LAPORAN AKHIR
PROJEK PENYELIDIKAN R & D
JANGKA PENDEK

NO GERAN: 304/PPSP/6131117

TAHUN: 2000

Semua laporan kemajuan dan laporan akhir yang dikemukakan kepada Bahagian Penyelidikan dan Pembangunan perlu terlebih dahulu disampaikan untuk penelitian dan perakuan Jawatankuasa Penyelidikan di Pusat Pengajian.
USM R&D/JP-04

LAPORAN AKHIR PROJEK PENYELIDIKAN

R&D JANGKA PENDEK

A. MAKLUMAT AM

Tajuk Projek:

Molecular detection of genetic defects in ambiguous genitalia (AG) and congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency.

Tajuk Program:

Tarikh Mula: 1 Januari 2000

Nama Penyelidik Utama: Dr. Fuziah Md. Zain
(berserta No. K/P) (No. K/P 590530-02-5698)

Nama Penyelidik Lain: 1. Prof. Madya Dr. Mohd Nizam Isa
(berserta No. K/P) (No. K/P 530514-03-5609)

2. Dr. Rus Anida Awang

(No. K/P 641027-03-5714)

B. PENCAPAIAN PROJEK:

(Sila tandakan [/] pada kotak yang bersesuaian dan terangkan secara ringkas di dalam ruang di bawah ini. Sekiranya perlu, sila gunakan kertas yang berasingan)

Penemuan asli/peningkatan pengetahuan

We have met the objective of the project which is to determine the presence of point mutations in patients affected with congenital adrenal hyperplasia.

Rekaan atau perkembangan produk baru,

(Sila beri penjelasan/makluman agar mudah dikomputerkan)

(1) Tiada

Mengembangkan proses atau teknik baru,

(Sila beri penjelasan/makluman agar mudah dikomputerkan)

(1) Gen SRY (Bahagian Seks Kromosom Y): Ia menentukan pembentukan testis yang berlokasi di lengan pendek pada kromosom Y.

(2) Allele Specific Oligonucleotide Hybridization (ASOH) : Ia merupakan salah satu kaedah untuk mengesan mutasi dalam penyakit CAH.

(3) Gen WNT-4 : Ianya sebahagian dari famili WNT. Fungsinya berlawanan dengan gen SRY di mana ia memainkan peranan mengawal pembentukan jantina perempuan dan memghalang pembentukan testis.

Memperbaiki/meningkatkan produk/proses/teknik yang sedia ada

(*Sila beri penjelasan/makluman agar mudah dikomputerkan*)

(1) *menggunakan teknikal yang telah sedia ada dengan sedikit modify*

Memperbaiki dalam teknik sitogenetik.

C. PEMINDAHAN TEKNOLOGI

Berjaya memindahkan teknologi.

Klien: *Tiada*

(*Nyatakan nama penerima pemindahan teknologi ini dan sama ada daripada pihak swasta ataupun sector awam*)

Berpotensi untuk pemindahan teknologi: *Tiada*

(*Nyatakan jenis klien yang mungkin berminat*)

Berpotensi untuk pemindahan teknologi: *Tiada*

(*Nyatakan jenis klien yang mungkin berminat*)

D. KOMERSIALISASI

Berjaya dikomersialkan.

Tiada

Nama Klien:

Tiada

Berpotensi untuk dikomersialkan.
(*Nyatakan jenis klien yang mungkin berminat*)

Tiada

E. PERKHIDMATAN PERUNDINGAN BERBANGKIT DARIPADA PROJEK

(*Klien dan jenis perundingan*)

Clinical services offered to patients with ambiguous genitalia, in patients confirmed as congenital adrenal hyperplasia and surgical correction. In CAH cases, counseling service was provided in terms of sex assignment or reassignment.

F. PATEN/SIJIL INOVASI UTILITI

(*Nyatakan nombor dan tarikh pendaftaran paten. Sekiranya paten/sijil inovasi utiliti telah dipohon tetapi masih belum didaftarkan, sila berikan nombor dan tarikh fail paten*).

Tiada

G. PENERBITAN HASIL DARIPADA PROJEK

i) PEMBENTANGAN DI PERSIDANGAN ATAU SEMINAR

1) First ASEAN Conference on Medical Sciences, 18-21 May 2001 at Renaissance Hotel, Kota Bharu, Kelantan. Poster presentation
Molecular Analysis in Gender Assignment and Management of Ambiguous Genitalia. Y.K. Muhamad, Fuziah MZ, Rus Anida A, M.R. Sidek, S.F. Fatimah Ramli, N.A. Adam, M.N. Isa.

2) 13th National Biotechnology Seminar, 10-13th November 2001, Bayview Hotel, Penang. Poster presentation.
Detection of Point Mutation (Pro30Leu) in Exon 1 of the 21-hydroxylase gene (CYP21) in patient with congenital adenal hyperplasia using digoxigenin system.
Y.K. Muhamad, Fuziah MZ, Rus Anida A, M.R. Sidek, S.F. Fatimah Ramli, N.A. Adam, M.N. Isa.

3) 41st Annual European Society for Paediatric Endocrinology Meeting, Madrid, Spain on 25-28th September 2002.
Molecular Analysis of CYP21 Gene in Patients Presenting with Ambiguous Genitalia. Poster presentation.
Fuziah MZ, Y.K. Muhamad, us Anida A, M.R. Sidek, S.F. Fatimah Ramli, M.N. Isa.

