การศึกษาผลของการกลายพันธุ์ของยีนซีอาร์ทีเอพีในผู้ป่วยโรคกระดูกเปราะกรรมพันธุ์

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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GENETIC AND FUNCTIONAL ANALYSES OF A NOVEL MUTATION IN *CRTAP* GENE IN OSTEOGENESIS IMPERFECTA

Miss Natnicha Houngngam

A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	GENETIC AND FUNCTIONAL ANALYSES OF A NOVEL MUTATION IN *CRTAP* GENE IN OSTEOGENESIS IMPERFECTA
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ณัฐณิชา ห่วงงาม : การศึกษาผลของการกลายพันธุ์ของยีนซีอาร์ทีเอพีในผู้ป่วยโรคกระดูกเปราะกรรมพันธุ์. (GENETIC AND FUNCTIONAL ANALYSES OF A NOVEL MUTATION IN *CRTAP* GENE IN OSTEOGENESIS IMPERFECTA) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.นพ.ธิติ สนับบุญ, 56 หน้า.

ที่มา โรคกระดูกเปราะกรรมพันธุ์ (Osteogenesis Imperfecta; OI) ที่มีรูปแบบการถ่ายทอดทางพันธุกรรมแบบลักษณะด้อย (Autosomal Recessive; AR) มีสาเหตุจากความผิดปกติจากการกลายพันธุ์ของยีนที่สร้างกลุ่มโปรตีน collagen-modifying complex ได้แก่ยีน cartilage-associated protein (*CRTAP*), prolyl 3-hydroxylase 1 (*LEPRE1*) and cyclophilin B (*PPIB*) ซึ่งทำหน้าที่ในกระบวนการ post-translation modification ที่กรดอะมิโน proline ตำแหน่ง 986 บนสายแอลฟาคอลลาเจนชนิดที่ 1

วิธีการศึกษา ศึกษาผลที่เกิดจากการกลายพันธุ์ทั้งในเชิงคุณภาพและเชิงปริมาณที่ส่งผลต่อคอลลาเจนชนิดที่ 1 จากผู้ป่วยหญิงอายุ 37 ปี และสมาชิกในครอบครัวที่มีการแต่งงานกันภายในเครือญาติ โดยสมาชิกทุกคนในครอบครัวไม่มีความผิดปกติจากการกลายพันธุ์จากยีนที่สร้างคอลลาเจนชนิดที่ 1 จึงได้ทำการศึกษาหาการกลายพันธุ์ของยีนที่เกี่ยวข้องกับโรค OI ที่มีการถ่ายทอดแบบลักษณะด้อย ได้แก่ยีน *CRTAP*, *LEPRE1* และ *PPIB* หลังจากนั้นศึกษาการทำหน้าที่จากผลที่เกิดจากการกลายพันธุ์ของยีนนี้

ผลการศึกษา พบการกลายพันธุ์ชนิดใหม่ในยีน CRTAP แบบ homozygous ตำแหน่ง c.1106delA ที่ exon 6 ส่งผลทำให้เกิดการเปลี่ยนแปลงของลำดับเบสเป็นตำแหน่งหยุดก่อนกำหนด (premature stop codon) จำนวน 10 กรดอะมิโน ทำให้กระบวนการ 3-hydroxylation ที่สายคอลลาเจนชนิดที่ 1 ที่กรดอะมิโน proline ตำแหน่ง 986 ลดลง 37% แต่ไม่พบความแตกต่างด้านปริมาณของโปรตีน CRTAP เมื่อเทียบกับคนปกติ แต่พบปริมาณ P3H1 ที่ลดลงที่ตำแหน่ง ER

สรุปผลการศึกษา ตำแหน่งที่พบการกลายพันธุ์ซึ่งเป็นส่วนท้ายของยีนอาจส่งผลทำให้เกิด

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2555 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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NATNICHA HOUNGNGAM: GENETIC AND FUNCTIONAL ANALYSES OF A NOVEL MUTATION IN *CRTAP* GENE IN OSTEOGENESIS IMPERFECTA.
ADVISOR: ASST.PROF.THITI SNABBOON, M.D.56 pp.

Background Autosomal recessive type of osteogenesis imperfecta (OI) is caused by mutation in the gene encoding the intracellular collagen-modifying complex: cartilage-associated protein (*CRTA*P), prolyl 3-hydroxylase 1 (*LEPRE1*) and cyclophilin B (*PPIB*). The complex is responsible for one step in collagen post-translational modification, the prolyl 3-hydroxylation of specific proline residues at position 986 of type I collagen chains.

Method We identified the mutation and its effect on the qualitative and quantitative defects in type I collagen production by fibroblasts. A proband, 37-year-old female from a consanguineous family, without defects in type I collagen genes was analyzed the mutation in genes responsible for recessive type of OI: *CRTAP*, *LEPRE1* and *PPIB*. Functional study was performed with Western blot, mass spectrophotometry and immunofluoresense microscopy.

Result A novel homozygous mutation of CRTAP gene, c.1106 delA in exon 6, was identified. Its predicted protein was expected to premature stop codon 10 amino acid downstream. The affected CRTAP protein showed 37% decreased in 3-hydroxylation at α1 (I) Pro 986 compared with the wild type. With normal quantitative analysis and the expected deletion part located on the tail of the protein and decreased P3H1 protein in the ER on immunofluorescence microscopy.

Conclusion These may correspond to its less severe in phenotype of our patient compared with those in recessive group.

Field of Study : Medical Science ..... Student's Signature .....

Academic Year : 2012 ..... Advisor's Signature .....

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LIST OF ABBREVIATIONS

AD	=	Autosomal Dominant
AR	=	Autosomal Recessive
COL1A1	=	Collagen, Type I, Alpha 1
COL1A2	=	Collagen, Type I, Alpha 2
CRTAP	=	Cartilage-associated protein
CYPB or PPIB	=	Peptidyl-prolyl cis–trans isomerase B (cyclophilin B)
DI	=	Dentinogenesis Imperfecta
ECM	=	Extracellular matrix
rER	=	rough Endoplasmic reticulum
Hyp	=	Hydroxyproline
LEPRE1	=	Leucine Proline-Enriched Proteoglycan (leprecan) 1
OI	=	Osteogenesis Imperfecta
Pro	=	Proline
P3H1	=	Prolyl 3-hydroxylase 1

CHAPTER I

INTRODUCTION

1. Background and Rationale

Osteogenesis Imperfecta (OI) or also known as “Brittle bone disease” is known a clinical heterogeneous heritable connective tissue disorder by low bone mass and bone fragility (1) caused by abnormalities of collagen, especially type I collagen that a major component of bone. The incidence of OI disease in occurs in approximately 1:10,000 – 1:20,000 (2). Mode of inheritance with heterogeneous heritable that mostly autosomal dominant (AD), while a few cases of autosomal recessive (AR) form were found about 5-7%.

The most of OI cases have inherited in AD type caused by mutations in *COL1A1* or *COL1A2* gene that two this genes encode type I collagen. Mutations in type I collagen gene result in qualitative and/or quantitative defects in type I collagen production (3, 4) that is overmodification caused by post-translation modification process longer time which play a critical effect for the triple helix, secretion and/or fibril formation including amount of collagen type 1 were decreased (5-7).

Moreover, in the recessive type of OI have identified several loci variants that causes of inherited forms of OI. In four groups of recessive genes following by

3-Hydroxylation defect ; *CRTAP*, *LEPRE1* and *PPIB*

Chaperon defects ; *FKBP10* and *SERPINH1*

Unclassified osteogenesis imperfecta-like or collagen-based disorders ; *PLOD2* and *SP7*

Mineralize defects ; possible *SERPINF1* (8)

In this study, we found the mutations in *CRTAP* gene that defected in the 3-hydroxylation and resulting in the symptoms of the disease are less severe and longevity when compared with previous report.

2. Research questions

Identify a novel mutation of *CRTAP* gene at position c.1106delA and defects in prolyl 3-hydroxylation of type 1 collagen?

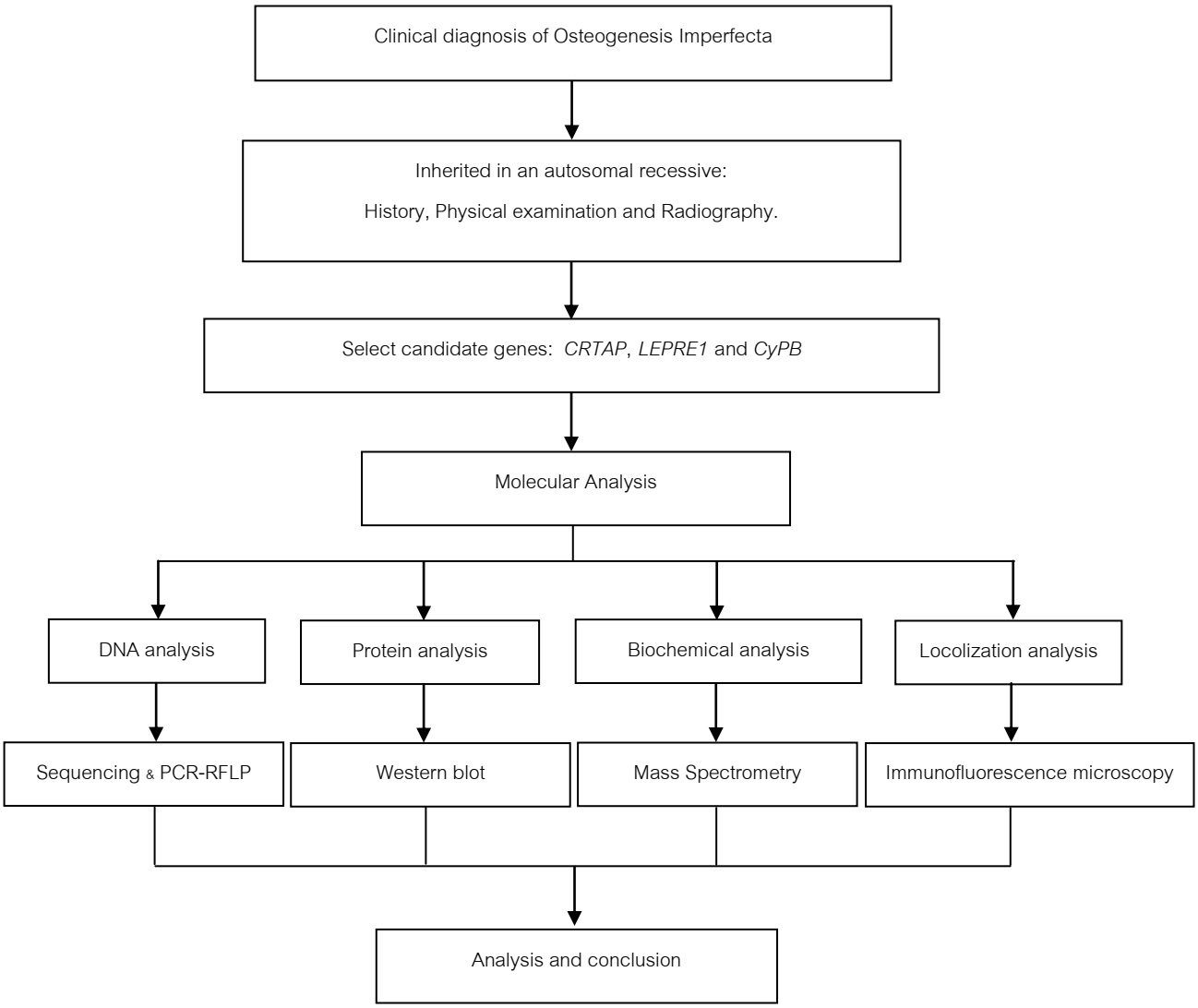
3. Objectives

To determine the effects of a novel mutation of *CRTAP* gene.

