MUTATION IDENTIFICATION AND FUNCTIONAL ANALYSIS OF MUTANT DAX-1 PROTEINS FOUND IN THAI PATIENTS WITH X-LINKED ADRENAL HYPOPLASIA CONGENITA

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Biological Sciences
Common Course
Faculty of Science
Chulalongkorn University
Academic Year 2018

การระบุการกลายและการวิเคราะห์หน้าที่ของโปรตีนสายพันธุ์กลาย DAX-1 ที่พบในผู้ป่วยชาวไทยที่มีภาวะต่อมหมวกไตฝ่อแต่กำเนิดแบบเอกซ์ลิงค์

น.ส.ชนิสรา สุทธิวรชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาศาสตร์ชีวภาพ ไม่สังกัดภาควิชา/เทียบเท่า
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2561

Dissertation Title	MUTATION IDENTIFICATION AND FUNCTIONAL ANALYSIS OF MUTANT DAX-1 PROTEINS FOUND IN THAI PATIENTS WITH X-LINKED ADRENAL HYPOPLASIA CONGENITA
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ชนิสรา สุทธิวรชัย : การระบุการกลายและการวิเคราะห์หน้าที่ของโปรตีนสายพันธุ์กลาย DAX-1 ที่พบในผู้ป่วยชาวไทยที่มีภาวะต่อมหมวกไตฝ่อแต่กำเนิดแบบเอกซ์ลิงค์. (MUTATION IDENTIFICATION AND FUNCTIONAL ANALYSIS OF MUTANT DAX-1 PROTEINS FOUND IN THAI PATIENTS WITH X-LINKED ADRENAL HYPOPLASIA CONGENITA) อ.ที่ปรึกษาหลัก : ผศ. ดร.รัชนีกร ธรรมโชติ , อ.ที่ปรึกษาร่วม : รศ. พญ.ธนินี สหกิจรุ่งเรือง

การกลายในยีน *DAX-1* ส่งผลให้เกิดภาวะต่อมหมวกไตฝ่อแต่กำเนิดแบบเอกซ์ลิงค์ ซึ่งเป็นภาวะที่พบได้น้อย โดยผู้ที่มีภาวะนี้จะมีความบกพร่องในการพัฒนาต่อมหมวกไตและการสังเคราะห์สเตียรอยด์ ภาวะนี้มักจะพบในทารกหรือในเด็กเพศชาย มักแสดงอาการจากการขาดฮอร์โมนจากต่อมหมวกไตอย่างรุนแรง ในบางรายพบมีภาวะพร่องโกนาโดโทรปินทำให้ไม่เข้าสู่วัยรุ่น ยีน *DAX-1* ร่วมกับยีน *SF-1* ทำหน้าที่ในการควบคุมการแสดงออกของโปรตีน StAR ซึ่งโปรตีน StAR ทำหน้าที่กระตุ้นขบวนการสังเคราะห์สเตียรอยด์ โดยยีน *DAX-1* ทำหน้าที่เป็นตัวยับยั้งการถอดรหัสยีนในขบวนการสังเคราะห์สเตียรอยด์ วัตถุประสงค์ของการศึกษานี้เพื่อศึกษาการกลายพันธุ์ในยีน *DAX-1* และเพื่อวิเคราะห์การทำงานของโปรตีน DAX-1 ที่กลายพันธุ์ ซึ่งพบในผู้ป่วยชาวไทยที่มีภาวะต่อมหมวกไตฝ่อแต่กำเนิดแบบเอกซ์ลิงค์ การศึกษานี้ได้มีการตรวจสอบการกลายพันธุ์ด้วยเทคนิคพีซีอาร์ และเทคนิควิเคราะห์หาลำดับเบสในผู้ป่วยชาวไทย 7 รายทั้งหมด 6 ครอบครัวที่มีภาวะต่อมหมวกไตฝ่อแต่กำเนิดแบบเอกซ์ลิงค์ และภาวะพร่องโกนาโดโทรปิน โดยผู้ป่วยบางรายมีภาวะอื่นร่วมด้วย พร้อมทั้งวิเคราะห์การทำงานของโปรตีน DAX-1 ที่กลายพันธุ์ด้วยเทคนิควิเคราะห์ลูซิเฟอเรส ผลการวิเคราะห์หาลำดับเบสในยีน *DAX-1* ในผู้ป่วยชาวไทยพบการกลายพันธุ์รูปแบบใหม่ 3 รูปแบบ ได้แก่ c.363delG (p.Gly122Valfs*142), c.1062delC (p.Ala355Profs*17), c.1156C>T (p.Leu386Phe) และพบการกลายพันธุ์ที่เคยรายงานมาก่อนหน้านี้ 3 รูปแบบ ได้แก่ c.805_807delGTC (p.Val269del), c.1148_1149delGG (p.Gly383Aspfs*5), c.501_502insG (p.Ala170Argfs*15) ผลการวิเคราะห์การทำงานของโปรตีน DAX-1 ที่มีการกลายพันธุ์พบว่ามีระดับการแสดงออกในการยับยั้งการทำงานของโปรโมเตอร์ในยีน StAR ต่ำกว่า เมื่อเปรียบเทียบกับโปรตีน DAX-1 ปกติ การศึกษานี้พบว่าความสัมพันธ์ระหว่างลักษณะอาการที่แสดงออกกับลักษณะทางพันธุกรรมมีแนวโน้มไปในทิศทางเดียวกัน ผลการศึกษานี้สนับสนุนการวินิจฉัยและเพิ่มองค์ความรู้ในรูปแบบลักษณะอาการที่แสดงออกของผู้ป่วย และรูปแบบการกลายพันธุ์ของยีน *DAX-1* นอกจากนี้การวิเคราะห์เชิงโมเลกุลและการศึกษาเชิงฟังก์ชันยังช่วยให้เข้าใจถึงสาเหตุของโรคได้ดียิ่งขึ้น

สาขาวิชา	วิทยาศาสตร์ชีวภาพ	ลายมือชื่อนิสิต .....
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5672904123 : MAJOR BIOLOGICAL SCIENCES
KEYWORD: X-LINKED ADRENAL HYPOPLASIA CONGENITA, DAX-1, MUTATIONS ANALYSIS, FUNCTIONAL ANALYSIS

Chanisara Suthiworachai : MUTATION IDENTIFICATION AND FUNCTIONAL ANALYSIS OF MUTANT DAX-1 PROTEINS FOUND IN THAI PATIENTS WITH X-LINKED ADRENAL HYPOPLASIA CONGENITA. Advisor: Asst. Prof. Rachaneekorn Tammachote, Ph.D.,Assoc. Prof. TANINEE PETWIJIT, M.D.

Mutations in the *DAX-1* gene result in X-linked adrenal hypoplasia congenita (X-linked AHC), a rare congenital disorder of adrenal insufficiency with defects in adrenal gland development and steroid biosynthesis. Male patients usually develop severe adrenal insufficiency in early infancy or childhood. In some cases, hypogonadotropic hypogonadonism (HH) is presented in puberty. *DAX-1* gene product, DAX-1 protein, acts as a transcriptional repressor of genes in the steroidogenic pathway by interacting with, and repressing the steroidogenic factor 1 (SF-1) protein. In contrast, the SF-1 protein transactivates the steroidogenic acute regulatory (StAR) protein that functions in the steroid biosynthetic pathway. The aim of this study is to identify mutations in the *DAX-1* gene and to analyze functions of mutant DAX-1 proteins found in Thai patients with X-linked AHC. Seven Thai patients in six families with typical and atypical symptoms of X-linked AHC and HH were investigated in the present study. The *DAX-1* gene was sequenced from all patients, and the mutant DAX-1 proteins were functionally analyzed by luciferase assays. Sequence data identified three novel mutations: c.363delG (p.Gly122Valfs*142), c.1062delC (p.Ala355Profs*17), c.1156C>T (p.Leu386Phe), and three known mutations: c.805_807delGTC (p.Val269del), c.1148_1149delGG (p.Gly383Aspfs*5), c.501_502insG (p.Ala170Argfs*15) in the *DAX-1* gene. DAX-1 mutants showed lower levels of repressor activity on the *StAR* gene promoter when compared with wild-type. Moderate genotype-phenotype correlation was observed in the patients. These results support the diagnosis, and extend the phenotypic and mutational spectrum of *DAX-1* mutations. Moreover, molecular analysis and functional studies pave ways to better understanding towards the disease pathogenesis.

Field of Study:	Biological Sciences	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....

ACKNOWLEDGEMENTS

Firstly, I would like to express my deepest gratitude to my advisor, Assistant Professor Dr. Rachaneekorn Tammachote, and my co-advisor, Associate Professor Taninee Sahakitrungruang, for all their guidance, understanding, motivation, helps and patience throughout the time during my graduate studies. They are the best advisors and mentors for my PhD. study.

I also would like to express my sincere gratitude to Prof. Vorasuk Shotelersuk and Prof. Dr. Kanya Suphapeetiporn who provided me an opportunity to join their great team and granting access to the laboratory and research facilities. I have learned many things from them. They advised and helped me in conducting this research. My works in this thesis would not accomplish without these persons.

I highly appreciate all of my thesis committee for their worthy thesis evaluation, corrections, suggestions and valuable time.

I am deeply grateful to everyone at the Center of Excellence for Medical Genetics, especially Mrs. Rungnapa Ittiwut and Mr. Chalurmpon Srichomthong, for their guidance, helps and supports. Without their superior knowledge and experience, it would be very hard for me to conduct this research. I would like to give my special thanks to the patients and their family for participation in this study.

Finally, and most importantly, I would like to express my gratitude toward my dear parents, husband, family and friends for their love, support, understanding, and encourage me to pursue all of my goals.

Financial support was provided by Ratchadapiseksomphot Endowment Fund of Chulalongkorn University, the Thailand Research Fund grant and The Human Resource Development in Science Project (Science Achievement Scholarship of Thailand; SAST).

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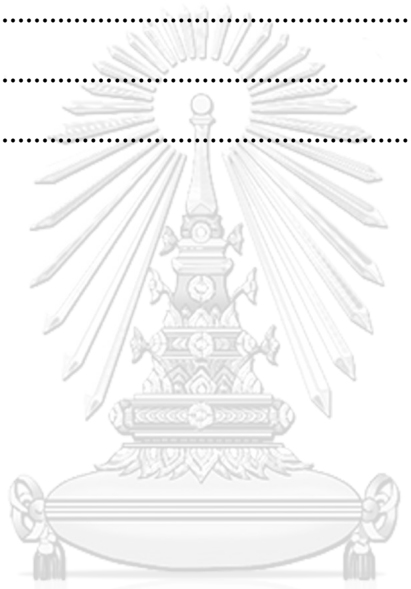

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AHC	Adrenal hypoplasia congenita
cDNA	Complementary DNA
CT	Computed tomography
CRH	Corticotropin releasing hormone
DAX-1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
DBD	DNA-binding domain
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DLR™	Dual-Luciferase Reporter
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	Ethylenediaminetetra acetic acid
FSH	Follicle-stimulating hormone
GFP	Green Fluorescent Protein
GIPP	GnRH-independent precocious puberty
GH	Growth hormone
GKD	Glycerol kinase deficiency
GnRH	Gonadotropin releasing hormone
HEK293T	Human embryonic kidney 293T cells
HGMD	Human Genome Mutation Database

HH	Hypogonadotropic hypogonadonism
HPA	Hypothalamic-pituitary-adrenal axis
HPG	Hypothalamic-pituitary-gonadal axis
LAR II	Luciferase Assay Reagent II
LBD	Ligand-binding domain
LH	Luteinizing hormone
LHRH	Luteinizing hormone-releasing hormone
LRH-1	Liver receptor homolog-1
MCR1	Melanocortin receptor type 1
MSH	Melanocyte stimulating hormone
NR0B1	Nuclear receptor subfamily 0 group B member 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLB	Passive Lysis Buffer
SEM	Standard error of the mean
SF-1	Steroidogenic factor 1
SST	Short synacthen test
StAR	Steroidogenic acute regulatory protein
VMH	Ventromedial hypothalamus

CHAPTER I
Introduction

1.1 Rationales, Theories, or Assumptions

Adrenal hypoplasia congenita (AHC) is a rare congenital disorder of adrenal insufficiency caused by defects in adrenal gland development and deficient in steroid biosynthesis (Achermann et al., 1999; Iyer and McCabe, 2004; Yu et al., 1998). General symptoms are salt wasting early in life, hyponatremia (the concentration of sodium level in blood is abnormally low), hyperkalemia (the concentration of potassium level in blood is higher than normal) and skin hyperpigmentation (darker the skin tone due to an overproduction of melanin). AHC can be inherited by a few modes of inheritance; one of them is X-linked recessive. X-linked AHC causes the adrenal glands to be defected in the permanent adult zone of the adrenal cortex, resulting in glucocorticoid and mineralocorticoid deficiency. Affected boys with X-linked AHC usually develop severe adrenal insufficiency in early infancy or childhood. Male patients also have apparent hypogonadotropic hypogonadonism (HH; delayed or absent pubertal development due to low levels of the sex steroids and gonadotropin secretion) in puberty resulted from impaired or absent production of gonadotropin releasing hormone (GnRH) from the hypothalamus and/or gonadotropins (Follicle-stimulating hormone; FSH and Luteinizing hormone; LH) from the anterior pituitary (Achermann et al., 1999; Iyer and McCabe, 2004; Jadhav et al., 2011; Lehmann et al., 2003).

X-linked AHC is characterized by a deficiency in the steroid hormones that is associated with a decrease in the blood level of serum glucocorticoid and aldosterone.

It is caused by mutations in a nuclear receptor gene called dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 *(DAX-1*) that located on Xp21. The *DAX-1* gene is also known as nuclear receptor subfamily 0 group B member 1 (*NR0B1)*. DAX-1 protein acts as a transcriptional repressor of other genes in the steroidogenic pathway at all levels of the hypothalamic-pituitary-gonadal (HPG) axis, thereby inhibits the steroid biosynthesis from the adrenal cortex (Clipsham and McCabe, 2003; Iyer and McCabe, 2004).

X-linked AHC has an estimated incidence of 1 in 12,500 live births (Laverty et al., 1973; Lin et al., 2006). In Thailand, there has been no report in the number of incidence. To understand the consequences of the mutations in the *DAX-1* gene, functional studies have been performed in patients with many ethnics. Up to date, several publications have described a broad mutational spectrum in the *DAX-1* gene in X-linked AHC patients in different ethnics, almost all which are nonsense or deletion mutations that caused frame-shift mutations leading to premature truncation of the protein. Moreover, missense mutations were detected (Ali et al., 2014; Li et al., 2010; Wang et al., 2014).

Genetic analysis of *DAX-1* mutations can lead to accurate diagnosis of X-linked AHC patients and results in suitable treatment for patients and prenatal diagnosis and genetic counseling for their family. Nowadays, genetic analysis of *DAX-1* is widely available as a clinical test in western nations, but has not been employed in Thailand. While this disease is an X-linked inherited, counseling is necessary to identify possibly affected males in the mother's family. Accurate and early diagnosis can help the patients to avoid life-threatening adrenal deficiency in

other family members, while prenatal diagnosis can prevent the adrenal crisis in the future pregnancies, especially the diagnosis in the pre-symptomatic stage (Ostermann et al., 2006). Therefore, *DAX1* sequencing plays an important role in the diagnosis of X-linked AHC with family history. While there have been a few reports on clinical and genotyping data of this gene in a few ethnics, only one is available in Thai patients (Boonyawat et al., 2017).

In collaboration with the Pediatrics Endocrine Disorders Unit at King Chulalongkorn Memorial Hospital which has collected samples from patients diagnosed with X-linked AHC, this research gained access to patient information and samples. Molecular genetics data is necessary to confirm the diagnosis and to provide accurate information to patients. Hence, the conditions of this disorder can be detected early and can reduce the symptoms and increase chance of patients' survival.

1.2 Research scope

Mutations in the *DAX-1* gene were studied in twelve Thai patients with X-linked AHC who were diagnosed by pediatric endocrinologist and genetic counseling. Moreover, the patient's parents, especially mother, were studied. Direct sequencing method were used to detect mutations. Nonsense, missense and frameshift mutations were identified. Effects of the mutations were functionally analyze by cloning the wild-type and mutant genes into a luciferase vector and quantify for the luciferase activity.

1.3 Limitations of research

The functional study was performed *in vitro*, as the *DAX-1* gene is expressed in the adrenal gland but not in the blood.

1.4 Expected outcomes

Knowledge gained from this study will provide information for physicians to accurately diagnose and provide treatment for patients with X-linked AHC. Moreover, results from functional studies will provide information on molecular pathogenesis of the mutations which can pave ways to better treatment, or individualized medicine in the future.

CHAPTER II
Literature review

2.1 Adrenal gland and steroid synthesis

2.1.1 Adrenal gland structure

The adrenal glands are situated above each kidney (Figure 1). The adrenal gland can be divided into two very distinct zones, an outer region called the adrenal cortex and an inner region called the adrenal medulla. Each of regions secretes different hormones (Molina, 2013).