4) 4th HUGO Pacific Meeting and 5th Asia Pacific Conference on Human Genetics at Pattaya, Thailand on 27-30th October 2002.
Mutations of Pro30Leu and Val281Leu of the CYP21 gene in patients diagnosed with ambiguous genitalia.
M.N. Isa, Y.K. Muhamad, Fuziah MZ, Rus Anida A, M.R. Sidek, S.F. Fatimah Ramli.

ii) PENERBITAN SAINTIFIK

1) First ASEAN Conference on Medical Sciences, 18-21 May 2001 at Renaissance Hotel, Kota Bharu, Kelantan. Poster presentation
Molecular Analysis in Gender Assignment and Management of Ambiguous Genitalia. Y.K. Muhamad, Fuziah MZ, Rus Anida A, M.R. Sidek, S.F. Fatimah Ramli, N.A. Adam, M.N. Isa.

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Publication : Hormone Research 2002 58 (suppl 2) 1-197 (international Journal of Experimental and Clinical Endocrinology) P1-160, p 48. ISSN : 0301-0163

4) 4th HUGO Pacific Meeting and 5th Asia Pacific Conference on Human Genetics at Pattaya, Thailand on 27-30th October 2002.
Mutations of Pro30Leu and Val281Leu of the CYP21 gene in patients diagnosed with ambiguous genitalia.
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Publication : Poster DY16, Abstract Book 4th HUGO Pacific Meeting and 5th Asia Pacific Conference on Human Genetics. ISBN : 974-05-0173-7

5) Molecular analysis in the management of congenital adrenal hyperplasia (CAH) and ambiguous genitalia. M.N. Isa, Y.K. Muhamad, Fuziah MZ, Rus Anida A, M.R. Sidek, S.F. Fatimah Ramli. Journal of Asean Federation of Endocrine Societies, Vol. 20, 1/2, Jan/Jul 2002, p 12-18. ISSN 0857-1074

H. HUBUNGAN DENGAN PENYELIDIK LAIN

(*sama ada dengan institusi tempatan ataupun di luar negara*)

(1). *Prof. Madya Dr. Mohd Nizam Isa*

(No. K/P 530514-03-5609)

(2). *Dr. Rus Anida Awang*

(No. K/P 641027-03-5714)

I. SUMBANGAN KEWANGAN DARI PIHAK LUAR

(*Nyatakan nama agensi dan nilai atau peralatan yang telah diberi*)

Tiada

J. PELAJAR IJAZAH LANJUTAN

(Nyatakan jumlah yang telah dilatih di dalam bidang berkaitan dan sama ada diperingkat sarjana atau Ph.D).

Nama Pelajar

Yulia Kesuma Muhamad

Sarjana

Sarjana Sains Genetik

Ph.D

Tiada

K. MAKLUMAT LAIN YANG BERKAITAN

pencapaian 0.K

Yang

Professor Zabidi Azhar Mohd. Hussin
Chairman of Research & Ethics Committee
School of Medical Sciences
Universiti Sains Malaysia
16150 Kubang Kerian,
KELANTAN, MALAYSIA.

Tarikh Tandatangan

TANDATANGAN PENGERUSI
JAWATANKUASA PENYELIDIKAN
PUSAT PENGAJIAN

Semua laporan kemajuan dan laporan akhir yang dikemukakan kepada Bahagian Penyelidikan dan Pembangunan perlu terlebih dahulu disampaikan untuk penelitian dan perakuan Jawatankuasa Penyelidikan di Pusat Pengajian

USM JP-06

BAHAGIAN PENYELIDIKAN
UNIVERSITI SAINS MALAYSIA

Laporan Akhir Projek Penyelidikan Jangka Pendek

Nama Penyelidik: *Dr. Fuziah Md Zain*

(berserta No. K/P) *(No. K/P 590530-02-5698)*

Nama Penyelidik-Penyelidik Lain: 1. *Prof. Madya Dr. Mohd Nizam Isa*

(Jika berkaitan) *(No. K/P 530514-03-5609)*

2. *Dr. Ros Anida*

(No. K/P 641027-03-5714)

1. **Pusat Pengajian/Pusat/Unit:** Pusat Pengajian Sains Perubatan

1. **Tajuk Projek:**

Molecular detection of genetic defects in ambiguous genitalia (AG) and congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency.

4. (a) **Penemuan Projek/Abstrak**

(Perlu disediakan makluman diantara 100-200 perkataan di dalam Bahasa Malaysia dan Bahasa Inggeris, ini kemudiannya akan dimuatkan ke dalam Laporan Tahunan Bahagian Penyelidikan & Pembangunan sebagai satu cara untuk menyampaikan dapatan projek tuan/puan kepada pihak Universiti.)

ABSTRACT

Congenital adrenal hyperplasia (CAH) is a group of inherited disorders affecting the enzymes catalyzing the synthesis of steroids in the adrenal cortex. Malfunction of 21-hydroxylase is the most frequent defect among these disorders. This enzyme is essential for the synthesis of cortisol and aldosterone. The decreased level of serum cortisol in patients with 21-hydroxylase deficiency stimulates ACTH secretion, which result in elevated levels of steroid precursors as 17-hydroxyprogesterone and increased production of adrenal androgens causing virilization.

Deficiency of the 21-hydroxylase (21-OH) enzyme is by far the most common form of CAH which arises as a result of deletions or deleterious mutations in the active gene (CYP21) located on chromosome 6p21.3. Many different mutations of the CYP21 gene cause varying degrees of impairment of 21-OH activity that results in a spectrum of disease statement. There is no sharp limit between the salt- wasting, the simple virilizing and the late onset forms.

The objective of our study was to determine the 21-OH deficiency mutation defects and correlate the genotype with their phenotypic statement of the disease.

