KYOTO INSTITUTE OF TECHNOLOGY

Ph.D. Thesis

BASIC AND CLINICAL INVESTIGATIONS ON PATIENTS WITH PORPHYRIAS: CONGENITAL ERYTHROPOIETIC PORPHYRIA, HEREDITARY COPROPORPHYRIA, AND HARDEROPORPHYRIA

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ABBREVIATIONS

AIP	Acute intermittent porphyria
ALAD	Aminolevulinic acid dehydratase
ALAS	Aminolevulinic acid synthase
BS^3	Bis[sulfosuccinimidyl]suberate
BMT	Bone marrow transplantation
CBB	Coomassie Brilliant Blue
cDNA	Complementary deoxyribonucleic acid
CEP	Congenital erythropoietic porphyria
CPOX	Coproporphyrinogen oxidase
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraaetic
EPP	Erythropoietic porphyria
FECH	Ferrochelatase
HLA	Human Leucocyte Antigen
HCT	Hematocrit
HCP	Hereditary coproporphyria
HPLC	High performance liquid chromatography
HSCT	Hematopoietic stem cell transplantation
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilo base
kDa	Kilo Dalton

MCH	Mean corpuscular hemoglobin
MCS	Multicloning site
PBS	Phosphat buffered saline
PCR	Polymerase chain reaction
PCT	Porphyria cutanea tarda
PPO	Proto porphyrinogen oxidase
PVDF	Polyvinylidene difluoride
RBC	Red blood cell
SDS –PAGE	Sodium dodecyl sulphate-polyacrylamide
UROD	Uroporphyrinogen III decarboxylase
UROS	Uroporphyrinogen III cosynthase
VP	Variegate porphyria
wt	Wild-type
XLP	X-linked protoporphyria

GENERAL INTRODUCTION

GENERAL INTRODUCTION

I. Introduction

Porphyria is not a single disease but it is a group of rare disorders with each specific porphyria resulting from mutations in one of eight genes encoding enzymes of heme biosynthetic pathway (Figure I.1) [1]. The result of these is increasing secretion of porphyrins and porphyrin precursors [2]. These enzymes are found in all organs in the body and are arranged sequentially in all steps of heme production, which is most active in the bone marrow and liver. As phototoxic compound, the accumulation of porphyrin in the skin causes photosensitivity and neurological feature results from excess porphyrin precursor. Porphyria can be inherited or (rarely) acquired [3]. However, the development of symptoms in some disorders of porphyria may be aggravated by environmental factors such as alcohol, smoking, certain drugs (barbiturates, sulfonamide antibiotics, birth control pills, sedatives, tranquilizers), menstrual hormones, exposure to sunlight, stress, dieting or fasting, infections, excess iron in the body. Researchers have classified porphyria as either hepatic or erythroid, depending on the principal site of expression of the specific enzymatic in each disorder. Some porphyrias have acute presentations (acute intermittent, variegate, hereditary coproporphyria), whereas others have a chronic, relatively stable presentation (congenital, erythropoietic) [4]. Another classification system distinguishes porphyrias according to the predominant symptoms. They are the neurovisceral or acute porphyrias with abdominal pain, neuropathy,

autonomic instability, and psychosis and the cutaneous porphyrias with symptoms of photosensitive lesions on the skin [5]. Aminolevulinic acid dehydratase (ALAD) porphyria and acute intermittent porphyria (AIP) cause predominately neurovisceral symptoms, whereas congenital erythropoietic porphyria (CEP), porphyria cutanea tarda (PCT) and erythropoietic porphyria (EPP) cause mostly cutaneous symptoms. Two porphyrias overlap these categories and can cause both neurovisceral and cutaneous symtomps, namely hereditary coproporphyria (HCP) and variegate porphyria (VP) [6]. Circulating porphyrins enter the skin where they interact with the light energy resulting in a phototoxic reaction. Therefore, cutaneous porphyrias are a unique group of photosensitivity diseases caused by the endogenous agent. The skin symptoms of porphyrias manifest clinically as an immediate or chronic photosensitivity depending on the type of porphyrin which accumulates in the skin. In contrast to other photodermatoses in which ultraviolet radiation is usually responsible for photosensitivity in porphyria visible light induces the skin symptoms due to the distinctive light absorbing spectra of porphyrin molecules [7]. Cutaneous porphyrias are characterized by skin blistering and fragility on sun-exposed areas of the body, typically the back of hands, forearms, face, ears and neck. Porphyrin levels are extremely high in most cases of CEP, and the more severe blistering skin lesions lead to infection and multilation with loss of facial features and fingers. EPP and a variant form of EPP, X-Linked protoporphyria (XLP) cause a distinctive, intermediate, painful but non blistering reaction to sunlight. Porphyrias often are misdiagnosed because patients have vague symptoms. Acute forms of porphyria can be life-threatening, so it is important to make an accurate diagnosis and initiate proper medical treatment. Nowadays, severe symptoms may be avoided and most of people affected by these disorders will have normal lives relatively if the physicians have a good management.

In the research process of cutaneous porphyria, I found the mutation of the uroporphyrinogen III cosynthase Gene in a Vietnamese patient and a molecular diagnosis for the identification of clinically asymptomatic heterozygous mutation carriers with congenital erythropoietic porphyria. Besides, I also provided evidence that the mutant homodimer results in harderoporphyria and heterodimer leads to hereditary coproporphyria. The finding proposes a new insight into hereditary coproporphyria disease.

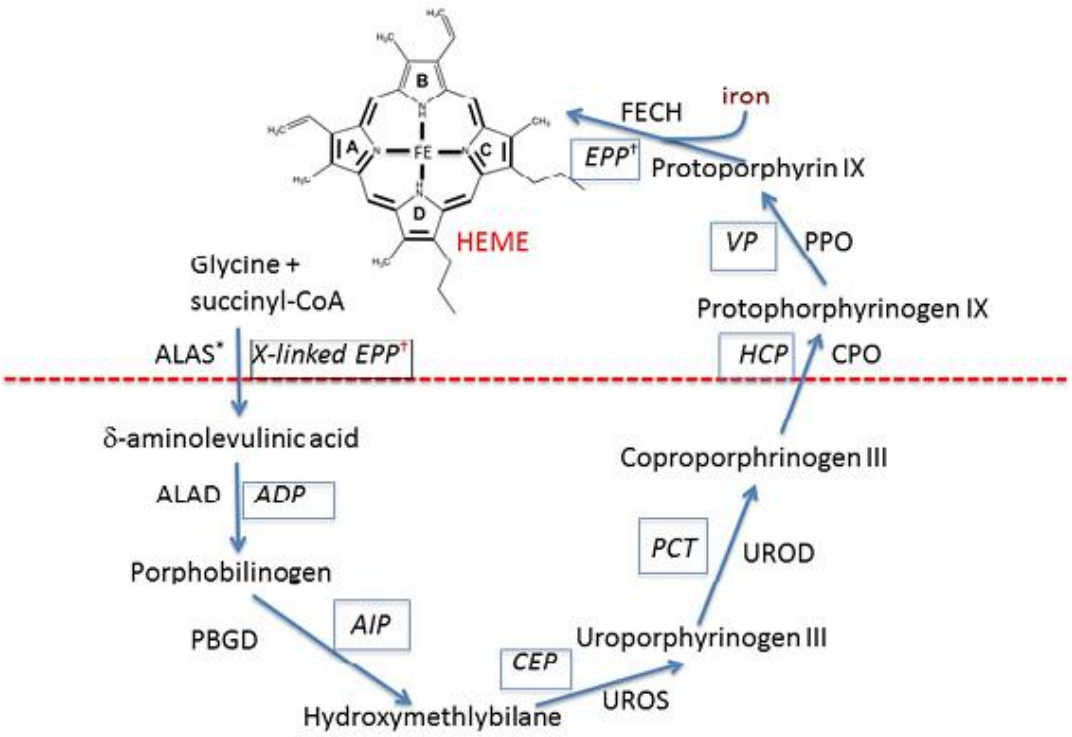

Figure I.1: The heme biosynthetic pathway. Reactions shown above the dashed line take place in the mitochondrion, while those below this line occur in the cytosol. At every step of the pathway, corresponding porphyria from the respective deficient enzyme is mentioned in italics and in rectangles.

ALAS: Aminolevulinic acid synthase; EPP: Erythropoietic protoporphyria; ALAD: ALA dehydratase; ADP: ALAD deficient porphyria; PBGD: Porphobilinogen deaminase; AIP: Acute intermittent porphyria; UROS: Uroporphyrinogen III synthase; CEP: Congenital erythropoietic porphyria; UROD: Uroporphyrinogen III decarboxylase; PCT: Porphyria cutanea tarda; CPO: Coproporphyrinogen oxidase; HCP: Hereditary coproporphyria; PPO: Protoporphyrinogen oxidase; VP: Variegate porphyria; FECH: Ferrochelatase Hepatoerythropoietic porphyria is due to autosomal recessive UROD mutation with severe deficiency of UROD in liver and red blood cells causes same phenotype as PCT with onset of disease in childhood.

** ALAS, rate limiting enzyme in heme biosynthesis has 2 isoforms: ALAS-1 is the general form present in all cells and ALAS2 is the erythroid specific variant present only in red blood cells.*

† X-linked EPP is caused by a gain-of-function mutation in ALAS2. EPP is also caused by mutations in FECH.

II. Culture and history

The word “porphyrus” means the color purple in Greek and the term “porphyria” and “porphyrin” are derived from this Greek word. In some cases, there is a presentation of reddish- purple coloring in the urine.

There has been porphyria since the human life. It appears at the beginning of time and has continued to alter throughout the ensuing generation. It was known as blood or liver disease during many centuries. The patients dying from liver failure or liver cancer is the last result of porphyria. According to American Porphyria Foundation, Dr. Schultz, a German graduate medical student, described the first actual clinical of the malaise and gave it the name in 1874. Hippocrate was acknowledged as the first person finding porphyria. However, the truly name is attributed to Schultz. Probably the first clinical description of acute porphyria was identified by Dr. B.J.Stokvis in 1889. CEP was found in 1923. In the year 1930, Hans Fisher, the Nobel laureate, described heme as the compound that make blood red and grass green. Up to 1937, Dr. Waldenstrom in Sweden published his findings. At one point AIP was seen as Swedish porphyria, or Waldenstrom’s porphyria. In the 1960’s porphyria was studied seriously in Europe and in the US. There was a deeply understanding when ALA and PBG were found by the scientists in the 1960’s. This is a summary overview of the top name in porphyria history. The modern techniques of molecular biology have been used to identify the mutations responsible for porphyria since 1989. At present, DNA testing for porphyria is available only through a few research laboratories [8].

III. Epidemiology

Up to day, the exact prevalence of porphyria is unknown. However, the Genetics Home Reference reveals that the ratio ranges from 1 in 500 to 1 in 50,000 people worldwide. In general, we don't know exactly the prevalence because many people who have a genetic mutation associated with the disease never spend signs or symptoms in some forms of porphyria.

Porphyria cutanea tarda is the most common type of porphyria [9]. The incidence rate of acute porphyria is 0.3 per 100,000 in Europe [10] but only few cases have been reported in Taiwan [11]. The most common form of acute porphyria in most of countries is acute intermittent porphyria. Hereditary coproporphyria, which is the other kind of the disorder has been reported in Europe and North America. According to Genetic Home Reference, there are about 3 in 1,000 people in the Afrikaner population of South Africa get Variegate porphyria.

Establishment of a specialist porphyria center network in Europe (European Porphyria Network [EP net]) estimated the risk of recurrent attacks at 4% of porphyia patients in Europe. The incidence of symptomatic porphyria was around 0.13 case per million case per year in most European countries, except Sweden, where it was 0.51 case per million per year [12, 13].

IV. Classification of the Major Human Porphyrias

There are several different classification systems in porphyrias. The most accurate classification is by the specific enzyme deficiency. Another classification system distinguished porphyrias that cause neurologic symptoms (acute porphyrias) from those that cause photosensitivity (cutaneous porphyrias). A third classification system is based on whether the excess precursors originate primarily in the liver (hepatic porphyrias) or primarily in the bone marrow (erythropoietic porphyrias) (Table IV.1). Some porphyrias are classified as more than one of these categories.

Table IV.1: Overview of Porphyria

	Affected Enzyme	***Inheritance Pattern***	***Non-blistering photosensitivity***	***Blistering photosensitivity***	***Acute neurovisceral symptoms***
Erythropoietic					
Erythropoiectic Protoporphyria (X-linked)	δ-Aminolevulinic acid Synthase-2* (ALAS2) (erythroid specific)	X-linked	Y	N	N
Erythropoiectic Protoporphyria (autosomal)	Ferrochelatase (FECH)	Autosomal recessive	Y	N	N
Congenital Erythropoietic Porphyria	Uroporphyrinogen synthase (UROS)	Autosomal recessive	Y	Y	N
Hepatic					
δ-aminolevulinic acid dehydratase porphyria	δ-Aminolevulinic acid dehydratase (ALAD)	Autosomal recessive	N	N	Y
Acute Intermittent Porphyria	Prophobilinogen deaminase (PBGD)	Autosomal dominant	N	N	Y
Hereditary Coproporphyria	Coproporphyrinogen oxidase (CPO)	Autosomal dominant	Y	Y	Y
Variegate porphyria	Protoporphyrinogen oxidase (CPO)	Autosomal dominant	Y	Y	Y
Porphyria cutanea tarda +	Uroporphyrinogen decarboxylase (UROD)	Autosomal recessive	Y	Y	N
Hepatoerythropoietic Porphyria (HEP)	Uroporphyrinogen decarboxylase	Autosomal recessive	Y	Y	N

** Gain of function mutation of ALAS2 causes X-linked eythropoietic protoporphyria*

+ Most cases (~80%) of porphyria cutanea tarda are sporadic or type I (no UROD mutation) due to an acquired inhibitor of UROD, but heterozigosity for mutant

All autosomal dominant porphyria have low penetrance

V. Photosensitivity in porphyrias

The photosensitizing properties of porphyrins were first demonstrated in 1913 by a physician Meyer-Betz, who injected himself intravenously haematoporphyrin [14]. Shortly thereafter he had marked erythema and oedema of the skin after the sun exposure resembling skin symptoms of EPP and later on chronic photosensitivity similar to PCT.

Porphyrins are phototoxic compounds due to their molecular configuration (Figure V.1). An extensive double-bond structure of tetrapyrrole ring and one of several ions at the centre render porphyrin strong absorbers of the light energy [15]. The most important absorption bands of porphyrins lie in the visible region between 400–410 nm (Soret band) and to smaller extent between 500-650 nm (Figure V.2). The action spectrum studies performed in patients with porphyric skin symptoms have shown similarly that a peak response eliciting cutaneous photosensitivity lies at around 400 nm and to a lesser extent between 500 and 600 nm [16, 17].