The adrenal gland is derived from mesodermal mesenchyme for cortex and from neural crest cells for medulla. During the 5^{th} week of fetus development, the mesothelium cells differentiate to fetal cortex or primitive adrenal cortex. At seven weeks, the medulla is derived from the embryonic neural crest cells. After eight weeks, mesenchymal cells begin to encapsulate the fetal cortex and derived to definitive (permanent) cortex. At the later stages of development, the permanent cortex differentiates into three zones. The permanent cortex is presented only two zones at birth; zona glomerulosa and fasciculata. The development of definitive cortex is regulated by adrenocorticotropic hormone (ACTH) from fetal pituitary gland. After birth, zona reticularis continues to develop and present when the child or kid is 3-4 years old (Moore and Persaud, 1998).

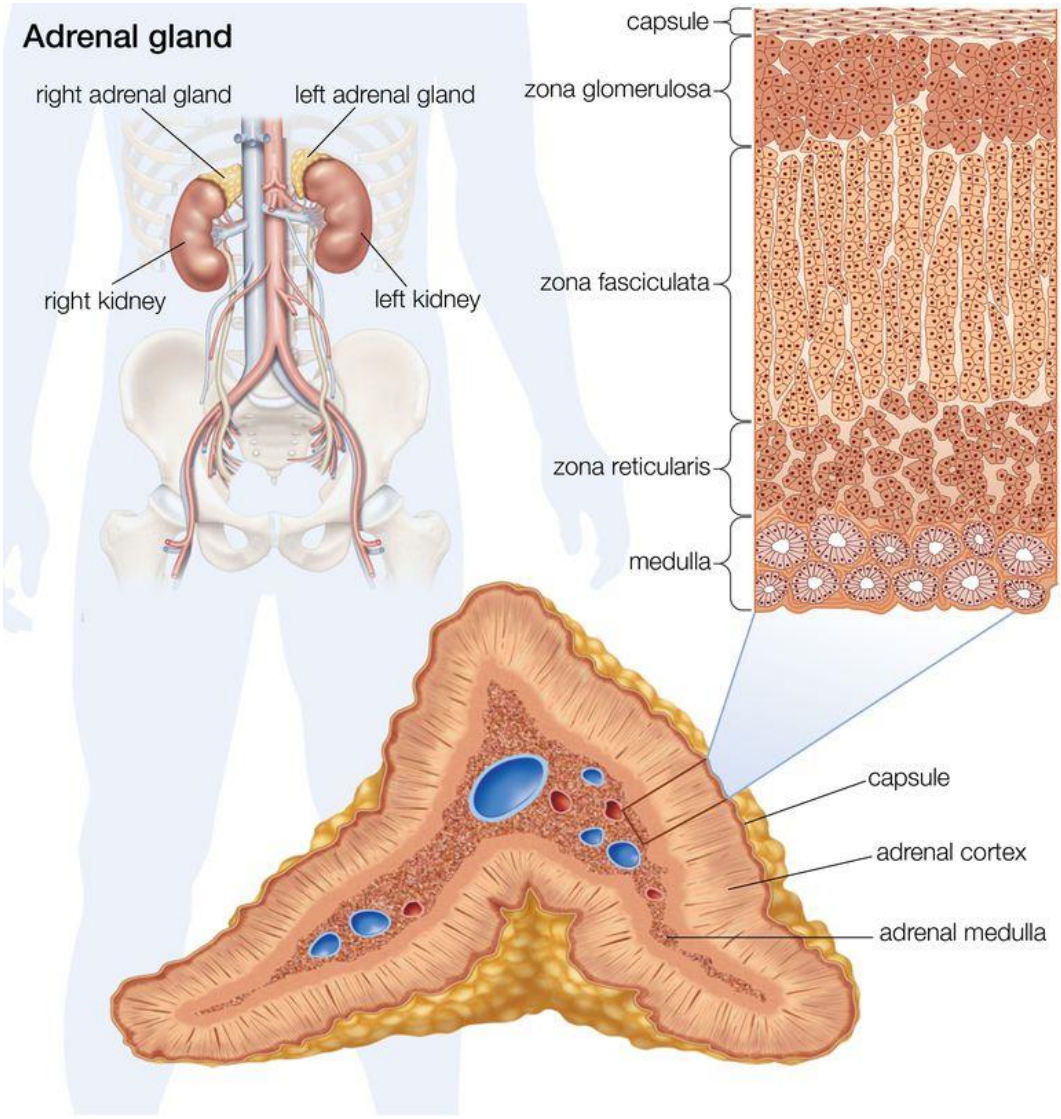


Figure 1 Anatomy of adrenal gland, cross section. The adrenal glands are situated above each kidney and are composed of an outer cortex and an inner medulla, all surrounded by capsule. The adrenal cortex comprises three layers that surround the medulla: zona glomerulosa, zona fasciculata, and zona reticularis.

(https://www.verywellmind.com/what-are-the-adrenal-glands-2794816)

Adrenal medulla produces and secretes amine hormones, adrenaline (epinephrine) and noradrenaline (norepinephrine). This region is stimulated by nerve impulses that originated in the hypothalamus and travels through the brain stem, spinal cord, and sympathetic nerves (Molina, 2013).

2.1.2 Steroid biosynthesis

Adrenal cortex makes up the majority of the gland and is stimulated by ACTH from the anterior pituitary. Three parts of the cortex (Figure 1) synthesize the different types of hormones (Jameson, 2010).

1. Zona glomerulosa lies directly beneath the capsule and stays in outer zone. The cells secrete mineralcorticoids, mainly aldosterone which maintains sodium and potassium levels in the body. It regulates blood volume and concentration of minerals in the blood. The main regulator is the kidney, via the renin-angiotensin-aldosterone system.
2. Zona fasciculata stays in the intermediate zone which is widest of the three zones. Cells secrete glucocorticoids, cortisol and corticosterone. Cortisol promotes the conversion of fatty acids (and sometimes from amino acids) into glucose. The secretion is regulated by negative-feedback of hypothalamic-pituitary-adrenal (HPA) axis via ACTH from pituitary gland and corticotropin releasing hormone (CRH) from hypothalamus (Figure 3).
3. Zona reticularis is the inner zone. Cells secrete androgens and estrogens which are sex hormones. The major androgen secreted by the adrenal gland is dehydroepiandrosterone (DHEA) and

dehydroepiandrosterone sulfate (DHEAS). The secretion is regulated by ACTH hormone and cortical androgen-stimulating hormone, but the mechanism is unclear.

Pathway of steroid biosynthesis

Steroid hormones are derived from cholesterol that transfered into the inner mitochondria membrane by steroidogenic acute regulatory protein (StAR). The pathways of steroid biosynthesis in the adrenal cortex are shown in Figure 2.

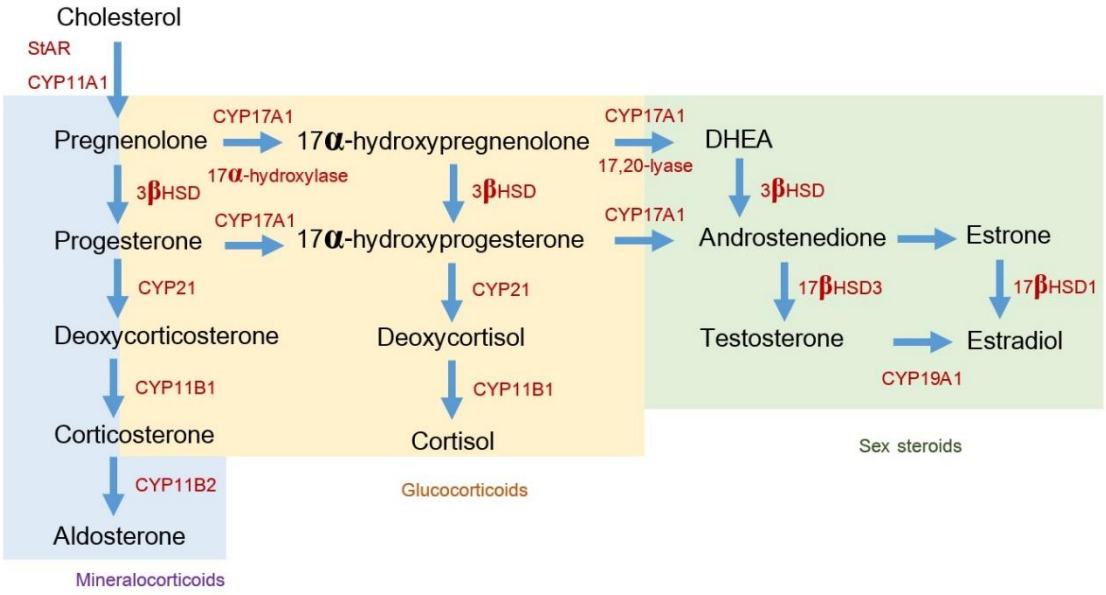


Figure 2 Schematic representation of pathway for the steroid biosynthesis in adrenal cortex. StAR is a transport protein that regulates cholesterol transfer within the mitochondria and response to agents that stimulate steroid production. Blue background colour show pathway secretion of the mineralocorticoids hormones that synthesized from zona glomerulosa zone. Pink background colour show pathway secretion of the glucocorticoids hormones that synthesized from zona fasciculata zone. Grey background colour show pathway secretion of the sex steroids hormones that synthesized from zona reticularis zone.

HPA axis

HPA axis is the regulation of synthesis hormones from the hypothalamus, the anterior pituitary and the adrenal cortex. Steroid hormone plays a key role in maintaining balance such as aldosterone and mineralocorticoid are essential for maintaining salt balance and blood pressure. Controlling the concentration of steroid hormone in the body is important by regulatory synthesis. CRH is synthesized by neurons in the hypothalamus, and is carried to the anterior pituitary where it stimulates the release of ACTH. CRH mediates the diurnal rhythm in ACTH secretion and the ACTH response to stress. Regulation of secretion of CRH and ACTH are controlled by negative feedback of circulating cortisol. Cortisol inhibits secretion of CRH from the hypothalamus and ACTH from the pituitary (Jameson, 2010).

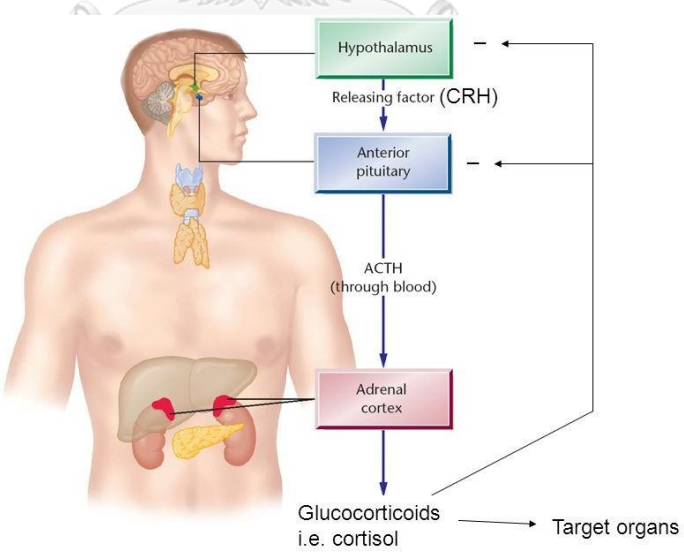


Figure 3. Schematic representation of the hypothalamic-pituitary-adrenal (HPA) axis (https://www.myownworstenemy.org/podcast/why-stress-destroys-us/)

2.2 Adrenal hypoplasia congenita (AHC)

Adrenal hypoplasia congenita (AHC) is a rare congenital disorder of adrenal insufficiency caused by defects in adrenal gland development and attributed to deficiency in steroid biosynthesis (Achermann et al., 1999; Iyer and McCabe, 2004; Yu et al., 1998).

2.2.1 Phenotype

Patients with AHC usually present in early infancy or childhood with congenital adrenal insufficiency that caused by disorder of the adrenal cortex. Cells in adrenal cortex are smaller than normal that caused the adrenal gland atrophy. In adrenal cortex, plasma concentrations of glucocorticoids and mineralocorticoids are decreased in AHC patients. This disease is divided into two forms, based on their adrenal morphologies, histologic appearance and pattern of inheritance: X-linked or autosomal recessive (Iyer and McCabe, 2004; Ostermann et al., 2006).

2.2.2 Inheritance

2.2.2.1 AHC with autosomal recessive inheritance

The autosomal recessive form has a ‘miniature adult’ adrenal phenotype that has small permanent zone of fetal cortex, but has normal structural zonation. This form is involved in abnormal central nervous system and impaired pituitary development and function. It is inherited by autosomal recessive mode or sporadic, although the molecular basis is currently unclear (Iyer and McCabe, 2004).

2.2.2.2 X-linked AHC

The X-linked form, also called cytomegalic, is X-linked recessive associated. X-linked AHC causes the adrenal glands to be defected in the permanent adult zone of the adrenal cortex. The adrenal cortex of these patients is absent and adjusted of the zona fasciculata, reticularis and glomerulosa, resulting in glucocorticoid and mineralocorticoid deficiency. Affected boys with X-linked AHC usually develop severe adrenal insufficiency in early infancy or childhood. Moreover, these patients had apparent hypogonadotropic hypogonadonism (HH) in puberty that impaired or absented production of gonadotropin releasing hormone (GnRH) from the hypothalamus and/or gonadotropins (FSH and LH) from the anterior pituitary (Achermann et al., 1999; Iyer and McCabe, 2004; Jadhav et al., 2011; Lehmann et al., 2003).

General symptoms of X-linked AHC are salt waste early in life, hyponatremia, hyperkalemia, skin hyperpigmentation, vomiting, feeding difficulty, dehydration, low level of serum glucocorticoid and aldosterone, but increase in plasma ACTH and plasma renin level. The skin hyperpigmentation is happened including the areas that not exposed to the sun such as nipple, elbow, foldable joint and etc. It affects from the increase in ACTH and melanocyte stimulating hormone (MSH) in anterior pituitary gland. The worst-possible outcome is shock caused by a salt-wasting condition resulting in

mortality (Achermann et al., 1999; Clipsham and McCabe, 2003; Jadhav et al., 2011; Niakan and McCabe, 2005).

X-linked AHC has an estimated incidence of 1 in 12,500 live births to 1:70,000 to 1:600,000 males (Laverty et al., 1973; Lin et al., 2006). In Thailand, there has been no report in the number of incidence.

X-linked AHC patients present heterogeneous phenotypes. Even among family members with the same mutation, the onset age of adrenal insufficiency, disease severity and manifestation could vary. However, effects of genetic, epigenetic, and non-genetic factors could also influence the severity of disease outcome (Jadhav et al., 2011).

2.2.3 Diagnosis

Diagnosis of AHC disease is complicated because some clinical symptoms of patients look like some other adrenal disorder. Moreover, adrenal insufficiency is relatively rare in children. For clinical diagnosis of this disease, it can be used following steps.

2.2.3.1 Collecting patient's information

Clinical signs and symptoms associated with AHC can include exhaustion, fatigue, muscle weakness, weight loss, increased pigmentation of the skin and low blood pressure. Other symptoms include nausea, vomiting (or feeding problems), salt wasting, and dry skin and lip. All of these symptoms are caused by impaired aldosterone

and cortisol production. In some cases, apparent HH in puberty that caused by impaired or absent production of GnRH from the hypothalamus and/or gonadotropins from the anterior pituitary (Achermann et al., 1999; Clipsham and McCabe, 2003; Jadhav et al., 2011; Niakan and McCabe, 2005). Moreover, pediatric endocrinologist and genetic counseling studied patients' family history and took these information to draw the pedigree.

2.2.3.2 Laboratory testing

Laboratory testing is performed to measure hormone levels and to check for health problems in adrenal insufficiency.

2.2.3.2.1 Basal hormone measurement

The diagnosis of AHC is made by measuring basal hormone levels for testing the HPA axis in the morning (8-9 AM.), serum cortisol measurement has gained interest as a screening test. On the other hand, ACTH level in the patients is lower compared to normal persons. This testing suggested that the patient has primary adrenal insufficiency (Bornstein et al., 2016; Park et al., 2016).

2.2.3.2.2 ACTH stimulation test

ACTH stimulation test should be performed because this test is a very reliable measurement in diagnosing and classifying adrenal insufficiency arising from adrenal or hypothalamus or pituitary gland disease. This test is also known

as a cosyntropin test or a short synacthen test (SST). This test measures the ability of the adrenal glands to produce cortisol in response to ACTH. Moreover, SST measures the pituitary hormone that regulates adrenal cortisol production. This test is to measure cortisol levels before and after the ACTH injection for 30-60 minutes. If the adrenal glands are impaired, cortisol production after injection cosyntropin is no change. By contrast, healthy adrenal glands (but impaired in hypothalamus or pituitary gland) are excess to produce this amount of cortisol (Bornstein et al., 2016; Park et al., 2016).

Table 1 Basal hormone and mineral levels in normal range and adrenal problems in AHC patient (Fuggle., 2018)

Cause	**Normal range**	**Adrenal problems**
ACTH	<46 pg/dL	↑
Cortisol	137-429 nmol/L	↓
Aldosterone	100-800 umol/L	↓
Sodium	133-146 mmol/L	↓
Potassium	3.5-5.3 mmol/L	↑

2.2.3.3 CT Scan

A computed tomography (CT) scan of the abdomen should be performed in some cases to figure the adrenal glands in size and shape (Bornstein et al., 2016).