We performed mutational analysis using Polymerase Chain Reaction-Allele Specific Oligonucleotide Hybridization (PCR-ASOH) technique on patients who were suspected to have CAH as they presented with ambiguous genitalia (AG) and or electrolyte derangement.
Among the 52 patients, 3 had Val281Leu mutation (5.76%), 2 had Pro30Leu mutation (3.85%), 1 had Gln318stop mutation (1.92%) and in 46 (88.46%) patients no mutation was detected.

The three patients with Val281Leu mutation had presented with adrenal crises during infancy and was classified as salt losers and treated with glucocorticoids and mineralocorticoids. These 3 patients could well be the other 40% who are categorized as salt-losers. The two patients with Pro30Leu mutations have normal male external genitalia and presented with hyponatraemia and hyperkalaemia. Only one patient required mineralocorticoid therapy that was given for about 5 months duration. Subsequently he had normal electrolytes level even without mineralocorticoid therapy. The Gln318stop mutation was identified in one patient who presented with ambiguous genitalia and adrenal crises.

Our study showed that the cases with genotype Val281Leu, Pro30Leu and Gln318stop mutations correlated with their phenotype. The mutation analysis of CYP21 gene proved to be a good complementary investigation and supportive to the diagnosis and management of our CAH patients.

ABSTRAK

Hiperplasia adrenal kongenital (CAH) merupakan kumpulan penyakit warisan yang melibatkan enzim mengkatalase sintesis steroid pada korteks adrenal. Ketakfungsian enzim 21-hidroksilase adalah merupakan kecacatan yang sering berlaku berbanding dengan enzim-enzim lain dalam masalah ini. Enzim ini penting untuk sintesis kortisol dan aldosteron. Akibat kekurangan hormon kortisol, perangsangan sekresi ACTH terjadi dan ini menyebabkan peningkatan paras precursor steroid seperti 17-hidroksiprogesteron dan produk androgen adrenal dan terjadinya virilisasi. Kekurangan enzim 21-hidroksilase merupakan penyebab utama dalam hiperplasia adrenal kongenital yang terjadi dari deletan dan mutasi gen aktif (CYP21) di kromosom 6p21.3.

Terdapat beberapa perbezaan mutasi pada gen CYP21 yang mengakibatkan kepelbagaian darjah kerosakan aktiviti enzim 21-hidroksilase mengakibatkan spectrum pada penyakit. Disini tiada batasan yang tepat antara "salt wasting", "simple virilising" dan "late onset form".

Objektif kajian ini adalah bagi menentukan jenis mutasi akibat kekurangan enzim 21-hidroksilase dan untuk melihat hubung kait antara genotip dan fenotip.

Kami menjalankan analisis mutasi menggunakan kaedah reaksi berantai polymerase-hibridisasi oligonukleotid spesifik alel (PCR-ASOH) pada pesakit yang mempunyai masalah keraguan jantina (AG) dan atau pun hiponatraemia dan hiperkalaemia. Mereka dianggap mempunyai hiperplasia adrenal kongenital. Mutasi Val281Leu dan Pro30Leu menyebabkan kadar aktiviti enzim antara 20-60% dari kadar normal. Kedua-duanya dikaitkan dengan "non classical CAH".

Mutasi Gln318stop dikategorikan sebagai "salt wasting". Dikalangan 52 sampel darah pesakit, 3 orang mengalami mutasi Val281Leu, 2 orang mutasi Pro30Leu dan seorang mutasi Gln318stop sementara tidak ada mutasi dikesan dalam 46 sampel.

Tiga orang pesakit dengan mutasi Val281Leu mengalami adrenal krisis semasa peringkat bayi dan dikategorikan sebagai "salt loser". Mereka menerima rawatan glukokortikoid dan mineralokortikoid. Ketiga-tiga pesakit ini mungkin mewakili 40% pesakit yang dikategorikan sebagai "salt wasting". Dua orang pesakit dengan mutasi Pro30Leu mempunyai jantina eksternal lelaki yang normal dan hadir

dengan hiponatraemia dan hiperkalaemia. Hanya seorang pesakit sahaja yang memerlukan terapi mineralokortikoid di mana rawatan ini telah diberikan dalam jangka waktu 5 bulan. Berikutnya, pesakit itu mempunyai paras elektrolit yang normal tanpa terapi mineralokortikoid. Mutasi Gln318stop telah dikenalpasti berlaku dikalangan pesakit yang mengalami keraguan jantina dan krisis adrenal.

Kajian ini menunjukkan bahawa pesakit-pesakit yang mempunyai genotip Val281Leu, Pro30leu dan Gln318stop mempunyai korelasi yang berhubungkait dengan fenotip. Analisis mutasi pada gen CYP21 merupakan satu kajian yang sangat baik dan membantu diagnosis dan pengurusan pesakit hiperplasia adrenal kongenital.