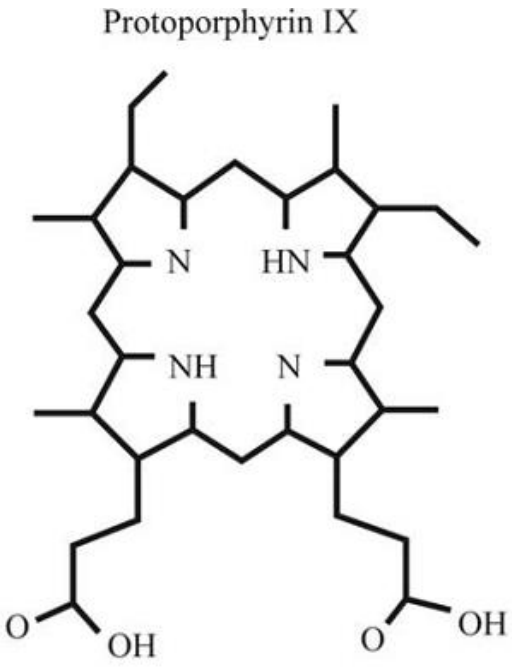

Figure V.1: The structure of protoporphyrin IX [18].

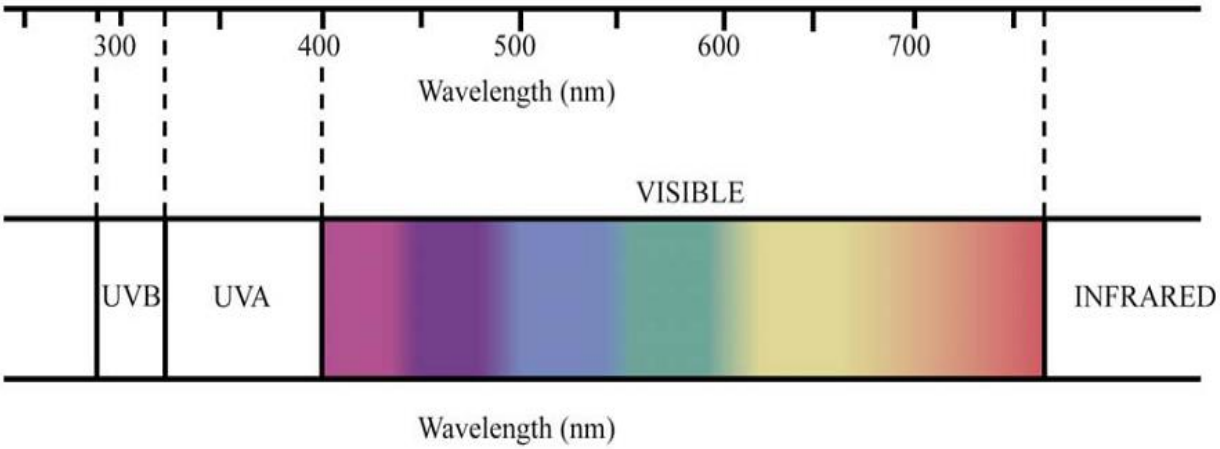

Figure V.2: The spectrum of the solar radiation [18].

Absorption of photon converts stable ground–state porphyrin molecule to the singlet or triplet excited state which is unstable of character (Figure V.3). Porphyrin sensitised photoreaction is classified into two types. In type I reaction, transfer of a hydrogen atom or electron from the singlet excited state porphyrin to molecular oxygen produces free radicals which cause cellular damage. The singlet excited state may return to ground status by emission of typical red fluorescence or it may be converted to the excited triplet state porphyrin.

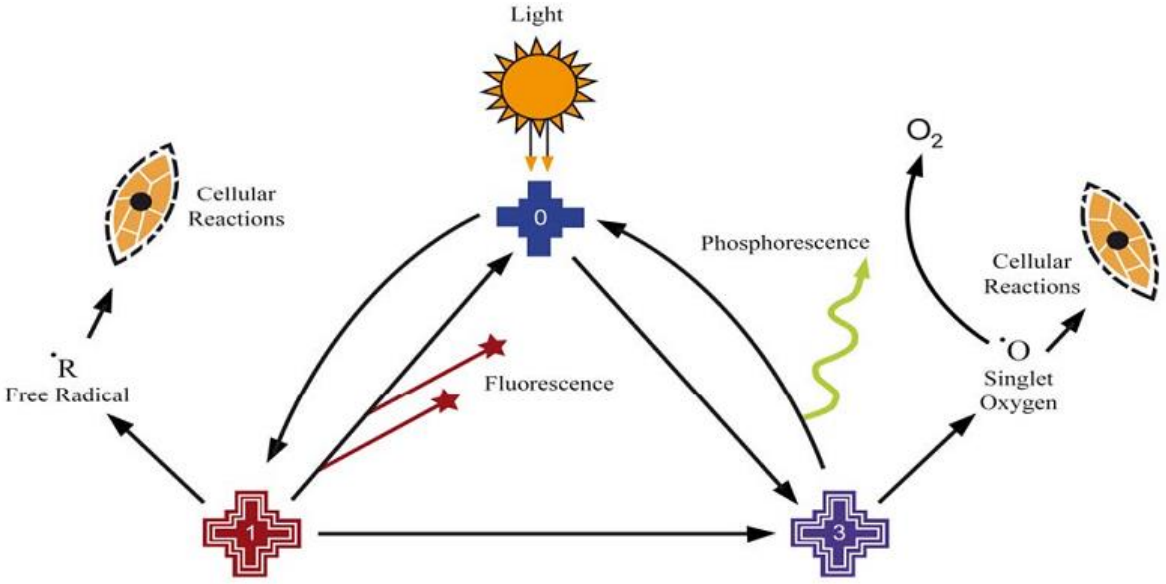

Figure V.3: Porphyrin-induced phototoxic reaction [18].

Type II photoreaction arises from the triplet excited state molecule which generates highly reactive singlet oxygen. Excited oxygen may react with hydrogen to form peroxides which are destructive for cellular structures such as cell membranes [15, 19].

Photosensitization via porphyrins produces two different clinical manifestations: acute skin reactions characterised by a burning sensation, oedema and erythema immediately after the sun exposure, and chronic skin lesions including blisters and erosions aggravated by mechanical trauma in continuously sun-exposed areas [20]. The first one occurs in EPP and CEP. Hepatic cutaneous porphyrias, HCP, PCT and VP are characterised by a chronic type of photosensitivity. CEP manifests with the most severe photosensitivity and is characterised by burning reaction with vesicles, bullae and erosions resulting in scarring and mutilations especially on the face and hands in addition to erythrodontia and severe anaemia [21].

The acute or chronic types of skin symptoms have been explained by different intracellular localization and solubility of porphyrins in the aqueous and lipid phases [22]. Protoporphyrin has high affinity for lipoproteins in cellular membranes and mitochondria because of its lipophilic nature. Uroporphyrin is water soluble and accumulates in lysosomes. Thus, the clinical manifestations may be associated with the intracellular site of the primary photodamage.

Porphyrins vary in their phototoxicity. It has been shown experimentally that protoporphyrin IX is the most potent photosensitizer [23]. Coproporphyrin III is less efficient than protoporphyrin IX and uroporphyrin III is the least potent. However, this is in contrast to the clinical experience that the most severe photosensitivity occurs in CEP with a drastic increase of accumulation of uroporphyrin I [21].

Photodynamic therapy used for the treatment of various malignant and premalignant tumors of the internal organs and the skin, is a clinical application of phototoxicity of porphyrins [24, 25]. Lethal cell damage is achieved by a systemic administration of haematoporphyrin derivatives or by topical application of porphyrin precursor ALA, which is converted intracellulary to protoporphyrin IX, and by subsequent irradiation with an appropriate light source.

VI. Signs and symptom

People with porphyria-causing gene mutations have latent porphyria. That means there are no symptoms of the disorder in some cases.

Acute porphyrias are characterized by attacks of pain and other signs of neurological distress, while the built-up of porphyrins and porphyrin precursors interacts with ultraviolet light from sun exposure cause the skin to become oversensitivity to sunlight. Moreover, symptoms of acute porphyrias can develop over hours or days and last for days or weeks. They can come and go over time, whereas cutaneous porphyrias tend to be more continuous.

Symptoms of acute porphyrias do not usually appear until the patient reach to the stage after puberty. They are seen more in women than men especially during menstruation. Attacks of acute porphyria may break out suddenly with the following symptoms:

- Anxiety, restlessness and insomnia
- Severe abdominal pain
- Pain in arms, legs or back
- Vomiting and constipation
- Hypertension
- Muscle pain, tingling, numbness, weakness or paralysis
- Confusion, hallucinations and seizures
- Respiratory paralysis
- Reddish coloured urine

The symptoms of cutaneous porphyrias and neurocutaneous porphyrias usually appear quickly following exposure to the sun light. These are pruritus, painful skin redness, edema, blistering (not associated with EPP) on exposed areas of the skin, abnormal hair growth on the face, reddish coloured urine.

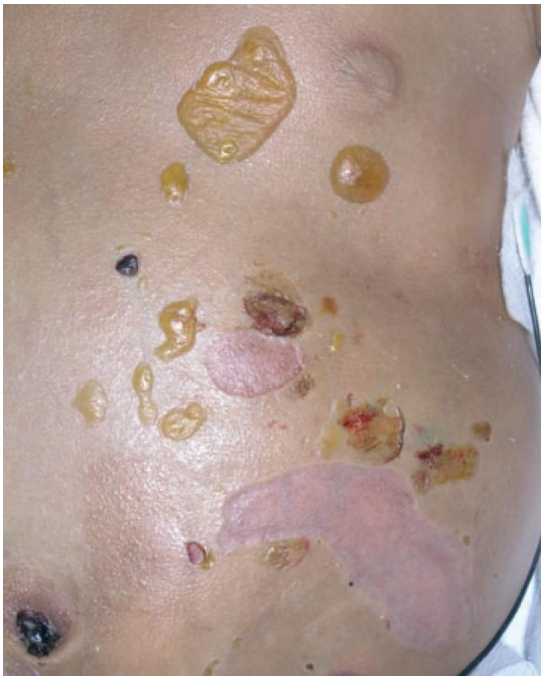

Figure VI.1: Photo-distributed bullae [26].

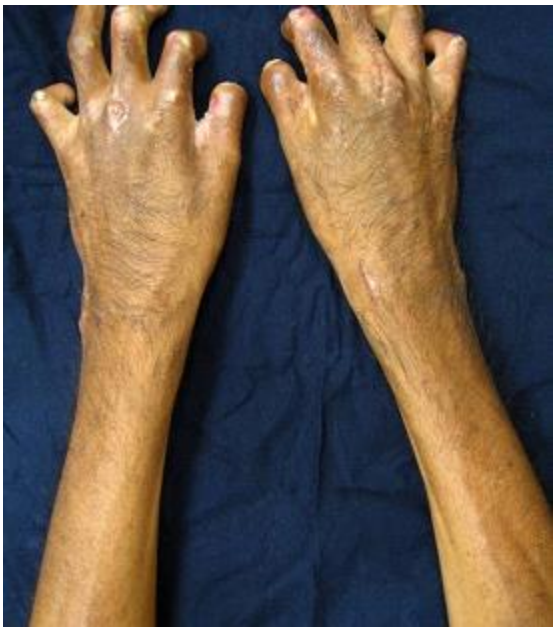

Figure VI.2: Severe mutilation of the fingers with scarring and hypertrichosis over sun-exposed areas [27].

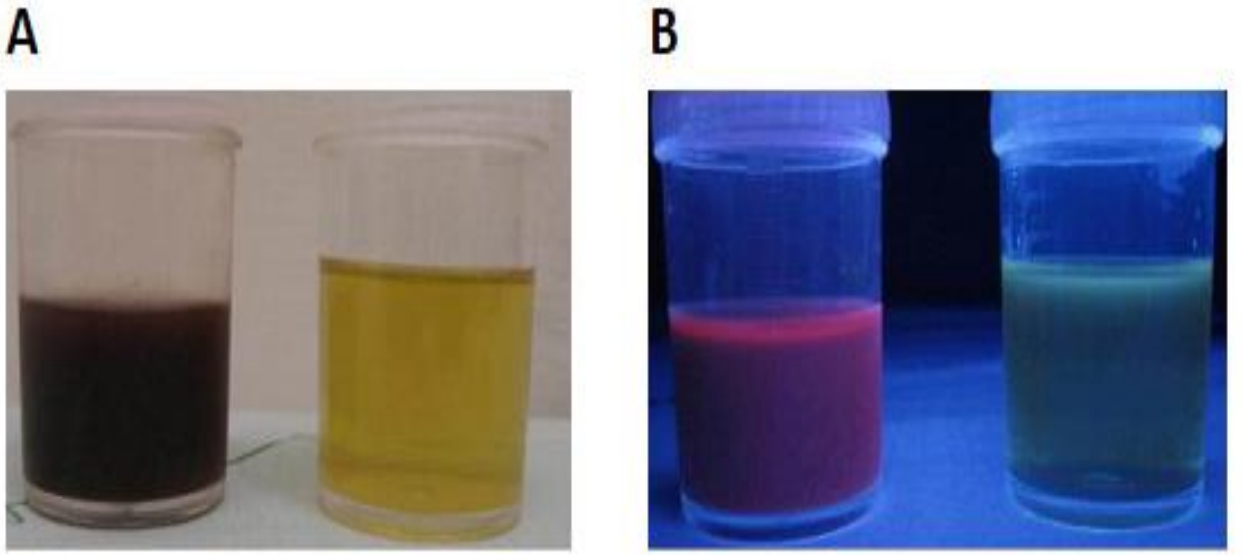

Figure VI.3: Comparison of the urine's color from patient and normal person [27].

(A) The dark purple urine of patient (left) compared with a normal person's light yellow urine (right)

(B) Pinkish fluorescence under WOOD's light detection (left) compared with the normal sample (right)

VII. Diagnosis

Porphyria is often missed or wrongly diagnosed by physicians because it is uncommon disease. Diagnosis is difficult because most symptoms are non-specific, such as abdominal pain, vomiting and constipation…The range of symptoms is common to many disorders.

The physicians not only examine physical symptoms but also carry out appropriate biochemical investigations such as urine, blood, stool sample test, erythrocyte uroporphyrinogen decarboxylase (diagnostic test for PCT), electrolytes (hyponatremia is typical) [28], genetic mutations and imaging (CT scanning of the abdomen and pelvis, MRI).

Since most types of porphyria are inherited, investigations should also be carried out on children and blood relatives of affected people.

VIII. Complication

Possible complications of porphyria include:

- Dehydration: Vomiting to an attack of acute porphyria can lead to dehyhration
- Breathing difficulties: acute porphyria can cause muscle weakness and paralysis, which can lead to respiratory failure.
- Hyponatremia: Low blood sodium may be a sign that porphyria has damaged kidneys in rare cases.
- Hypertension: It is the result of the kidneys were damaged by porphyrin buildup.
- Chronic kidney failure: Porphyrin buildup may cause the kidneys to gradually lose their ability to function.
- Liver damage: Some forms of porphyria cause excessive porphyrins in the liver which may lead to severe liver damage.
- Permanent skin damage: After cutaneous porphyria, the skin may have milia, pigmentation, scar.

IX. Treatment and management

IX.1. Acute porphyria

Treatment of acute porphyrias focuses on eliminating symptoms. Severe cases require hospitalization.