4. Hypothesis

Recessive Osteogenesis Imperfecta caused by mutations in the *CRTAP*, *LEPRE1* or *CypB* gene affected the collagen prolyl 3-hydroxylation complex, and the process of prolyl 3-hydroxylation were reduced in collagen type 1

5. Conceptual Framework



6. Assumption

No

7. Limitation

A patient and all family members of patients

8. Expected Benefits and Applications

8.1. This study explains the pathology of OI disease with effect of *CRTAP* gene mutation.

8.2. Classification type of OI patient by use mutation of this gene.

8.3. For genetic counseling, education to parents about risk for having children with genetic defects and preventing of disease.

8.4. This research may lead to improved patient health and may reduce the severity of disease.

9. Ethical Consideration

This study used data from the patient and the patient's personal history information has been kept confidential. Explanation regarding process, methods, potential benefits and risks. Medical personnel will drawed blood and Skin biopsy of patient to study for genetic abnormalities in the laboratories. The only side effect is a slight pain at the of the blood draw, the patient has pain and may have complications including bruising or infection. If complications occur, the researcher is responsible for all aspects. This study was performed only in patients with volunteers who understanded and agreed in inform consent.

This study was approved by the ethics committee of the chulalongkorn memorial hospital.

CHAPTER II

LITERATURE REVIEWS

1. Osteogenesis Imperfecta

Brittle bone disease or Osteogenesis Imperfecta (OI) is one of skeletal dysplasias with a genetic heterogeneity disorder that caused by a multifunction of the process in biosynthesis of collagen type 1, resulting in bone fragility and deformity. It's characterized by connective tissue abnormalities by fragile bones, osteoporosis, grey-blue sclera color, hyperextensible joints, dentinogenesis imperfect (DI) and adult-onset hearing loss (9, 10). The first report in 1979 by David Sillence et al. have been classified into four groups; type I, II, III and IV (1), see table 1.

OI dominant form is due to defects in type I collagen genes (*COL1A1* and *COL1A2*) with a collagen structural mutation. In five years, many genetic defects result in recessive form by mutation in several genes as shown in table 1. The mutations in this genes is common biochemical abnormalities in type I collagen biosynthesis.

Table 1: Nosology of osteogenesis imperfect (2, 11, 12)

Osteogenesis Imperfecta Type	Inheritance	Phenotype	Gene defects
Classical Silence Types			
I	AD	Mild	Null *COL1A1* allele
II	AD	Lethal	*COL1A1* or *COL1A2*
III	AD	Progressive deforming	*COL1A1* or *COL1A2*
IV	AD	Moderate	*COL1A1* or *COL1A2*
Unknown Type			
V	AD	Distinctive histology	*IFITM5(2, 12)*
Mineralization defect			
VI	AR	Mineralization defect, distinctive histology	*SERPINF1*
3-Hydroxylation defects			
VII	AR	Severe (hypomorphic),Lethal (null)	*CRTAP*
VIII	AR	Severe to lethal	*LEPRE1*
IX	AR	Moderate to lethal	*PPIB*
Chaperon defects			
X	AR	Severe to lethal	*SERPINH1*
XI	AR	Progressive deforming (Bruck syndrome 1)	*FKBP10*
Unclassified osteogenesis imperfecta-like or collagen-based disorders			
Bruck syndrome2	AR	Joint contractures	*PLOD2*
Caffey disease	AD	Cortical hyperostosis	*COL1A1*
Osteoblast maturation defects	AR	Moderate	*SP7*

Today, The International Skeletal Dysplasia Society or ISDS is made the grouping of skeletal system disorders in forty groups (as shown in table 2) that defined by molecular, biochemical and/or radiographic criteria and the OI disease arranged into groups 25; Osteogenesis Imperfecta and decreased bone density group (13).

Table 2: Osteogenesis imperfecta and decreased bone density group

Gene / name of disorder	Inheritance	Gene	Protein
Osteogenesis Imperfecta, non-deforming form (OI type 1)	AD	*COL1A1*, *COL1A2*	COL1A1; collagen 1 alpha-1 chain COL1A2; collagen 1 alpha-2 chain
Osteogenesis Imperfecta, perinatal lethal form (OI type 2)	AD, AR	*COL1A1*, *COL1A2*, *CRTAP*, *LEPRE1*, *PPIB*	COL1A1; collagen 1 alpha-1 chain COL1A2; collagen 1 alpha-2 chain CRTAP; cartilage-associated protein, LEPRE1: leucine proline-enriched proteoglycan (leprecan) 1, PPIB: Peptidyl-prolyl cis–trans isomerase B (cyclophilin B)
Osteogenesis Imperfecta, progressively deforming type (OI type 3)	AD, AR	*COL1A1*, *COL1A2*, *CRTAP*, *LEPRE1*,	COL1A1; collagen 1 alpha-1 chain COL1A2; collagen 1 alpha-2 chain CRTAP; cartilage-associated protein, LEPRE1: leucine proline-enriched proteoglycan (leprecan) 1,

		PPIB, *FKBP10*, *SERPINH1*	PPIB: Peptidyl-prolyl cis–trans isomerase B (cyclophilin B) FKBP10: FK506 binding protein 10, SERPINH: serpin peptidase inhibitor clade H 1 *Note : also Bruck syndrome type 1*
Osteogenesis imperfecta, moderate form (OI type 4)	AD, AR	*COL1A1*, *COL1A2*, *CRTAP*, *FKBP10*, *SP7*	COL1A1; collagen 1 alpha-1 chain COL1A2; collagen 1 alpha-2 chain CRTAP; cartilage-associated protein, FKBP10; FK506 binding protein 10, SP7; SP7 transcription factor (Osterix)
Osteogenesis imperfecta with calcification of the interosseous membranes and/or hypertrophic callus (OI type 5)	AD		
Osteogenesis imperfecta, other types Bruck syndrome type 1 (BS1)	AR	*FKBP10*	FK506 binding protein 10 *Note* : intrafamilial variability between OI3 and BS1 Documented
Bruck syndrome type 2 (BS2)	AR	*PLOD2*	Procollagen lysyl hydroxylase 2

2. Inheritance and genetics of OI disease

A heterogeneous group of OI characterized by bone fragility, susceptibility to fractures, deforming and growth deficiency of bone which defects in type 1 collagen biosynthesis, abnormality of collagen structure and including lacking quantity protein in post-translational modification, protein folding and transport to extracellular matrix (14).

2.1 Autosomal Dominant; AD

Autosomal dominant osteogenesis imperfecta was found in type I-IV, defected in *COL1A1* or *COL1A2* gene. Current, mutations of two genes found that more than 1,000 variants (15). For OI type V, mutation in *IFITM5* gene at position c.-14C> T (2, 12). Nearly 90% of all cases of OI type I-IV have mutations in *COL1A1* or *COL1A2* gene (16, 17).

2.2 Autosomal Recessive; AR

Recently, many group of genes involved causes of recessive OI (18, 19). Currently, known nine genes (table 3) that causes recessive mutation by clinical or radiological phenotype compared with distinguish between OI type II, III or IV that caused by type I collagen gene; *COL1A1* and *COL1A2* gene (20). M. LABUDA, *et al*. have discovered the transmission form of a novel recessive OI that was detected in a small First Nation community from northern Quebec that don't have mutations in the *COL1A1* or *COL1A2* gene and called this OI type VII, recessive variant were mapped on chromosome 3p22-24.1 (21)**.** Following, L.M. Ward, *et al*. has been described the phenotype of OI type VII is very mild to very severe with characteristic by fractures

at birth, bluish sclera, deformity of the lower extremities, osteopenia and histopathology of iliac crest bone were similar to OI type I (22).