(b) **Senaraikan Kata Kunci yang digunakan di dalam abstrak:**

Bahasa Malaysia	**Bahasa Inggeris**
Hiperplasia Adrenal Kongenital	*Congenital Adrenal Hyperplasia (CAH)*
Gen CYP21	*CYP21 gene*
Keraguan Jantina	*Ambiguous Genitalia (AG)*
Penentuan seks(SRY)	*Sex Determination Region-Y (SRY).*

5. Output Dan Faedah Projek

(a) Penerbitan (*termasuk laporan/kertas seminar*)
(Sila nyatakan jenis, tajuk, pengarang, tahun terbitan dan di mana telah diterbitkan/dibentangkan)

1) First ASEAN Conference on Medical Sciences, 18-21 May 2001 at Renaissance Hotel, Kota Bharu, Kelantan. Poster presentation. Molecular Analysis in Gender Assignment and Management of Ambiguous Genitalia. Y.K. Muhamad, Fuziah MZ, Rus Anida A, M.R. Sidek, S.F. Fatimah Ramli, N.A. Adam, M.N. Isa. **Poster dilampirkan.**

2) 13th National Biotechnology Seminar, 10-13th November 2001, Bayview Hotel, Penang. Poster presentation.
Detection of Point Mutation (Pro30Leu) in Exon 1 of the 21-hydroxylase gene (CYP21) in patient with congenital adenal hyperplasia using digoxigenin system. Poster dilampirkan. Y.K. Muhamad, Fuziah MZ, Rus Anida A, M.R. Sidek, S.F. Fatimah Ramli, N.A. Adam, M.N. Isa. **Poster dilampirkan.**

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Publication : Hormone Research 2002 58 (suppl 2) 1-197 (international Journal of Experimental and Clinical Endocrinology) P1-160, p 48. ISSN : 0301-0163.
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4) 4th HUGO Pacific Meeting and 5th Asia Pacific Conference on Human Genetics at Pattaya, Thailand on 27-30th October 2002.
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Publication : Poster DY16, Abstract Book 4th HUGO Pacific Meeting and 5th Asia Pacific Conference on Human Genetics. ISBN : 974-05-0173-7. **Poster dilampirkan.**

5) Molecular analysis in the management of congenital adrenal hyperplasia (CAH) and ambiguous genitalia. M.N. Isa, Y.K. Muhamad. Fuziah MZ. Rus Anida A. M.R. Sidek. S.F. Fatimah Ramli. Journal of Asean Federation of Endocrine Societies. Vol. 20. 1/2. Jan/Jul 2002. p 12-18. ISSN 0857-1074. **Salinan penerbitan dilampirkan.**

(b) Faedah-Faedah Lain Seperti Perkembangan Produk, Prospek Komersialisasi Dan Pendaftaran Paten
(*Jika ada dan jika perlu, sila gunakan kertas berasingan*)

Tiada

(c) Latihan Gunatenaga Manusia

i) Pelajar Siswazah: *Yulia Kesuma Muhamad*
Sarjana Sains Genetik

ii) Pelajar Prasiswazah: Tiada

iii) Lain-lain: Tiada

11. Peralatan Yang Telah Dibeli:

Tiada

UNTUK KEGUNAAN JAWATANKUASA PENYELIDIKAN UNIVERSITI

TANDATANGAN PENGERUSI
JAWATANKUASA PENYELIDIKAN
PUSAT PENGAJIAN
fn:borang/adlinaimc/nak

Semua laporan kemajuan dan laporan akhir yang dikemukakan kepada Bahagian Penyelidikan dan Pembangunan perlu terlebih dahulu disampaikan untuk penelitian dan perakuan Jawatankuasa Penyelidikan di Pusat Pengajian.
USM R&D/JP-04

LAPORAN AKHIR PROJEK PENYELIDIKAN

R&D JANGKA PENDEK

A. MAKLUMAT AM

Tajuk Projek:

Molecular detection of genetic defects in ambiguous genitalia (AG) and congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency.

Tajuk Program:

Tarikh Mula: 1 Januari 2000

Nama Penyelidik Utama: Dr. Fuziah Md. Zain
(berserta No. K/P) (No. K/P 590530-02-5698)

Nama Penyelidik Lain: 1. Prof. Madya Dr. Mohd Nizam Isa
(berserta No. K/P) (No. K/P 530514-03-5609)

2. Dr. Rus Anida Awang

(No. K/P 641027-03-5714)

MOLECULAR DETECTION
OF GENETIC DEFECTS IN AMBIGUOUS GENITALIA (AG)
AND
CONGENITAL ADRENAL HYPERPLASIA (CAH)
DUE TO 21-HYDROXYLASE DEFICIENCY

by

Dr. Fuziah Md.Zain

Prof. Mohd.Nizam Isa

Dr. Rus Anida Awang

Yulia Kesuma Muhamad

[1]Human Genome Centre,

[2]Department of Paediatrics, School of Medicine,

Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.

[3]Department of Paediatrics, Hospital Kota Bharu, Kelantan, Malaysia.

Introduction:

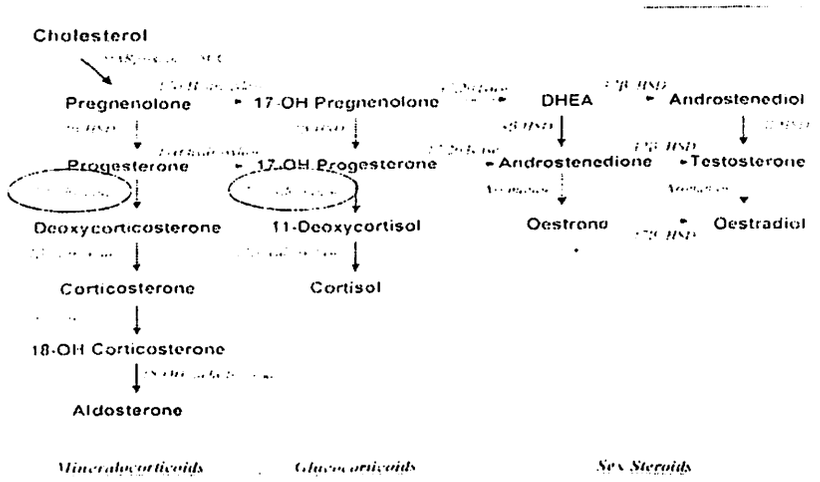
CAH due to 21-(OH) deficiency :

- Group of recessively inherited diseases
- More than 95% of all cases of CAH
- CAH exist in a very wide severity :

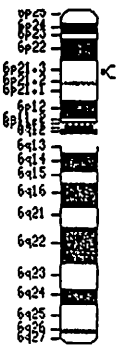
  i. Salt Wasting
  ii. Simple Virilizing } Classical
  iii. Non Classical

- Incidence 1: 10,000-17,000 in Western Europe & USA.
World wide 1: 14,000 births.