2.2.4 Treatments

2.2.4.1 Treatments prior to laboratory testing

Emergency patients with suspected adrenal crisis should be treated as soon as possible before receiving the results of laboratory testing for confirmation (Table 2). The first choice medicine for patients with adrenal crisis or insufficiency is hydrocortisone that provides both glucocorticoid and mineralocorticoid effects. In some cases fludrocortisone and DHEA may also be considered (Tucci and Sokari, 2014).

Table 2 Treatment guide before laboratory testing for emergency patients with suspected adrenal crisis (Tucci and Sokari, 2014)

Treatment	Adult	Pediatric
Fluids	5% dextrose in normal saline or normal saline boluses may be used	Shock and hypotension is addressed with standard fluid resuscitation measures: 20 mL/kg normal saline boluses up to a maximum of 60 mL/kg over 1 hour
Vasopressors	May be necessary if shock is refractory	May be necessary if shock is refractory
Steroids	Hydrocortisone, 100 mg bolus Followed by daily doses of 100 mg divided two to three times per day	Hydrocortisone Infants and toddlers to age 3: 25 mg/V Children ages 3–12: 50 mg IV Adolescents older than 12: 100 mg/V
Glucose	D50 may be used as necessary	Infants and children to age 12: 2.5 mL/kg of 10% dextrose Adolescents older than 12: 1 mL/kg of 25% dextrose

2.2.4.2 Treatments after laboratory testing

After laboratory testing is confirmed for the patients to have X-linked AHC, they should be treated based on the guideline provided in Table 3 (Tucci and Sokari, 2014).

Table 3 Treatment options for patients with adrenal disorders (Tucci and Sokari, 2014)

Treatment	**Primary**	**Secondary**
Glucocorticoid	20–25 mg hydrocortisone per 24 hours divided in two to three doses	15–20 mg hydrocortisone per 24 hours divided in two to three doses
Mineralcorticoid	0.1 mg per day	Not required
Androgen	May consider DHEA 25–50 mg and using transdermal testosterone	Not required

2.3 *DAX-1* gene

The *DAX-1* gene (*NR0B1*) located on X-chromosome Xp21, has 2 exons that encode 470 amino acid protein products (Figure 4). The gene is expressed in the adrenal cortex, gonads, hypothalamus and anterior pituitary. More than *DAX*-1 mutations have been described in different ethnic groups (http://www.hgmd.org). Most of these mutations are nonsense or frameshift mutations leading to premature truncation of the protein. Missense mutations are mainly clustered at the carboxyl terminus of the putative ligand-binding region of *DAX-1* (Suntharalingham et al., 2015). However, it was the association of X-linked AHC with glycerol kinase deficiency (GKD) and Duchenne muscular dystrophy (DMD) as a contiguous gene syndrome caused by deletion of multiple genes on Xp21. The DAX-1 protein consists of two regions. The amino-terminal region contains repeating units of 65-67 amino acids that comprises three LXXLL sequence motifs. This motif has common character as nuclear co-activators that is significantly involved in protein-protein interactions. Moreover, this protein lacks a typical zinc finger DNA binding domain that is a common feature of members of the nuclear receptor superfamily. The carboxyl-terminal region has high homology with the ligand-binding domain that belongs to the other members of this family which contains transcriptional regulatory sequences. However, no ligand has been identified for DAX-1 protein. From this reason, it is classified in the group of the orphan receptors (Iyer and McCabe, 2004; Reutens et al., 1999).

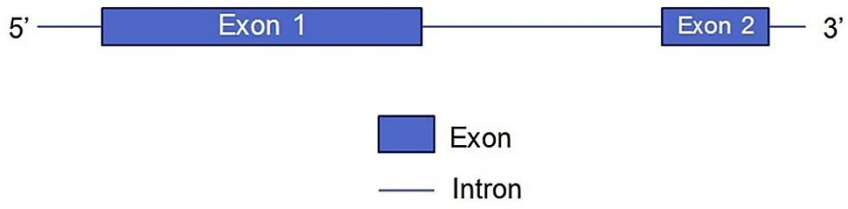


Figure 4 Structure of the *DAX-1* gene

The mechanism of DAX-1 action remains unclear, but most studies indicate that DAX-1 acts as a transcriptional repressor of genes involved in the steroidogenic pathway (Figure 2) and at all levels of the HPG axis *in vitro* (Clipsham and McCabe, 2003) (Figure 5). The DAX-1 protein inhibits the steroid biosynthesis from the adrenal cortex (Iyer and McCabe, 2004). It is characterized by a deficiency in the steroid hormones that is associated with a decrease in the blood level of serum glucocorticoid and aldosterone. Moreover, DAX-1 protein encodes an orphan nuclear receptor that interacts with steroidogenic factor 1 (SF-1; encoded by *SF-1* also known as *NR5A1*). DAX-1 and SF-1 are interacting proteins and found to bind to the promoters of *StAR* gene (Figure 6). The protein-protein interaction inhibits the development of the HPG axis and reproduction, and functional activation of the steroid biosynthesis from the adrenal cortex (El-Khairi et al., 2011).

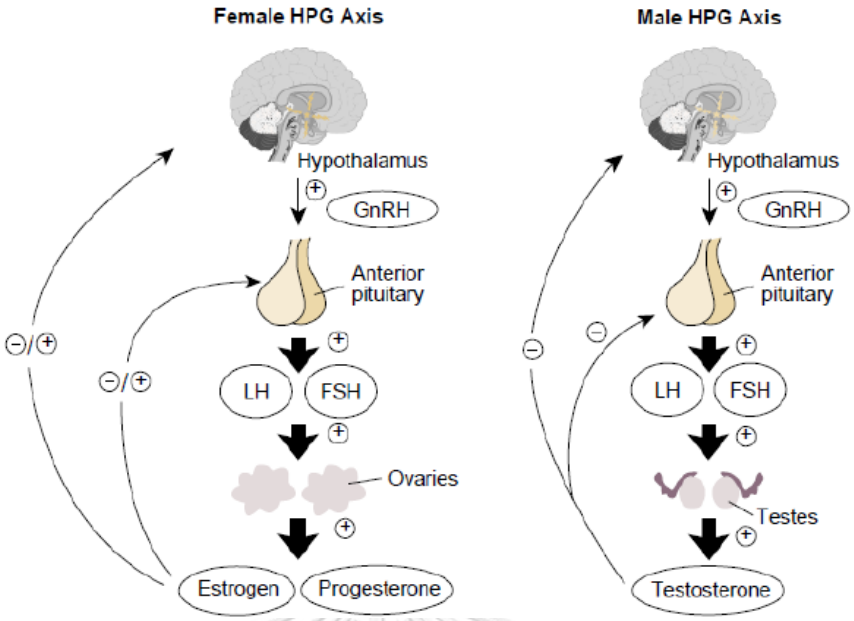


Figure 5 Schematic representation of the hypothalamic-pituitary-gonadal (HPG) axis (https://www.researchgate.net/figure/Schematic-representation-of-the-hypothalamic-pituitary-gonadal-HPG-axes_fig5_268791949)

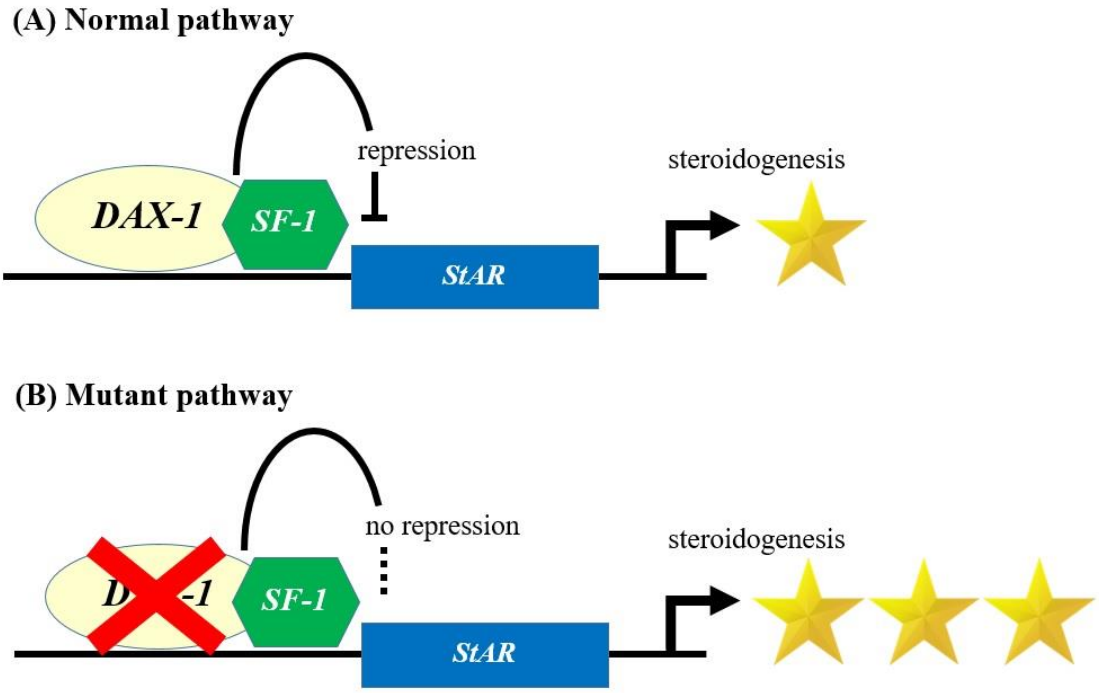


Figure 6 Mechanism of *DAX-1* gene expression in normal pathway and mutant pathway. (A) Normal pathway, DAX-1 and SF-1 are interacting proteins and bind to the promoter of *StAR* gene. DAX-1 acts as a transcriptional repressor, but SF-1 acts as a transcriptional activator of *StAR* gene expression. DAX-1 and SF-1 are transcription factors that play a key role in human adrenal and reproductive development. Steroid hormone biosynthesis occurs in the delivery of the substrate for all steroid hormones, cholesterol, from the outer to the inner mitochondrial membrane by *StAR* gene. (B) Mutant pathway, the mutations in *DAX-1* gene that caused X-linked AHC disease had the effect to loss of control in *SF-1* gene. Therefore, DAX-1 could not regulate expression of the *StAR* gene that caused *StAR* gene expression more than normal. Star symbol represents *StAR* gene expression.

2.4 Mutation analysis

Mutation is the genetic changing in nucleotide sequence that can be happened at chromosomal or DNA levels. Mutations have many possible causes depending on where they occur and alter the function of essential proteins. Some mutations happen spontaneously without any outside influence, when mistakes are made during DNA replication or transcription. In this study, focus on germline mutations that can be transmitted to offspring and every cell in the offspring will have the mutation. Mutations can occur either in non-coding or coding sequences that remarked as an inherited disorder or disease.

2.4.1 Mutation

2.4.1.1 Mutation in the DNA level

In the DNA level, many types of mutations can occur as follows.

- Substitution mutation is a change in single nucleotide base to the other nucleotide base.

- Insertion mutations is the adding one or more base pairs in DNA.
- Deletion mutations is the loss of one or more base pairs in DNA.

2.4.1.2 Mutation in the protein level

The mutation occurs in DNA from the transcription, the amino acids from the translation will change and impact on protein function.

- Nonsense mutation is a nucleotide substitution that changes a codon resulting in codon codes for a stop codon. The resulting protein is

incomplete and premature termination of translation. Hence, most nonsense mutations result in nonfunctional proteins.

- Silent mutation is a nucleotide substitution that usually occurs in the third location of the mRNA codon but an amino acid does not change. The result causes no change in the activity of the protein.

- Missense mutation is an amino acid changes to another amino acid. This may or may not affect protein function, depending on the amino acid that changed is conservative or non-conservative, and the property of amino acid.

- Frameshift mutations are insertions or deletions in the genome that are not in multiples of three nucleotides. These mutations caused premature stop codons, and caused the protein changes and may not function properly. If in the insertions or deletions are multiples of three nucleotides, it will not disrupt the reading frame (shift the frame) called in-frame mutation that caused the amino acid shorter.

The mutations are a cause of disease which affected to function of protein. It can be divided into two types: loss-of-function and gain-of-function mutations. Loss-of-function can result in either reduced or completed protein's ability to work. Type of mutations that caused loss-of-function mutations such as frameshift and missense mutation. Gain-of-function is increase in the protein's function (or increased levels of gene expression), or the development of a new function of protein. (Lodish, 2000)

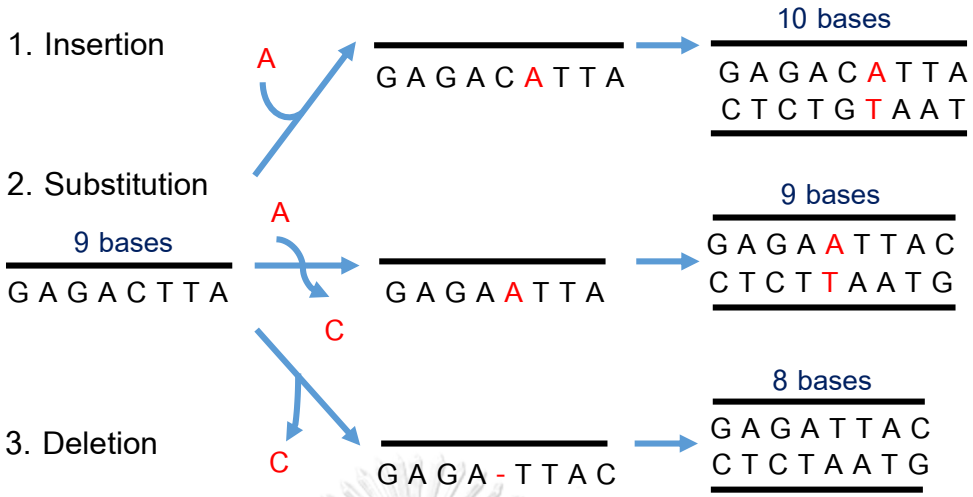


Figure 7 Type of mutations

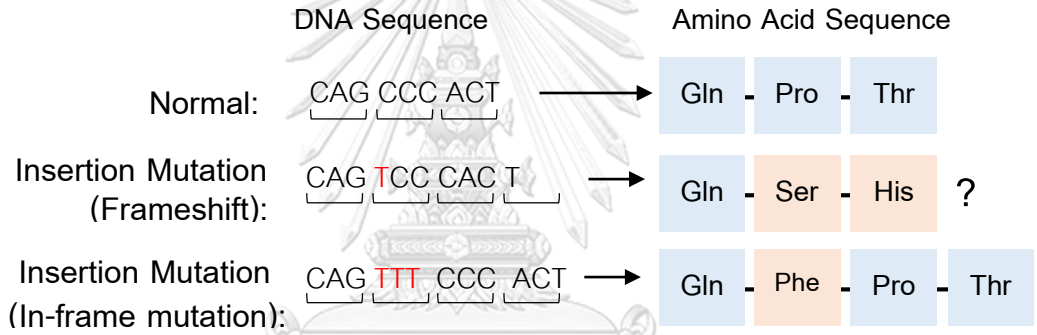


Figure 8 Insert mutations that caused frameshift mutation and in-frame mutation

2.4.2 Methodology for studying mutation analysis

Basically, method used in the clinical diagnosis laboratory is direct DNA sequencing. It is easy to identify when the gene causing a particular disease. Direct DNA sequencing is performed for screening of point mutation including single nucleotide changes and a few nucleotides deletion or insertion. This suitable technique represents the most widely used method to detect small regions of interest. Hence, because *DAX-1* gene has 2 exons, the direct sequencing method was used to detect all mutations. Moreover, this

method is basic, inexpensive and less time consuming as it can reduce morbidity and mortality of affected boys (Mahdieh and Rabbani, 2013).

2.4.3 Prediction programs

The prediction tools are software used for predicting non-synonymous substitutions (missense mutations). The best method for earlier assessing the pathogenicity of novel mutations is used these advancing computational tools. These technologies are provided fast results and inexpensive methods. The efficient approaches for predicting the functional effects on protein stability, structure, function, and located of the protein that stays at highly conserved genomic regions across species. In the present study, eight prediction tools which widely used in medical genetics studies, including SIFT, PolyPhen2, M-CAP, Mutation taster, CADD, PMut, Panther and ClastalX, were employed (Adzhubei et al., 2010; Jagadeesh et al., 2016; Kircher et al., 2014; Kumar et al., 2009; López-Ferrando et al., 2017; Schwarz et al., 2014; Tang and Thomas, 2016; Thompson et al., 1997). For SIFT, PolyPhen2, M-CAP, Mutation taster, CADD and PMut tools, they are commonly used to analyze for the stability and structure of protein that are key factors affecting in activity, function, and regulation of proteins. Moreover, these programs analyzed for conformational changes that can impair the protein folding, protein-protein interaction and stability of protein molecules by using the basis of three-dimensional structure and molecular dynamics (Adzhubei et al., 2010; Jagadeesh et al., 2016; Kircher et al., 2014; Kumar et al., 2009; López-Ferrando et al., 2017; Schwarz et al., 2014). Panther and ClastalX program is

performed for predicting in relation to the evolutionary conservation in a specific amino acids by using multiple alignments of homologous sequences (Tang and Thomas, 2016; Thompson et al., 2002; Thompson et al., 1997).