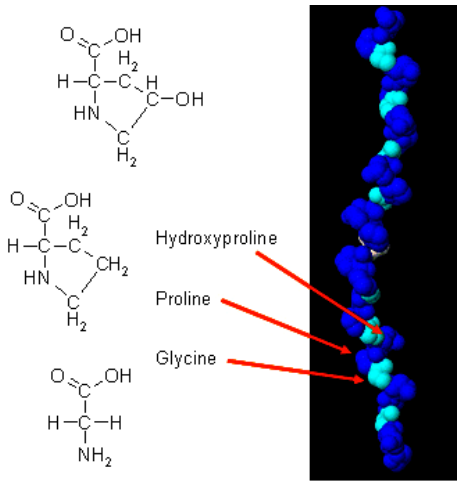
Table 3: Name of the analyzed genes and models recessive form of OI

Gene/Protein	Locus	Models	
		Mouse	**Human**
CRTAP	3p22.3	*Crtap* knockout; severe not lethal (23)	OI type VII; moderate to severe
LEPRE1/P3H1	1p34.2	P3H1 null; knockout is not lethal (severe) (24)	OI type II,III; severe to lethal
PPIB/CYPB	15q22.31	*Ppib* knockout; appeared normal at birth but with reduced body size and weight, kyphoscoliosis at eight weeks, death between 40 and 50 weeks of unknown etiology (25)	OI type II, III, and III/IV ; moderate to lethal
FKBP10/ FKBP65	17q21.2	No mouse model reported	OI type III/IV ; moderate severe
SERPINF1/ PEDF	17p13.3	Serpinf1-/- mice lacking exons 3-6 (26)	OI type VI
SERPINH1/ *HSP47*	11q13.5	*Hsp47* knockout; Embryonic lethal (27, 28)	OI type III; severe
PLOD2/LH2	3q24	No mouse model reported	Bruck syndrome
SP7/ Osterix	12q13.13	Osx knockout	OI type IV (29)
BMP1/BMP1	8p21.3	*Bmp1* knockout; Perinatal lethal (30)	OI type III

3. Type I collagen Biosynthesis

Collagen is the main protein of connective tissue found in endothermic animals and mammals. The collagen structures are macromolecule in the form of long fibers, mostly found in fibrous tissues, it abundant 90% of bone (5). The fibroblast cell is the most common cell which synthesizes collagen and extracellular matrix (31). The morphology and properties of collagen are associated with scaffolding structure and high tensile strength that is a important component of cartilage, bones, tendons, ligament, cornea and skin (32, 33)

Type I collagen is a heterotrimeric structure which built up from three alpha polypeptides chains; 2 alpha1 and 1 alpha 2 chain to form triple helix structure, that each chain with a left-handed helical conformation and coiled around to form characteristic right-handed. Each alpha polypeptide chain has the repeating structure Gly-X-Y triplet, which the X and Y positions can be any amino acid but high content of proline and hydroxyproline (see figure 1) (33, 34).



Figures 1: Collagen triple helix and it's formula structure (35)

Type I collagen I is the most abundant collagen synthesized by fibroblasts cell or osteoblasts, multistep process to starts with transcription, translation of individual collagen type I gene and post-translational modifications of them being unique to collagen proteins. The forming of fibril collagens were synthesized as procollagens by *COL1A1* and *COL1A2* genes; pro-α1 (I) and pro-α1 (II). Found that nearly 90% of patients OI are caused by mutation of *COL1A1* or *COL1A2* genes (4, 36). After the synthesis of procollagens, it translocated to the lumen of the rough endoplasmic reticulum (rER) (37) and modifications to removal of signal peptides and post-translational modification process by chaperon machinery. The post-translational modifications of collagen by hydroxylation of the proline and lysine residues occurs on the the alpha chain before formation of a triple helix as demonstrated in figure 2. Hydroxylation of lysine is necessary for cross-link of intermolecular fiber molecules (38, 39). For the proline, proline 4-hydroxylase is required to convert proline residues to hydroxyproline residues (40, 41) and proline 3-hydroxylase is less know well but shown to unfolded collagen or procollagen is retained within the ER (6, 42-44).

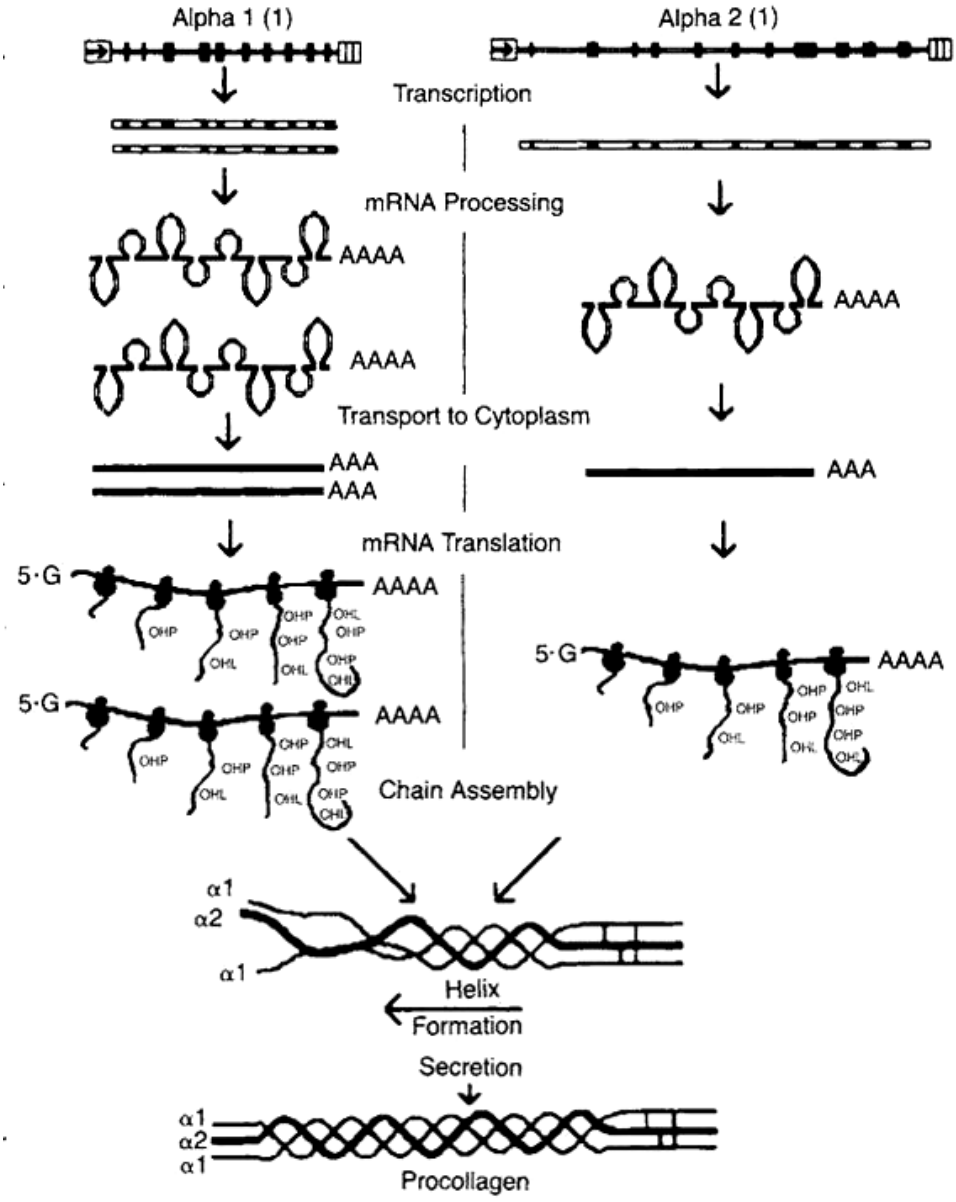


Figure 2: Collagen type I biosynthesis and the post-translation modifications of collagen chain when procollagen molecule is completed and secreted from the cell to extracellular matrix (ECM) (9).

4. The collagen 3-hydroxylation complex

Components of the collagen 3-hydroxylation complex including Prolyl 3-hydroxylase 1 (P3H1 or LEPRE1), Cartilage-associated protein (CRTAP) and Peptidyl-prolyl *cis-trans* isomerase B (PPIB or cyclophilin B) (45). The complex was corresponds into a 1:1:1 ratio within the ER for hydroxylation in the post-translational modification process, that specific only one proline at residue 986 in the alpha I chains (see figure 3) (23).

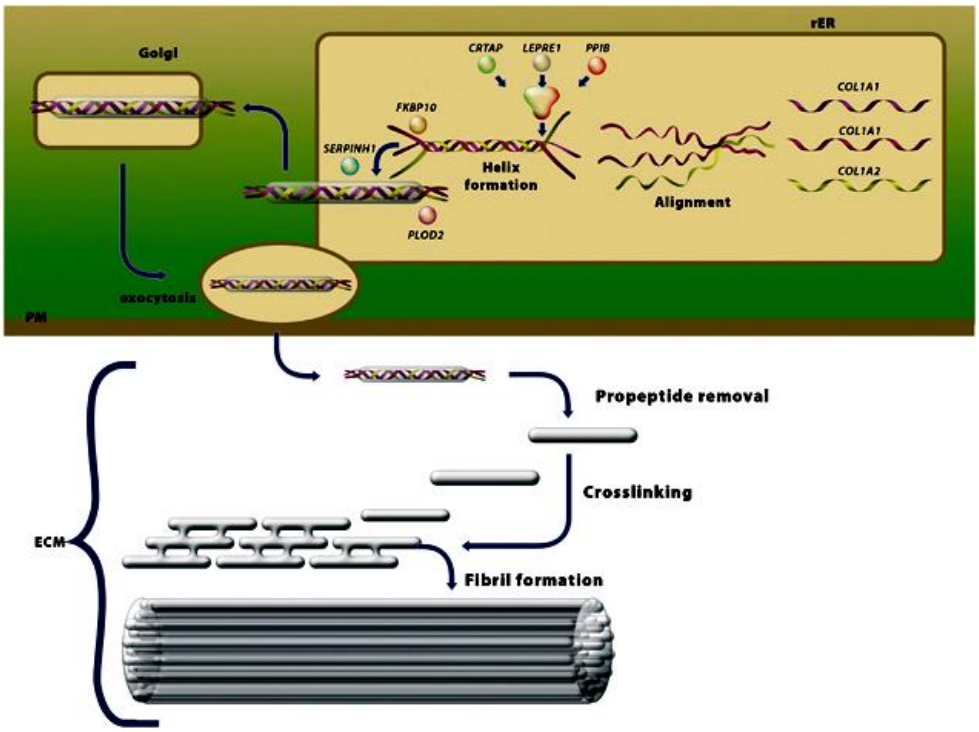


Figure 3: Collagen type I biosynthesis and indication of genes known to be involved in OI (18)

4.1 Prolyl 3-hydroxylase 1; LEPRE1

Prolyl 3-hydroxylase1 or P3H1 is the enzymatic component of collagen 3-hydroxylation complex that encoded by *LEPRE1* gene. The P3H1 have contains a KDEL sequence for endoplasmic reticulum retrieval and it's crucial for collagen modification (46, 47). The genetic defects by P3H1 deficiency with OI type VIII and phenotypic characteristics overlap with the type II or type III. The patient of OI type VIII is also presents severe or lethal autosomal recessive form and has the distinguishing features of white sclera, undertubulated long bones and rhizomelia like type VII. In *LEPRE1* knockout mice, found that significant hearing loss also common problem in patient OI but milder phenotype than patient OI type VIII (24).