- Stopping medications that may have triggered symptoms
- Supportive care such as fluid, electrolytes, and nutrition is paramount
- Monitor for hyponatremia or hypomagnesemia, and treat vigorously is found.
- Aggressively treat respiratory failure, which may ensue once muscle weakness involves the diaphragm, and ventilate in an intensive care unit as appropriate.
- Administer phenothiazines for nausea, vomiting, agitation, etc. Ondansetron or related drugs can also be used for nausea [29].
- Treat pain with parenteral narcotics. Complicated and debilitating chronic cases may require celiac plexus injection [30].
- Prompt treatment of infections or other illness that may have cause symptoms
- Promptly start glucose infusion in the form of 10% dextrose. At least 300-400g should be given in 24 hours. The infusion is a time-buying measure to bridge the patient to more definitive treatment with hemin; by itself, glucose infusion is only effective for mild symptoms [31].
- Intravenous fluids to combat dehydration.
- Hemin or hematin injections which can reduce the burden placed on the body to produced heme, thus lowering porphyrin levels (1-4 mg/kg/d for up to 14 days). Both heme arginate and heme hydroxide have been used [29].

IX.2. Cutaneous porphyria

Treatment of cutaneous porphyrias focuses on reducing the amount of porphyrins in the body to help eliminate symptoms. Avoidance of sunlight is the key in treating cutaneous porphyrias [32].

- Phlebotomy reduces the iron in the body, which decreases porphyrins. Porphyria cutanea tarda can be effectively treated by phlebotomy [33]. Low-dose chloroquine appears at least as effective as phlebotomy [34].
- Iron-chelation therapy with Deferasirox has been tested in a pilot study of 10 patients with porphyria cutanea tarda in the United States, with reduction in new blisters and ferritin levels in most patients [35].
- Medication: hydroxychloroquine and chloroquine can absorb excess porphyrins and help the body get rid of them more quickly than usual. These medications are used only in patients who can’t tolerate phlebotomy.
- Beta carotene may increase the skin’ tolerance to sunlight in cutaneous porphyria.
- Hematopoietic stem cell transplantation (HSCT) has been applied with success in severe cases of CEP and EPP, especially in the setting of symptomatic hemolytic anemia and thrombocytopenia [36-40].

Liver transplantation may be needed for recurrent and/or life threatening acute attack in acute intermittent porphyria or acute liver failure or end-stage chronic liver disease in erythropoietic protoporphyria [1].

X. Prevention

Educate patients about their porphyria disease, inheritance, precipitating drugs and events, and the importance of seeking treatment in early stages of attacks.

A high risk of primary hepatocellular carcinoma has been demonstrated in acute hepatic porphyrias, and periodic surveillance is recommended [41].

XI. Outline of this thesis

In this thesis, I studied on clinical and basic aspects of patients with two porphyrias.

Firstly, congenital erythropoietic porphyria (CEP) arises from an autosomal recessive inherited disorder of the porphyrin metabolism, which leads to the accumulation of uroporphyrinogen I in bone marrow, skin and several other tissues by a deficiency of uroporphyrinogen III cosynthase (UROS). I studied a Vietnamese patient and her family suffering from severe cutaneous photosensitivity with skin fragility, bullous lesions and hypertrichosis on light-exposed areas. A missense mutation in the UROS gene was identified as a transversion of G to T at nucleotide 11,776, resulting in a substitution of valine by phenylalanine at codon 3 of exon 2. The patient showed a homozygous mutant profile, and the heterozygous state was observed in the parents. The activity of mutated UROS expressed in *Escherichia coli* was less than 16.1% that of the control, indicating that the markedly reduced activity of UROS is responsible for CEP. I described for the first time a mutation in the UROS gene in a Southeast Asian patient and a molecular diagnosis for the identification of clinically asymptomatic heterozygous mutation carriers and families with CEP.

Secondly, I examined hereditary coproporphyria (HCP), an autosomal dominant-inherited disease of haem biosynthesis caused by partial deficiency of the enzyme coproporphyrinogen oxidase (CPOX). Patients with HCP show <50% of normal activity and those with the rare autosomal recessive harderoporphyria accumulate harderoporphyrinogen, an intermediate porphyrin of the CPOX reaction. To clarify the relationship of the low enzyme activity with these diseases, I expressed mutant CPOX carrying His-tag from these porphyria patients and co-expressed mutant CPOX carrying His-tag and normal CPOX carrying HA-tag in a tandem fashion in Escherichia coli. Purification of

the His-tag-containing enzyme revealed that the His-enzyme forms a heterodimer in association with the HA-enzyme, and analysis using a cross-link reagent confirmed that the enzyme is a dimer (~70 kDa). Then, I expressed homo- and heterodimers composed of the wild-type (wt) and engineered mutants of the enzyme or mutants from HCP patients. The monomer form of mutated CPOX did not show any activity and homodimeric enzymes derived from HCP mutant showed low activity (<20% of the control). Some mutations of amino acids 401-404 were associated with marked accumulation of harderoporphyrinogen, with decrease in the production of protoporphyrinogen, whereas K404E derived from patients with harderoporphyria produced less harderoporphyrinogen. The heterodimers with wt and mutated subunits from HCP patients showed low protoporphyrinogen producing activity. These results show that the substitution of amino acids from R401 to K404 results in extremely low enzyme activity with either mutant homodimer or heterodimers containing normal and mutated subunits and can be linked to HCP disease.

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CHAPTER 1

Identification of mutation in the Uroporphyrinogen III Cosynthase gene in a Vietnamese patient with Congenital Erythropoietic Porphyria.

CHAPTER 1

1.1. Introduction

Congenital erythropoietic porphyria (CEP), also known as “Gunther’s disease” is transmitted as an autosomal recessive trait in which the activity of uroporphyrinogen III cosynthase (UROS) is severely reduced. It leads to excessive levels of uroporphyrins in erythrocytes and results in increased osmotic fragility, subsequent hemolysis, and deposition of metabolites in various organs such as the skin, teeth and bone [1-3]. The enzyme is localized in the cytosol and catalyzed the conversion of the linear tetrapyrrol hydroxylmethybilane to the cyclic tetrapyrrol uroporphyrinogen III [4].

UROS is encoded by the UROS gene located on chromosome 10q26. The gene has 10 exons and 2 alternative promoters, which are responsible for erythroid specific and ubiquitous expression of the same polypeptide [2]. Biochemically the disease is characterized by the accumulation of excess uroporphyrinogen and coproporphyrin in plasma, tissues and red cells [2, 5], and by the excretion of these porphyrins in urine and feces. Clinically CEP is characterized by severe photosensitivity with skin fragility, bullous dermatitis, and scarring because of recurrent cycles of infections and healing on light-exposed areas. Additional manifestations of include hypertrichosis, alopecia and erythrodontia. The diagnosis is confirmed by the demonstration of markedly deficient URO-synthase activity or the identification of specific mutatations in the UROS gene [6]. Early diagnosis of CEP is essentials, but some nonspecialists may miss it because porphyrins in the amniotic fluid or urine may be confused with meconium staining in some circumstances [7].

CEP is an extremely rare disorder with prevalence estimated at 1 in 1,000,000 or less [8]. There is no clear racial or sexual predominance. At that time of writing, a precise record of the incidence of CEP has not been established, however, it is approximately 200 cases reported worldwide [2, 9-11]. Furthermore, at least 40 different mutations have been reported in the UROS gene till date [12, 13], including 25 missense/nonsense [5, 12, 14-20], 2 splice sites [18, 20], 4 regulatory mutations [21], 3 small deletions/insertions [18, 22], 3 gross insertions/deletions [14, 18], and 1 complex rearrangement [18]. Individuals with CEP can be either homozygous with the same mutation or heterozygous with two different mutations and severity of the disease is inversely related to the residual enzyme activity of UROS [2, 5, 23]. In individuals suffering from CEP, UROS enzyme activity is markedly decrease but not fully absent. Complete absence of UROS would likely result in death. The degree of severity of clinical symtomps mainly depends on the amount of residual UROS activity [5]. Patients with CEP are classified based on UROS activity to one of three phenotypes: severe phenotype with extremely low enzymatic activity (<1% of wildtype), moderate phenotype (2-8% of the wild type) and mild phenotype (8-35% of the wild type) [1, 2, 12]. On the other hand, the severity is graded according to the age of onset, degree of hemolytic anemia, extent of cutaneous involvement, presence of splenomegaly, and osteopenia [2]. The patient who suffers from CEP usually has the reduction of life expectancy, depending on the severity of hemolysis and management of secondary infection.

This is the first reported case of CEP from Vietnam as well as Southeast Asia. To clarify the first molecular basis of Vietnamese family, identification of UROS mutation, and measurement activity of Uro'gen III cosynthase in a patient with CEP were performed.

1.2. Materials and methods

1.2.1. Detection of URO-cosynthase mutation in the UROS gene

From the proband patient and two asymptomatic family members origin blood samples were collected in tubes containing ethylenediaminetetraaetic (EDTA). Genomic DNA was isolated according to standard technique. Amplification by 9 primer sets was carried out in a thermal cycle. 50 µl reactions containing buffer for KOD FX 25 µl, dNTP 10µl, primer mix 1.5µl, DNA 1µl, KOD FX 1µl, sterilized water 11.5 µl. All primer sequences and annealing conditions are described in Table 1. PCR for amplification of the coding regions of the UROS gene was carried out according to the following program: initial denaturation at 94^{o}C for 2 min; followed by 35 cycles of denaturation at 98^{o}C for 10 sec, annealing at primer specific temperatures as outlined in Table I.1 for 30 sec, and extension at 68^{o}C for 1 min. The PCR products were purified in 1% agarose gel electrophoresis (Invitrogen™). Subsequently, PCR fragments were sequenced with Big Dye, and denatured with Hi-Di™ Formamide using an ABI Prism 310 Genetic Analyzer from Applied Biosystem Inc (Applied Biosystems, Foster City, CA).

Table 1.1: Primer sequences and annealing conditions used for PCR

Amplified region	Annealing temperature	Primer sequence	Direction
Exon 2	55ºC	TTGCTTAGGAAGAGTCT	forward
		CTGTGGGATAAGGAGTC	reverse
Exon 3	55ºC	TTTGCAAAACCTCAGA	forward
		GTCCCTCTCTGGCTTCA	reverse
Exon 4	55ºC	CTATTGTGAAGCATTTGGAA	forward
		TCTCCCAGCAGGAGAAATAA	reverse
Exon 5	55ºC	ATAGTTTGCTTTGCTCACA	forward
		CTGCATTCTTATCAGTAGT	reverse
Exon 6	57ºC	ATACCTGTGTATTTGCACG	forward
		CAAGAATGCACTGAGGAAA	reverse
Exon 7	55ºC	CTGAGTCCTAGAAGCAGAG	forward
		CACCCACTTCTATCACTGC	reverse
Exon 8	50ºC	AGGAGGGTGGTGCTGTATT	forward
		GGGACAGTGAAACCACATA	reverse
Exon 9	57ºC	GGCTTGAGGTCTTGATGCG	forward
		TAAGGCACCTGCTAGGCCA	reverse
Exon 10	50ºC	CAGGCTTGAGGCAGGAGTCT	forward
		AGTAACGTCCAACCGCAAAG	reverse

1.2.2. Expression of the mutant UROS-cDNA in *E. coli*

To express the mutant UROS (V3F) in bacteria, the cDNA (pET UROS 2.3) was amplified with the primers as follows: Forward primer (URO-mut: GCCTGCAGGTCGACAAGCTTAAGTTTCTTT) and Reverse primer (UROS-

-P-R: CATTATGCGGCCGCAAGCTTCAGCAGCAGCCATG). The resulting DNA fragment was inserted into EcoRI/HindIII and digested pET duet-1 by in-fusion. The obtained plasmid (pET-UROS) was transformed into *E.coli* strain BL 21. Protein expression was induced by incubation with 300µM IPTG and in LB medium at 25ºC for 10 hours.

1.2.3. Expression of the wild type UROS-cDNA in *E.coli*

The cDNA of wildtype UROS also was amplified by PCR. The PCR primers were designed as follows: forward primer (UROS-HF: GCCTGCAGGTCGACAAGCTTAAGGTTCTTTTA) and reverse primer (UROS--P-R: CATTATGCGGCCGCAAGCTTCAGCAGCAGCCATG). The PCR product was digested with EcoRI and Hind III, inserted to the cloning site of an expression vector pET-duet-1, then transformed and cloned into *E.coli* BL 21. Protein expression was induced by incubation with 300µM IPTG and LB medium at 25ºC for 10 hours.

1.2.4. Enzyme assay

1.2.4.1. Purification of mutant Uro'gen cosynthase and wild type UROS

The cells were suspended with Buffer I, II containing 20 mM Tris/ 10% glycerol pH8.0, 1 mM DTT, 0.1% Tween 20, 20mM imidazole, and 0.3M NaCl. The cells were disrupted by sonication, and the soluble fraction was obtained by centrifugation. The expressed enzyme was purified with 60µl of nickel ions-beads (Quiagen Co. Ltd) and washed with Buffer I, II for five times.

1.2.4.2. Partial purification of HMBS synthase (HMBS)

50 mg of bacteria transformed by the plasmid carrying HMBS-cDNA from Drs. M. Sato and M. Noguchi (Kurume Medical University) was suspended in PBS and disrupted by sonication. The cell lysis was heat-denatured at 65ºC for 10

mins, and insoluble material was removed by centrifugation. The supernatant was fractionated by ammonium sulfate (30-60%). The collected precipitate was dissolved in 20µl TE solution.

1.2.4.3. Measurement enzyme activities

After purification of the expressed enzyme with nickel ion-beads, the activity of mutated UROS (V3F) was compared with that of normal enzyme by the method of Wright and Lim [24].

Reaction samples were prepared as follow:

	Mutant UROS	**Wildtype UROS**
20 mM potassium phosphate buffer, pH 7.5	90 µl	90 µl
HMBS (100 µg protein)	1µl	1µl
Porphobilinogen 50mM	1µl	1µl
Buffer III		1µl
Purified mutant UROS	5µl	
Purified wildtype UROS		4µl
Total	97µl	97µl

The mixtures were incubated at 37^{o}C for 10 mins. The reactions were stopped by the addition of 200 µl of 10% trichloroacetic acid (TCA) containing 0.5% $I_{2,}$ and 100 µl of water. After centrifugation, the amount of enzyme in the supernatants was measured using a HITACHI MPF4 Fluorescence Spectrophotometer with an excitation wavelength of 400 nm and an emission wavelength of 580-650 nm.

1.3. Results

A 5-year-old girl owned a history of pink-stained urine while she was as a newborn. Onset of the disease occurred at the tenth month of life. Her deciduous teeth sprouted with a red-brown color and revealed reddish fluorescence on WOOD's lamp examination (Fig 1.1a). She also suffered from episodes of burning sensation, erythema and edema on her face, neck, arms and lower legs within minutes following exposure to direct sunlight in her infancy (Fig 1.1b). Vesicles or subepidermal bullous lesions progressed to crusted erosions, which healed with scarring and either hyperpigmentation or hypopigmentation (Fig1.1a). They appeared constantly and got worse during the spring and summer times. Hypertrichosis developed on unprotected skin areas. Spleen enlargement was also observed. The liver and kidney function tests were normal. The evidence of hemolysis was unclear. Urine porphyrins were extremely elevated to16 μmol/dl with a predominance of coproporphyrins and uroporphyrins. However, the detailed concentration of each porphyrin was not analyzed. Severe cutaneous photosensitivity, blistering of light-exposed skin, history of pink-stained diapers, accumulation of porphyrins in the blood and various other tissues, particularly erythrodontia (Fig 1.1b), suggested the diagnosis of CEP. The clinical and biochemical data of the patient are summarized in Table 1.1. With strict avoidance of UV and visible light, use of topical sunscreen, oral treatment with β-carotene, and antibiotic for preventing bacterial infection of the skin, the bullae healed, leaving milia and hyperpigmented scars without the need of a blood transfusion.