2.5 Functional analysis of gene mutations

The principle methods to evaluate the pathogenicity of novel mutations are performed in genotype-phenotype correlation studies, and functional analysis studies in an *in vitro* system or animal models. The functional study in this thesis was performed *in vitro*, as the *DAX-1* gene is expressed in the adrenal gland, but not in the blood. Therefore, functional analysis cannot be studied in the adrenal gland or studied by using RNA extraction technique. Thus, in this study the mutations were analyzed by producing the wild-type plasmid construction, the mutant plasmid construction by using site-directed mutagenesis method, and the luciferase reporter construction. After that, the gene expression levels were analyzed in mammalian cells by luciferase assay technique.

2.5.1 Site-directed mutagenesis

This method can be used to introduce specific nucleotide substitutions, insertion and deletion by using PCR based methods. This useful technique is made specific mutations in the DNA. The point mutations can be introduced to plasmids by using mutagenic primers that contained the desired mutations. The nucleotide substitution can be designed by having one of the primers contains the desired mutation in the middle of the primer. The deletions can be designed to eliminate a desired nucleotide sequence in the target sequence. This sequence is situated apart from the flanking region by primers, it would not be amplified during PCR. The insertions can be designed one of the primers contains a desired nucleotide sequence at the 5’ end of the primer. The inserted sequence may remain attached to the amplicon. The desired mutation

and the entire plasmid template is amplified by PCR. The non-mutated parental DNA template and methylated are digested using a methylation-dependent endonuclease such as *Dpn*I. The nuclease-resistant nicked plasmid or the PCR product is transformed to competent cells. After transformation, the competent cells are repaired the nicks in the mutated plasmid. The plasmids are screened and isolated from the resulting colonies for selecting the positive clones. The positive clones are sequenced for confirming the desired modification (Liu and Naismith, 2008).

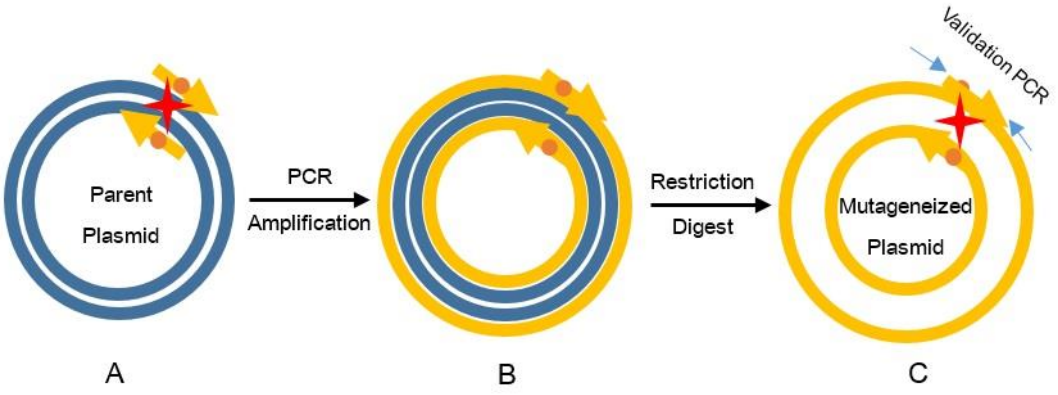


Figure 9 Schematic representation of site-directed mutagenesis. (A) The mutant strand is synthesize by denaturing DNA template and annealing the mutagenic primers. (B) The mutagenic primers are extended and the nicks are ligated, (C) a methylation-dependent endonuclease digests non-mutated template DNA.

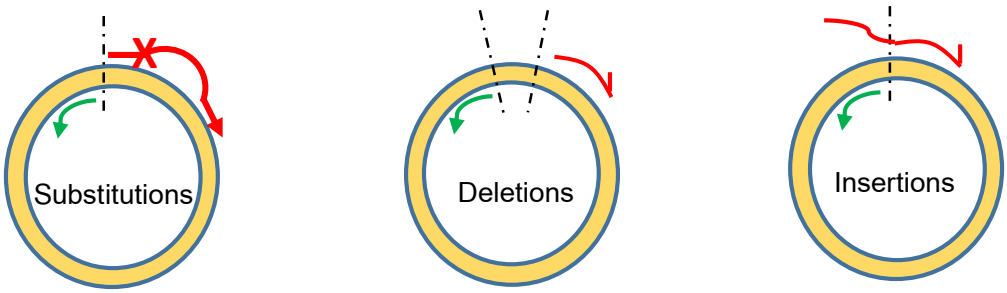


Figure 10 Primers designed for site-directed mutagenesis for either substitutions, deletions or insertions. Red line represents one of the primers containing the desired mutations, green line represents non-mutating primer, and dashed lines represent the site that contains the mutations.

2.5.2 Transient transfection

Transfection is a process for introducing nucleic acids into eukaryotic cells by using various chemical, lipid or physical methods. The purpose of this technique is to study gene function and/or regulation, protein function and protein expression in mammalian cells. The principle of this technique is to introduce negatively charged molecules (phosphate backbones of DNA and RNA) into cells with a negatively charged membrane. For chemical methods, the chemicals had a positive charge to the molecule that made easier for the DNA transfection reagent complex to cross the membrane. Especially, for lipids that have a “fusogenic” component can be easier fused the lipid-based reagents coat the DNA into the cell with the lipid bilayer. Various transfection methods have been developed to have high transfection efficiency, low cell toxicity, minimal effects on normal physiology, easy to use and reproducible (Table 4) (Kim and Eberwine, 2010).

In this study, the chemical methods of gene delivery in transfection, lipofection (or liposome mediated gene transfer) technique, was employed.

Cationic lipid transfection reagents is the most commonly used. The principle is a polycationic head group that consists of a positively charged. These cationics are attached to a lipid backbone structure for delivering the nucleic acids that adsorbed to these vesicles/packed structure into eukaryotic cells. The advantages of cationic-lipid mediated transfection are high efficiency of their ability to transfect a wide range of cell types that were mainly adherent cell lines, fast, easy, low costs, and the successful delivery of DNA of all sizes (Kim and Eberwine, 2010).

Table 4 Conventional transfection methods

Class	Methods	Advantages	Disadvantages	Examples
Biological	- Virus-mediated	- High-efficiency - Easy to use - Effective on dissociated cells, slices, and *in vivo*	- Potential hazard to laboratory personne - Insertional mutagenesis - Immunogenicity - DNA package size limit	Herpes simplex virus, Adeno virus, Adeno-associated virus, Vaccinia virus, Sindbis virus
Chemical	- Cationic polymer - Calcium phosphate - Cationic lipid	- No viral vector - High-efficiency - Easy to use - Effective on dissociated cells and slices - Plenty of commercially available products - No package size limit	- Chemical toxicity to some cell types DEAE - Variable transfection efficiency by cell type or condition - Hard to target specific cells	DEAE-dextran, polyethyleneimine, dendrimer, polybrene, calcium phosphate, lipofectin, DOTAP, lipofectamine, CTAB/DOPE, DOTMA
Physical	- Direct injection - Biolistic particle delivery - Electroporation - Laser-irradiation - Sonoporation - Magnetic nanoparticle	- Simple principle and straightforward - Physical relocation of nucleic acids into cell - No need for vector - Less dependent on cell type and condition - Single-cell transfection	- Needs special instruments - Vulnerable nucleic acids - Demands experimenter skill, laborious procedure	Micro-needle, AFM tip, Gene Gun, Amaxa Nucleofector, phototransfection, Magnetofection

2.5.3 Luciferase activity assay

Reporter-gene assays are widely used as indicators to study of eukaryotic gene expression and cellular physiology. Detection in the expression of gene is directly measured by the reporter protein or the enzymatic activity of the reporter protein from the cells of interest or in the organism. The dual reporter assay is used to measure the expression of two individual reporter enzymes within a single assay. One reporter is used to study the effect of specific acitivity of an experimental reporter that is observed the luminescent properties of firefly luciferase. While the luminescent properties of *Renilla* luciferase is observed to study at baseline response or internal control of activity in co-transfected assays. The advantages of internal control is to reduce the variance between assays such as cell viability, transfection efficiency, cell lysis efficiency and assay efficiency. Hence, dual-reporter assays provide more reliable interpretation of the experimental data and commonly used to improve experimental accuracy by reducing extraneous influences. Moreover, these techniques have more applications including the study of transcription factors, receptor activity, intracellular signaling, protein folding and mRNA processing (Allard et al., 2008).

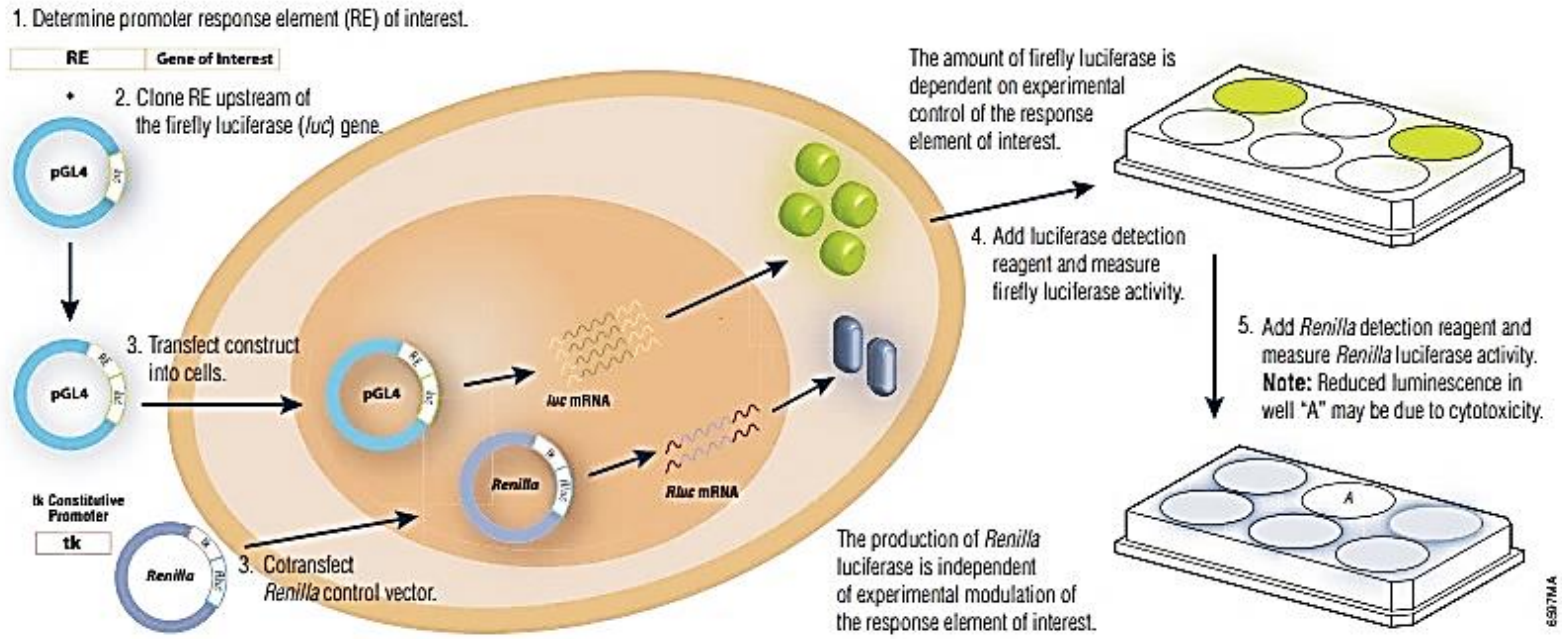


Figure 11 Schematic of dual-luciferase reporter assay system (Allard et al., 2008)

CHAPTER III
Methodology

3.1 Sample collection

3.1.1 Ethic approval

The protocol for taking blood sample from X-linked AHC patients was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB No. 426/59 and COA no. 954/2016). All research participants provided written informed consents after study procedures were explained.

3.1.2 Patients

The subjects of this study were 10 unrelated patients (Patient III-XII) and 2 cousins (Patient I and Patient II) with clinical and hormonal findings suggesting AHC who were referred for *DAX-1* mutation analysis. The data were obtained from the Division of Pediatric Endocrinology, King Chulalongkorn Memorial Hospital, during 2015-2017.

Patient I was the only child of non-consanguineous parents of southern Thailand origin. He was born with a normal birth weight of 3.0 kg. The first symptom was at one month of age with diarrhea and poor feeding. He had salt-wasting crisis and had multiple seizure episodes due to hypoglycemia. The infant had normal male genitalia. The patient recovered slowly due to nosocomial pneumonia complication. He was maintained with oral hydrocortisone and fludrocortisone. An ACTH stimulation test at age 3.5 months failed to increase serum levels of progesterone, 17OHP, and cortisol.

Hypogonadotropic hypogonadism was diagnosed at the age of 15 years and testosterone therapy was started. At the time of this thesis writing, he is 20 years old with rather good physical health after treatment.

Patient II was the first cousin of Patient I. He was born at term with a birth weight of 3.15 kg. His skin had to be severe dark from birth. He had drowsiness, and was found to have hypoglycemia and salt-wasting at 2 days old. An ACTH stimulation test was confirmed primary adrenal insufficiency. The patient recovered under treatment with hydrocortisone. Ultrasound of abdomen could not identify both adrenal glands.

Patient III was born from non-consanguinous parents at term with a birth weight of 3.17 kg. At birth, he had initially diagnosed Klebsiella pneumonia (infection in bacteria names *Klebsiella pneumonia)* and hypovolemic shock (an emergency condition that makes the heart unable to pump enough blood to the body from severe loss of blood or fluid). At age 24 days, he had intractable seizure and was admitted to NICU. His laboratory results were shown that he had salt-wasting crisis and hypoglycemia. Physical examination was unremarkable except hyperpigmentation. Hydrocortisone and NaCl were given. Adrenal ultrasound was shown both adrenal glands were very small without evidence of mass lesion. His pedigree was highly suggestive of X-link recessive transmission as nine males in previous generations had unexplained death during infancy (Figure 12).

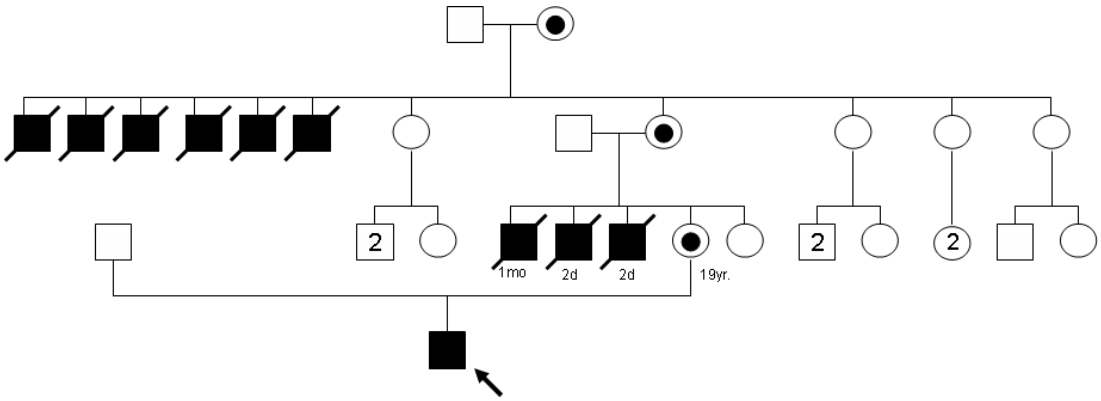


Figure 12 Pedigree of Patient III (Arrow represents the index case)

Patient IV was the second son from the three sons of non-consanguineous parents of northern Thailand origin. The first symptoms were presented at age 17 year olds with progressive hyperpigmentation over the past two years. He was presented with hypogonadotropic hypogonadism. At that time, it was noted that he had not yet developed secondary sexual characteristics. His genitalia was normal pre-pubertal male, and his testes were small at 2.5 cm in diameter. His electrolytes were normal. He denied serious past medical history. From abdomen CT, the bilateral adrenal glands were presented hypoplasia. Moreover, the MRI result showed a small size of pituitary gland.

Patient V was a 1-month-old boy born from non-consanguineous parents and was presented with poor feeding, diarrhea and lethargy for 8 days. Physical examination revealed poor weight gain, hypotension, severe dehydration, hyperpigmentation and normal male genitalia (penile length was 4 cm) with descended testes. Electrolytes documented were shown the patient had salt-wasting, but basal cortisol and testosterone levels were normal. Oral hydrocortisone was given and remained well with normal electrolytes. At the

age of 10 months, he developed acne, rapid growth with penile enlargement (penile length 8.5 cm, testes 3 mL bilaterally) without bone age advancement. Moreover, GnRH-independent precocious puberty was observed in this patient that is atypical symptom in this disease. MRI pituitary was revealed normal and abdomen MRI showed that the adrenals glands were not enlarged. At the time of this thesis writing, he is 11 years old, and all steroidogenic precursors suggested AHC.

Patient VI was onset at 3.5 years old with progressive hyperpigmentation and symptoms of salt-wasting (nausea, vomiting, and diarrhea). His electrolytes were confirmed salt-wasting. An ACTH stimulation test failed to increase the cortisol. CT scan was revealed no visible bilateral adrenal glands. He was given hydrocortisone and fludrocortisone replacement and remained well. He had hypogonadotropic hypogonadonism at the age of 14 years.