4.2 Peptidyl-prolyl *cis–trans* isomerase B; PPIB or CYPB

Cyclophilin B is a member of the cyclophilin family or prolyl *cis-trans* isomerases and highly related family member that expression within the ER (48, 49). The CYPB form a complex with the P3H1 and CRTAP proteins for prolyl 3-hydroxylation complex at position 986 prolyl residue of triple helix. The activity of peptidyl-prolyl *cis-trans* isomerase all isoforms, to changed the structure of cis conformation of prolines to the trans conformation (50). This process suggests the role to facilitate the folding of the procollagen biosynthesis (51). Mutations in *PPIB* found in OI type IX cause premature termination codons or misfolded protein and result to moderate to lethal OI (52, 53). The phenotype of defects in *PPIB* are similar to OI type VII, VIII but rhizomelia not included. However, absence of PPIB in activity of collagen 3-hydroxylation complex is not enough to Pro986 α1 (I) 3-hydroxylation (54). Lacking the $Ppib^{-/-}$ gene in mice found abnormal morphology of

collagen, developed kyphosis, severe osteoporosis and needful to interaction of the P3H1-CRTAP-CYPB complex for formation of collagen (25).

4.3 Cartilage-associated protein; CRTAP

In human, the cartilage-associated protein is proteins that encoded by the *CRTAP* gene, located on chromosome 3p22.3 and encode protein with 401 amino acid residues (55). The CRTAP is the helper protein of the collagen 3-hydroxylation complex that highly homologous amino of P3H1 but in the C-terminus is lacking of catalytic domain or enzymatic active site (7, 23, 56, 57). The CRTAP is localized mainly in the endoplasmic reticulum and expressed in the skeletal system by chondrocytes, osteoblasts and osteoclasts (58). The *Crtap*$^{-/-}$ deficiency in mouse has characterized by shortened proximal segment of limbs (rhizomelia), growth deficiency, curvature of the spine (kyphosis) and osteopenia, that moderately severe connective tissue disorder (59). For human, CRTAP deficiency has showed moderate to lethal recessive; OI type VII that presents with osteochondrodystrophy, rhizomelia, growth deficiency, severe osteoporosis and also with white sclera (23, 58, 60-62). The *CRTAP* mutations that almost all reported cause with framshifts mutation result in nonsense-mediated mRNA decay (NMD), loss of CRTAP protein and absence of the α1(I) 3-hydroxylation (58) see table 4.

Table 4: The *CRTAP* database all sequence variants (15, 58)

Pathogenic	Exon	DNA change	Mutation effects/ Protein	Disease	Ethnic origin	Reference
Pathogenic	1	c.3G>A+c.278_293dup	Other	OI II	German	Barnes et al., 2006
Pathogenic	1	c.21_22dup+c.21_22dup	Frameshift / p.(Ala8Glyfs*6)	OI II/III	Caucasian	van Dijk et al., 2009
Pathogenic	1	c.22dupG+c.22dupG	Frameshift / p.(Ala8Glyfs*153)	OI VII	Pakistani	Italy:Verona
Pathogenic	1	c.24_31del+c.24_31del	Frameshift/ p.(Ala10Serfs*148)	OI II/III	Lebanese	Baldridge et al., 2008
Unknown	1	c.38C>A+c.469A>G, c.923-2A>G	Missense / p.(Ala13Glu)	OI III	Caucasian	van Dijk et al., 2009
Pathogenic	1	c.118_133delinsTACCC + c.118_133delinsTACCC	Frameshift/ p.(Glu40Tyrfs*117)	OI VII	Egyptian	Valli et al., 2011
Pathogenic	1	c.198C>A + c.471+2C>A	Nonsense / p.(Tyr66*)	OI	Caucasian	van Dijk et al., 2009
Pathogenic	1	c.200T>C+c.200T>C	Missense / p.(Leu67Pro)	OI III	Iranian	Baldridge et al., 2008
Pathogenic	1	c.278_293dup	Frameshift / p.(Gly99Alafs*67)	OI II/III	Caucasian	Baldridge et al., 2008
Pathogenic	1	c.278_293dup + c.3G>A	Frameshift / p.(Gly99Alafs*67)	OI II	German	Barnes et al., 2006
Pathogenic	1	c.404delG+c.404delG	Frameshift / p.Ser135Thrfs*39	OI	Moroccan	van Dijk et al., 2009
Pathogenic	1	c.469A>G+c.38C>A, c.923-2A>G	Missense / p.(Lys157Glu)	OI III	Caucasian	van Dijk et al., 2009
Pathogenic	1i	c.471+1G>C + c.471+1G>C	Splice site	OI II	Pakistani	Barnes et al., 2006
Pathogenic	1i	c.471+1G>C + c.471+1G>C	Splice site	OI II	Icelandic	Bodian et al., 2009
Pathogenic	1i	c.471+2C>A+c.198C>A	Splice site	OI IIC/III	Caucasian	van Dijk et al., 2009
Pathogenic	1i	c.471+2C>A+c.471+2C>A	Splice site	OI II/III	-	van Dijk et al., 2009
Pathogenic	1i	c.471+2C>A+ c.471+2C>A	Splice site	OI II	-	Italy:Bologna
Pathogenic	1i	c.472-1021C>G + c.472-1021C>G	Other	OI VII	First Nations Canadian	Morello et al., 2006
No known Pathogenicity	2	c.534C>T	Silent / p.(=)		-	-

No known Pathogenic	2	c.558A>G	Silent / p.(=)		-	-
pathogenic	2	c.561T>G+ c.561T>G	Nonsense / p.(Tyr187*)	OI	Saudi Arabian	Shaheen et al., 2012
pathogenic	3	c.634C>T+ c.826C>T	Nonsense / p.(Arg212*)	OI VII	African American	Chang et al., 2010
pathogenic	4	c.804_809del+ c.804_809del	In-frame deletion / p.Glu269_Val270del	OI III	Sudanese	Ben Amor et al., 2011
pathogenic	4	c.822_826delinsT+ c.278_293dup	Frameshift/p.(Lys274Asnfs*11)	OI	Caucasian	Baldridge et al., 2008
pathogenic	4	c.826C>T+ c.826C>T	Nonsense / p.(Gln276*)	OI VII	African American	Barnes et al., 2006
pathogenic	4	c.826C>T+ c.826C>T	Nonsense / p.(Gln276*)	OI VII	African American	Chang et al., 2010
pathogenic	4	c.879delT+ c.879delT	Frameshift / p.Phe293Leufs*16	OI II	Indian	Morello et al., 2006
pathogenic	4i	c.923-2A>G+c.38C>A, c.469A>G	Frameshift / r.923_936del	OI III	Caucasian	van Dijk et al., 2009
No known Pathogenicity	5	c.1032T>G	Silent / p.(=)	-	-	dbSNP
No known Pathogenicity	5	c.1044G>A	Silent / p.(=)	-	-	dbSNP

CHAPTER III

MATERIALS AND METHODS

1. PCR and Sequence Analysis

The patient and all members in her family were selected for screening in *CRTAP* gene and no mutation in *COL1A1*, *COL1A2*, *LEPRE1* and *PPIB* genes. Genomic DNA (gDNA) was extracted from the proband and her siblings using peripheral blood by phenol-chloroform method. The seven exons of *CRTAP* gene and flanking intronic sequences were sequenced (Macrogen Inc., Korea). The *CRTAP* gene was selected because of recurrence in this family of consanguinity. The patient was skin biopsy and cultured by informed. These studies have been approved by the Institutional Review Boards of Faculty of Medicine, King Chulalongkorn Memorial Hospital.

1.1 PCR reaction contained the following:

Stock	Final concentration
10X PCR buffer	1x
25 mM $MgCl_2$	1.5 mM
10 mM dNTP	200 µM
10 pmole forward primer	0.2 pmole
10 pmole reverse primer	0.2 pmole
Tag DNA polymerase	2 Units
DNase, RNase-free H_2O	up to a final volume of 20 ml

The PCR condition was performed in MJ MiniTM Gradient Thermal Cycler, BIO-RAD, USA as the following:

Step	Time/Temperature	Cycle
Denaturation	5 min at 94 ^{o}C	1
Denaturation	30 sec at 94 ^{o}C	38
Annealing	30 sec at 60 ^{o}C	
Extension	30 sec at 72 ^{o}C	
Final extension	7 min at 72 ^{o}C	1

After finish, the PCR products were checked by 1% agarose gel electrophoresis.

Table 5: PCR primers for *CRTAP* amplification and sequencing

Exon	Forward Oligo (5' to 3')	Reverse Oligo (5' to 3')	Size (bp)
1	CACCGTCCTCTTTCCTTTCC	TATCAGGCACTTTGTTGGGT	819
2	AAGTTATAGCAACACAAAGT	TAAAAGTCA ATCATT TCTAT	405
3	CTTGGTTCCCTTTGAGATTT	AGAAACCAGACATCTAAGGA	315
4	TTTTCATTTGGGCAGGAG	AGCATAGCACCAGGGTTGTC	338
5	GAAGAAGGACTGTGGAGAAG	ATGAAGAGAAAAGGGAAGGT	297
6	CAGCCATTACCACTATCTA	GTCCCCTCTGTTTCTTACT	327
7	AAAGCAGACAGTGGTGATG	TGGGAAAAGGACAAAGAAT	223

2. PCR-RFLP

The PCR products were confirmed by restriction enzyme digestion (*BseR*I, New England Biolabs, U.S.)

Reaction contained the following:

Stock	Final concentration
10x NEB Buffer4	1X
*BseR*I	1 Unite
PCR products	5 µl
DNase, RNase-free H_2O	up to a final volume of 15 ml

The PCR-RFLP condition was performed in 1 hour at 37°C and after finish; the products were checked by 2.5 % agarose gel electrophoresis.

3. Primary fibroblast cell culture

Primary dermal fribroblasts from proband wer cultured in DMEM (Invitrogen, UK) and supplemented with 10% FBS (HyClone, U.S.). For 3-hydroxylation, media contained with DMEM free FBS.

4. Western blotting

Preparation protein lysate from primary fibroblasts cell culture were analyzed by SDS-PAGE, 6X sample loading buffer were added to protein samples. The

mixtures of protein into the wells of the SDS-PAGE gel, run the gel for 1 hour at 100 voltages in 1X running buffer (see appendix F).