Mutation analysis of the UROS gene of the patient with CEP was performed. The DNA of exons 2–10 including splicing junctions of all exons was amplified

by PCR and analyzed by agarose gel. The analysis of exon 1 was not performed since protein coding region is not located at exon 1. The obtain DNAs were sequenced. A single missense mutation corresponding to a G-to-T transversion (11,776 g>t) in exon 2 was found, leading to an amino acid change from valine at position 3 to phenylalanine (Fig 1.2) and any other mutations were not found. The result indicated that the patient was homozygous for this mutation. And then, I also analyzed the UROS genes of her parents who were shown to be heterozygote carriers of this mutation (Fig 1.3). Her brother had died of a disease similar to hers in terms of symptoms and abnormalities, suggesting that he had the same gene mutation (V3F). Conversely, on the basis of the pedigree analysis spanning three generations, no other symptomatic patients were found. To confirm the genotype-phenotype relationship, I constructed a pET-duet vector [25] carrying wild type or mutated cDNA of UROS and transformed them into an *Escherichia coli* BL21 strain. The expression of UROS in bacteria was induced at 25°C. After the expressed enzyme was purified with nickel ion-beads (Qiagen Inc., Valencia, Calif., USA), the activity of mutant UROS (V3F) was compared with that of normal enzyme. The activity of the mutant UROS was less than 16.1% that of the wild type control (Fig 1.4).

1.4. Discussion

In my study, the mutational analysis of the UROS gene revealed a mutation (V3F) in the genomic DNA of a CEP patient from Vietnam. The patient was homozygous for the missense mutation in V3F and her parents were shown to be heterozygous carriers of the same mutation. The possibility of consanguineous parents has to be considered in this patient. No further family members were available for molecular analysis, but her brother had already died in association with symptoms similar to those of the patient. The family was hesitant to undergo additional biochemical studies to assess residual enzymatic activity to expand the genotype-phenotype assessment. The deficiency, but not absence, of UROS results primarily in the accumulation of porphyrins in various tissues. The complete absence of UROS would likely lead to death. I constructed mutated cDNA and expressed the recombinant protein in *E. coli*. The mutant UROS (V3F) showed low enzyme activity with 16.1% of the control. Although the age of onset and clinical manifestations in CEP are highly variable, from severe to milder forms of hemolytic anemia with only cutaneous lesions among adult CEP patients, photosensitivity was observed soon after birth in the present case. Furthermore, severe clinical manifestations in infancy were observed in my patient, which were derived from the reduced enzymatic activity of UROS. The substitution of valine with phenylalanine at position 3 causes a change of the side chain of the hydrophobic amino acid, which likely leads to structural alterations at the N-terminus of UROS [26]. This mutation has been previously described in a Japanese patient [23] and a L4F mutation was found in American patients [2], suggesting that the N-terminal region of the enzyme is quite important for the catalytic reaction. The amino acid sequence MKVLLL at the N-terminus of UROS is well conserved among mammals and many other organisms (Fig.1.7) [26], which also supports the assertion that it is

an indispensable domain. On the basis of the 3-D structure of UROS [26], the conserved amino acids at the N-terminus form a β -sheet associated with domains of α-helix and other β-sheets of the enzyme and may play an important role in the maintenance of the basic structure of UROS. The present study showed that the change of the isopropyl group from valine to the phenyl group of phenylalanine markedly reduced the enzyme activity, and other investigators reported that the change of the isopropyl group from leucine to a phenyl group at the next amino acid (L4F) also reduced the activity [2]. Thus, small structural alterations such as changes of a hydrophobic-hydrophobic group in the N-terminus of the protein can result in a dramatic reduction in enzyme activity.

CEP is a genetic disease for which therapy includes avoidance of visible light, especially the Soret band wavelengths (400–410 nm), and blood transfusions to decrease porphyrin production. The treatment modalities, such as zinc sunscreens, oral β-carotene, oral charcoal and other porphyrin binders, have been prescribed with limited effects [2, 27-29]. On the other hand, allogeneic bone marrow transplantation (BMT) has been successful for the treatment of CEP [30]. It has been reported that BMT in several patients resulted in a decrease in porphyrin levels and photosensitivity reduction [30]. Moreover, BMT significantly reduces splenomegaly and porphyrin excretion [31], and it decreases the cutaneous lesions caused by sun exposure in CEP patients [30, 32]. Therefore, BMT is one of the most rational therapies for CEP patients, although it needs adjustment for HLA-matched donors. Conversely, it should be noted that patients with CEP face increased risk of graft rejection when a donor is HLA-mismatched, and then the risk of BMT must be balanced with the severity of the disease. In the future, the use of stem cells engineered with vector-mediated expression of a correct copy of the UROS cDNA can be a treatment option [33]. Successful bone marrow or hematopoietic stem cell

transplantation is essentially curative but it associated with increased morbidity and mortality [31, 34-38]. Because of the rarity of the disease, the limits of human experimentation and the varying clinical severity of CEP patient, efforts have been directed to develop murine models of CEP to investigate the disease pathophysiology and evaluate various therapeutic strategies in recent study [39]. Besides, gene transfer experiments in mouse models have shown promising results and may prove to be a valuable option in the future, in the human disease before the development of devastating cutaneous lesions [40]. Although the amount of residual of UROS activity is not too low, the early onset and severe cutaneous photosensitivity are the significant signs in this case. So we should establish the strict supervise and have a good management to maintain her life.

At the time of writing, about 40 different mutations have been reported in UROS. Among them, C73R is the most frequent mutation leading to a severe phenotype in CEP patients. This mutation has been observed in one third of CEP patients and in half of the disease alleles in European countries[2, 5, 13-15, 22, 41]; however, it has not been reported in Asian countries. L237P and S47P have been observed in Western Asia [42]. Six mutations of UROS were found in Japanese CEP patients, in whom V3F was observed in the homozygote state. All other studied patients were either compound heterozygotes or homozygous for other mutations in the middle of the primary structure of UROS [26]. This is the first analysis of a CEP patient in Southeast Asia, and no further data of molecular defects in the UROS gene are currently available in relation to the genotype of the UROS gene in this area. The mutation V3F has not been identified in Caucasian populations, but its presence was found in a Japanese patient [23]. The reason for the same V3F mutation in Vietnamese and Japanese patients is not clear since these countries are far apart and separated by the sea; however, a common ancestry between these countries may be suggested. Recently, I also checked CEP of the second Vietnamese family. As shown in

Fig 1.5, individuals express the CEP phenotype and were homozygous for the V3F mutation. Both parents of two patients were heterozygous (carriers) of the V3F mutation and no other mutations were detected in the UROS gene. The pedigree reveals that all patients were born in consanguineous families (Fig 1.6). It is not surprising since they were suspected to be a strong consanguinity based on the epidemiological which is the same geographic area in the countryside of North Vietnam.

Taken together, the mutational analysis of gene causing CEP is important in the patient's disease management. Genetic counseling of the family and prenatal diagnosis is strongly recommended in this disease.

Table 1.2: Clinical and biochemical features in a patient with CEP

Clinical features		**Biochemical features**	
Anemia	+	RBC	4.78 million/mm^3
Splenomegaly	+	HCT	34.6f%
Erythrodontia	+	Hemoglobin	10.3 g/dl
Hypertrichosis	face	MCH	22.2 pg
		Urine total porphyrins	16 µmol/dl
		Control	<10 nmol/dl
Skin photosensitivity	severe cutaneous lesions, blistering, scarring		
Erythrodontia	+		
Hyperpigmentation	+		
Hemolysis	±		
Liver function	normal		

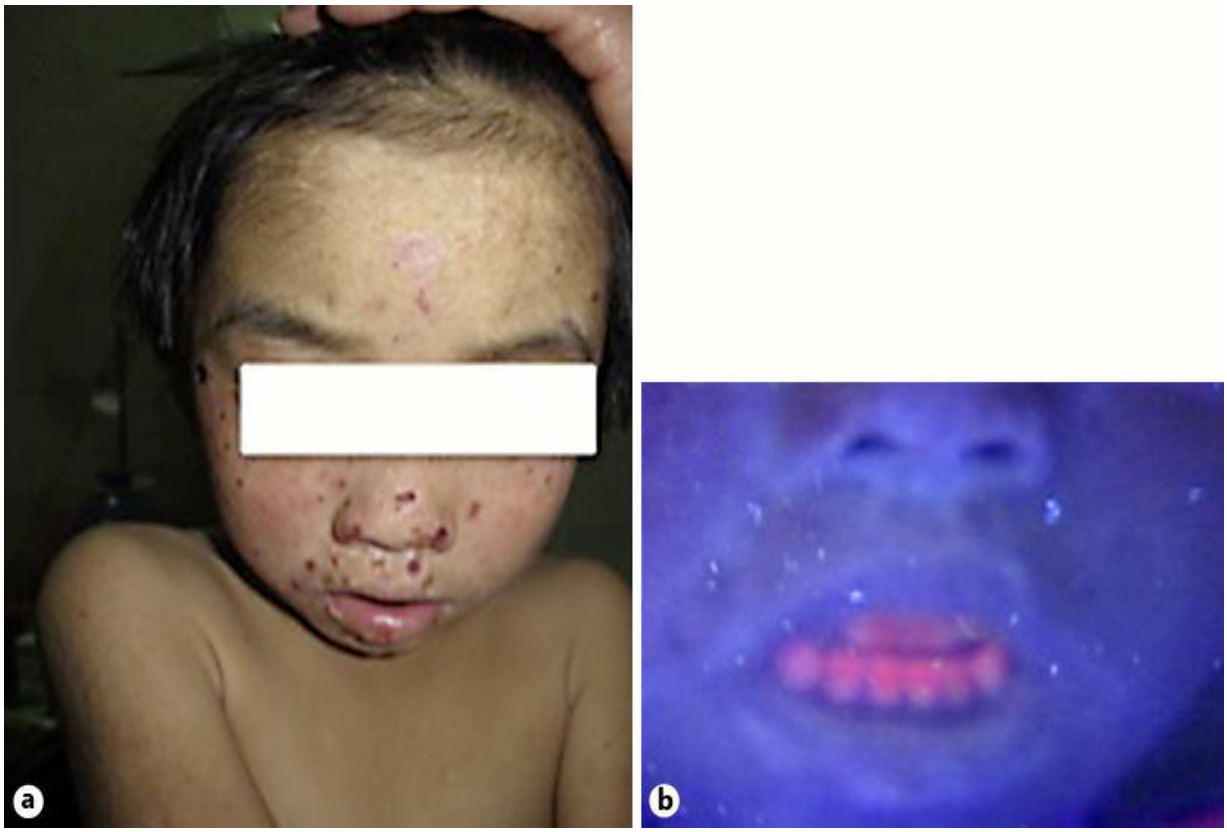

Fig 1.1: Photograph of a patient with CEP.

a. Crusted erosions and hypertrichosis on the forehead.

b. Red-brown teeth with intense red fluorescence under WOOD's light detection.

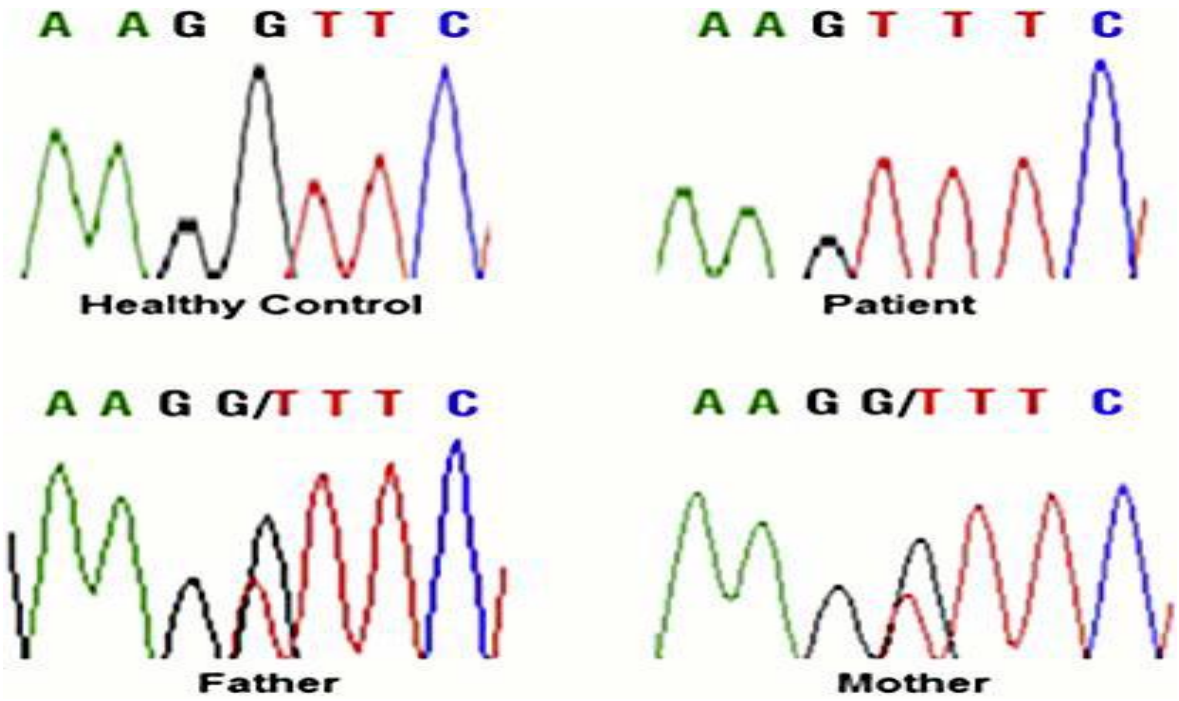

Fig 1.2: Nucleotide sequences of exon 2 of the UROS gene of the patient, her parents and a healthy control. Missense V3F mutation comprising a homozygous G-to-T transversion.

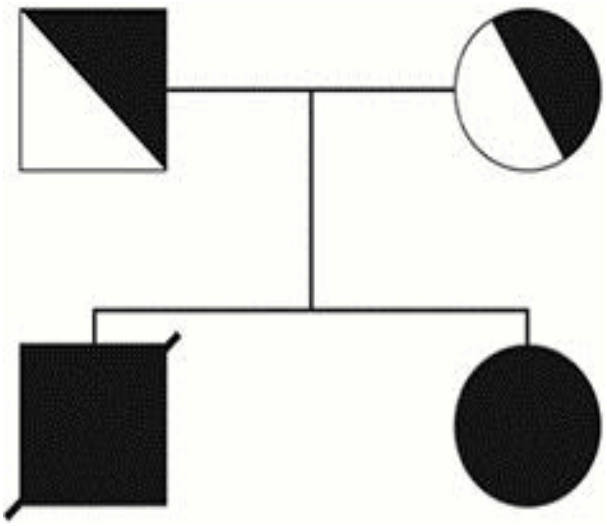

Fig 1.3: Family pedigree of the first CEP patient. The patient was homozygous for V3F. Her brother had died of a disease similar to that of the patient, and her parents were asymptomatic heterozygotes.

