers of genotypes at each locus).

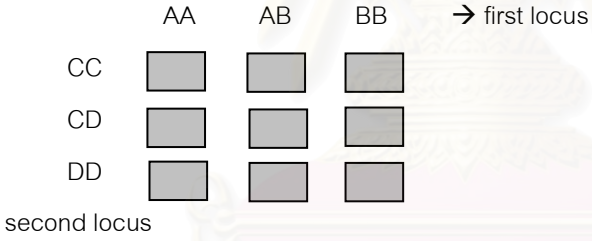
EH.DAT, EH.OUT Sample input and output files.

Protocol for using EH consisted of 2 steps.

1.Create the data file (.dat)

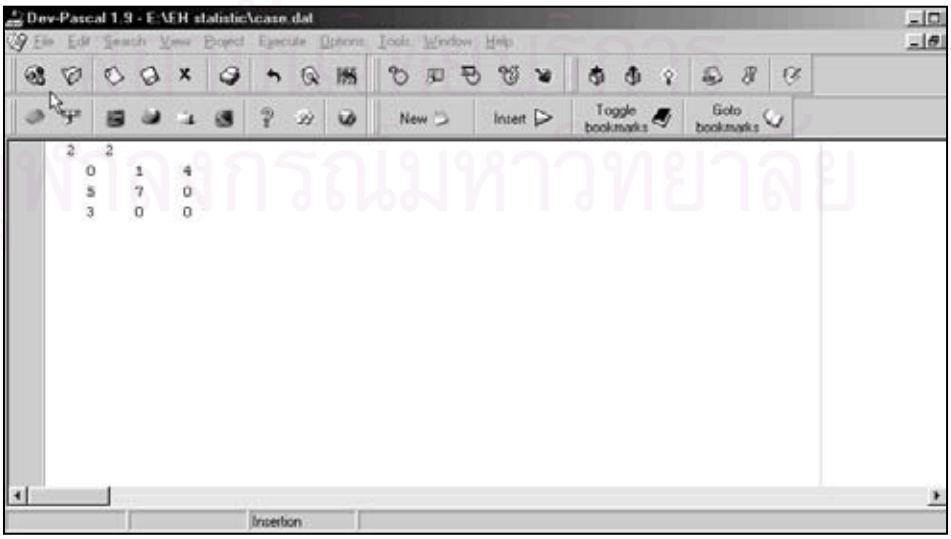
We created the data file in the Pascal program. The first line was the number of alleles at the first locus, number of alleles at the second locus, and so on. Assuming you have 2 loci, each locus has two alleles A & B and C & D, respectively.

The possible haplotypes.

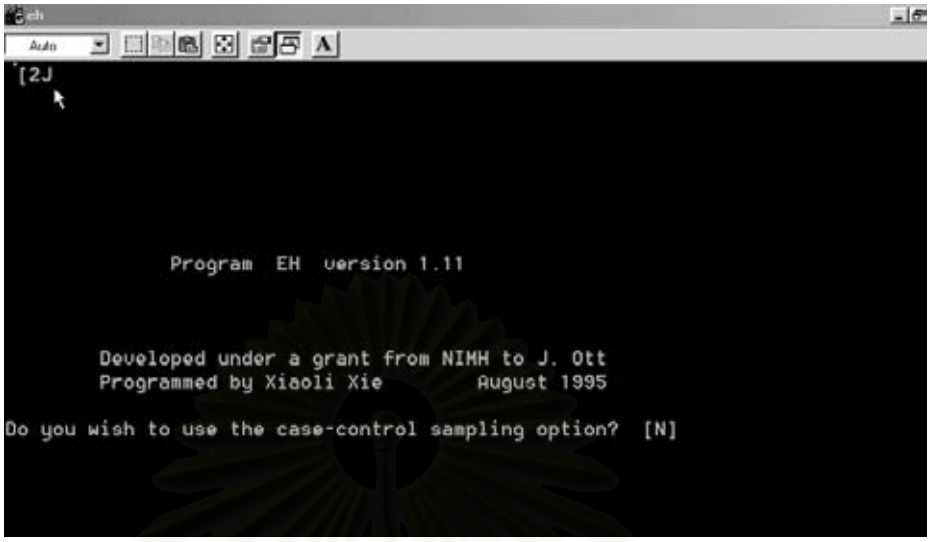


The number of haplotype in the box was filled in Pascal program and save unit as a .dat file.

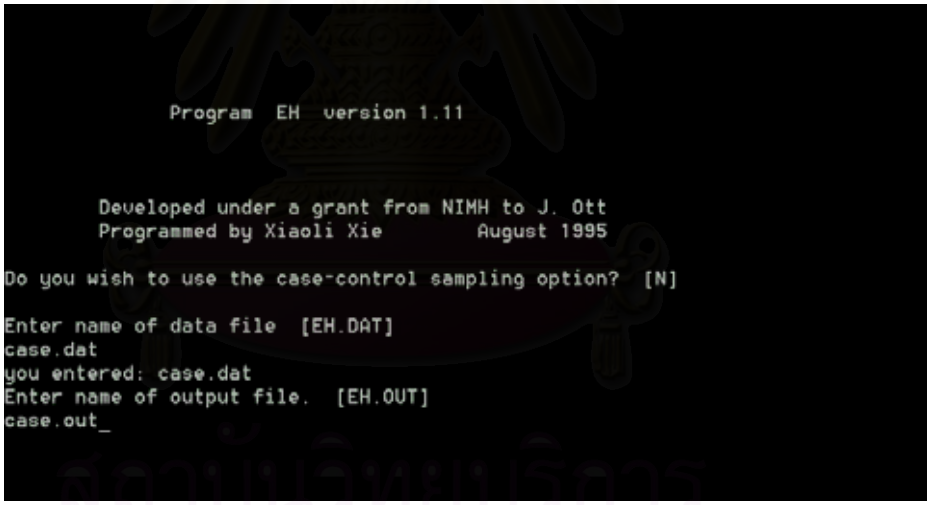
2.Running the EH program



-Running EH program showed the window as below.



-Type your data filename and output filename.



-The output file presented the haplotype frequencies in two kind. “Independent” these are obtained from the allele frequencies at the individual loci. That is, these haplotype frequencies are not estimated but calculated from allele frequencies under the assumption of no association. “w/Association” these are estimated from the data, allowing for association (linkage disequilibrium), assuming Hardy Weinberg equilibrium.

BIOLOGRAPHY

Ms. Suphawee Janklat was born in Bangkok, the capital city of Thailand, in April 14th,1977. In 2001, She reseived her bachelor degree in science (Med.Tech.) from Faculty of Allied Health Science. Chulalongkorn University.

Publication

Shotelersuk, V.Janklat, S.Siriwan, P.Tongkobpetch, S. De novo missense mutation, S541Y, in the p63 gene underlying Rapp-Hodgkin ectodermal dysplasia syndrome. Clin Exp Dermatol 2005;30(3):282-5.