Patient VII was born with a normal birth weight (2.7 kg). The patient was a 12-year old boy was diagnosed with congenital adrenal insufficiency and severe short stature (height 112 cm, weight 35 kg, bone age 7 years). In the first month of life, he had salt wasting crisis and multiple seizure episodes due to hypoglycemia. Moreover, he had early onset adrenal insufficiency, hyperpigmentation and multiple pituitary hormone deficiency (thyroid hormone and gonadotropins). MRI of pituitary was revealed as normal. At age of 13 years, he showed no signs of puberty with small testes. Growth hormone (GH) provocative tests were performed and shown that the patient had GH

deficiency. GH therapy was started with a good clinical response, but sex steroid therapy was delayed to maximize final height outcome.

Patient VIII-XII have no clinical data.

3.2 Method

3.2.1 Genotyping

3.2.1.1 DNA extraction

The peripheral blood leukocyte samples were collected from patients and diagnosed to have X-linked AHC and their family by using standard venipuncture and stored at 4°C in tubes containing ethylenediaminetetra acetic acid (EDTA) until use. Leukocyte genomic DNA was extracted using QIAamp®DNA Blood Mini Kit (Qiagen, USA) according to the manufacturer's instruction. The genomic DNA concentration and purity were estimated by Nano Drop 1000 (Thermo Scientific, USA). After, the genomic DNA was stored in 4°C and diluted to the working concentration of 50 ng/µL.

3.2.1.2 Mutation analysis

The two exons of the *DAX-1* gene in all patients were amplified by polymerase chain reaction (PCR) amplification with specific oligonucleotide primer pairs (Table 5). The primers were designed with Primer3 program (http://bioinfo.ut.ee/ primer3-0.4.0/). The locations of all primers are presented in Figure 13. The PCR condition was shown in Appendix. The PCR products were gel electrophoresis in 1% agarose by using 110 volts in 60 minutes, and visualized by Redsafe Nucleic Acid Staining Solution (iNtRON Biotechnology, South Korea) and ultraviolet light transillumination. Then the PCR product was treated with ExoSAP-IT (USP Corporation, Cleveland,

OH), and sent for direct sequencing at Macrogen Inc. (Seoul, South Korea). The sequencing results were analyzed by Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI). All the sequencing results of seven patients were analyzed by comparing with the published human *DAX-1* sequence (accession no.: NM_000475).

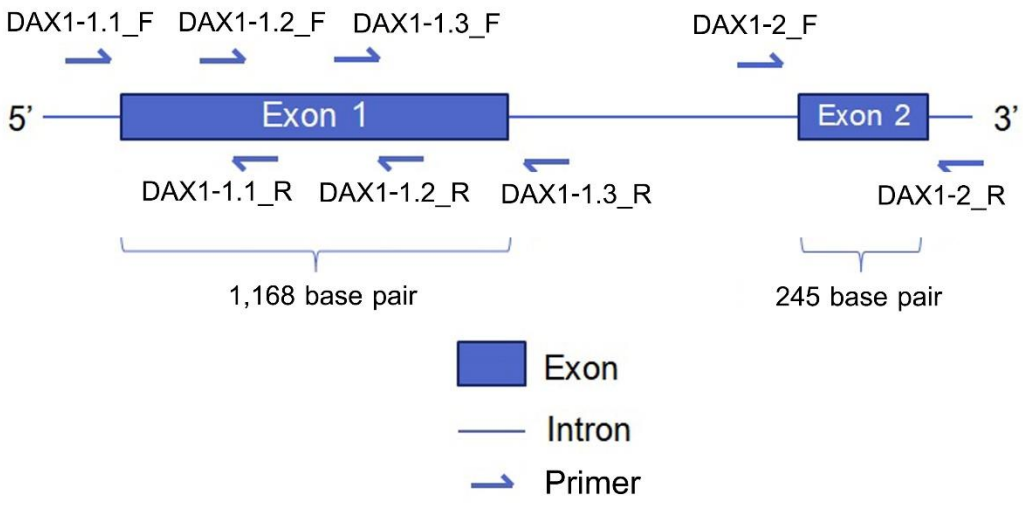


Figure 13 Location of all primers

Table 5 PCR primers used to amplify genomic DNA of *DAX-1*

Gene/exon amplified#	**Primer sequence (5' to 3')**	**Product size (bp)**
g. *DAX-1* Exon 1	DAX1-1.1_F: GCT CCC ACG CTG CTG TTC TTC DAX1-1.1_R: CCG CCC ACC CGG AAG CCC CGC	605
	DAX1-1.2_F: CGA AGG CGC CCG AGG CGA CGC DAX1-1.2_R: GGA CGC CCA GCA GTT GCG CAC	599
	DAX1-1.3_F: CGC TTC GTC AAG TAC TTG CCC DAX1-1.3_R: GTG TAG AGA GCC AAG TAC	624
g. *DAX-1* Exon 2	DAX1-2_F: TAC CCT TTT AAC CGG GAA GC DAX1-2_R: GCT ACC TGT TGG CAA ATG TC	585

3.2.1.3 Mutation interpretation

Data from the mutation analysis in the patient samples were interpreted in positions of possible mutations. Then, the mutations were genotyped in the mother of patients and compared with Thai exome database (Center of Excellence in Medical Genetic Research, King Chulalongkorn Memorial Hospital) which comprises more than 130 healthy Thai controls. For the missense mutations detected were subjected to mutation prediction analyzes: SIFT (http://sift.jcvi.org/), Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), M-CAP (http://bejerano.stanford.edu/mcap/), Mutation Taster (http://www.mutationtaster.org/), CADD (https://cadd.gs.washington.edu/snv), PMut (http://mm

b.irbbarcelona.org/PMut/analyses/new/) and Panther (http://www.pantherdb.org/tools/csnpScore.do) programs to analyze whether the mutations had any effects to the structure of the DAX-1 protein, and ClustalX program (http://clustalx.software.informer.com) for evolutionary conserved amino acids.

3.2.2 Functional studies

3.2.2.1 Gene cloning

Human *DAX-1*, *SF-1* complementary DNA (cDNA) clones were obtained from Prof. Han-Wook Yoo (University of Ulsan College of Medicine Seoul, South Korea) that he purchased human *DAX-1* and *SF-1* complementary DNA (cDNA) clones from OriGene (Rockville, USA) and, generated the coding regions by PCR amplification, were subcloned into pcDNA3.1 (+) expression vector (Invitrogen, USA) (Figure 14). *StAR* promoter clone (+3 to -1222) was obtained from Prof. Han-Wook Yoo (University of Ulsan College of Medicine, Seoul, South Korea). The StAR promoter was then cloned into the pGL4.10 vector (Promega, USA) which encodes the luciferase reporter gene *luc2* using *BglII* and *XhoI* restriction enzyme (Promega, USA) (Figure 15). The clones were rechecked via sequencing analysis at Macrogen Inc. (Seoul, South Korea).

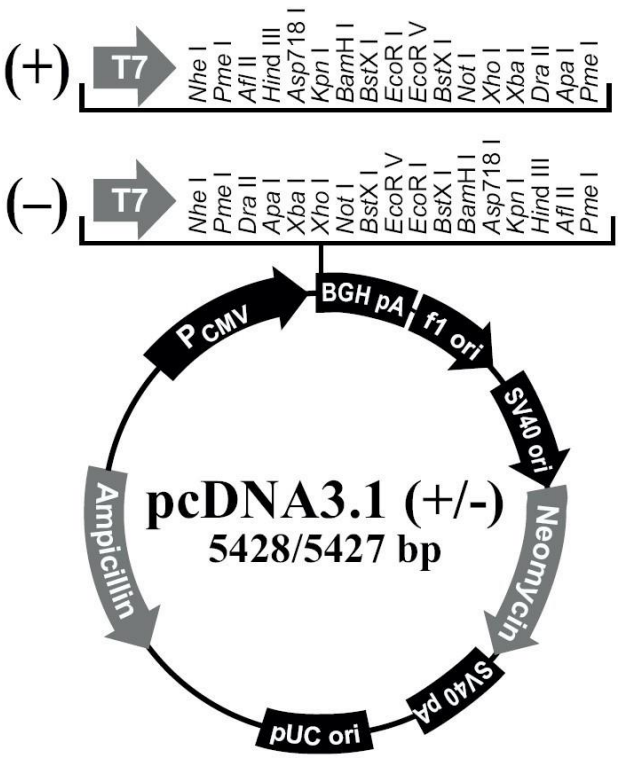


Figure 14 Component of pcDNA3.1+ mammalian expression vector

(http://www.biofeng.com/zaiti/buru/pcDNA3.1(-).html)

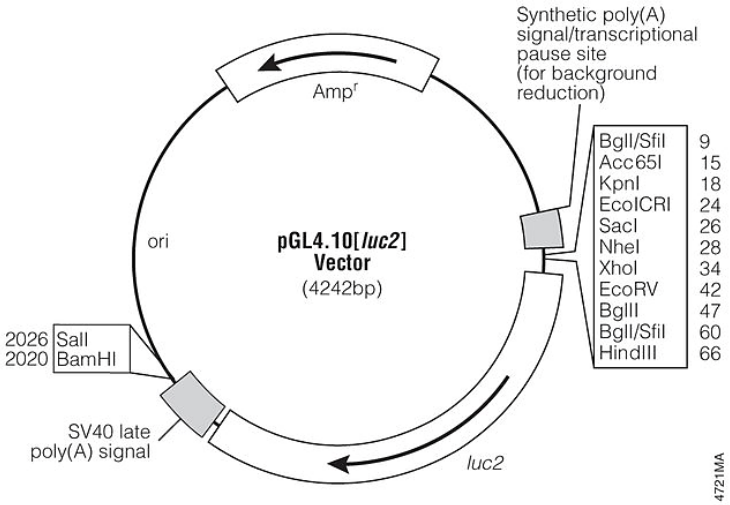


Figure 15 Component of the pGL4.10 vector encoding the luciferase reporter gene,

luc2 (https://www.addgene.org/60873/)

3.2.2.2 Site-directed mutagenesis

All mutant DAX-1 expression vectors were created to mimic the patients' mutations by QuickChange® Site-Directed Mutagenesis Kit (Agilent Technologies, USA) according to the manufacturer's instruction. Full-length human *DAX-1* cDNA was used as a template and overlapping PCR strategy with primers containing the appropriate nucleotide substitutions was employed (Appendix). Mutagenic primers were designed to create the mutations corresponding to the mutation in the nucleotide sequence of *DAX-1* from each patient. The primers were designed by Primer3 Program (Table 6). After temperature cycling, the products were treated with *Dpn*I 1 µL at 37ºC for 1 hour to digest the methylated, non-mutated parental DNA template. After, aliquots 1 µL of the *Dpn*I-treated DNA from each control and sample reaction were transformed into the 50 µL XL1-Blue super-competent cells and incubated the reactions on ice for 30 minutes. Then the mutagenized plasmid, along with the wild-type plasmid, were separated by heat shock transformation of QuickChange® Site-Directed Mutagenesis Kit (Agilent Technologies, USA) according to the manufacturer's instruction. The media was prepared by adding 490 µL SOC media, 5 µL $MgCl_2$ and 5 µL glucose were placed into the reaction and incubated the transformation reactions at 37°C for 1 hour with shaking at 225–250 rpm. Then, the transformation reaction was plated on agar plates containing the ampicillin antibiotics and incubated the transformation plates at 37°C for 16-18 hours. The transformed

colonies were selected and the plasmids were extracted by High-Speed Plasmid Mini Kit (Geneaid, Taiwan) according to the manufacturer's instruction. Mutation sites and the accuracy of cloned plasmids were confirmed via sequence analysis at Macrogen Inc. by using the Sequencher program (version 4.2; Gene Codes Corporation, Ann Arbor, MI).

Table 6 Mutagenic primers were designed to create the mutations corresponding to the mutation in the nucleotide sequence of *DAX-1* from each patient. The primers were designed by Primer3 Program.

Primers	**Sequence 5'->3'**
PatientI&II_sense	AAG ACG CTG CGC TTC AAG TAC TTG CCC TGC
PatientI&II_antisense	GCA GGG CAA GTA CTT GAA GCG CAG CGT CTT
PatientIII_sense	TAC GCC TAC CTC AAG GAC CGT GCT CTT TAA CC
PatientIII_antisense	GGT TAA AGA GCA CGG TCC TTG AGG TAG GCG TA
PatientIV_sense	TAC CCG GGT TAA AGA ACA CGG TCC CCT TGA G
PatientIV _antisense	CTC AAG GGG ACC GTG TTC TTT AAC CCG GGT A
PatientV_sense	GCG GGG CTT CCG GTG GGC GGC C
PatientV_antisense	GGC CGC CCA CCG GAA GCC CCG C
PatientVI_sense	GAA GGT GCC CTC GCC TCC CAG GTC
PatientVI_antisense	GAC CTG GGA GGC GAG GGC ACC TTC
PatientVII_sense	GGG GGG CGC GTG GTG GGA C
PatientVII_antisense	TGG CCG TGC CTC GGG CGC

3.2.2.3 Transient transfection

The verified plasmids were transiently transfected into Human embryonic kidney 293T cells (HEK293T) by X-tremeGENE9 DNA transfection reagent (Sigma-Aldrich, Singapore) according to the manufacturer's instruction. HEK293T cells were thawed in cold D10 that containing 89% DMEM (Dulbecco's Modified Eagle's Medium),

10% fetal bovine serum and 1% streptomycin/penicillin in T75 flask. The cells were cultured at 37°C in 5% CO_2 atmosphere. The day before transfection, the cells were counted for preparing the transient transfection by designing the experimental as shown in the Appendix. The cells were removed the growth medium and washed with 10 mL PBS (Phosphate buffered saline) 2 times. Then, the cells were added trypsin-EDTA 3 mL and incubated at 37°C in 5% CO_2 atmosphere for 5 minutes. After, the cells were replaced 6 mL D10 and removed growth medium with the cells into 15 mL tubes. The cells were counted with automated cell counters and separated in 12-well plates that seeded at 200,000 cells per well and incubated at 37°C in 5% CO_2 atmosphere for 24 hours. The day of transfection, the X-tremeGENE 9 DNA Transfection Reagent was prepared with a concentration of 3:1 ratio of µL Reagent to µg DNA. In 1000 µL tube, 90.8 µL medium and 1.2 µL X-tremeGENE 9 DNA Transfection Reagent were added and gentle mixed. Then, the verified plasmids were transiently co-transfected (including; 100 ng wild-type or 100 ng mutant DAX-1 vector or 100 ng empty vector with 100 ng SF-1 vector, 100 ng StAR promoter reporter vector and 100 ng *Renilla* expression vector) into HEK293T with gently mixed, and incubated at room temperature for 15 minutes. After, the transfection complex was added to the cells in a drop wise manner and incubated at 37°C in 5% CO_2 atmosphere for 48 hours before measured protein expression by luciferase assay activity.

3.2.2.4 Luciferase activity assay

At approximately 48 hours post transfection, the transfected cells, were extracted for protein by removing the transfection complex, washed with PBS, treated with 1X PLB (Passive Lysis Buffer) and shaked the culture vessel for 15 minutes at room temperature. After, the cells were scraped from culture dishes and transferred lysate to a tube and frozen at -80^{o}C for 24 hours.

The luciferase activities of StAR from each experiment were determined by using Dual-Luciferase Reporter (DLR^{TM}) Assay System (Promega, USA) with the SpectraMax Microplate Reader (Molecular Devices, USA). In 96-well plates, 20 µL of cell lysate and 100 µL of LAR II (Luciferase Assay Reagent II) were added in each well. The reagents were mixed by pipetting 2 or 3 times and placed into the luminometer to initiate measuring the firefly luciferase activity. After measurement, the sample tubes were removed from the luminometer and 100 µL of Stop & Glo® Reagent was added by pipetting and the sample was replaced in the luminometer for initiate measuring the *Renilla* luciferase activity (Figure 16). All transfections were performed in three independent experiments in triplicates.