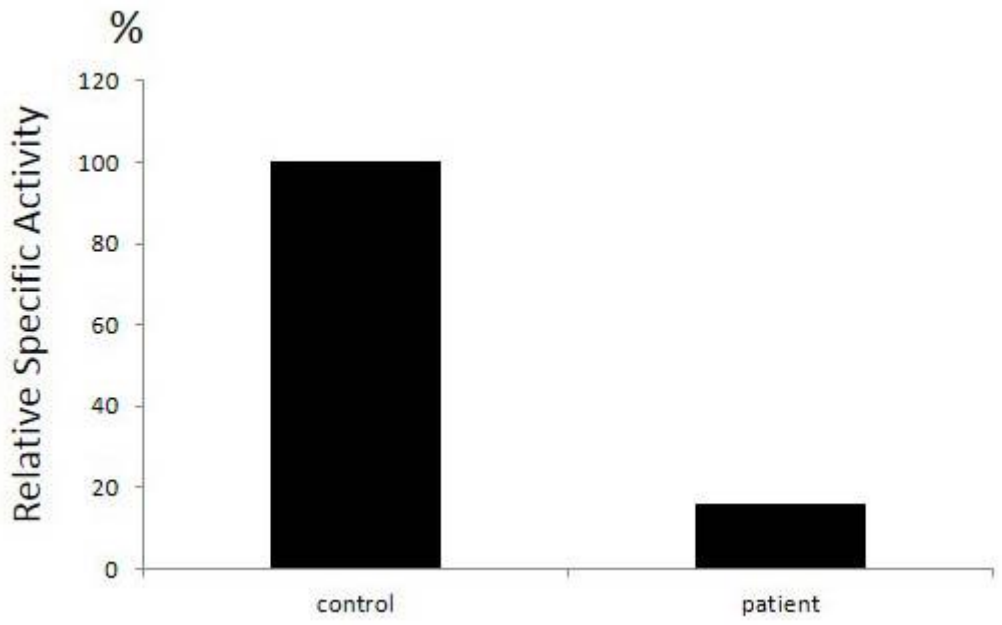

Fig 1.4: Expression of the residual enzymatic UROS activity.

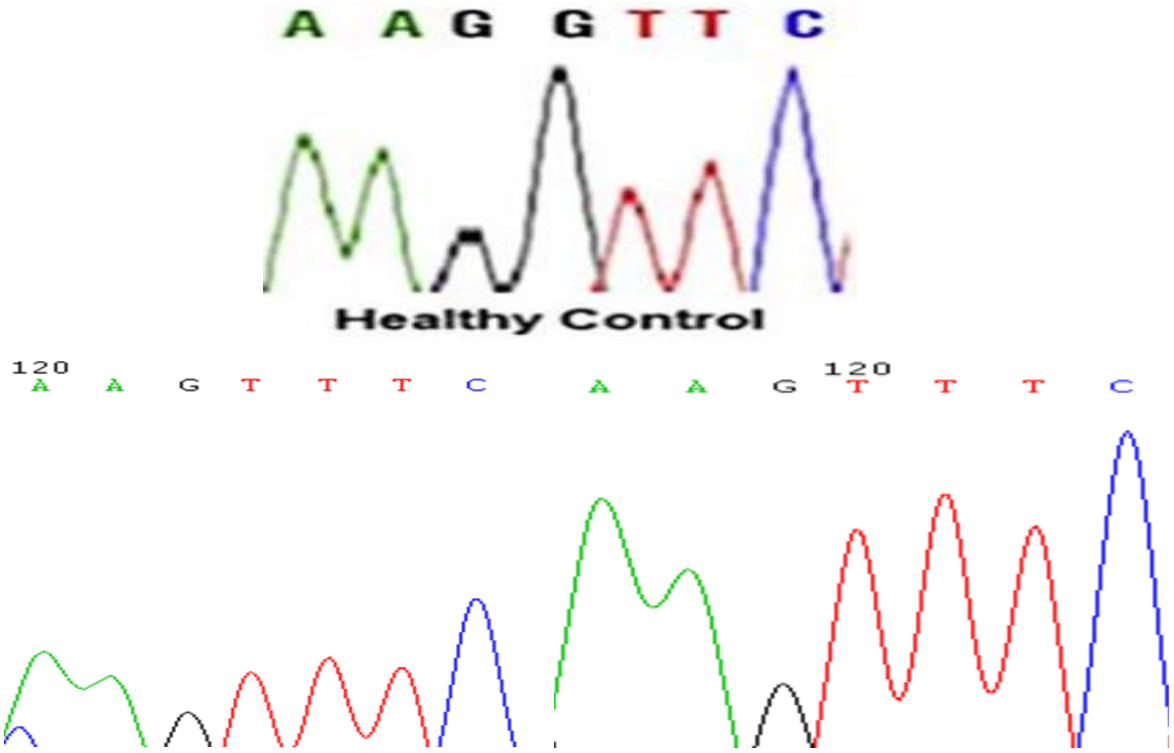

Fig 1.5: Nucleotide sequences of exon 2 of the UROS gene of the second and third patient and a healthy control. Missense V3F mutation comprising a homozygous G-to-T transversion.

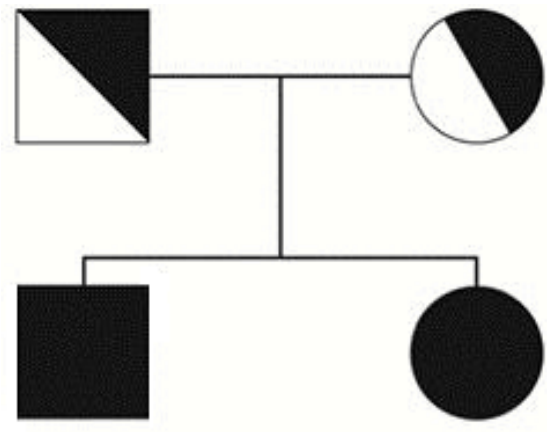

Fig 1.6: Family pedigree of the second and third CEP patients in Vietnam. The patients were homozygous for V3F and their parents were asymptomatic heterozygotes.

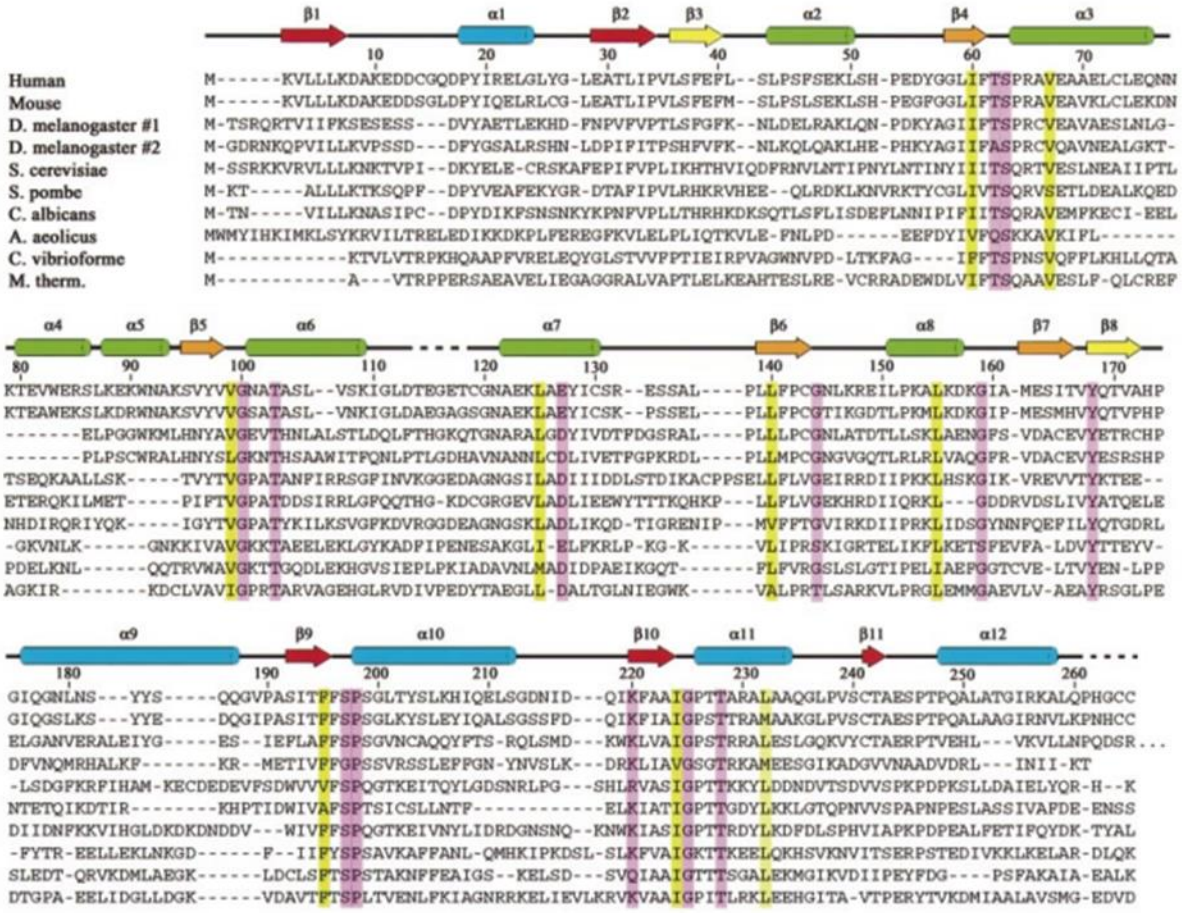

Fig 1.7: Alignment of U3S protein sequences. Alignment was performed using the Clustal method (Higgins and Sharp, 1989). Every 10th residue is indicated using the numbering of the human sequence. Conserved residues are highlighted: surface exposed, magenta; buried, yellow. DDBJ/EMBL/Gene Bank data base entries shown are: human, *Homo sapiens* (A40483); mouse, *Mus musculus* (A56838), *Drosophila melanogaster* (AAF46419 and AAF55222); *Saccharomyces cerevisiae* (NP_014921); *Schizosaccharomyces pombe* (P87214); *Candida albicans* (CAA22001); *Aquifex aeolicus* (E70452); *Chlorobium vibrioforme* ; *Methanobacterium thermoautotrophicum* (O26268).

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CHAPTER 2

Contribution to a new insight of hereditary coproporphyria and harderoporphyria via enzyme engineering of mutant homodimer and heterodimer of CPOX.

CHAPTER 2

2. 1. Introduction

In living organisms, heme plays fundamental roles in oxygen transport and storage. Furthermore, the heme–containing cytochrome function in electron transport in the mitochondrial membrane of eukaryotic cells [1]. There are eight enzyme-catalyzed reactions in the heme biosynthetic pathway. It is found that different models of overproduction, accumulation, and excretion of intermediates of heme synthesis depend on the specific enzymatic defect.

In the course of heme biosynthesis, the sixth enzyme, converting coproporphyrinogen III to protoporphyrinogen IX through two sequential steps of oxidative decarboxylation, is coproporphyrinogen oxidase (CPOX) (Fig. 2.1A) [2]. Human CPOX is a mitochondrial enzyme encoded by a 14 kb CPO gene containing 7 exons located on chromosome 3q11.2 [3-5]. The enzyme catalyses the stepwise oxidative decarboxylation of the heme precursor, coproporphyrinogen III to protoporphyrinogen IX, via a tricarboxylic intermediate known as "harderoporphyrinogen" (Fig. 2.1A). In mammals, CPOX is expressed as a 40 kDa precursor, and contains an amino terminal mitochondrial targeting signal [6]. After proteolytic processing, the protein is present as a mature 37 kDa form [7-11]. Hereditary coproporphyria (HCP) and harderoporphyria are two phenotypically separate disorders that concern partial deficiency of this enzyme. Neurovisceral symptomatology is the predominant manifestation in most of the acute polysymtomatic syndrome of HCP. Additionally, it may be associated with abdominal pain and/or skin photosensitivity. Nearly 20% of patients may experience photodermatosis with skin fragility and bullae in sun-exposed areas that result in depigmented scars in

some reports [12-14]. Hyper-excretion of coproporphyrin III in urine and faeces has been recorded in biochemical laboratory investigations [11]. In the majority of heterozygous HCP patients, CPOX activity is reduced to ~ 50% [9, 10, 15]. In rare homozygous cases, enzyme activity decreases to <10% [8, 14, 16]. HCP is a mixed form of chronic (cutaneous) porphyria and acute (hepatic) porphyria. As in the other acute porhyrias, there is an increased risk to develop hepatocellular carcinoma above the age of 50 years [17]. HCP is inherited in an autosomal dominant manner whereas harderoporphyria is an autosomal recessive fashion. Harderoporphyria is a rare erythropoietic variant form of HCP. Clinically, this form presents in infancy with neonatal jaundice and hemolytic anemia. Sometimes the presence of skin lesions is also described. Biochemical finding is a marked faecal excretion of harderoporphyrin in harderoporphyric patients.

To date, over 50 mutations in the CPO gene have been identified in HCP families (The Human Gene Mutation Database, http://portal.biobase-international .com/hgmd/pro/start.php) [18]. Most of these mutations result in substitution of amino acid residues within the structural framework of CPOX [19]. In terms of the molecular basis of HCP and harderoporphyria mutations of CPOX in patients with harderoporphyria were demonstrated in the region of exon 6, where mutations in those with HCP were also identified [20, 21]. As only patients with mutation in this region (K404E) would develop harderoporphyria, this mutation led to diminishment of the second step of the decarboxylation reaction during the conversion of coproporphyrinogen to protoporphyrinogen, implying that the active site of the enzyme involved in the second step of decarboxylation is located in exon 6 [18]. For this reason, it is so interesting to clarify the catalytic mechanism of CPOX between HCP and harderoporphyria by engineering the substitution of amino acids in exon 6. Moreover, CPOX exists as a homodimer and all mutant studies on CPOX have

been carried out by expressing homodimeric forms of mutant enzymes in bacteria [8, 14], although the heterodimeric form consisting of mutant/normal subunits is produced in patients with autosomal dominant-inherited disease HCP. However, the heterodimeric form of the enzyme has not yet been characterized. Furthermore, the homodimeric form consisting of the same mutant subunit accumulates as an intermediate, harderoporphyrinogen of the CPOX reaction in patients with harderoporphyria, an autosomal recessive disease.

To widen the knowledge of catalysis manifested by CPOX, an understanding of binding of substrate, active site structure or the formations of protoporphyrinogen, harderoporphyrinogen is essential for researching the enzyme activity. Therefore, I first established mutant CPOX carrying His-tag containing mutant enzyme derived from these porphyria patients and co-expressed the heterodimeic CPOX carrying His-tag containing mutant and HA-tag normal enzymes in *Escherichia coli.* My research now reveals that the mutation of amino acid residues at 401-404 of human CPOX caused diminishment of the second step of the reaction, resulting in the accumulation of harderoporphyrinogen. Taken together, these results demonstrate that the human CPOX in the heterodimeric form containing mutant and wild-type (wt) subunits was active, not only contributing to elucidate the molecular mechanism of CPOX but also providing a new insight into HCP and harderoporphyria.

2.2 Materials and methods

2.2.1. Materials

Restriction endonucleases and DNA modifying enzymes were from Takara Co. (Tokyo, Japan) and Toyobo Co. (Tokyo, Japan). Coproporphyrin was from Frontier Scientific Co. (Logan, UT) and harderoporphyrin was a kind gift of Dr. Seiyo Sano. Antibodies for CPOX were as previously described [22]. Monoclonal antibody for HA-tag was from Nacalai Tesuque (Kyoto, Japan). All other chemicals were of analytical grade.