After electrophoresis, the proteins from the gel were transferred to PVDF membranes (Millipore, USA) and filter papers were equilibrated in 1X transfer buffer in 20% Metanol (see appendix F) with constants voltage at 100 voltages for 90 min. After blotting, block the membrane with 5% skim milk in 1X PBS buffer (see appendix F) overnight at 4°C. Optimization of the primary antibody dilution 1: 1000 of monoclonal primary antibody to CRTAP (Abcam, UK) in 1X PBS/0.1% tween20 for 1 hour at RT then washed with 1XPBS/0.1%tween20 for 15 minutes, 3 times. For secondary antibody; goat anti-mouse IgG (Millipore, USA) conjugated with horseradish peroxidase (HRP) dilution 1: 5000 in 1X PBS/0.1% tween20 were incubated at RT for 1 hour.

The detection step, HRP substrate (Millipore,USA) based on Chemiluminesence (xRS system, BIO-RAD) reaction was performed.

4.1 Stripping and Reprobing membrane

The membrane was submerged in 50 ml stripping buffer (see appendix F) and incubated at 50°C for 30 min. Wash the membrane 10 minutes for 3 times with 30 ml of 1X PBS/0.1%tween20. Blocking the membrane with 5% skim milk in 1X PBS/0.1%tween20. Incubate normalize primary antibody with dilution 1:1000 of GADPH anti rabbit and secondary antibody, 1:5000 of goat anti-rabbit. And the detection step, HRP substrate based on Chemiluminesence (xRS system, BIO-RAD) reaction was performed.

5. Biochemical analysis

For 3-hydroxylation of proline at position 986, used media from primary fibroblast of proband and control were digested with pepsin at 4 °C for 24 hrs. Precipitate collagens with 1 M NaCl and separated by SDS-PAGE, gel band stained with Coomassie Blue.

5.1 In-gel Tryptic Digest for Protein

The collagen gel band was prepared for mass spectrometry by David Miyamoto 2/12/2002;

5.1.1. Excise band from Coomassie gel and cut gel band into 1 mm tubes using clean blade on a clean glass surface. Transfer to 1.5 ml eppendorf tube.

5.1.2. Remove excess water with pipette. Add 25-35 µl acetonitrile to tube to cover gel pieces and incubate 10 minutes at room temperate to dehydrate and shrink gel pieces.

5.1.3. Acetonitrile was removed with pipette and speed-vac to dryness for 10 minutes.

5.1.4 Swell gel particles in 150 µl 10 mM DTT in 100 mM ammonium bicarbonate and incubate 1 hr at 56°C.

5.1.5 Cool to RT. Replace DTT solution with 150 µl 55 mM iodoacetamide in 100 mM ammonium bicarbonate and incubate 45 minutes at RT in the dark with occasional vortexing.

5.1.6 Remove solution and wash gel pieces with150 µl 100 mM ammonium bicarbonate and incubate 10 minutes at RT.

5.1.7 Ammonium bicarbonate was removed with pipette and adds 150 µl acetonitrile to dehydrate gel pieces and incubates 10 minutes at RT.

5.1.8 Repeat wash steps 6-7. Remove acetonitrile and speed-vac to dryness for 10 minutes.

5.1.9. Place tubes in ice water bath and swell gel particles in 25-35 µl digestion buffer. Incubate 45 minutes in ice water bath. Digestion buffer consists of 12.5 ng/µL trypsin (Promega, USA) in 50 mM ammonium bicarbonate.

5.1.10 Remove trypsin-containing buffer. Add 5-10 ul mM ammonium bicarbonate without trypsin to keep pieces wet during cleavage. Incubate overnight at 37°C

5.1.11 Spin down gel pieces. Save supernatent in a separate PCR tube

5.1.12 Add 20 µl 20 mM ammonium bicarbonate to cover gel pieces and incubate 10 minutes at RT. Transfer supernatant to the PCR tube from step 11

5.1.13 Add 25 µl 5% formic acid, 50% acetonitrile to the gel pieces and incubate 20 minute at RT.

5.1.14. Spin down to remove formic acid/acetonitrile solution and save in the same PCR tube from step 11

5.1.15 Repeat formic acid extraction (step 13-14) twice more.

5.1.16 Dry PCR tube in speed-vac to complete dryness and store at -20°C until analysis.

6. Locolization analysis

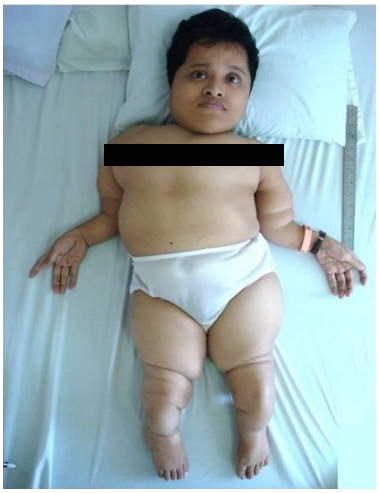
Primary fibroblast cell from proband and control was cultured on 8 chamber slides and grew for 2 days. Washing with PBS and fixed cell in 4% paraformaldehyde for 15 min. Permeabilized with 0.25%TrixonX-100 in 1x PBS at 4°C for 10 min. Blocking step with 1%BSA in 1xPBS at 4°C for 1 hr. Then incubated with primary antibody; CRTAP (Abcam, ab56651), LEPRE1(Millipore, ST1622-100UG), Type I collagen (Abcam, ab90395) and PDI-ER marker (Abcam, ab3672) at 4°C for 1 hr. Secondary antibody Alexa Fluor ® 488 goat anti-rabbit IgG (H+L) (Invitrogen A11004, UK), Alexa Fluor® 568 goat anti-mouse IgG (H+L) (Invitrogen A11008, UK) at 4°C for 1 hr. Nucleus were counter stained with 4, 6-diamino-2-phenylindole hydrochloride (DAPI) (Invitrogen, UK). Stained cells were visualized under confocal microscope (Zeiss LSM 510 Inverted Meta confocal microscope, Germany**)**

CHAPTER IV

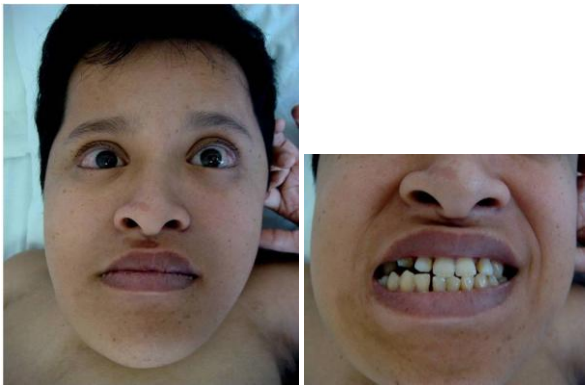
RESULTS

1. Clinical report

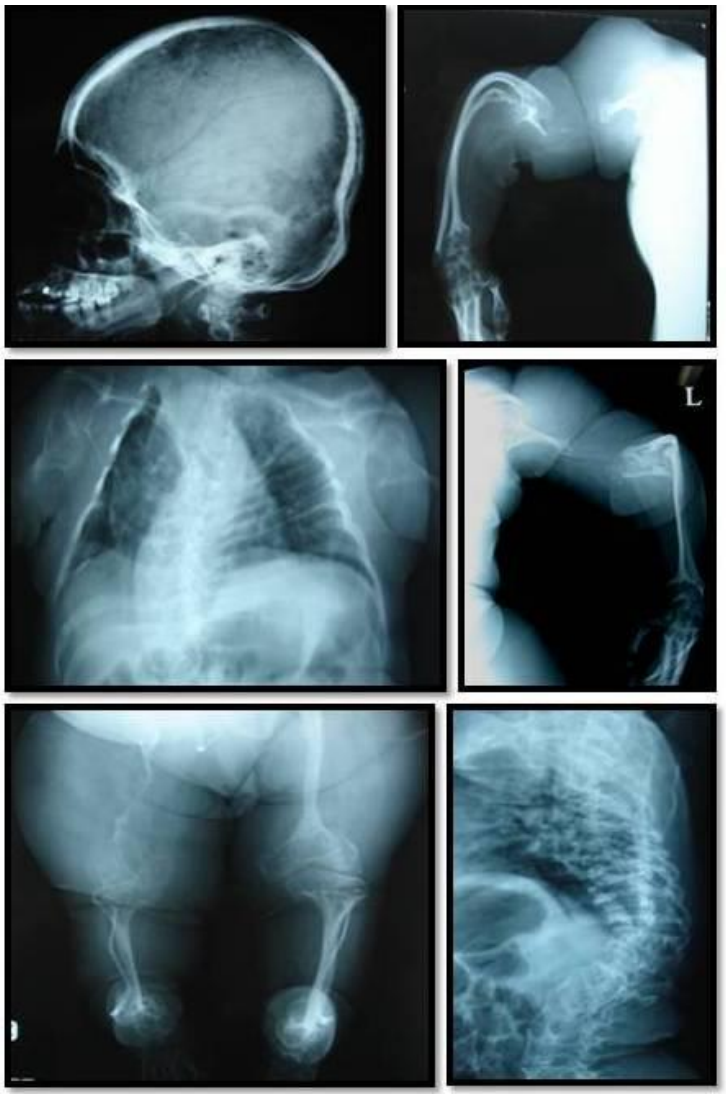
A patient with multiple fractures of both upper and lower extremities at different stages of healing, no blue sclera, no yellow or bluish-grey discoloration of teeth, diffuse osteopenia, short and deformed long bones with broad metaphyses and thin diaphyses, kyphoscoliosis with compressed vertebral bodies numerous wormian bones.