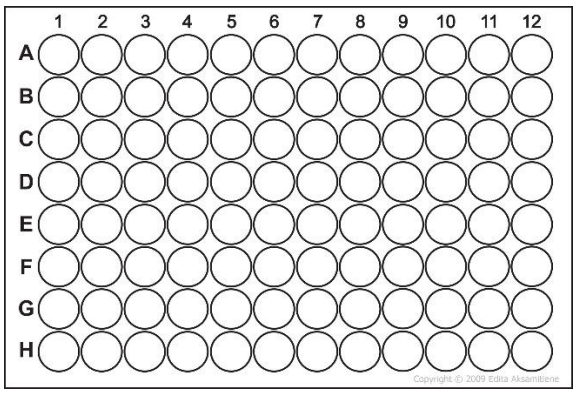
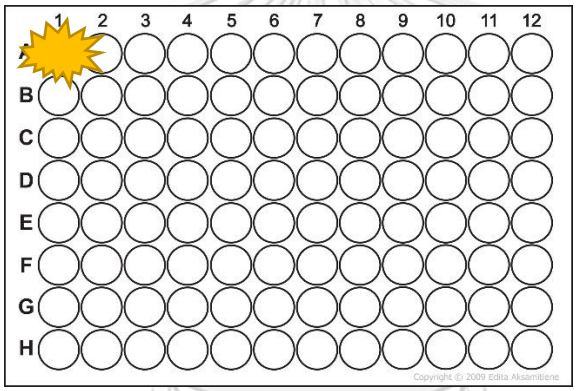


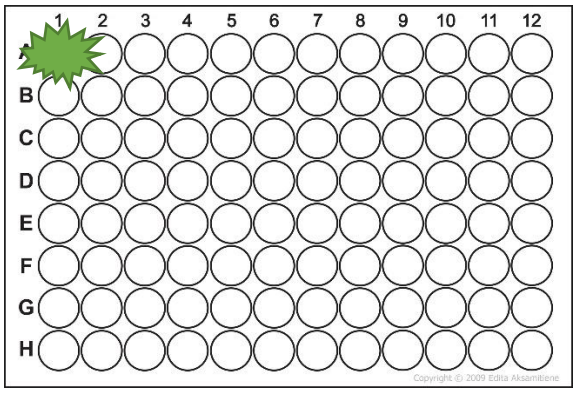
Plate with 20 µL lysate per well

Inject 100 µL firefly working solution (LARII)



Measure firefly luciferase activity

Inject 100 µL *Renilla* working solution
(Stop & Glo® Reagent)



Measure *Renilla* luciferase activity

Repeat loop for remaining assay wells

Figure 16 Flow chart Dual-luciferase assay protocol with 96 well-plate. In 96-well plates, 20 µL of cell lysate and 100 µL of LAR II (Luciferase Assay Reagent II) were added in each well. The firefly luciferase activity was measured by the luminometer. After measurement, the sample tubes were removed from the luminometer and 100 µL of Stop & Glo® Reagent was added by pipetting and the sample was replaced in the luminometer for initiate measuring the *Renilla* luciferase activity.

3.2.2.5 Statistical analysis

Results were expressed as mean ± SEM of each triplicate reaction that is a percentage of the DAX-1 empty vector control for that study to allow comparison of the statistical significance of repressive activity of mutants with wild type activity. This studied was normally distributed and the variances between the two groups are equal. The statistical analysis was performed by the independent-samples *t-test* in the SPSS Statistic program (IBM Corporation, USA) at 95% confidence.

CHAPTER IV
Result

4.1 Case reports

The results of clinical feature and molecular data of the Thai patients with X-linked AHC were shown in Table 7. Individuals from eleven families with twelve suspected X-linked AHC were studied. The symptoms in majority of the patients presented in the neonatal period with adrenal insufficiency and salt wasting crisis. However, unusual symptoms including late-onset Addison disease, precocious puberty and growth hormone deficiency were observed in this study.

Table 7. The clinical features and mutational profiles of patients. Based on GenBank accession No. NM_000475.4.

Patient No.	Sex	Age at onset	Clinical features				Genotypes		Unusual phenotypes
			P	H	SW	HH	DNA mutation	Protein change	
I	M	1 month	X	X	X	X	c.805_807delGTC	p.Val269del	-
II	M	2 days	X	X	X	N/A	c.805_807delGTC	p.Val269del	-
III	M	24 days	X	X	X	N/A	c.1148_1149delGG	p.Gly383Aspfs*5	-
IV	M	17 years	X	-	-	X	**c.1156C>T**	**p.Leu386Phe**	Late-onset Addison
V	M	1 month	X	-	X	N/A	**c.363delG**	**p.Gly122Valfs*142**	Precocious puberty
VI	M	3.5 years	X	-	X	X	**c.1062delC**	**p.Ala355Profs*17**	-
VII	M	3 days	X	X	X	X	c.501_502insG	p.Ala170Argfs*15	Growth hormone deficiency
VIII-XII	M	-	-	-	-	-	-	-	-

Notes: P, hyperpigmentation; H, hypoglycemia; SW, salt-wasting; HH, hypogonadotropic hypogonadism; N/A, not applicable

Novel mutations are indicated in **bold**.

4.2 Mutation analysis

4.2.1 Amplification of the *DAX-1* gene

PCR was used to amplified exon 1 and 2 of the *DAX-1* gene. As exon 1 is quite large (1,168 bp), the PCR was separated into 3 parts. PCR products separated by gel electrophoresis are shown in Figure 17.

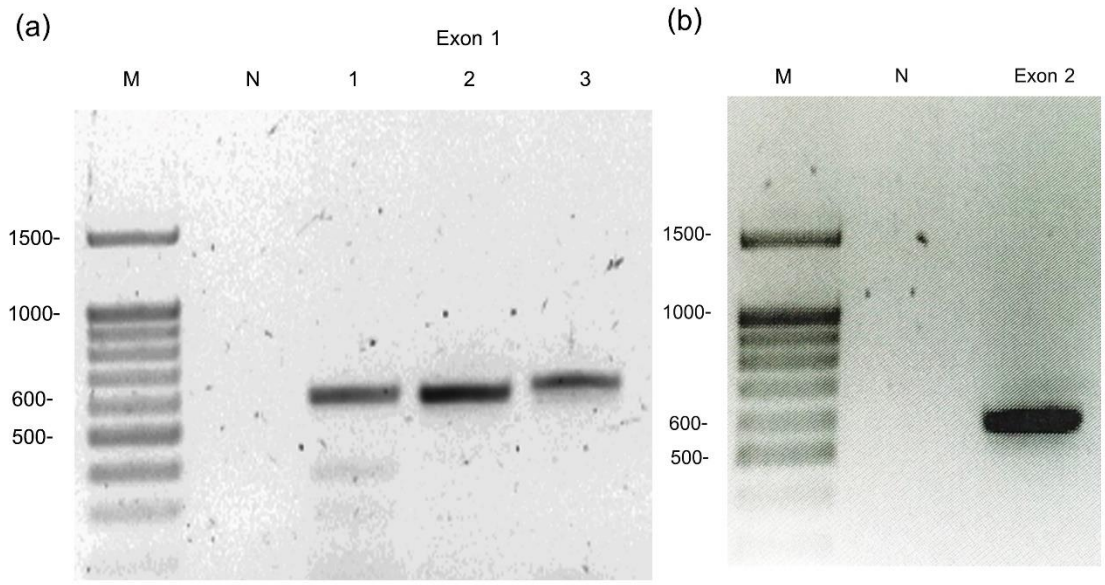


Figure 17 Gel electrophoresis results (a) PCR product of exon 1-1 (605 bp), exon 1-2 (599 bp) and exon 1-3 (624 bp), (b) PCR product of exon 2 (585 bp), (M = 100 bp DNA ladder, N = Negative Control)

4.2.2 Mutation identification

DNA sequencing analysis of the *DAX-1* gene coding region in Patients I, II (cousins) revealed three nucleotides deletion mutations (c.805_807delGTC, p.Val269del) (Figure 18 (a)). For Patient III, two nucleotides deletion mutations (c.1148_1149delGG, p.Gly383Aspfs*5) were

identified (Figure 18 (b)). Patient IV presented substitution mutations (c.1156C>T, p.Leu386Phe) (Figure 18 (c)). Patient V, one nucleotide deletion mutations (c.363delG, p.Gly122Valfs*142) was identified (Figure 18 (d)). Also, Patient VI showed one nucleotide deletion mutation (c.1062delC, p.Ala355Profs*17) (Figure 18 (e)). Lastly, Patient VII revealed one nucleotide insertion mutation (c.501_502insG, p.Ala170Argfs*15) (Figure 18 (f)). All mutations identified in seven patients were located in the exon 1. Patients VIII-XII were not find mutations in *DAX-1* gene. The results of deletion mutation would cause frameshift mutations, produce premature stop codon and subsequently, truncated proteins. Mutations of Patient I, II (cousins) and III were caused by known frameshift mutations. The c.805_807delGTC mutation was previously described in a European boy (Muscatelli, Strom, Walker, Zanaria, Recan, Meindl, Bardoni, Guioli, Zehetner, Rabl, & et al., 1994). For c.1148_1149delGG has just been reported last year in a Thai patient (Boonyawat et al., 2017). Interestingly, sequencing data of Patients V and VI presented novel frameshift mutations; c.363delG, and c.1062delC, respectively (NCBI cDNA reference sequence NM_000475.4) (Figure 18 (d-e)). Moreover, sequencing data identified a novel missense mutation, c.1156C>T, in Patient IV that would change the leucine residue 386 to phenylalanine (p.Leu386Phe) (Figure 18 (c)). Another case (Patient VII) was found to have a known insertion mutation, (c.502_503insC). This mutation was previously described in a Spanish boy who had complete adrenal failure, hypogonadotropic hypogonadism and pathological short stature in the neonatal period (Pérez Rodríguez et al., 2006).

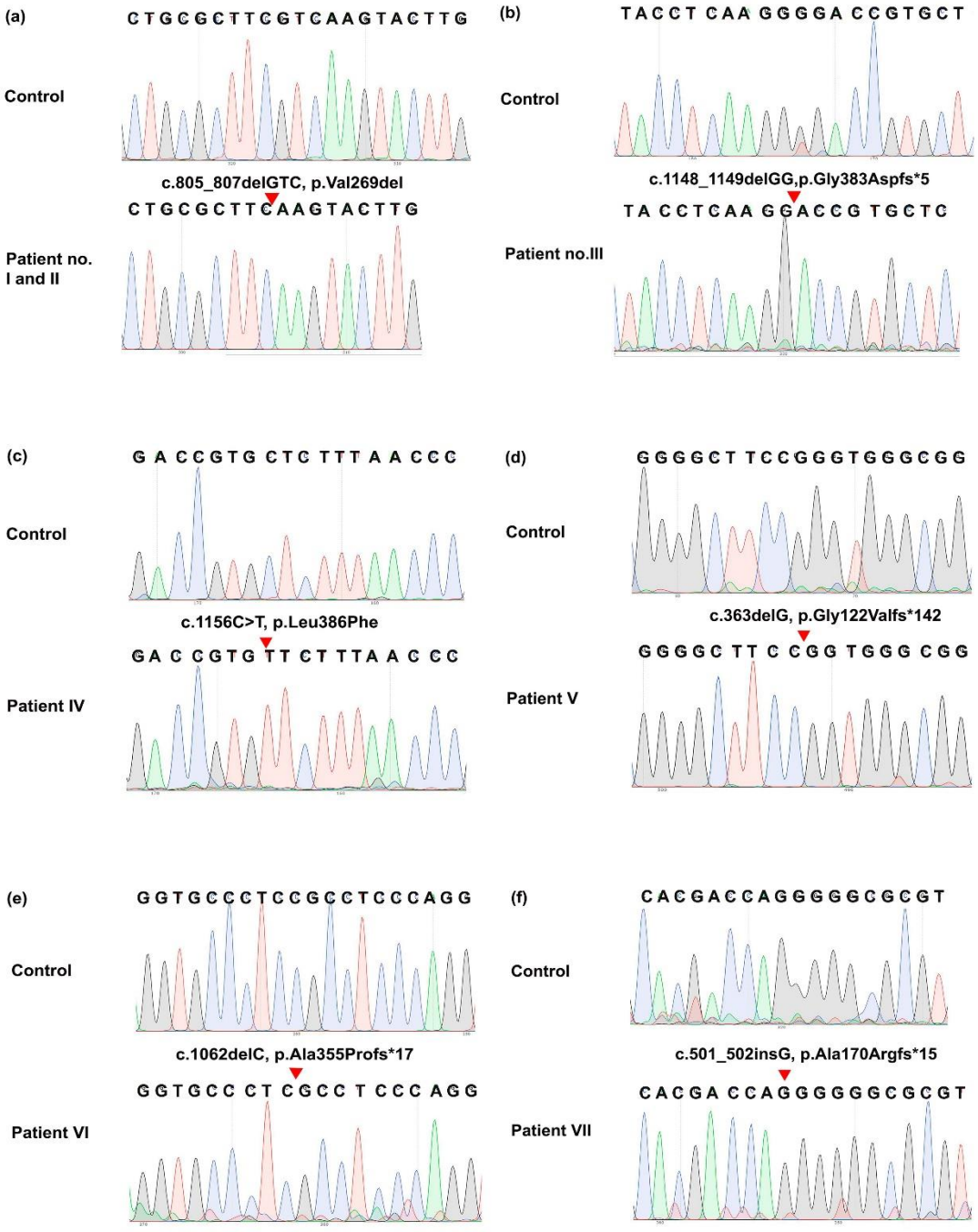


Figure 18 Sequencing data identified *DAX-1* mutations in all patients. (a) Chromatogram of patient I, II showing the c.805_807delGTC mutation that causes

p.Val269del. (b) Chromatogram of patient III showing the c.1148_1149delGG mutation that causes p.Gly383Aspfs*5. (c) Chromatogram of patient IV showing the c.1156C>T mutation that causes p.Leu386Phe. (d) Chromatogram of patient V showing the c.363delG mutation that causes p.Gly122Valfs*142. (e) Chromatogram of patient VI showing the c.1062delC mutation that causes p.Ala355Profs*17. (f) Chromatogram of patient VII showing the c.501_502insG leading to p.Ala170Argfs*15.

4.3 Mutation interpretation

4.3.1 Comparing the sequencing data with their mother

As X-linked AHC is inherited in an X-linked recessive manner, we would like to verify whether the patients inherited the mutations from their mothers or whether they were *de novo* mutations. Therefore, mothers of some patients were recruited to analyze for the mutations. However, only two pairs of mother-patient were able to recruit. Direct DNA sequencing of Patient IV's mother was not the same as that of Patient IV, but the same as control (Figure 19), suggesting that the mutation in Patient IV was a *de novo* mutation. On the other hand, DNA sequencing of Patient V was identical with his mother, suggesting that the patient inherited it from his mother. Therefore, the mother is heterozygous carrier of the mutation (Figure 20).

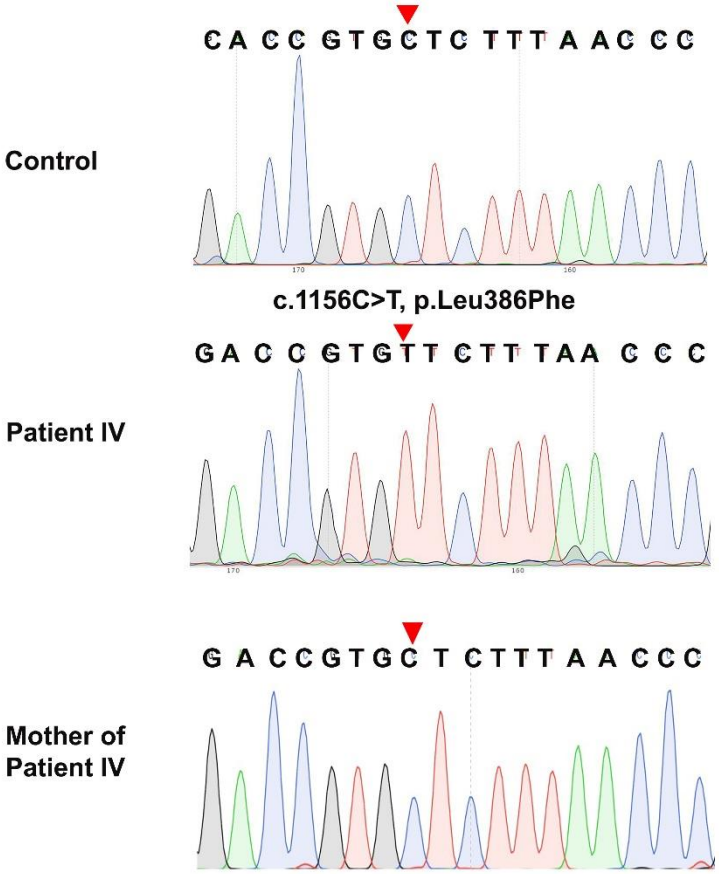


Figure 19 DNA sequencing of Patient IV comparing with his mother and a normal control.

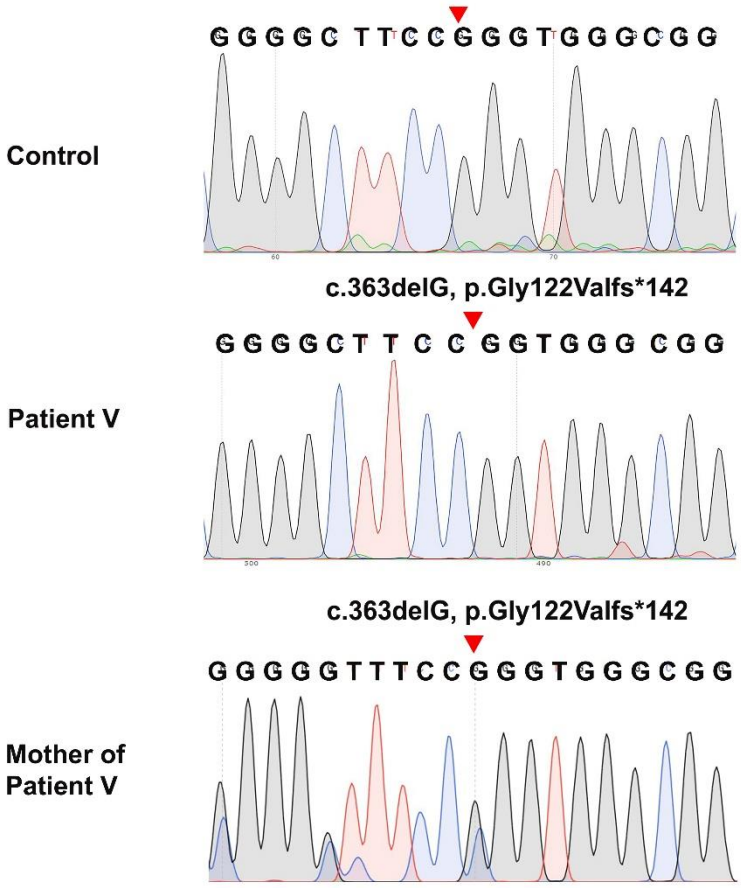


Figure 20. DNA sequencing of Patient V comparing with his mother and a normal control.