2.2.2. Construction of CPOX expression plasmids

The cDNA for human CPOX with a N-terminal truncation [11] was subcloned into the SalI-HindIII site of multicloning site of MCS1of the expression vector pET-Duet-1 (EMD Biosciences, Darmstadt, Germany). Then to make human CPOX containing a HA-tag at the N-terminus, the truncated CPOX was ligated into the XbaI-HindIII site of the vector pCG-HA [23]. The insert was amplified by adding NdeI and EcoRV cloning sites, after which it was ligated into the NedI-EcoRV site of the MCS2 of pETDuet-1. Thus, the co-expression vector carrying wild-type His-CPOX/wild-type HA-CPOX (pET-His-wt/HA-wt) was obtained. cDNAs for mutated human CPOX K404E, R391W and R401W were inserted into pET-Duet. pET-K404E/-, -K404E/wt, -R401W/-, -wt/R401W, -R401W/wt, -R391W/-, and -R391W/wt were thus obtained. cDNAs for mutated human CPOX K404E and R401W were prepared as follows: In the first round of PCR, I used a pUC-CPOX plasmid [11] as a template. Primer pairs used for the amino acid substitution are shown in Table 2.1. In the second round, the (A) and (B) primer pairs were used to amplify the full-length human CPOX with the

mutation, and the DNA fragments were purified, sequenced, and inserted into pET-Duet as described above, to give pET-Duet -R388W/-, -R388W/wt, D400A/-, D400A/wt, R401K/- and R401K/wt. All plasmids were introduced into *E. coli* strain BL21. Proteins were overexpressed by the addition of 0.3 mM IPTG and 1 mM ALA at 24ºC [23].

Table 2.1: A list of primers for amplification of human CPOX cDNA

MCS I-F (Sal I)	**5'-AAGTCGACACTTCGCTGGGGAGGCC**
MCS I-R (Hind III)	5'-AAAAGCTTCTGCCTGCATCAACG
MCS II (Nde I)	5'-AACATATGGCTTCTAGCTATC
MCS II (EcoRV)	5'-AAGATATCCTGCCTGCCTGCATCAACGCA
R388W-F	5'-CAGCAGCTCTGGAGAGGACGG
R388W-R	5'-CCGTCCTCTCCAGAGCTGCTG
R391W-F	5'-GAAGAGGATGGTATGTA
R391W-R	5'-TACATACCATCCTCTTC
D400A-F	5'-GCTGTATGCTCGGGGCT
D400A-R	5'-AGCCCCGAGCATACAGC
R401W-F	5'-TGTATGATTGGGGCACAAA
R401W-R	5'-TTTGTGCCCCAATCATACA
R401G-F	5'-CTGTATGATGGGGGCACAAA
R401G-R	5'-TTTGTGCCCCCATCATACAG
R401K-F	5'-CTGTATGATAAGGGCACAAA
R401K-R	5'-TTTGTGCCCTTATCATACAG
G402D-F	5'-TGATCGGGACACAAAGT
G402D-R	5'-ACTTTGTGTCCCCGATCA
K404E-F	5'-GGGGCACAGAGTTTGGC
K404E-R	5'-GCCAAACTCTGTGCCCC
K404H-F	5'-GGGGCACATAGTTTGGC
K404H-R	5'-GCCAAACTATGTGCCCC
K404Q-F	5'-GGGGCACACAGTTTGGC
K404Q-R	5'-GCCAAACTGTGTGCCCC

2.2.3. Purification of recombinant CPOX

Wild-type, mutated, and chimeric mutated CPOX expressed with a six-His tag at the *N*-terminus were purified using Ni^{2+}-NTA Agarose (Quagen Inc. Valencia, CA), according to the manufacturer's recommendations. The elution of the enzyme from Ni^{2+}- beads was performed with 10 mM potassium phosphate, pH 7.4 containing 250 mM imidazole and 10% glycerol. The eluted proteins were dialyzed against 10 mM potassium phosphate, pH 7.4 containing 10%

mercaptoethanol. The purified CPOX were treated with 2 mM a cross-linker, BS^3. The resulting CPOX were analyzed by SDS-PAGE, followed by immunoblotting with anti-CPOX.

2.2.4. Immunoblots

The purified CPOX were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene difluoride (PVDF) membrane. Immunoblotting was carried out with anti-CPOX and anti-HA as the primary antibodies [23, 24].

2.2.5 Enzyme assay

CPOX activity was measured using coproporphyrinogen as previously described [22, 25]. The CPOX activity was designated as 1 nmol of protoporphyrinogen formed per minute at 37^{o}C. Harderoporphyrin and protoporphyrin were separated from coproporphyrin by high performance liquid chromatography (HPLC) with a Cosmosil 5C18-AR column (0.46×15cm, Nacalai Tesque, Kyoto Japan; Fig 1B) [25]. The elution was performed with 1M ammonium acetate (pH 5.1)/methanol (12:88, v/v) at a flow rate of 1.0 ml/min. Harderoporphyrin and protoporphyrin were determined spectrophotometrically at 400 nm, using porphyrin standard. The protein concentration was estimated by the method of Bradford [26].

2.2.6. Statistical anylysis

Values are expressed as mean ± SD (n ranging from 3 to 5 for independent experiments). Statistical significance was estimated by one-way or two-way analysis of variance or Student's t-test. A P-value of <0.05 was considered significant.

2.3. Results

2.3.1. Expression of recombinant CPOX as homodimer in *E. coli*

Several strategies for the expression of recombinant human CPOX in bacteria have been developed [20, 22, 27, 28]. As CPOX is a homodimer, the patients with HCP produce the heterodimeric form of CPOX from two alleles. However, no study has been conducted with heterodimeric enzyme. To examine the catalytic mechanisms of mutant CPOX involved in the formations of protoporphyrin and harderoporphyrin, we first prepared a bicistronic construct expressing CPOX containing a His-tag and an HA-tag. Figure 2.2A shows the Coomassie Brilliant Blue (CBB) staining of homogenates and immunoblot analysis of recombinant CPOX with anti-CPOX antibody. His-tag CPOX was precisely expressed. Anti-HA only reacted with the protein when His-/HA-tag CPOX were co-expressed. When CPOX was purified with Ni^{2+} - beads, and the amount of enzyme was evaluated by CBB staining, the CPOX containing the His-tag was purified (Fig 2.2B). Interestingly, anti-HA also reacted with the protein HA-tags (lane 3), suggesting that the enzyme formed a heteromer. To confirm whether CPOX was expressed as a dimer, purified CPOX was treated with BS^3, a cross-link reagent, followed by analysis with SDS-PAGE. Treatment of the purified enzyme with the cross-linker resulted in the formation of a new band with a molecular mass of 74 kDa, indicating that the recombinant CPOX is a homodimer (Fig 2.2C). I then examined the activity as the production of protoporphyrin with purified enzymebetween His-tag and His-tag/HA-tag CPOX. As shown in Fig. 2D, His-tag enzyme showed activity similar to that of the His/HA-tag heterophilic form.

2.3.2. Functional dimer containing mutant subunit.

Figure 2.3 shows the alignment of amino acid residues of CPOX among various species. CPOX among various species exhibits several perfectly conserved regions of amino acid sequence. Other investigators [20] reported that the mutation of amino acids 388-404 corresponding to the α-helix H9 band of X-ray analysis can be responsible for the accumulation of harderoporphyrin in severe cases of HCP. It was also reported that patients with HCP as an autosomal dominant trait expressed mutants R391W and R401W [20]. Patients with harderoporphyria reported as an autosomal recessive disease produced K404E mutant enzyme, which caused the accumulation of harderoporphyrin [21, 28]. Next, we expressed R391W, R401W and K404E with His-tag and examined the enzyme activity with purified enzymes. As shown in Fig. 2.4A, the production of protoporphyrin of R391W, R401W and K404E exhibited 20-30% of the level of the wt enzyme. The proportion of harderoporphyrin to protoporphyrin in R401W enzymes was markedly high (Figs. 2.1B and 2.4A), although patients with R401W mutation are reported to have typical HCP [20]. When the time course of the production of harderoporphyrinogen with R401W was examined, the amount of harderoporphyrinogen increased with time, while the production of protoporphyrinogen almost stopped, indicating that the mutation diminished the second decarboxylation reaction from harderoporphyrinogen to protoporphyrinogen (Fig. 2.1C). The production of harderoporphyrinogen with K404E was high, but this is less common. I next constructed two mutant CPOXs with the amino acid substitution at arginine at 401 and expressed R401K and R401G. R401G showed low activity, but the production activity of harderoporphyrinogen was extremely high. R401K produced more harderoporphyrinogen, but less protoporphyrinogen than the control (Fig. 2.4B). To clarify whether the lysine residue at 404 is required for the activity, the

activities of K404H and K404Q were examined. The production activity of protoporphyrinogen with K404Q showed normal activity, but K404H produced a high level of harderoporphyrinogen with low production of protoporphyrinogen (Fig. 2.4C). These results suggest that mutation at 401 and 404 is involved in the second decarboxylation reaction, producing harderoporphyrin. We further made the mutants R388W, D400A and G402D with amino acid substitution of conserved amino acids at the α-helix H9 region [19]. G402D and R388W showed low activity, but G402D produced a considerable amount of harderoporphyrinogen. No activity was observed with D400A. I then examined whether the mutant enzymes formed homodimers. Wt, R388W, R391W, D400A, R401W, G402D and K404E enzymes were treated with a cross-linker BS^3. R388W, R391W, R401W, G402D and K404E formed dimers. D400A remained as a monomeric form (Fig. 2.4D). Aspartic acid at 400 can be a prerequisite for dimerization of CPOX, and the mutant in the monomeric form is inactive.

2.3.3. Functional analysis of heterodimer of mutant/ wild-type complex

To clarify whether the wt and mutated subunits can form a heterodimer, we then expressed heterophilic forms of His-R388W/HA-wt, His-R391W/HA-wt, His-D400A/HA-wt, His-R401W/HA-wt, His-G402D/HA-wt and His-K404E/HA-wt. Immunoblot analysis with antibody for CPOX revealed that all enzymes purified with nickel beads exhibited two bands, and the migration of mutated enzymes in the gel was different depending on the substitution of amino acids (Fig. 2.5). All mutated enzymes purified with nickel ion beads reacted with antibody to HA (Fig. 2.5), indicating that heterophilic forms of mutant/wt form dimers. These results indicated that all mutant/wt forms expressed formed heterodimers. We then examined their enzyme activities. Interestingly, purified R388W/wt, R391W/wt, D400A/wt and K404E/wt enzymes exhibited 35-55% of the activity of the wt enzyme and produced relatively low levels of

harderoporphyrinogen (Figs. 2.6A and B). It was found that the activity of R401W/wt was very low. Purified R401G/wt, R401K/wt and G402D/wt showed low activity for protoporphyrinogen and the proportion of harderoporphyrinogen to protoporphyrinogen was relatively high (Fig. 2.6B). I finally examined the activity of K404H/wt and K404Q/wt. K404Q/wt showed normal activity, whereas K404H showed lower production of protoporphyrin with an increase in harderoporphyrinogen (Fig. 2.6C). These results indicate that heterodimers of mutant/wt CPOX are active, and those consisting of mutants that produced a high level of harderoporphyrinogen also showed high activity for the production of harderoporphyrinogen.

2.4. Discussion

This study demonstrated the relationship between the enzyme activity and protein structure of homophilic and heterophilic forms of mutated CPOX. I found that mutant CPOX D400A did not show any activity, which was consistent with the previous observations by Stephenson et al. [1] that the formation of protoporphyrinogen with mutation of aspartic acid at 400 was not detected. As the experiments with the cross-linker did not show the dimeric form of D400A (Fig. 2.4D), the monomer of CPOX is inactive. On the other hand, when D400A/wt was expressed, purified enzyme with the dimeric form showed high activity, suggesting that one subunit of the heterophilic complex is the wt enzyme, which leads to dimerization. Thus, in the case of the presence of one aspartic acid residue at 400 in two subunits, the heterophilic enzyme was able to adopt a dimeric form. Among the mutants tested, all enzymes exhibiting the activity formed dimers. As the heterodimer was able to produce protoporphyrinogen, each subunit acts in an independent reaction. From the structure of human CPOX determined by X-ray analysis [19], α-helices H9 of the homodimer face each other and aspartic acid at 400 in one subunit is associated with that in the other subunit. Therefore, the aspartic acid at 400 is essential for dimerization of CPOX.

The amino acid residues 388-397 of human CPOX are perfectly conserved among species (Fig. 2.3), suggesting that arginine at positions 388 and 391 is required for proper catalytic activity. The mutant R388W enzyme exhibited markedly decreased activity. Lamoril et al. [20] reported that R391W was found in a patient with HCP and we also found the decreased activity with R391W (Fig. 2.4A). Very recently, Mori et al [29] found that cataracts in the BALB/c-nct/nct mouse were caused by a hypomorphic mutation in exon 5 of the mouse CPOX gene, resulting in the production of the R380L enzyme: R380 in mouse

CPOX corresponds to R391 in human CPOX. The CPOX activity in liver homogenates of homozygote mutant mice was <15% of that of control mice, with the marked accumulation of coproporphyrin in liver, serum and lens. No accumulation of coproporphyrin was observed in the heterozygote mice. When the mouse R380L enzyme was expressed in *E. coli* and the activity was examined, decreased activity (<15%) compared with the control, similar to that in liver, was found. In both liver and *E. coli*, the mutant CPOX did produce harderoporphyrinogen at a low level. Furthermore, no accumulation of harderoporphyrin was observed in liver of nct/nct mice [29]. These results suggest that the mutation of conserved arginine residues in exon 5 may not be responsible for the formation of harderoporphyrinogen.

It is reported that harderoporphyria is a rare auto-somal recessive inherited disease with very low activity of CPOX [14, 19]. Only H327R and K404E were found in Caucasian families [21, 28]. We showed that a considerable amount of harderoporphyrinogen with purified K404E was produced, but not in much accordance with the accumulation in patients with harderoporphyria [21, 28]. The production of harderoporphyrinogen by K404E was much less than that due to K404H (Figs 2.4A and 2.6). H327R did not accumulate the harderoporphyrinogen (data not shown). The difference between the activities of mutant enzyme is not explained, but may be due to different conditions of the expression of mutated CPOX. As harderoporphyria is an autosomal recessive-inherited disease, patients with harderoporphyria produce mutant CPOX with the homophilic form as K404E.

On the basis of the present observations that K404H but not K404Q, was associated with marked accumulation of harderoporphyrinogen, the mutation at K404 can change the structure of CPOX, possibly leading to cessation of the second decarboxylation reaction of the enzyme. Although it is possible that the mutation of lysine at 404 to another amino acid diminished the second step of

the reaction, lysine at 404 is not conserved among species. Therefore, the reason for the accumulation of harderoporphyrinogen in patients with K404E may be derived from different conditions of the unique enzyme reaction of K404E in the mitochondria of mammalian cells. The present data showed that change of amino acids at 400-404 to several other amino acids gives rise to the accumulation of harderoporphyrinogen. On the basis of the three-dimension a structure of human CPOX, this region showed close association with the other subunit [19]. Thus, this region may be adjacent to or form part of its active site [30] and a mutation may impair the decarboxylation of harderoporphyrinogen [20, 21, 31].