Figures 4: Characteristics of the patient



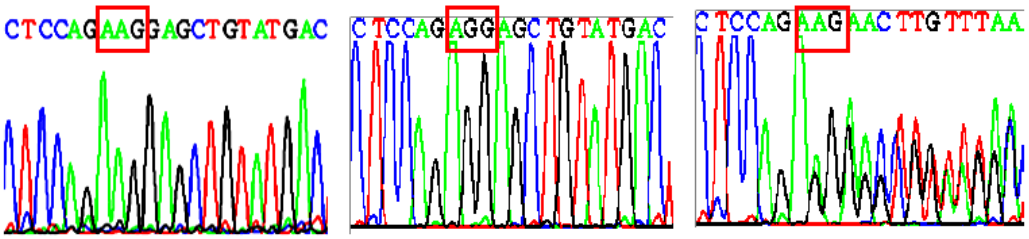
Figures 5: No yellow or bluish-gray discoloration of teeth



Figures 6: The radiograph of patient consistent with homozygous at c.1106delA of *CRTAP* gene (*p.Lys369Argfx*10*) this mutation may correspond to less severe compared with those in recessive group

2. Mutation of *CRTAP* gene

The patient was a novel homozygous at c.1106delA at position 369 in exon 6 of *CRTAP* gene. The father and all members in her family; III-2, IV-1, IV-4, IV-5, IV-6, IV-8, IV-9, IV-10, V-3 were heterozygous for this mutation that confirmed by PCR-RFLP (Figure 10). The homologous mutation amino acid sequence alignment with clustalw2 tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) causes framshift with a premature termination codon at downstream for 10 amino acids (Figure 8, 9).



Normal | Patient; c.1106delA Homozygous | Siblings; c.1106delA Heterozygous

Figures 7: Sequencing of patient, gDNA shows a novel homozygous in exon 6 of *CRTAP* gene. This mutation deleted 1 nucleotides result in frameshift and the exon 7 was losing.

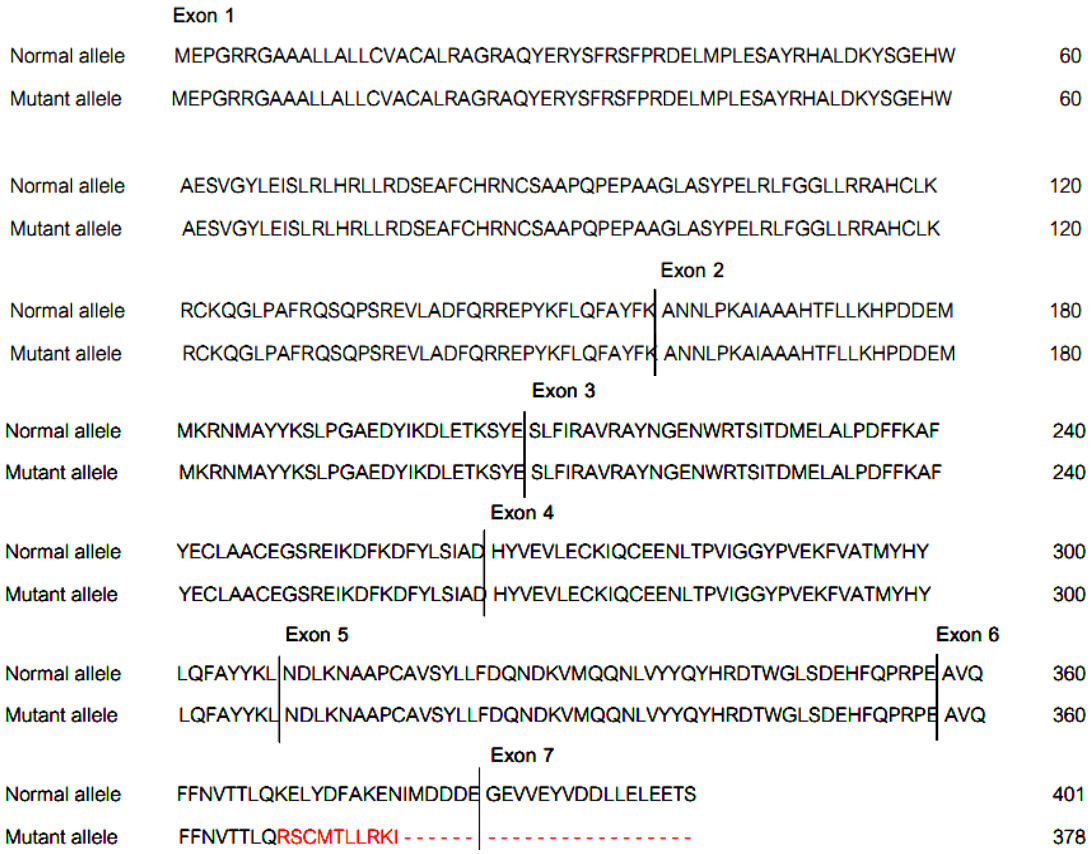
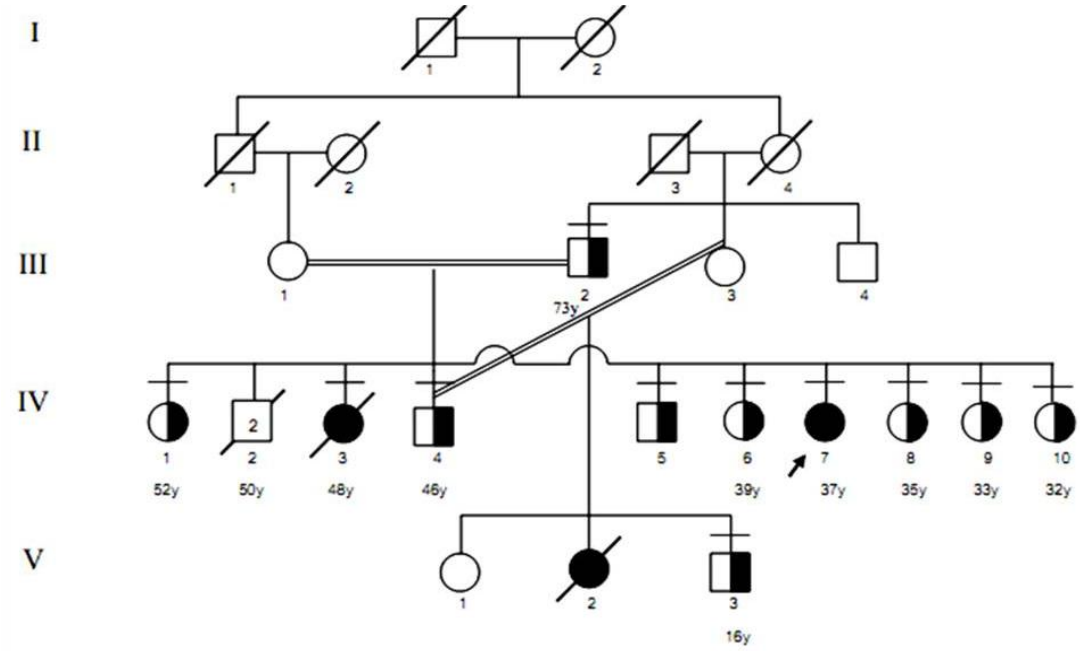
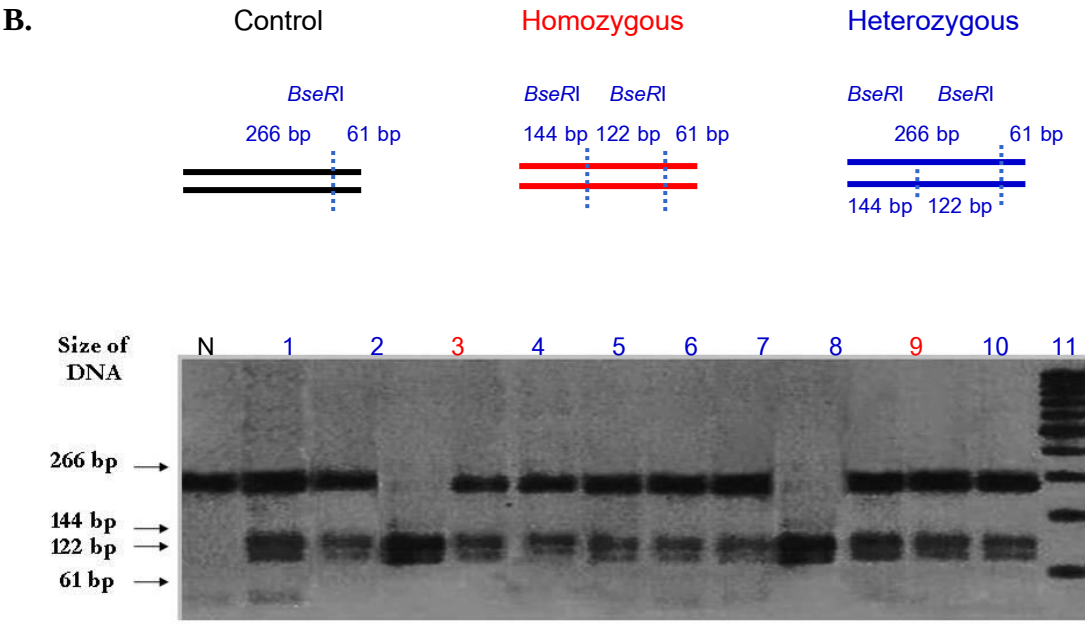


Figure 8: Amino acid sequence alignment result in frameshift with premature stop codon 10 amino acids downstream (*p.Lys369Argfx*10*) and all exon 7 was deleted.

A.

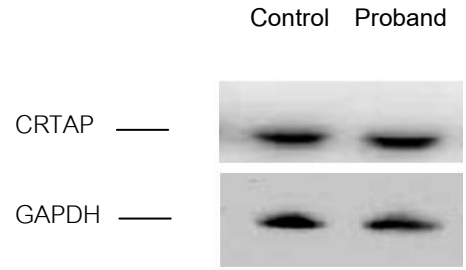


B.