Adrenal and Gonadal Steroidogenesis



CAH due to 21-(OH) deficiency :



- Arises as a result of deletions or deleterious mutations in the active gene (CYP21) located on chromosome 6p.
- Many different mutations of the CYP21 gene have been identified causing varying degrees of impairment of 21-hydroxylase activity that result in a spectrum of disease expression.

Introduction: Clinical Picture

i. Salt Wasting

- The most severe form of CAH
- Salt wasting crisis in the first 2 weeks of life
- First sign of the disease:
  Girls - born with ambiguous genitalia
  Boys - hypovolemia, acidosis, hyponatremia, and hyperkalemia (adrenal crisis)

Introduction: Clinical Picture

ii. Simple Virilizing

- Diagnosed as virilization at 3-7 yr
- Female - pubic hair, phallic enlargement, increase muscle mass, and advanced bone age.
  Boys - testicular size remains pre pubertal in CAH but increases in central precocious puberty.

Introduction: Clinical Picture

iii. Non Classical

- The mild NC form of 21-OHD.
- Females - diagnosed at or after adolescene
  - present with hirsutism, acne, irregular menses, infertility
- Male - not recognized.

- Phenotype graded according to clinical severity

OBJECTIVE:

- To determine the presence of point mutations in patients affected with congenital adrenal hyperplasia

Genotype and Phenotype Relationships

- CYP21 mutations can be grouped into 3 categories according to enzyme activity
- The relationship between genotype and phenotype in the common mutation of the CYP21 gene.

Genotype	Phenotype	Activity enzyme (%)
Arg 356Trp Gln 318Stop Ilel172Asn	Salt wasting	± 2
Pro30Leu	Simple virilizing	18±9
Val281Leu	Non-classic	30-60
	Normal	

Genotyping can be used to predict the degree of disease severity patients affected with CAH

a. P30L

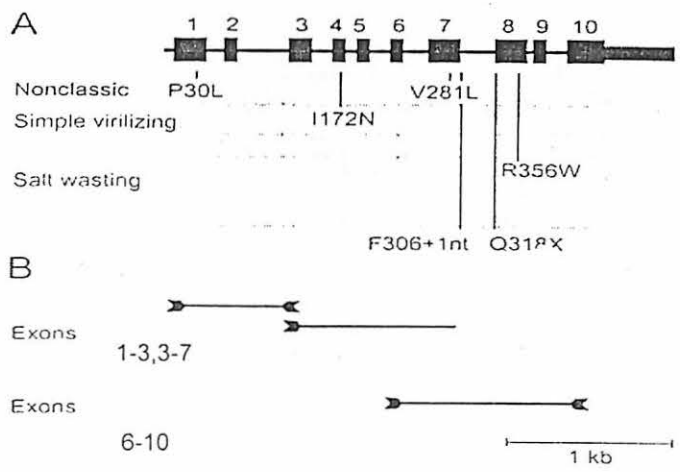
- The mutations in exon 1
- CCG (Proline) → to CTG (Leucine)
- Associated with nonclassical 21-hydroxylase deficiency

b. V281L

- The mutations in exon 7
- A change in codon 281 from GTG (Valine) → TTG (Leucine)
- The codon 281 mutation is associated with nonclassical 21-hydroxylase deficiency

c. Q318X

- The mutations in exon 8
- Codon 318 in this gene is changed from CAG (glutamine) → TAG stop codon
- The codon 318 mutation is associated with salt wasting form in CAH



Schematic representation of 21-hydroxylase deficiency (CYP21) gene structure and location of the common mutations of the gene. The numbers outside the box indicate exon number.

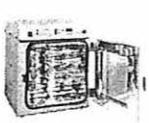
METHODS

Blood samples were obtained from patients referred to Hospital Universiti Sains Malaysia, Kelantan, Malaysia during 1995-2002. A thorough clinical examination and hormonal analyses were performed. A total of 52 samples included patients with external genitalia or electrolyte imbalances.

DNA extraction (non-phenol chloroform standard procedure)

Polymerase Chain Reaction (PCR)

PCR-ASOH (Allele Specific Oligonucleotide Hybridization) mutational analysis using PCR-ASOH technique



DNA sequencing

RESULTS

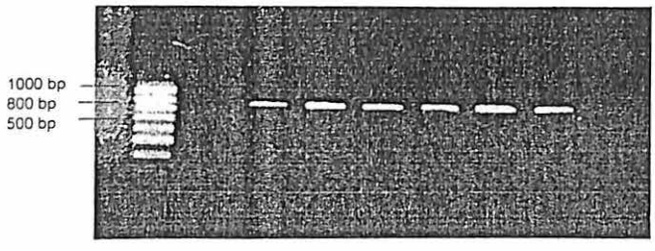


Figure 1: Presence of PCR product for CYP21 gene exon 1-3 using 2.0% agarose gel electrophoresis. Lane M : 100 bp. DNA ladder, lane N : negative control, lane 1: normal samples, lane 2-6 : samples showing PCR amplification products for CYP21 gene.