The mutant R401W is a typical enzyme associated with HCP [20]. The recombinant R401W showed 30% of the protoporphyrinogen-producing activity of wt CPOX, but was associated with high level of harderoporphyrinogen. The finding of harderoporphyrinogen production with R401W was also previously reported [20]. The activity of R401W was not as low as those of other mutant enzymes from patients with HCP. HCP patient with R401W did not accumulate harderoporphyrinogen [19]. This study on heterodimer R401W/wt showed extremely low activity without the accumulation of harderoporphyrinogen (Fig 2.6). Owing to this low activity, R401W/wt possibly acts as a dominant negative type. Considering that HCP with R401W was reported to be a heterozygote in an autosomal dominant trait [20] and recombinant enzyme can form heterodimer in *E. coli* (Fig 2.6), the HCP patients with R401W can produce heterodimer CPOX consisting of mutant and wt subunits. Owing to the low activity of R401W/wt enzyme, total activities of homophilic and heterophilic CPOX become <50% of that of the control.

Among the mutant enzymes constructed by mutagenesis, the mutants R401K/- and R401G/- accumulated a marked amount of harderoporphyrinogen with a decrease of the production of protoporphyrinogen. As heterodimers of

R401K/wt and R401G/wt expressed in *E. coli* showed low activity, the change of arginine at 401 may lead to the development of a serious form of HCP or harderoporphyria in patients, depending on the combination of dimeric forms of CPOX.

My research strongly demonstrated that CPOX is active only when the mutant is in dimeric form. The short region extending from amino acid 401 to 404 is important for catalysis of the oxidative decarboxylation of harderoporphyrinogen to produce protoporphyrinogen. Furthermore, the types and positions of mutations in the human CPOX gene may predict the clinical outcome of the disease with manifestations of HCP or harderoporphyria. I provided evidence that the mutant homodimer results in harderoporphyria and heterodimer leads to HCP.

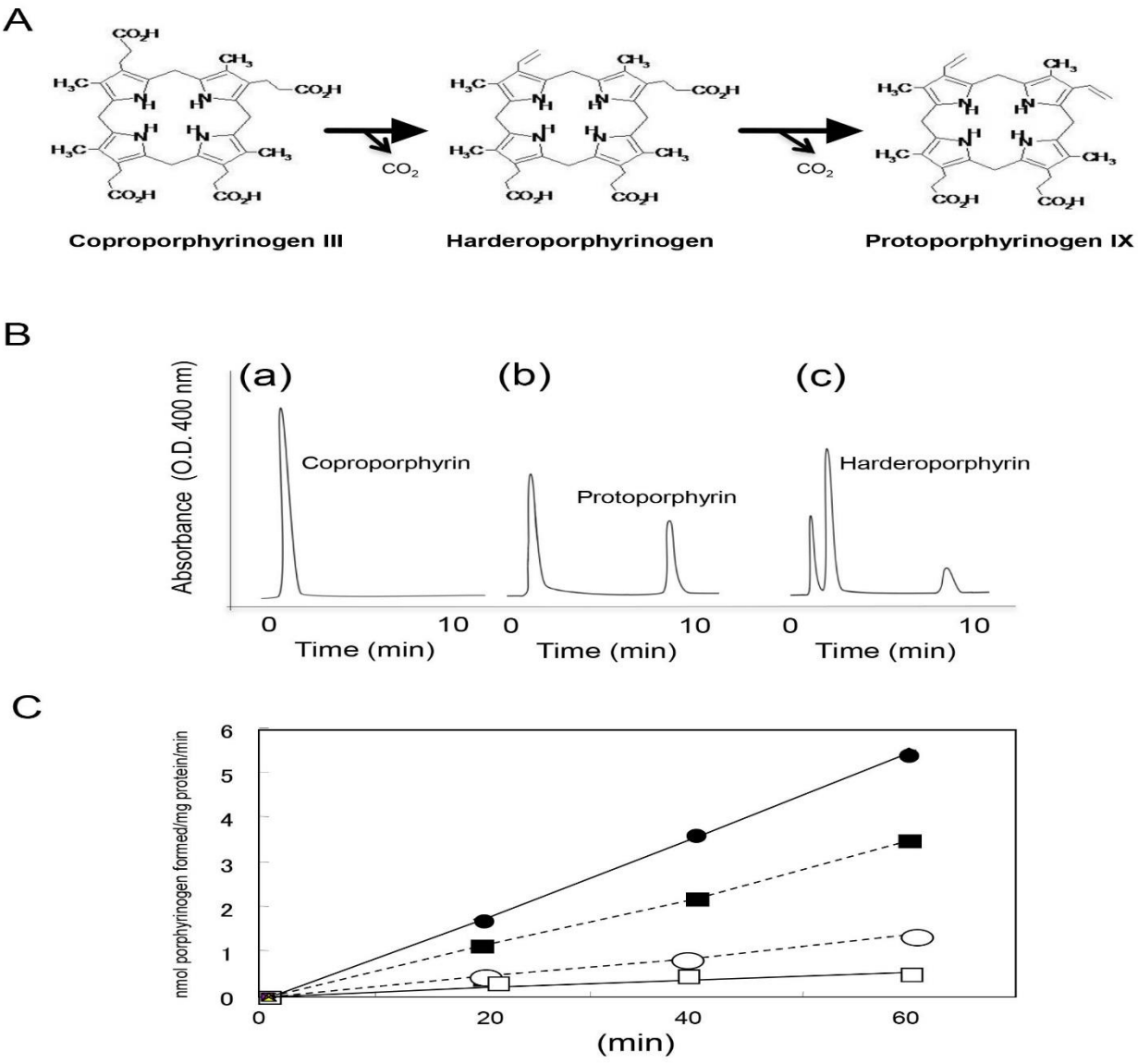

Fig 2.1 (A) Stepwise decarboxylation of coproporphyrinogen III to form protoporphyrinogen IX catalysed by CPOX. (B) HPLC profile of porphyrins before and after incubation with the recombinant purified CPOX. Porphyrins were formed before (a) or after incubation at 37^{o}C for 1h with wt CPOX (b) and R401W CPOX (c). After photo-oxidation of porphyrinogens, HPLC analysis of porphyrins was performed by measuring absorbance at 400 nm. (C) Time course of harderoporphyrinogen and protoporphyrinogen with wt (solid line) and mutant (dotted line) CPOX. Circle denotes protoporphyrinogen and square denotes harderoporphyrinogen.

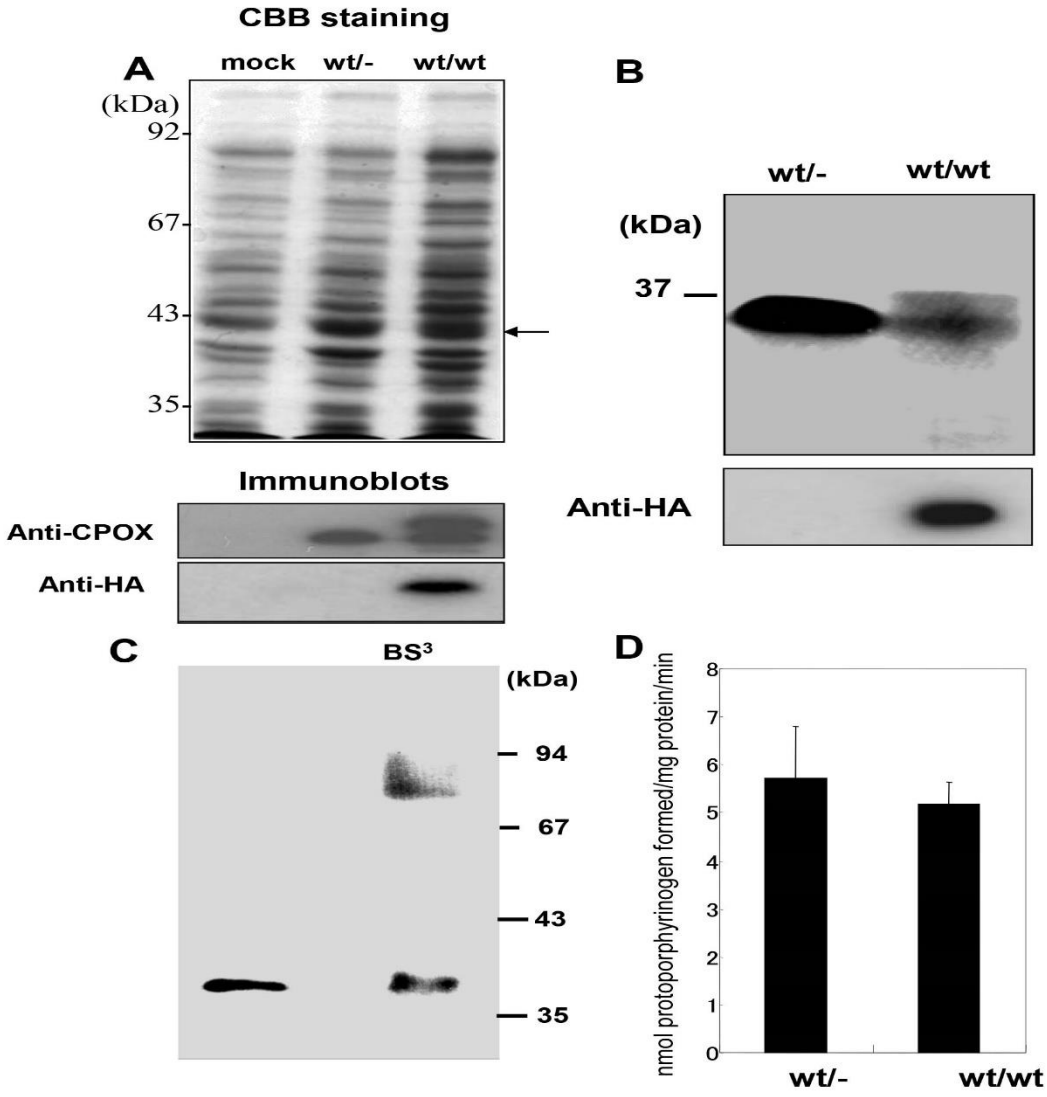

Fig 2.2: SDS-PAGE analysis of recombinant CPOX expressed in *E. coli*. (**A**) CBB staining of cell lysates of *E. coli* not expressing or expressing His-tag (wt/-) and His-/HA-tag (wt/wt) CPOX (upper panel). Immunoblotting was performed with anti-CPOX and anti-HA (lower panel). (**B**) CBB staining of CPOX (wt/-; wt/wt) purified with nickel ion beads (upper panel). Immunoblotting was performed with anti-HA (lower panel). (**C**) SDS-PAGE analysis of CPOX treated with a cross-linker BS^3 (2mM). (**D**) The CPOX activity with purified enzymes. The purified wt/- and wt/wt enzymes were incubated with coproporphyrinogen (5 nmol) at 37°C for 30 min. The activity is shown as the production of protoporphyrinogen (nmol) per milligram protein per minute at 37°C (n=3).

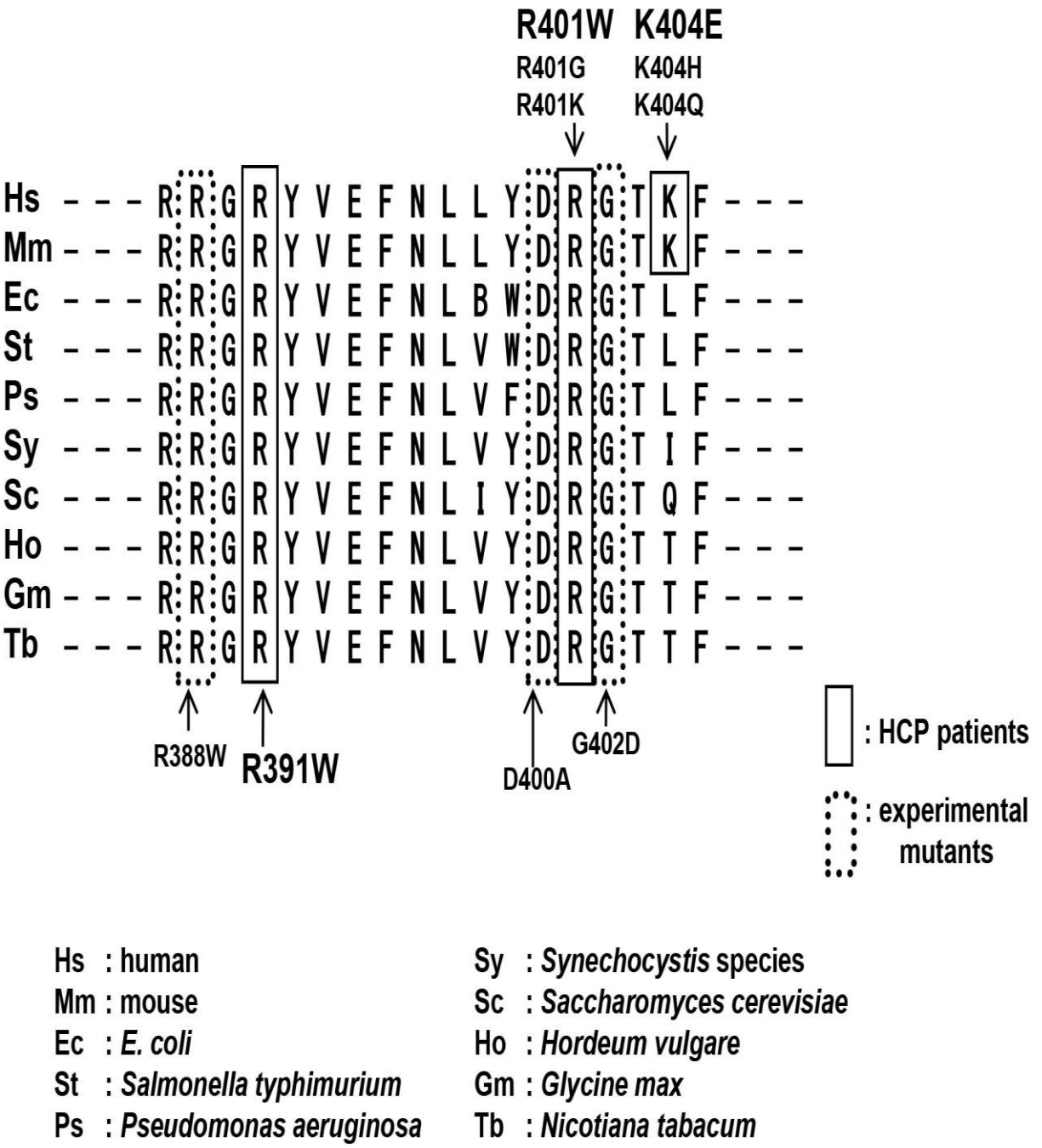

Fig 2.3: Comparison of amino acid sequences from human (*Homo sapiens*), mouse (*Mus musculus*), *E. coli*, yeast (*Saccharomyces cerevisiae*), *Salmonella typhimurium*, *Hordeum vulgare*, Soybean (*Glycine max*), *Synechocystis* sp., *Pseudomonas aeruginosa* and *Nicotiana tabacum*. Boxes (solid line) are amino acid residues mutated in patients with HCP, and with those a dotted line show amino acid residues constructed in this study.