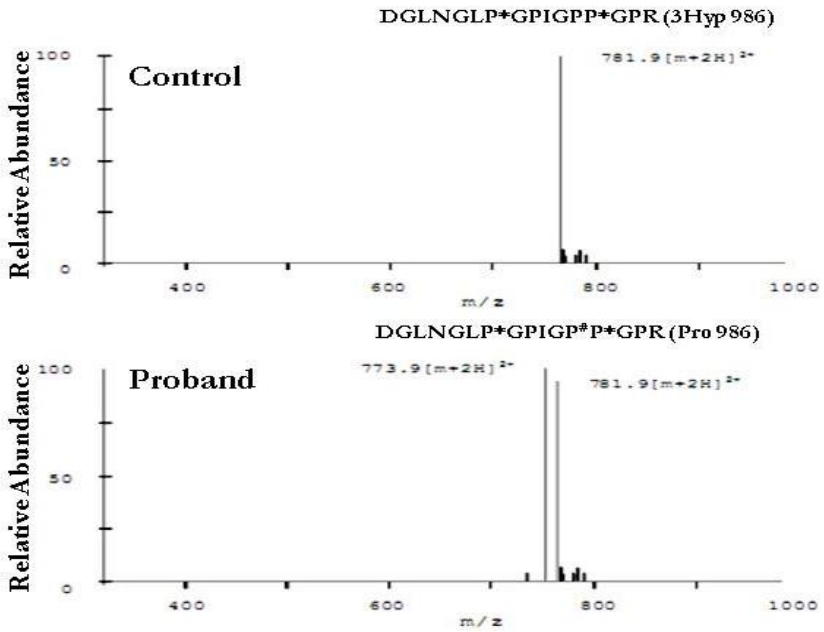


Figures 9: (**A)** Pedigree of the patient with obligate heterozygotes; III-2, IV-1, IV-4, IV-5, IV-6, IV-8, IV-9, IV-10, V-3 and affected individual; IV-3, V-3. **(B)** A homozygous mutation, *p.Lys369Argfx*10* was confirmed by PCR-RFLP.

3. Protein analysis

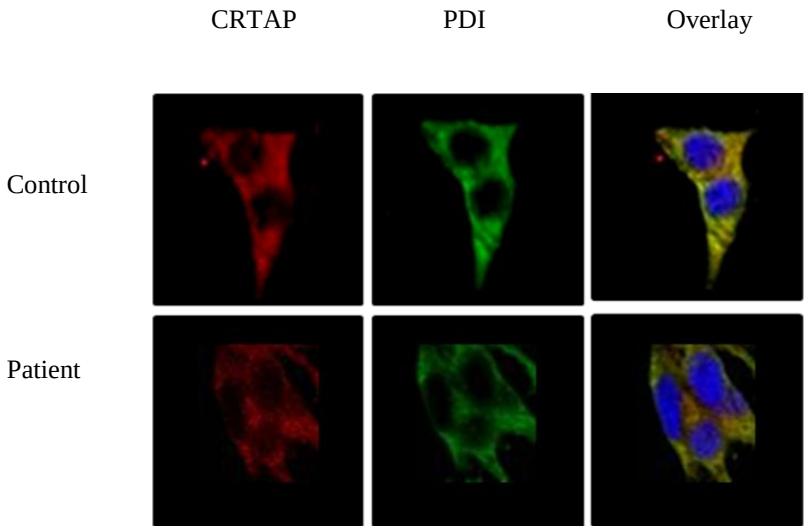


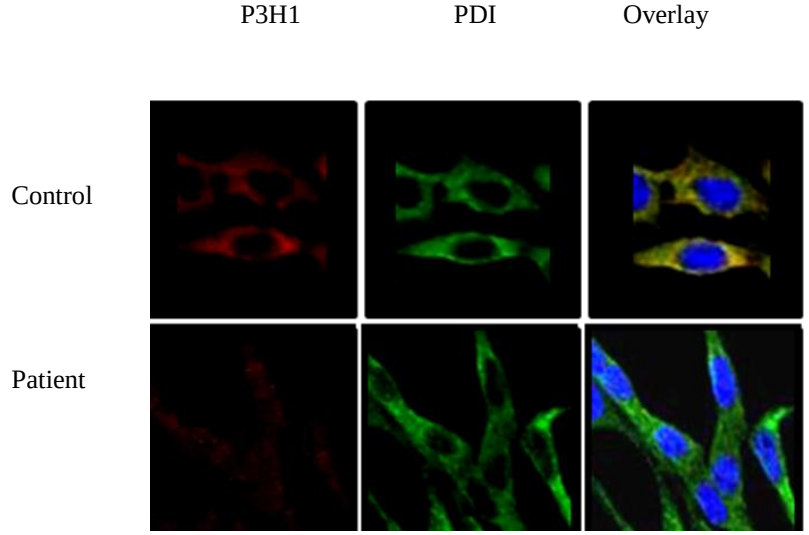
Figures 10: The expression of CRTAP protein level on the basis of western blotting analysis was not difference when compared to the control.

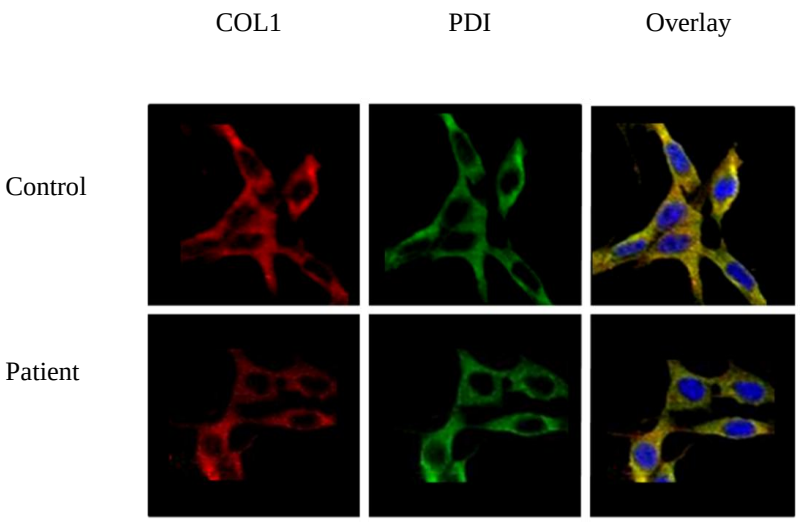


Figures 11: The 3-hydroxylation at α1 (I) at proline position 986 showed 37% decreased

4. Localization analysis







Figures 12: Immunofluoresence detection of fibroblasts of the patient and control, we can see the small amount of P3H1 localized in PDI-ER in cell patient, CRTAP and COL1 protein were similar localized to the ER.

CHAPTER V

DISCUSSION AND CONCLUSION

In the present study, we have identified a novel mutation in *CRTAP* gene in proband affected by less severe OI, caused by a homozygous c.1106delA at position 369 in exon 6 of *CRTAP* gene and determine to the function of CRTAP.

The CRTAP a homozygous c.1106delA (*p.Lys369Argfx*10*) in proband IV-3 and affected in V-3 demonstrates that interruption of the amino acid sequence of CRTAP that can lead to recessive OI. Many mutations in this gene have a broad range of clinical presentation since milder phenotype to lethal in OI (23). The deficiency of the components of the collagen 3-hydroxylation complex is caused of recessive OI due to mutation in all three members, *CRTAP*, *LEPRE1* or *PPIB* gene (53, 58, 60, 62).

In this case, the patient had presented at 12 weeks with disproportionate short stature and deformed of long bones. Generalized osteopenia with multiple fractures and deforming extremities; short and deformed long bones; broad metaphyses and thinner diaphyses was evident. Kyphoscoliosis with compressed vertebral body and thin ribs was found. No yellow or bluish-grey discoloration of teeth, no blue sclera and wormain bones of skull was shown. Currently, the proband is still alive and has been continuously supportive treatment. In this family have significant consanguinity and an increased prevalence of genetic condition with recessive type of OI inheritance.

We sequenced the exons and flanking regions of *CRTAP* gene in proband IV-3 who had less severe osteogenesis imperfecta with a clinical diagnosis of type VII and without mutation in type I collagen gene. The presence of a homozygous

c.1106delA in this proband IV-3 has affect post-translation modification of type I collagen, has 3-hydroxylation at α1 (I) at proline position 986 showed 37% decreased, however, the level of hydroxylation was still higher than other types of mutations (60, 62). This mutation in the *CRTAP* caused a frameshift and premature stop codon 10 amino acids downstream and expected deletion part located on the tail of the protein, for this reason, the function of protein was expressed. The CRTAP acts as part of a collagen 3-hydroxylation complex (6) and crucial for triple helix folding. The function of the CRTAP is with P3H1 and PPIB in the rER by form a complex for post-translational modification, the effect of mutation in CRTAP is defect in procollagen retained in the endoplasmic reticulum (42, 44). However, for the mutation of *CRTAP* result in normal CRTAP protein level on western blots with functional defect indentified by decreased P3H1 protein on immunofluorescence microscopy. Although CRTAP mainly localized in the rER, it's secreted and might have a matrix function (11, 23, 56, 63). Further, the hypothesis showed homozygous c.1106delA was associated with reduced in the level of P3H1 in the rER that indicate that crucial stabilize P3H1(64).

In summary, we report the patient that mutations in CRTAP with autosomal recessive osteogenesis imperfecta**.** These may correspond to less severe phenotype in our patient compared with those in recessive group. However, for the heterozygous mutation in all member of her family do not symptoms of disease. The molecular studies supported the genotype and phenotype correlation.

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APPENDICES

APPENDIX A

Inform Consent

ใบยินยอมเข้ารับการเจาะเลือดและตัดชิ้นเนื้อเพื่อการวิจัย
การศึกษาผลของการกลายพันธุ์ของยีนซีอาร์ทีเอฟ๓ในผู้ป่วยโรคกระดูกเปราะกรรมพันธุ์

วันให้คำยินยอม วันที่.....เดือน.....พ.ศ.....