4.3.2 Comparing the sequencing data with Thai Exome database

When compared the patients' sequencing data with the Thai Exome database, the three novel mutations were absent in 100 control alleles, suggesting that these mutations were unlikely to be polymorphisms.

4.3.3 Analyzing effects of mutations with prediction programs

Mutation prediction programs were useful for studying the effects of mutation on protein stability, function and in evolution was important in predicting the tendency of violence to occur in disease. In this study, the only

missense mutation (c.1156C>T, p.Leu386Phe) was analyzed, as frameshift mutations would highly likely to cause deleterious effect to the protein and therefore are pathogenic mutations. The amino acid sequence of human DAX-1 protein (Genbank accession No. NP_000466) was analyzed by ClustalX Program by comparing with 9 other species including *Mus musculus* (NP_031456.1), *Rattus norvegicus* (NP445769.1), *Pan troglodytes* (NP_001181864.1), *Macaca mulatta* (XP_002806222.1), *Canis lupus familiaris* (XP_003640302.1), *Bos Taurus* (NP_001192786.1), *Xenopus tropicalis* (XP_002933661.1), *Danio rerio* (NP_001076416.1) and *Gallus gallus* (NP_989924.1) as shown in Figure 21. The result revealed that the leucine amino acid at position 386 was a highly conserved amino acid through evolution.

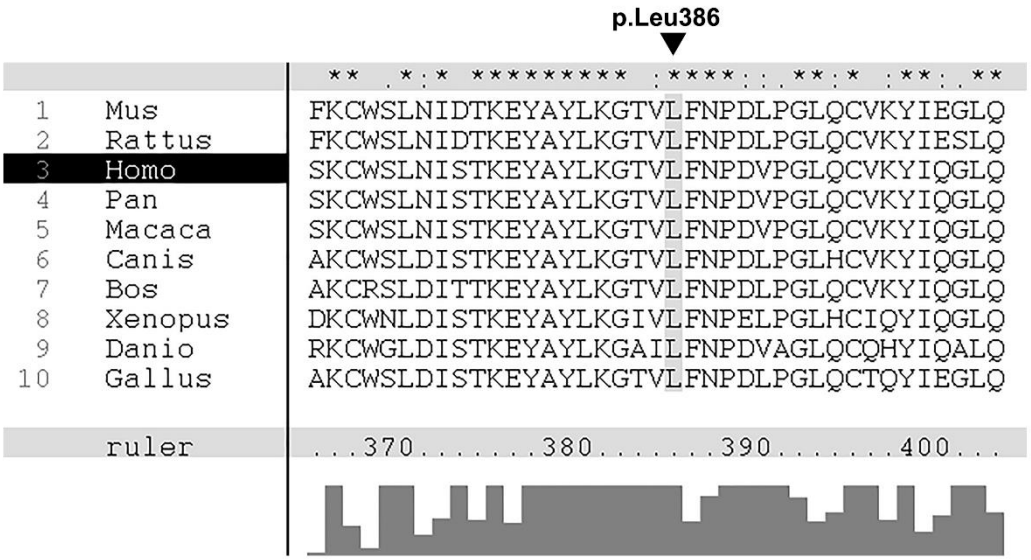


Figure 21. Amino acid sequence alignment of DAX-1 at position 386 by ClustalX program revealed that the leucine amino acid at position 386 was a highly conserved amino acid through evolution.

In mutation prediction programs, *in silico* analysis by SIFT, Polyphen-2, M-CAP, Mutation taster, CADD, PMut and Panther predicted p.Leu386Phe to be damaging (Table 8).

Table 8. Missense mutation (c.1156C>T, p.Leu386Phe) was analyzed by mutation prediction programs, *in silico* analysis.

***In silico* analysis programs**		**Prediction**	**Score**	**Note**
Name	**Feature**			
SIFT	The sequence preservation over the evolutionary time in predicting the effect of residue substitutions on function.	deleterious	0.001	below/equal to 0.05
Polyphen-2	Sequence and structure based method that predicts the possible impact of an amino acid substitution on the structure and function of a protein.	probably damaging	0.999	below/equal to 0.5
M-CAP	The pathogenicity classifier by combining previous pathogenicity scores (including SIFT, Polyphen-2 and CADD) with novel features and a powerful model.	pathogenic	0.9114	greater than 0.025
Mutation taster	The pathogenicity classifier by looking DNA sequence conservation, splice site prediction, mRNA stability prediction and protein feature annotations.	disease causing	-	-
CADD	The pathogenicity classifier by using 63 distinct variant annotation retrieved from Ensembl Variant Effect Predictor (VEP), data from the ENCODE project and information from UCSC genome browser tracks.	deleterious	32	greater than 15
PMut	The annotation and prediction of pathological variants on proteins.	disease	0.88 (92%)	-
Panther	Protein family and subfamily database that predicts the frequency of occurrence of amino acid at a particular position in evolutionary related protein sequences by using the conservation time and the likelihood of functional impact.	probably damaging	-	-

4.4 Functional analysis

4.4.1 Site-directed mutagenesis

After site-directed mutagenesis, clones of all mutants including c.805_807delGTC, c.1148_1149delGG, c.1156C>T, c.363delG, c.1062delC and c.501_502insG were confirmed by sequence analysis. An example of a selected clone resulted from site-directed mutagenesis is shown in Figure 22.

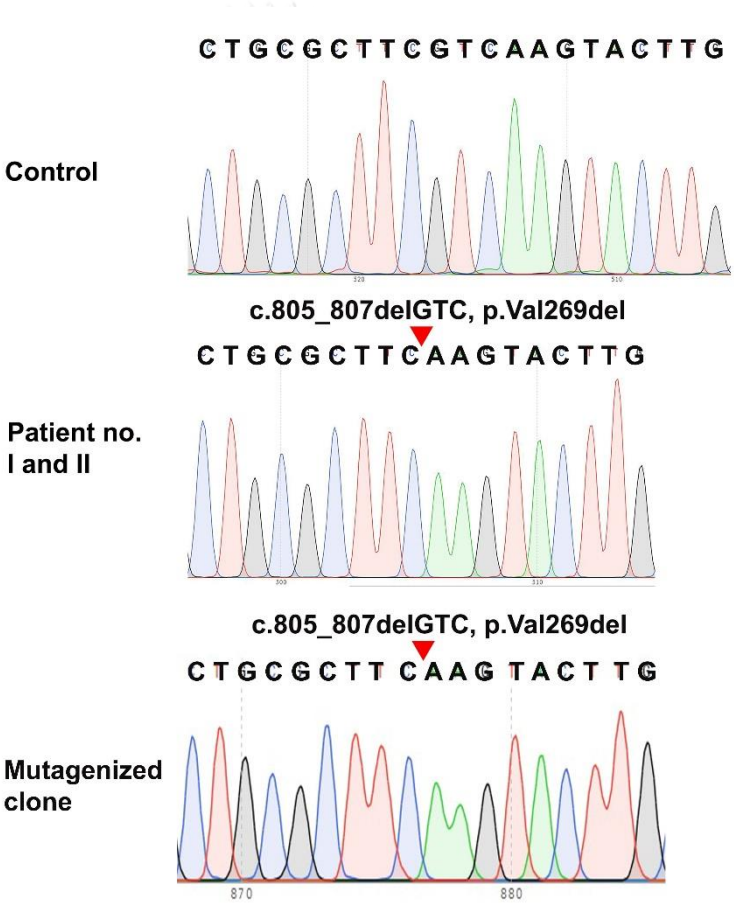


Figure 22 The example chromatogram of c.805_807delGTC, p.Val269del from site-directed mutagenesis clone comparing with control.

4.4.2 Transient transfection

After 24 hours of transfection, the transiently transfected HEK293T cells were identified transfection efficiency by using Green Fluorescent Protein (GFP) as a reporter gene and were observed by using confocal fluorescent microscope. The results showed that the transfection rate about 70-90%.

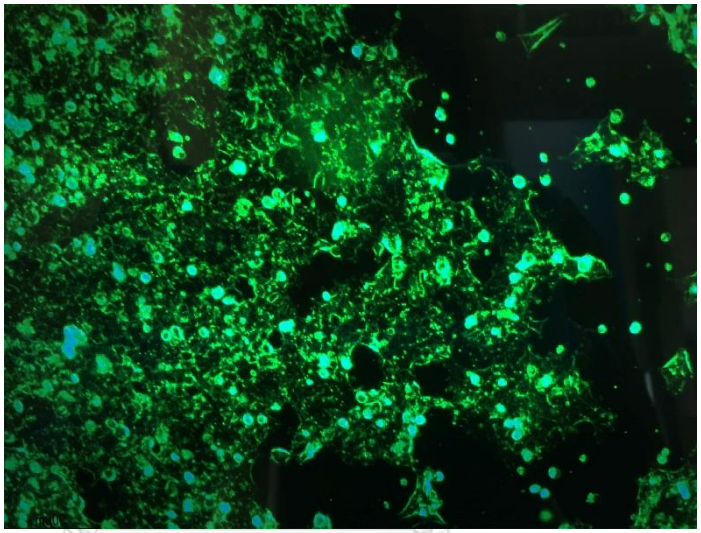


Figure 23 HEK293T cells (green) were transfected with GFP. Confocal fluorescent microscope was taken 24 hours after transfection (400X).

4.4.3 Effects of that mutations on luciferase activity

DAX-1 has been suggested to be a repressor regulator which acts by repressing the SF-1 mediated transactivation of steroidogenesis genes. One of the downsteam genes is *StAR*. Thus, in this study, the *StAR* gene promoter was used. A luciferase assay was performed to investigate whether the mutations impaired the repressor function of DAX-1. Values of the luciferase activity were expressed as mean ± standard error of the mean (SEM) from three independent transient transfection assays, each performed in triplicate. The

luciferase activities of mutants and wild type DAX-1 proteins by using the two-tailed Student's t-test (Table 9). The results showed high expression in the negative control (no *DAX-1*). However, co-expression of wild-type DAX-1 greatly repressed SF-1 activity on the promoter of the *StAR* gene. Wild-type DAX-1 suppressed the luciferase activity of pcDNA3.1 vector and the value was only 9%. Compared to empty vector, the relative luciferase activities were increased to 74% with p.Val269del and 15% with p.Gly383Aspfs*5, but this activity was suppressed down to 92% with p.Ala170Argfs*15, 46% with p.Ala355Profs*17, 67% with p.Gly122Valfs*142, and 76% with p.Leu386Phe. For all mutations shown the differences between the mutation and wild-type in statistically significant at 0.05 that abolished the repression activity of both SF-1 and *StAR* gene promoter activation (Figure 24).

Table 9. Luciferase activity detected in cells transfected with empty plasmid, wild-type DAX-1 or mutant DAX-1.

Plasmid	**Mean (%)**	**SEM (%)**	**P-Value**
pcDNA3.1	100	0	-
DAX-1(WT)	9	1.39	-
805_807delGTC	174	27.03	0.000
1148_1149delGG	115	10.67	0.000
1156C>T	76	14.98	0.001
363delG	67	23.85	0.026
1062delC	46	13.15	0.022
501_502insG	92	24.78	0.015

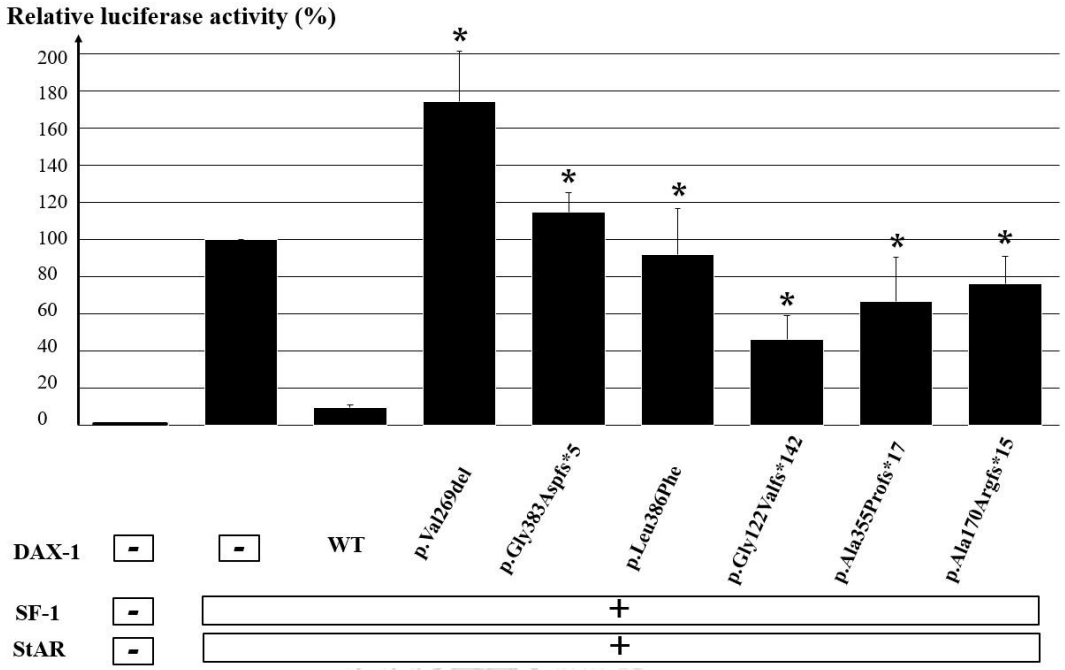


Figure 24 Functional effects of DAX-1 mutants measured by relative luciferase activities in cells transfected with SF-1, *StAR* and either wild-type or mutant DAX-1. Relative luciferase activities are expressed as the mean ± SEM for three independent experiments, each performed in triplicate. A star symbol on each bar represents the significant differences of luciferase activities between the mutants and WT DAX-1 (p-value <0.05).

CHAPTER V
Discussion and Conclusion

X-linked AHC is caused by mutations in a nuclear receptor gene called *DAX-1* gene, also known as *NR0B1* gene. The DAX-1 protein that is a member of the nuclear hormone receptor superfamily acts as a transcriptional repressor of genes involved in the steroidogenic pathway and at all levels of the HPG axis *in vitro* (Clipsham and McCabe, 2003). In this study reported seven patients with X-linked AHC from six families. The patients present typical and atypical clinical characteristics including late-onset Addison disease, precocious puberty, growth hormone deficiency, but most of them are present in the neonatal period. Six patients had typical phenotype with complete adrenal failure in early infancy, and Patient IV had late-onset Addison disease during adolescence. Patients V and VII had atypical phenotype that had GnRH-independent precocious puberty and severe growth hormone deficiency, respectively. This data confirmed that X-linked AHC patients present heterogeneous phenotypes. Moreover, genotype-phenotype correlations within families (Patients I and II) in this study demonstrated the similarity. Furthermore, Patient VII who had a known frameshift mutation had a clinical phenotype similar to a previously-reported Spanish boy with the same mutation. Both of them were presented with early-onset adrenal failure with complete growth hormone deficiency (Pérez Rodríguez et al., 2006). However, some *DAX-1* mutations do not have good phenotype-genotype correlation, even among family members with the same mutation, such as the onset age of adrenal insufficiency, disease severity and manifestation (Li et al., 2010). In this study, Patient III had severe adrenal failure, bilaterally small adrenal glands and

the earlier age onset of adrenal insufficiency than the previously-reported Thai boy (Boonyawat et al., 2017). It may be possible that epigenetics, non-genetic and environmental factors have influence to disease outcome (Jadhav et al., 2011).

Until now, almost 200 different mutations in *DAX-1* have been described, most of which are nonsense or frameshift mutations that cause premature truncation of the protein. Moreover, these mutations were found throughout the gene and are mostly related with severe phenotypes. On the other hand, missense mutations have been reported in the ligand-binding domain with several hotspots, suggesting that this domain is functionally essential (Suntharalingham et al., 2015). In-frame deletion mutations are relatively rare, but in multiple kindred have been reported the mutation in Patient I and II is one of the few mutations hotspots of *DAX-1* mutation (Ahmad et al., 2007). Moreover, other mutations have been reported in this region that had some specific changes associated with a late-onset phenotype (Kyriakakis et al., 2017; Mantovani et al., 2002; Tabarin et al., 2000). The DAX-1 protein consists of 2 regions that are the DNA-binding domain (DBD) and the ligand-binding domain (LBD). The DBD contains repeating units (hydrophobic LXXLL motifs) that have been originally recognized as a common feature in nuclear co-activators, which is essential for their interactions with nuclear receptors and other proteins. Moreover, the LBD plays a key role in the ligand structure that acts as a molecular switch by conformational changing and causes conversion of the receptor in a transcriptional repressor to activator (Clipsham and McCabe, 2003; Muscatelli, Strom, Walker, Zanaria, Recan, Meindl, Bardoni, Guioli, Zehetner, Rabl, Schwarz, et al., 1994).