RESULTS

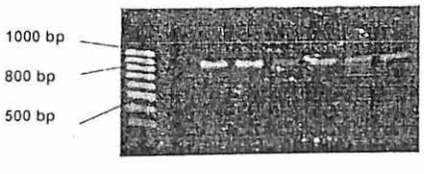


Figure 2: Presence of PCR product for CYP21 gene exon 4-7 using 2.0% agarose gel electrophoresis. Lane M : 100 bp. DNA ladder, lane 1 : negative control, lane 2 : normal samples, lane 3,4,5,6,7 : samples showing PCR amplification products for CYP21 gene.

RESULTS:

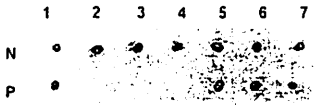
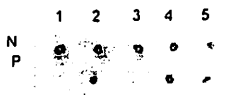


Figure 3: Dot blotting was performed using 1 µg genomic DNA. The blot was hybridized with 100 pmol/ml of the digoxigenin labeled antiphosphatase (DIG-AP) specific probe from samples of patient 1-7. ASOH of PCR product from CAH patients was performed with the probe V281L corresponding to the site exon. The status N: normal, P: patient.

RESULTS



- Figure 4: Pro30Leu hybridization DNA samples. Dot blotting was performed using 1 µg genomic DNA. The blot was hybridized with 100 pmol /ml of the digoxigenin labeled antiphosphatase (DIG-AP) specific probe Pro30Leu. ASOH of PCR product from CAH patients was performed with the probe (Pro30Leu) corresponding to the site exon 1. The status N: normal, P: patient.

RESULTS

- Figure 5: Q318X hybridization DNA samples. Dot blotting was performed using 1 µg genomic DNA. The blot was hybridized with 100 pmol /ml of the digoxigenin labeled antiphosphatase (DIG-AP) specific probe Q318X. ASOH of PCR product from CAH patients was performed with the probe Q318X corresponding to the site exon 8. The status N: normal, P: patient.

RESULTS

Table 1 : Identified mutations in samples analysed

Exon	Codon	Nucleotide alteration	Amino acid changes	No. of samples
1	30	C<u>C</u>G→C<u>T</u>G	Proline→ Leusine	2
7	281	C<u>G</u>T→C<u>T</u>T	Valine → Leusine	3
8	318	G<u>C</u>A→G<u>T</u>A	Glutamin → Stop codon	1

RESULTS

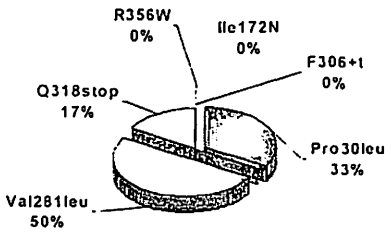


Figure 6 : A pie chart showing percentage of CYP21 gene mutation according to each exon.

RESULTS

Table 2: Frequency of CYP21 gene mutation in patients diagnosed with congenital adrenal hyperplasia (CAH).

Diagnosis of CAH	Mutation	Percentage (%)
Pro30Leu	2/52	3.85
Val281Leu	3/52	5.77
Q318Stop	1/52	1.92
TOTAL	6/52	11.54

RESULTS

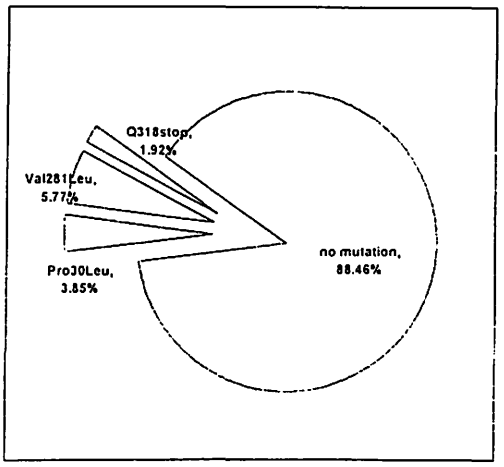
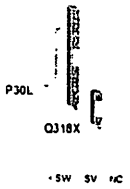


Figure 7 : A pie chart showing percentage of frequency for CYP21 gene mutation in patients diagnosed with CAH.

RESULTS

Table 3: Relations between genotype & phenotype

Genotype	Salt wasting (SW)	Simple virilizing (SV)	Non-classical (NC)
Pro30Leu	0	0	2
Val281Leu	3	0	0
Q318stop	1	0	0
Total	4 (66.67%)	0	2 (33.33%)



Frequency of common mutations among 21-hydroxylase deficiency alleles in different populations

Nationality	Total no.patients	P30L	I172N	V281L	Q318X	R356W
USA	394	2	10	9	4	4
Sweden	400	2	20	6	2	3
England	220	2	14	7	-	-
France	258	NA	9	17	4	NA
Finland	102	-	29	3	2	-
Italy	146	3	6	11	8	-
Italy (south)	50	0	6	6	4	4
Malaysia (USM,Kelantan)	52	2	0	3	1	0
Spain	58*	2	2	17	3	3
Japan	102	0	13	1	0	13
China	40	-	28	-	8	10
Chile	126	-	7	-	9	11
Mexico	94	9	12	9	4	7
Brazil	74	-	19	4	11	8
Argentina	72	-	15	-	14	6

CONCLUSION

- Some variability in clinical expression can occasionally be seen among patients with the same genotype but in the majority of cases good relationships between CYP21 genotype and CAH phenotype are found.
- Therefore we find genotyping of CYP21 to be very useful for prediction of clinical outcome in CAH patients.
- The possibility to predict disease outcome in CAH patients by mutations analyses has had several implications for treatment.
- Our findings showed that patients with Pro30Leu mutations were associated with non-classical form of CAH whereas Val281Leu mutations were associated with salt wasting form of CAH.