ข้าพเจ้านาย/นาง/นางสาว..... ได้อ่านรายละเอียดจากเอกสารข้อมูลสำหรับผู้เข้าร่วมโครงการวิจัยที่แนบมา และข้าพเจ้ายินยอมเข้าร่วมโครงการวิจัยโดยสมัครใจ

ข้าพเจ้าได้รับสำเนาใบแสดงความยินยอมเข้าร่วมโครงการวิจัยที่ข้าพเจ้าได้ลงนามและวันที่พร้อมด้วยเอกสารข้อมูลสำหรับผู้เข้าร่วมโครงการวิจัย ทั้งนี้ก่อนที่จะลงนามในใบยินยอมให้ทำการวิจัยนี้ ข้าพเจ้าได้รับการอธิบายจากผู้วิจัยถึงวัตถุประสงค์ของการวิจัย ระยะเวลาของการทำวิจัย วิธีการวิจัย อันตราย หรืออาการที่อาจเกิดขึ้นจากการวิจัย รวมทั้งประโยชน์ที่จะเกิดขึ้นจากการวิจัย ข้าพเจ้ามีเวลาและโอกาสเพียงพอในการซักถามข้อสงสัยจนมีความเข้าใจอย่างดีแล้ว โดยผู้วิจัยได้ตอบคำถามต่างๆด้วยความเต็มใจไม่ปิดบังซ่อนเร้นจนข้าพเจ้าพอใจ

ข้าพเจ้ามีสิทธิที่จะบอกเลิกเข้าร่วมในโครงการวิจัยเมื่อใดก็ได้ โดยไม่จำเป็นต้องแจ้งเหตุผล และการบอกเลิกการเข้าร่วมการวิจัยนี้ จะไม่มีผลต่อการรักษาโรคหรือสิทธิอื่นๆที่ข้าพเจ้าจะพึงได้รับต่อไป

ผู้วิจัยรับรองว่าจะเก็บข้อมูลส่วนตัวของข้าพเจ้าเป็นความลับและจะเปิดเผยได้เฉพาะเมื่อได้รับการยินยอมจากข้าพเจ้าเท่านั้น บุคคลอื่นในนามของบริษัทผู้สนับสนุนการวิจัย คณะกรรมการพิจารณาจริยธรรมการวิจัยหรือผู้ได้รับอำนาจมอบหมายให้เข้ามาตรวจและประมวลข้อมูลของผู้เข้าร่วมวิจัย ทั้งนี้จะต้องกระทำไปเพื่อวัตถุประสงค์เพื่อตรวจสอบความถูกต้องของข้อมูลเท่านั้น โดยการตกลงที่จะเข้าร่วมการศึกษานี้ข้าพเจ้าได้ให้คำยินยอมที่จะให้มีการตรวจสอบข้อมูลประวัติทางการแพทย์ของผู้เข้าร่วมวิจัยได้

ผู้วิจัยรับรองว่าจะไม่มีการเก็บข้อมูลใดๆของผู้เข้าร่วมวิจัยเพิ่มเติม หลังจากที่ข้าพเจ้าขอยกเลิกการเข้าร่วมโครงการวิจัยและต้องการให้ทำลายเอกสารและ/หรือตัวอย่างที่ใช้ตรวจสอบทั้งหมดที่สามารถสืบค้นถึงตัวข้าพเจ้าได้

ข้าพเจ้าเข้าใจว่า ข้าพเจ้ามีสิทธิที่จะตรวจสอบหรือแก้ไขข้อมูลส่วนตัวของข้าพเจ้าและสามารถเลิกการให้สิทธิในการใช้ข้อมูลส่วนตัวของข้าพเจ้าได้โดยต้องแจ้งให้ผู้วิจัยรับทราบ

ข้าพเจ้าได้ตระหนักว่าข้อมูลในการวิจัยรวมถึงข้อมูลทางการแพทย์ที่ไม่มีการเปิดเผยชื่อ จะผ่านกระบวนการต่างๆ เช่น การเก็บข้อมูล การบันทึกข้อมูลในคอมพิวเตอร์ การตรวจสอบ การวิเคราะห์ และการรายงานเพื่อวัตถุประสงค์ทางวิทยาศาสตร์ รวมทั้งการใช้ข้อมูลทางการแพทย์ในอนาคต หรือการวิจัยทางด้านเภสัชภัณฑ์ เท่านั้น

ข้าพเจ้ายินดีลงนามในใบยินยอมนี้เพื่อเข้าร่วมการวิจัยด้วยความเต็มใจ

…….…….. ลงนามผู้ยินยอม

(……) ชื่อผู้ยินยอมตัวบรรจง

วันที่.....เดือน.....พ.ศ.....

ข้าพเจ้าได้อธิบายถึงวัตถุประสงค์ของการวิจัย วิธีการวิจัย อันตราย หรืออาการที่อาจเกิดขึ้นจากการวิจัย หรือจากยาที่ใช้ รวมทั้งประโยชน์ที่จะเกิดขึ้นจากการวิจัยอย่างละเอียด ให้ผู้เข้าร่วมในโครงการวิจัยตามนามข้างต้นได้ทราบและมีความเข้าใจดีแล้ว พร้อมลงนามลงในเอกสารแสดงความยินยอมด้วยความ
เต็มใจ

ลงชื่อ …… (อาสาสมัคร)

(……..…..)

…… (แพทย์ผู้ทำการวิจัย)

(……..…..)

…… (พยาน)

(……..)

วันที่ …… / …… / ……..

APPENDIX B

Chemicals and Reagents

1. Agarose, Calbiochem, USA
2. PCR reagents, Fermentas, USA
3. PCR Gel Purification kit, QIAGEN, USA
4. ExoSAP-IT® For PCR Product Clean-Up, USB, USA
5. HANKS' balance salt solution, Invitrogen, USA
6. Bio- AMF-3 culture complete medium, Biological Industries Ltd, Israel
7. Fetal bovine serum, Gibco, USA
8. DMEM, Invitrogen, Gibco, USA
9. ECL Western Blotting Substrate, Thermo Scientific, USA
10. Sequencing Grade Modified Trypsin, Promega, USA
11. Iodoacetamide ≥ 99% (HPLC), Sigma, U.S.
12. 1,4-dithiotreitol (DTT) > 99%, Merck, Germany
13. Ammonium bicarbonate, Sigma, U.S.
14. Trypsin, Promega, USA

APPENDIX C

Enzymes

1. *Taq* DNA polymerase, Fermentas, USA
2. Collagenase, Invitrogen, USA
3. *BseR*I, New England Biolabs, U.S.

DNA & Protein markers

1. GeneRuler 1 kb DNA Ladder, Fermentas, USA
2. 40-300 kDa Spectra ^{TM} Multicolor High Rang Protein Ladder, Fermentas, USA

APPENDIX D

Instruments

1. MJ MiniTM Gradient Thermal Cycler, BIO-RAD, USA
2. Automatic Pipettes, Gilson, France
3. Spectophotometer, Bio-Rad, USA.
4. Vortex Mixer, Stuart scientific, UK.
5. Chemiluminesence, xRS system,Gel Doc, PC BIO-RAD, USA
6. Biological Safety Carbinet class II A2, Heal Force, China
7. Sonics vibra cell sonicator, USA
8. Autoclave, Hirayama, Japan
9. Drying ovens, BINDER, Germany
10. Biofreezer(-80 ^{o}C), Sanyo, Japan
11. Biofreezer (-20 ^{o}C), Sanyo, Japan
12. MiniSpin centrifuge, Eppendorf, USA
13. Refrigerated centrifuge, Eppendorf**,** USA
14. CO_2 humidified incubator, Thermo Scienctific, USA.
15. Horizontal DNA Electrophoresis C & Systems, Bio-Rad, USA

16. Vertical Electrophoresis and Blotting & Systems, Bio-Rad, USA
17. Filter paper 0.2 μm, Millipore, USA
18. PVDF membranes, Millipore, USA
19. Amicon Ultra 3k- 3,000MWCO, Millipore, Ireland
20. Heat block, Scientific, USA.
21. Inverted microscope, Nikon, Japan.
22. Nanodrop, Thermo scientific, USA.
23. Water bath 37 ^{o}C, Mammert, USA.
24. Zeiss LSM 510 Inverted Meta Confocal microscope และ LSM510 software, Germany
25. nano LC-MSMS, Bruker Daltanic, Germany
26. Automatic Environmental SpeedVac System, AES1010, Savant, U.S.

APPENDIX E

Punch Biopsy of the Skin

1. Nonsterile Tray for Anesthesia
2. Sterile Tray for the Procedure
3. Nonsterile gloves
4. Sterile gloves
5. Punch biopsy instrument (2 or 3 mm)
6. Needle holder for suturing
7. Desired size of suture 5–0 nylon
8. Iris scissors
9. Sterile fenestrated drape
10. Sterile conical centrifuge tubes 15 ml

APPENDIX F

Reagents and Buffers

1. 6X DNA loading dye (5 mL)

30 g glycerol

6 mL 0.5 M EDTA, pH 8

5 mg bromophenol blue

Adjust volume to 5 ml

2. 10x TBE Electrophoresis buffer (1,000 ml)

108 g of Tris base (tris(hydroxymethyl)aminomethane)

55 g of boric acid

7.5 g of EDTA, disodium salt

Adjust volume to 1,000 ml with distilled water

3. 10x Transfer buffer

30.3 g Tris base

144 g glycine

Adjust volume to 1,000 ml with distilled water, Chill to 4°C

4. 1X transfer buffer / 20%Metanol

100 ml 10X Transfer buffer

500 ml H_2O

200 ml methanol

Adjust volume to 1,000 ml with distilled water, Chill to 4°C

5. 10x Phosphate buffer saline (PBS), pH 7.3

80 g of NaCl (58.44 g mol^{-1})

2 g of KCl (74.55 g mol^{-1})

11.5 g of Na_2HPO_4 (141.96 g mol^{-1})

2 g of KH_2PO_4 (136.09 g mol^{-1})

Adjust volume to 1,000 ml with distilled water, Chill to 4°C

6. Blocking buffer

5% (w/v) Skim Milk (Becton Dickinson, France) in

1xPBS/0.1%tween20

10x running buffer, pH 8.3

250 mM Tris-HCl, pH 8.2

1920 mM Glycine

10% (w/v) SDS

Adjust volume to 1,000 ml with distilled water

7. Stripping buffer

100 mM 2-mercaptoethanol

2% SDS

62.5 mM Tris-HCl pH 6.7

8. 25 mM Ammonium bicarbonate (10 ml)

250 ul of 1 M ammonium bicarbonate

9,750 ul of milliQ water

9. 6M Urea

1 g of Urea (purity > 99%)

2.5 ml of 25 mM ammonium bicarbonate

10. 200 mM DTT (Reducing reagent)

30 mg of DTT

1 ml of 25 mM ammonium bicarbonate

11. 200 mM IAA (Alkylating reagent)

36 mg Iodoacetamide (IAA)

1 ml of 25 mM ammonium bicarbonate

12. Trypsin solution (1 ug/ul)

20 ug of trypsin

(1 vial; Sequencing grade modified trypsin,Promega, USA)

20 ul of buffer provided with Promega's trypsin

BIOGRAPHY

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Academic/Conference Attended:

2009 Wattanachanya L, Bunworasate U, Plengpanich W, **Houngngam N**, Buranasupkajorn P, Sunthornyothin S, Shotelersuk V, Snabboon T*. Bilateral pheochromocytoma during the postpartum period. Arch Gynecol Obstet. Arch Gynecol Obstet. 2009 Dec; 280(6):1055-8. Epub 2009 Apr 2.

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