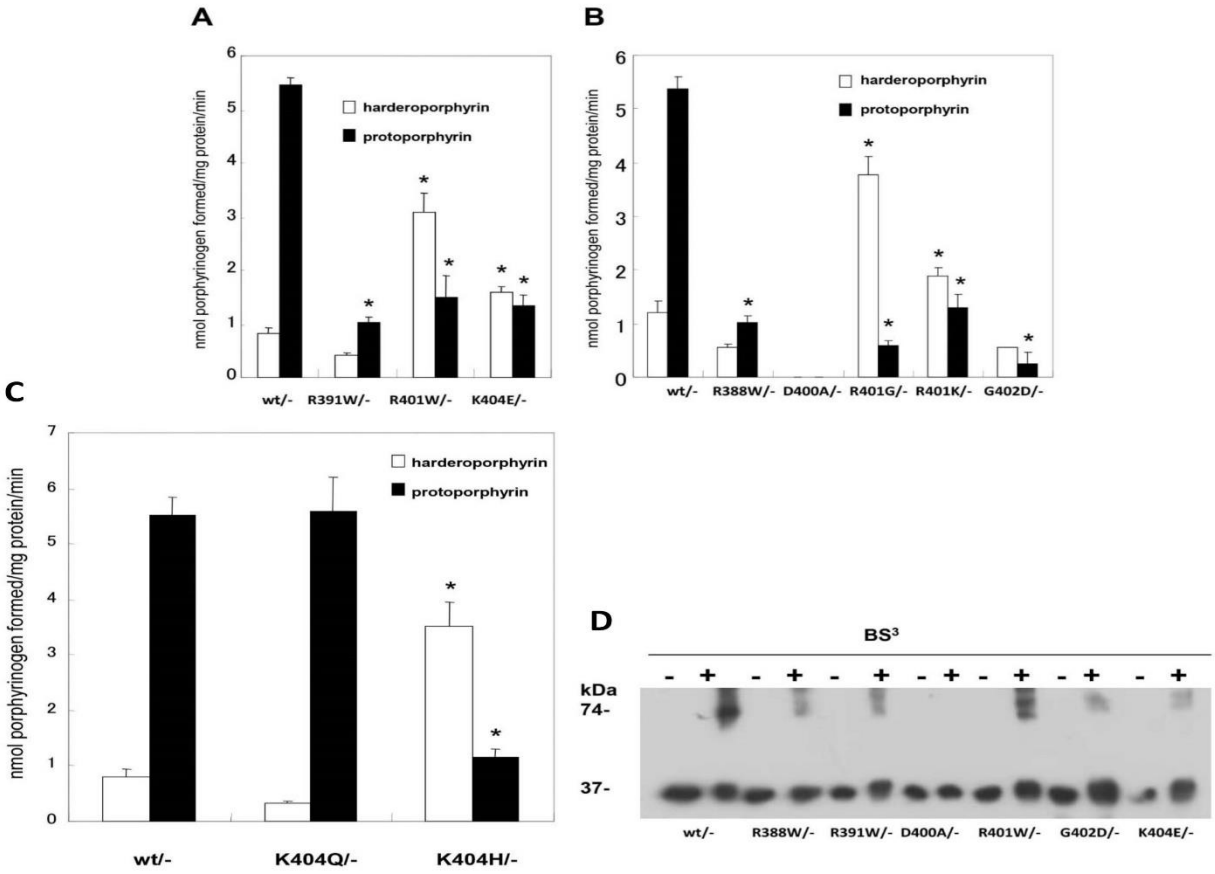

Fig 2.4: The harderoporphyrinogen and protoporphyrinogen producing activities of homodimer of wt and mutant CPOX.

Wt/-, R388W/-, R391W/-, D400A/-, R401W/-, R401G/-, R401K/-, G402D/-, K404E/-, K404Q/- and K404H/- enzymes were expressed in strain BL21. After purification of enzymes with nickel ion beads, purified His-tag enzymes were incubated with coproporphyrinogen (5 nmol) at 37°C for 30min. Comparison of the activities of mutant enzyme derived from patients with HCP (**A**) and constructed by mutagenesis (**B**, **C**). The activities with R388W, D400A, R401G, R401K and G402D (B), and K404Q and K404H (C) are shown as the production of harderoporphyrinogen and protoporphyrinogen (nmol) per milligram protein per minute at 37°C (n=4). *P<0.01, different from control (wt). (**D**) The coupling of mutant enzymes with BS^3. Mutant enzymes were treated with 2mM BS^3 for 30 min, and analysed by SDS-PAGE.

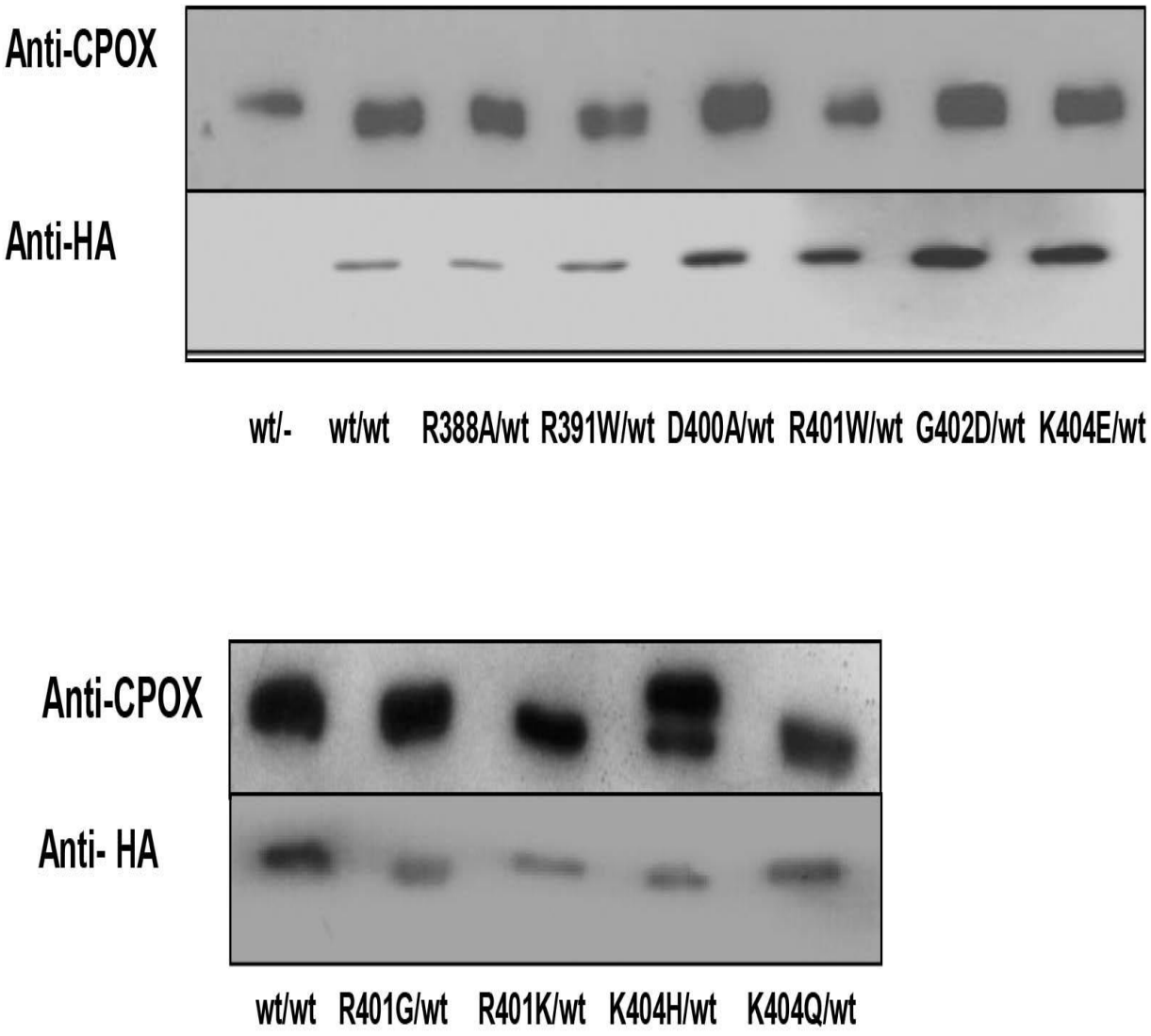

Fig 2. 5: Immunoblot analysis of His-tag and His-/HA-tags CPOX.

The indicated wt and mutant enzymes were expressed in strain BL21. After purification with nickel ion beads, the proteins were analysed by SDS-PAGE. Immunoblotting was performed with anti-CPOX and anti-HA.

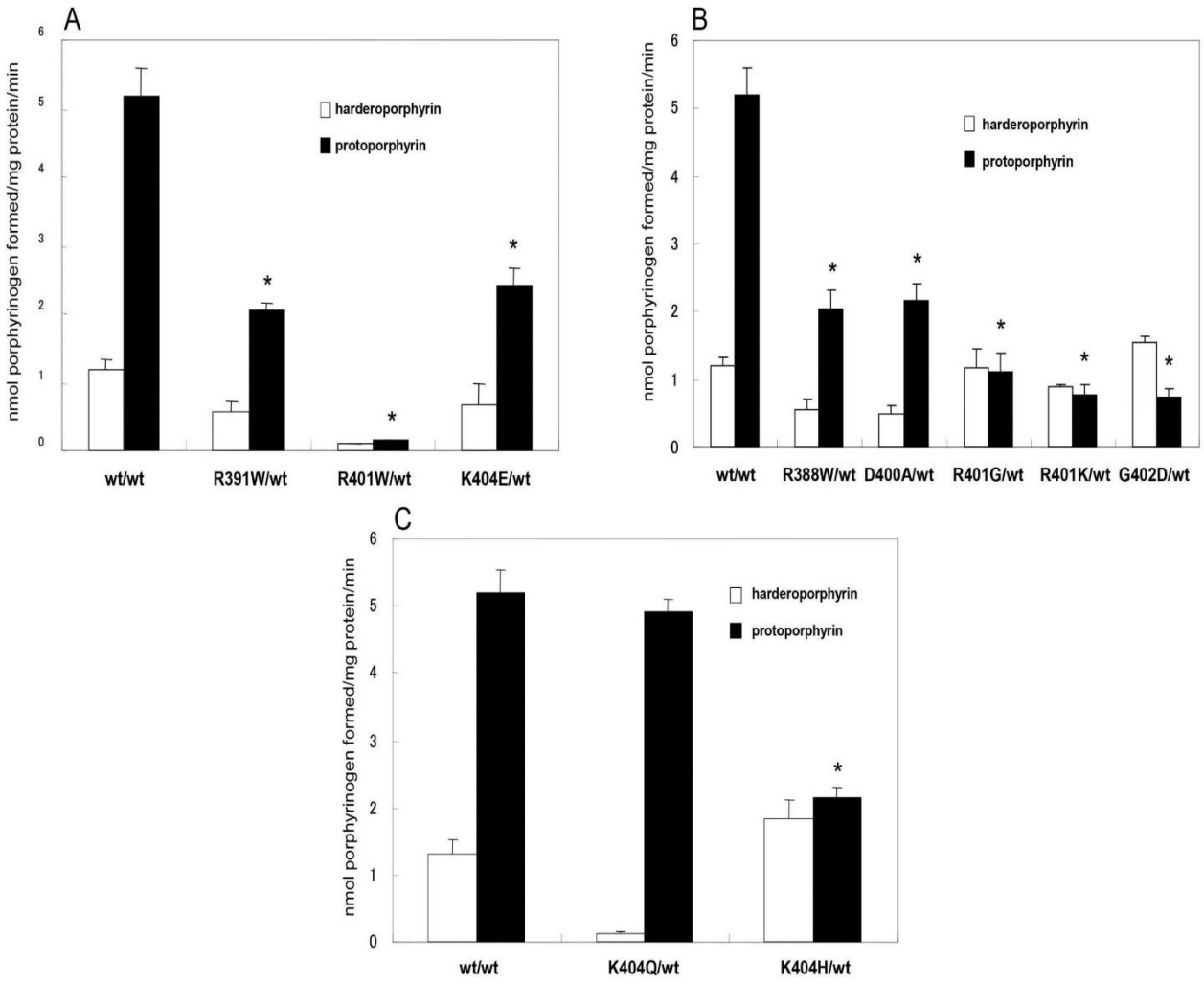

Fig 2.6: The harderoporphyrinogen and protoporphyrinogen producing activities of heterodimer of wt and mutant CPOX.

Wt/wt, R391W/wt, D400A/wt, R401W/wt, R401G/wt, R401K/wt, G402D/wt, K404E/wt, K404Q/wt and K404H/wt enzymes were expressed in strain BL21. The purified His-tag and His-/HA-tag enzymes were incubated with coproporphyrinogen (5 nmol) at 37ºC for 30min. Comparison of the activities of mutant enzyme derived from patients with HCP (**A**) and constructed by experimental mutagenesis (**B**, **C**). The activity is shown as the production of harderoporphyrinogen and protoporphyrinogen (nmol) per milligram protein per minute at 37ºC (n=5). *P<0.01, different from control (wt).

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CONCLUSION

CONCLUSIONS

Of eight inherited porphyria diseases in the heme-biosynthetic pathway, my study provides an understanding of a very rare disease which is the first description of a mutation in the UROS gene in a Southeast Asian patient and a molecular diagnosis for the identification of clinically asymptomatic heterozygous mutation carriers and families with CEP. A missense mutation in the UROS gene was identified as a transversion of G to T at nucleotide 11,776, resulting in a substitution of valine by phenylalanine at codon 3 of exon 2. The patient showed a homozygous mutant profile, and the heterozygous state was observed in the parents. The activity of mutated UROS expressed in *E. coli* was less than 16.1% that of the control, indicating that the markedly reduced activity of UROS is responsible for CEP. To date, 3 Vietnamese patients living in the North countryside have been diagnosed with the same mutation. Second, I clarified responsibility of different phenotypes between HCP and harderoporphyria, both of which are the mutations of the same enzyme, CPOX. My results show that the substitution of amino acids from R401 to K404 results in extremely low enzyme activity with either mutant homodimer or heterodimers containing normal and mutated subunits and can be linked to HCP disease. Thus, I provided evidence that the mutant homodimer results in harderoporphyria and heterodimer leads to HCP. Defining the severity of phenotype is important for the choice of therapeutic management. More deaths occurred because of a delay in diagnosis and in appropriate treatment of porphyria. However, the focus of management with cutaneous porphyria included emphasizing adherence to general precautions for minimizing photosensitivity by prevention of sun and UV exposure. Avoiding factors that provoke acute attacks can also be advised in HCP and harderoporphyria patients. Furthermore, all of CEP, HCP and Haderoporphyria are genetic

diseases so genetic counseling is strongly recommended in these types of porphyria. In areas of the North countryside of Vietnam with a high rate of consanguineous, it is a critical to conduct comprehensive genetic counseling of the affected families. I hope that the recent and future advances would markedly improve diagnosis and management of these diseases.

LIST OF PUBLICATIONS

1. **Congenital Erythropoietic Porphyria: Mutation of the Uroporphyrinogen III Cosynthase Gene in a Vietnamese Patient**

   Dao Hoang Thien Kim, Asako Kawazoe, Pham Dang Bang, Nguyen Tien Thanh and Shigeru Taketani

   Case Rep Dermatol. 2013; 5:105–110

2. **The enzyme engineering of mutant homodimer and heterodimer of coproporphyrinogen oxidase contributes to new insight into hereditary coproporphyria and harderoporphyria**

   Dao Hoang Thien Kim, Ryoko Hino, Yuka Adachi, Akio Kobori and Shigeru Taketani

   J. Biochem. 2013; 154 (6): 551-559

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