PRESENTATION / PUBLICATION

1) **Molecular Analysis of CYP21 Gene in Patients Presenting with Ambiguous Genitalia.** MZ Fuziah, KM Yulia, A Rus Anida, MR Sidek, SF Ramli SF, MN Isa. Poster presentation at European Society for Paediatric Endocrinology 41st Annual Meeting, Madrid on 25-28 September 2002.

Publication : Hormone Research 2002, 58 (suppl 2):1-197 (International Journal of Experimental and Clinical Endocrinology) P1-160, page 48.
ISSN 0301-0163

2) **Mutations of Pro30Leu and Val281Leu of the CYP21 Gene in Patients Diagnosed with Ambiguous Genitalia.**
MN Isa, Y.K. Muhamad, Fuziah MZ, Rus Anida A, M. Ros Sidek, S.F. Ramli.
Poster presentation at 4th HUGO Pacific Meeting and 5th Asia- Pacific Conference on Human Genetics on 27-30.10.02 at Pattaya, Thailand.

Publication : Poster DY 16, abstract book 4th HUGO Pacific Meeting and 5th Asia- Pacific Conference on Human Genetics
ISBN : 974-05-0173-7

PRESENTATION / PUBLICATION

3) **Molecular Analysis in the Management of Congenital Adrenal Hyperplasia (CAH) and Ambiguous Genitalia.**
MN Isa, Y.K. Muhamad, Fuziah MZ. Rus Anisa A, M. Ros Sidek, S.F. Ramli, N. Adam.
Journal of the Asean Federation of Endocrine Societies, Vol 20, No 1/2, Jan/July 2002 (12-18). ISSN0857-1074

4) **Detection of Point Mutation (Pro30Leu) in Exon 1 of the 21-hydroxylase gene(CYP21) in patient with Congenital Adrenal Hyperplasia using Digoxigenin system.**
Y.K. Muhamad, Fuziah MZ, Rus Anda A, M.R. Sidek , S.F., Ramli . N.A. Adam, M. N. Isa. Poster presentation at 13th National Bioatechnology Seminar ,10-13th November 2001, Penang.

5) **Molecular Analysis in Gender Assignment and Management of Ambiguous Genitalia.** Y.K. Muhamad . Fuziah MZ , Rus Anda A, , M.R. Sidek , S.F., Ramli, N.A. Adam, M. N. Isa
Poster presentation at First Asean Conference On Medical Sciences, , 18-21 May 2001,Kota Bharu, Kelantan.

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Abstracts

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KARGER

P1-160 Poster Session 1

MOLECULAR ANALYSIS OF CYP21 GENE IN PATIENTS PRESENTING WITH AMBIGUOUS GENITALIA

F. Md. Zain[1]; Y. KM[2]; R. A[3]; M. Sidek[2]; S. Ramli[2]; M. Isa[2]
[1]Paediatrics, School of Medicine, Health Campus, Universiti Sains Malaysia, Kelantan, Malaysia; [2]Human Genome Centre, School of Medicine, Health Campus, Universiti Sains Malaysia, Kelantan, Malaysia; [3]Paediatrics, Hospital Kota Bharu, Kelantan, Malaysia

Introduction: Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders of adrenal steroidogenesis. The genes of the steroidogenic enzymes and the mutations involved have been described. Deficiency of the 21-hydroxylase (21-OH) enzyme is by far the most common form of CAH which arises as a result of deletions or deleterious mutations in the active gene (CYP21) located on chromosome 6p. Many different mutations of the CYP21 gene cause varying degrees in impairment of 21-OH activity that results in a spectrum of disease expression. There is no sharp limit between the salt-wasting, the simple virilizing and the late onset forms. **Objective:** To determine the 21-OH deficiency mutation defects and correlate the genotype with their phenotypic expression of the disease. **Patients/Material and Methods:** We performed mutational analysis using Polymerase Chain Reaction - Allele Specific Oligonucleotide Hybridization (PCR-ASOH) technique on 6 patients who presented with ambiguous genitalia (AG) and or electrolyte derangement as hyponatraemia and hyperkalaemia, suspected to have CAH. The Val281Leu and Pro30Leu mutations result in enzymes with 20-60% of normal activity and both are associated with the non-classical form of CAH. The Gln318stop mutation is categorized under the salt-wasting type. **Results:** Among the 6 patients, 3 had Val281Leu mutation, 2 had Pro30Leu mutation and 1 had Gln318stop mutation. The 3 patients with Val281Leu mutation had presented with adrenal crises during infancy and was classified as salt losers and treated with glucocorticoids and mineralocorticoids. These 3 patients could well be the other 40% who are categorized as salt losers. The 2 patients with Pro30Leu mutations have normal male external genitalia and presented with hyponatraemia and hyperkalaemia. Only 1 of them required mineralocorticoids that was given for about 5 months duration. Subsequently he had normal electrolytes even without mineralocorticoid therapy. The Gln318stop mutation was identified in one patient who presented with AG and adrenal crises. **Conclusions:** Our study showed that the patients with genotype Val281Leu, Pro30Leu and Gln318stop mutations correlated with their phenotype. The mutation analysis of CYP21 gene proved to be a good complementary investigation and supportive to the diagnosis and management of our CAH patients.