Here, this study report mutations in seven patients. Among these, three were novel mutations, c.363delG (p.Gly122Valfs*142), c.1062delC (p.Ala355Profs*17), c.1156C>T (p.Leu386Phe), and three were known mutations, c.805_807delGTC (p.Val269del), c.1148_1149delGG (p.Gly383Aspfs*5), c.501_502insG (p.Ala170Argfs*15). Most of the mutations identified in this study were in the ligand-binding domain (Figure 25). Mutation identified in Patients I and II (c.805_807delGTC, p.Val269del) who were from related families, was suggested to disrupt the function of DAX-1 due to an in-frame deletion. Moreover, mutations in Patients III, IV, V and VI was suggested to disrupt the DAX-1 function by causing frameshift mutations resulting in a shorter deduced protein product.

The disruptive outcome of all mutations are confirmed by impairing the repressor function of DAX-1 in an *in vitro* assay. Therefore, *DAX-1* mutations did not suppress on the *SF-1* that mediated the *StAR* promoter activity in the luciferase assay. The protein with in-frame deletion (Patient I and II) presented higher expression activity than the frameshift mutation that is similar with the other studies. Hypothesis this result, this deletion will interrupt the tertiary structure of the DAX-1 protein. Moreover, the others have reported that this position is a critical function region that conserved hydrophobic residues belonging to the core of nuclear receptor and conserved among many vertebrate species in the evolution (Achermann et al., 2001; Ahmad et al., 2007; Lalli et al., 1997; Lehmann et al., 2003). However, frameshift mutations led to atypical protein products that changed the reading frame. The atypical protein had an incorrect amino acid sequence, it could be either longer or shorter sequence than the wildtype protein. Therefore, the function of normal protein of DAX-1 abolished the repression activity when have mutations. However, the

clinical manifestations and severity of disease differed substantially among these patients.

Patients I and II (cousins) who presented with classic, severe phenotypes at similar onset from in-frame single codon deletion that extremely loss of repression of *StAR* transcriptional activities *in vitro* assay. Patient III, V, VII also had severe disease outcome from carrying frameshift mutations that reduced the activities assay. The functional assays of these frameshift mutations provided fairly correlation with the clinical phenotype. Patient IV identified novel missense mutation (p.Leu386Phe) who had a milder phenotypes presenting hyperpigmentation and hypogonadotropic hypogonadism at 17 years old. Moreover, this patient showed unusual symptoms of late-onset Addison disease. Although X-linked AHC was commonly observed during infancy, some rare cases presented in adolescence or adulthood. Only ten previously-reported cases presented with late-onset Addison disease manifesting on later childhood (Kyriakakis et al., 2017). This position was taken in ClustalX program for checking the conservation of this position and found this position is highly conserved in the evolution (Figure 21). This amino acid will take a necessary part in structure of the DAX-1 protein that was located in LBD structure (Figure 25). The mutation changes codon 386 from leucine to phenylalanine which has not previously been reported on the Human Genome Mutation Database (HGMD). This position is the majority of *DAX-1* mutations that have been reported to date and also in cluster. LBD structure contains a key part in DAX-1 nuclear localization and transcriptional repression (Jadhav et al., 2011). By assessing the crystal structure of DAX-1 bound to its target nuclear receptor liver receptor homolog-1 (LRH-1) (Sablin et al., 2008), Leu386 is associated with the hydrophobic core of the protein. Hypothesis this result,

the bulkier phenylalanine residue would interfere with a function of the conserved leucine. Therefore, substitution of Phe for Leu may affect protein complex interactions or nuclear localization. Several specific mutations have been associated with late-onset phenotypes included p.Q37X, p.W39X, p.S259P, p.P279L, p.Gln305Hisfs*67 (c.915delG), p.Y380D, and p.I439S (Guclu et al., 2010; Kyriakakis et al., 2017; Mantovani et al., 2002; Oh et al., 2017; Ozisik et al., 2003; Raffin-Sanson et al., 2013; Sekiguchi et al., 2007; Tabarin et al., 2000). Some of these mutations were tested *in vitro* and retained partial function in different gene transcription assays associated with the milder phenotypes (Mantovani et al., 2002; Oh et al., 2017; Ozisik et al., 2003; Tabarin et al., 2000). The results can be added to previously described cases of atypical clinical in late-onset of AHC and expanded the genotypic spectrum of *DAX-1* mutations. Recently, Kyriakakis et al. (2017) diagnosed two probands with late-onset Addison disease. Both adults (age 19 and 30 years) presented primary adrenal insufficiency with hypogonadotropic hypogonadism. The probands had p.Ser259Pro and p.Pro279Leu mutations respectively that caused the structure of the repression helix domain of DAX-1 changing and affected protein complex interactions. However, *DAX-1* mutation with late-onset Addison disease and hypogonadotropic hypogonadism should be considered with X-linked AHC clinical manifestation.

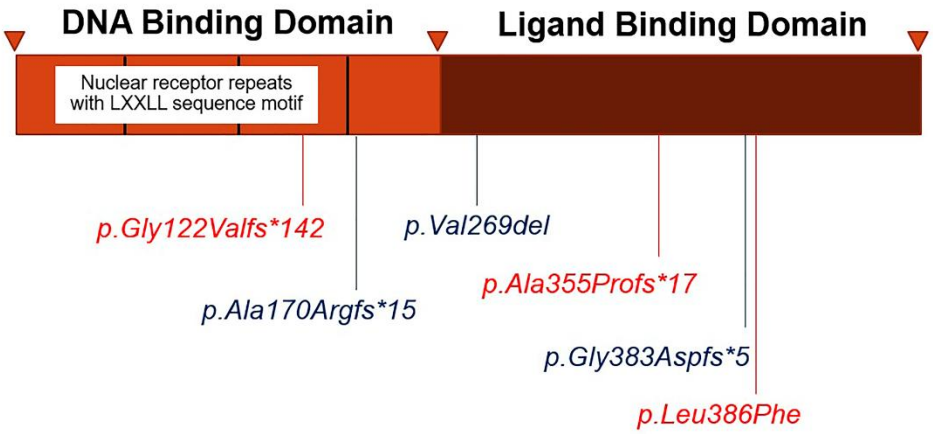


Figure 25 Schematic representation of the position of all *DAX-1* mutations identified in this study (novel mutations are represented in red label and known mutations are represented in blue label).

In general, *DAX-1* mutations with adult-onset AHC also had hypogonadotropic hypogonadism. However, *DAX-1* mutations appeared to be rare in men with sign of early puberty such as enlarge penis at birth. Interestingly, Patient V (c.363delG, p.Gly122Valfs*142) were diagnosed with early-onset of primary adrenal insufficiency in infancy and had signs of developed puberty (acne, penile enlargement) at 10 months of age. This manifestation has a rare case presented with pseudo-precocious puberty in early childhood and atypical presentation of *DAX-1* mutations which may complicate the diagnosis. The luteinizing hormone-releasing hormone (LHRH) stimulation test was performed to identify cause of sexual precocity and demonstrated that this patient had GnRH-independent precocious puberty (GIPP) with high testosterone levels. After increment of hydrocortisone dose, acne and penile enlargement were reversed and testosterone levels were normalized indicating a

potential ACTH-dependent pseudo-precocious puberty. To date, there have been only a few case reports of GnRH-independent in boys with *DAX-1* mutations. Domenice et al. (2001) reported a Brazilian boy with X-linked AHC who had pubic hair and enlarged penis at age 2 years. Similarly, Yeste et al. (2009) described a 9-month-old boy with classic AHC and early sexual development. The GnRH test supported the diagnosis of GIPP in both cases. However, these two previous studies presented mild testicular enlargement in a boy who were stable on steroid replacement. Moreover, these two cases were no evidence of true central precocious puberty with a prepubertal GnRH test. Although the exact mechanism of premature sexual development in this condition is still unclear, there are some literatures were described the hypotheses that high ACTH stimulated testicular steroidogenesis via melanocortin receptor type 1 (MCR1) and may be stimulated secretion of Leydig cells in testes. Likely, Ahmad et al. (2007) described autonomous Leydig cell hyperplasia in testes can induced GIPP in patient with X-linked AHC. Moreover, Jeffs et al. (2001) demonstrated that *Dax1*-knockout male mice for *Ahch* found the *Dax-1* deletion may be damaged the testicular developmental structure present abnormal seminiferous tubules and Leydig cell hyperplasia. In the same way, the results from this study in transient gene expression studies presented that the *DAX-1* inhibited the *SF-1* activity that play a critical role to control the expression of steroidogenic enzyme genes. Moreover, *DAX-1* bound to DNA hairpin secondary structures and blocked steroidogenesis in adrenal cells by transcriptional repression of *StAR* promoter (Lalli et al., 1997). Thus, the lack of DAX-1 repression was resulting in the overexpression of these two steroidogenesis activators. From this review suggesting the severe reduction of DAX-1 repression activity and extremely elevated ACTH

levels can be caused GIPP in some boy patient with *DAX-1* mutation (Domenice et al., 2001). However, further studies are needed to confirm all of these hypotheses.

The mutations of Patient VII (c.501_502insG, p.Ala170Argfs*15) presented frameshift insertion that has previously described in Spain country (Pérez Rodríguez et al., 2006). An 18-years-old revealed complete adrenal failure in the neonatal period (first admitted to hospital at the age of 16 days), hypogonadotropic hypogonadism and pathological short stature. The short stature may have the effect from the growth hormone deficiency in patients with X-linked AHC. Like Patient VII, a 3-day-old patient presented with hyperpigmentation, hypoglycemia, salt-wasting, and its association with growth hormone deficiency that is very uncommon. Hormonal tests confirmed complete growth hormone deficiency and central hypothyroidism in this case. The clinical characteristics were found similar that are the age onset in neonatal and has growth hormone deficiency with the same mutation, but different in some clinical manifestations such as hypogonadotropic hypogonadism. Up to now, growth hormone deficiency has not been a characteristic feature of patients with classic AHC. However, short stature with growth hormone deficiency has been recently reported in a few children with *DAX-1* mutations (Chung et al., 2015; Pérez Rodríguez et al., 2006). The mechanism of growth hormone deficiency in AHC patient is still unknown. However, *DAX-1* plays a key role in the development of the adrenal gland and hypothalamic-pituitary-gonadal axis in pituitary. Therefore, it should be associated with underlying defect in pituitary development, especially in other pituitary cell differentiation and maturation (Iyer and McCabe, 2004). Moreover, *DAX-1* is expressed in embryonic stem cells, steroidogenic tissues (including; gonads, adrenals), the ventromedial hypothalamus (VMH), and pituitary early in development

(Jadhav et al., 2011). It could be one of the important pituitary transcription factors and may affected other pituitary cell differentiation. However, the exact mechanisms of pituitary somatotropes or thyrotropes specifically respond to *DAX-1* remains unknown. Hence, further studies would be warranted to elucidate the role of *DAX-1* in other axes of pituitary development. The potential discovery of more patients with selective or combined hypopituitarism carrying *DAX-1* mutations could be most informative, and this is a potentially new clinical finding of AHC.

In conclusions, this study described clinical, molecular, and functional characterizations of three novel *DAX-1* mutations and three known mutations. The results supported fair genotype-phenotype correlation in patients with *DAX-1* mutations, even though *in vitro* functional assays may not fully reflect the complexity events *in vivo*. The study also extends the phenotypic and mutational spectrum of *DAX-1* mutations to include late-onset Addison disease, precocious puberty and growth hormone deficiency. This study provides insight regarding the new potential role of *DAX-1* in the development of the hypothalamus-pituitary-growth/thyroid axis. Growth velocity and thyroid function should be carefully monitored in patients with AHC. Further molecular analysis and functional studies should be performed to elucidate the process of the DAX-1 protein in the development with the atypical manifestations.

Recommendations of this study

- Shows the importance of molecular genetic analysis for diagnosis.
- Extends the phenotypic and mutational spectrum of *DAX-1* mutations.

- Provides information for physicians to accurately diagnose and provide treatment for patients with X-linked AHC.
- Provides information on molecular pathogenesis of the mutations which can pave ways to better treatment, or individualized medicine in the future.

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APPENDIX

Appendix 1: PCR condition of *DAX-1* mutations analysis

Component	**Final Concentration**	**Volume for 1 reaction (µl)**
10X *Taq* DNA polymerase Buffer (KCl)	1X	2
25 mM $MgCl_2$	0.5 mM	2
1mM dNTPs	0.2 mM	0.4
10 µM Forward Primer	0.15 µM	0.3
10 µM Reverse Primer	0.15 µM	0.3
5U/µl Taq DNA polymerase		0.1
Nuclease-Free Water		12.9
50 ng/µl DNA Template		2
Total Volume		20

Appendix 2: PCR program of *DAX-1* mutations analysis

Step	Temperature	Time
Initial Denaturation	96 C	5 minutes
Denaturation	94 C	30 second
Annealing	53,63 C	30 second
Extension	72 C	90 second
		35 cycles
Final Extension	72 C	2 minutes
Hold	15 C	∞

Appendix 3: Site-directed mutagenesis amplification condition

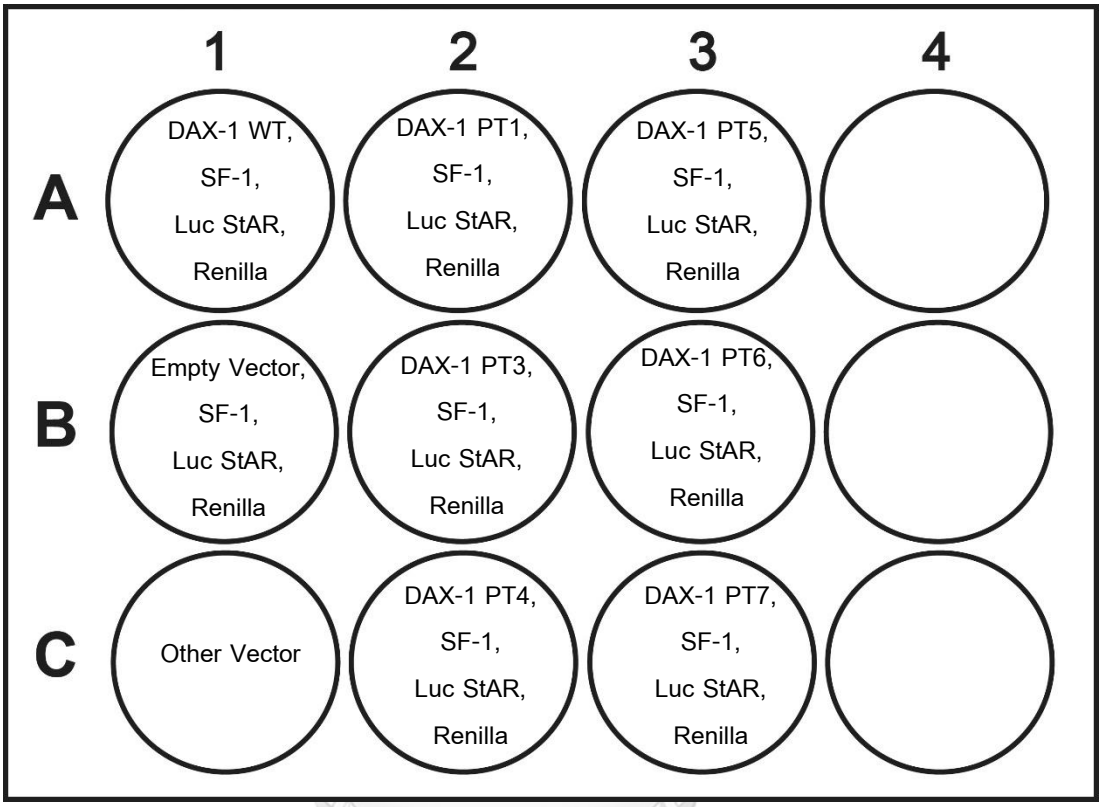
Component	**Final Concentration**	**Volume for 1 reaction (µl)**
10X Reaction Buffer	1X	5
1mM dNTPs	1 µl	1
100 ng/µl Patient_Sense Primer		1.25 µl
100 ng/µl Patient_Antisense Primer		1.25 µl
2.5 U/µl *PfuUltra* HF DNA polymerase		1
double-distilled water (ddH_2O)		38.5
5 ng/µl DNA Template		2
Total Volume		50

Appendix 4: Site-directed mutagenesis amplification program

Step	**Temperature**	**Time**
Initial Denaturation	95 C	30 second
Denaturation	95 C	30 second ◄
Annealing	55 C	1 minute
Extension	68 C	5 minutes ┘ 12-18 cycles*

* Type of mutation desired: Point mutations = 12 cycles, Single amino acid changes = 16 cycles, Multiple amino acid deletions or insertions = 18 cycles.

Appendix 5: Diagram illustrating the transient transfection plan used in this thesis. All transient transfections were performed in three independent experiments in triplicate.



Appendix 6: S.O.C. Medium suitable for use in the final step of bacterial cell transformation to obtain maximal transformation efficiency of *E. coli*.

Reagent	**Volume**
1M NaCl (MW=58.44 g)	0.5 g
1M KCl (MW=74.55 g)	0.125 ml
Peptone (Cesein Hydrolysate Enxymatic Digest)	1 g
Yeast Extract Powder	0.25 g
dH_2O	50 ml
Total	**50 ml**

VITA

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