P1-161 Poster Session 1

PROPOSAL FOR PRENATAL MANAGEMENT OF CONGENITAL ADRENAL HYPERPLASIA (CAH) IN EUROPE BASED ON THE EXPECTED NUMBER OF

MOLECULAR ANALYSIS of CYP21 GENE in PATIENTS PRESENTING with AMBIGUOUS GENITALIA

MZ FUZIAH[1], KM YULIA[2], A. RUS ANIDA[3], MR SIDEK[2], SF RAMLI[2], MN ISA[4]

[1] Department of Paediatrics ,[2] Human Genome Centre, School of Medicine, USM, 16150 Kubang Kerian, [3]Department of Paediatrics, Hospital Kota Bharu, Kelantan, [4]International Medical University, Putrajaya, Malaysia.

ABSTRACT

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders of adrenal steroidogenesis. The genes of the steroidogenic enzymes and the mutations involved have been described. Deficiency of the 21-hydroxylase (21-OH) enzyme is by far the most common form of CAH which arises as a result of deletions or deleterious mutations in the active gene (CYP21) located on chromosome 6p. Many different mutations of the CYP21 gene cause varying degrees of impairment of 21-OH activity that results in a spectrum of disease _expression. There is no sharp limit between the salt- wasting, the simple virilizing and the late onset forms.

The objective of our study was to determine the 21-OH deficiency mutation defects and correlate the genotype with their phenotypic _expression of the disease.

We performed mutational analysis using PCR-ASOH (Polymerase Chain Reaction - Allele Specific Oligonucleotide Hybridization) technique on six patients who presented with ambiguous genitalia (AG) and or electrolyte derangement as hyponatraemia and hyperkalaemia, suspected to have CAH. The Val281Leu and Pro30Leu mutations result in enzymes with 20-60% of normal activity and both are associated with the non-classical form of CAH. The Gln318stop mutation is categorized under the salt-wasting type.

Among the six patients, three had Val281Leu mutation, two had Pro30Leu mutation and one had Gln318stop mutation. The three patients with Val281Leu mutation had presented with adrenal crises during infancy and was classified as salt losers and treated with glucocorticoids and mineralocorticoids. These 3 patients could well be the other 40% who are categorized as salt-losers. The two patients with Pro30Leu mutations have normal male external genitalia and presented with hyponatraemia and hyperkalaemia. Only one patient required mineralocorticoid therapy that was given for about 5 months duration. Subsequently he had normal electrolytes level even without mineralocorticoid therapy. The Gln318stop mutation was identified in one patient who presented with ambiguous genitalia and adrenal crises.

Our study showed that the patients with genotype Val281Leu, Pro30Leu and Gln318stop mutations correlated with their phenotype. The mutation analysis of CYP21 gene proved to be a good complementary investigation and supportive to the diagnosis and management of our CAH patients.

Key words: ambiguous genitalia, congenital adrenal hyperplasia, CYP21 gene.

INTRODUCTION

Ambiguous genitalia (AG) are one of the clinical presentations of congenital adrenal hyperplasia (CAH). Congenital adrenal hyperplasia is an autosomal recessive disease caused by loss or severe decrease in the activity of 21-hydroxylase (21-OH). This enzyme is one of the five enzymes necessary for cortisol biosynthesis. Deficiency of the 21-OH is the most common form of CAH accounting for 90-95% of all cases of CAH[1,6].

Congenital adrenal hyperplasia presents a wide spectrum of clinical manifestations and patients are divided into 3 groups: salt wasting (SW), simple virilizing (SV) and non-classical (NC). Salt wasting patients manifest as neonatal electrolyte disturbances together with virilization of external genitalia at birth in girls and early pseudoprecocious puberty in boys, while SV patients present the same manifestation as SW patients, but without electrolyte disturbances. Non- classical patients present with late onset symptoms of androgen excess, ranging from progressive virilization and pseudoprecocious puberty in childhood to menstrual disturbances, infertility and hirsutism in adult women[2].

It was reported that more than 90% of cases of CAH are caused by mutation of the CYP21 gene. This 21-OH (CYP21) gene is located in the HLA class III gene region on chromosome 6p21.3 and consist of 10 exons[3]. Majority of the mutations on the CYP21 gene are Val281Leu and Pro30Leu. These mutations were reported to result in 20-60% of normal enzyme activity and both are associated with the non-classical form of CAH[4,5].

AIM OF THE STUDY

To detect the presence of point mutations in Pro30Leu, Ile172Asn & Val281Leu of CYP21 gene

RESULTS

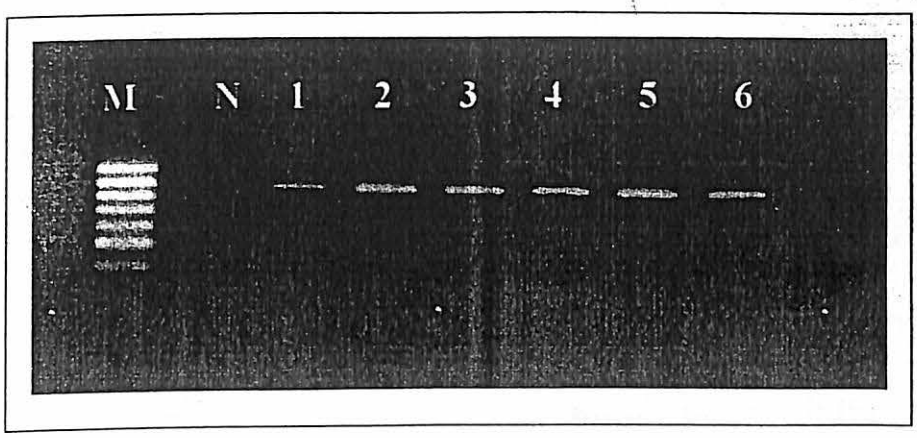

Figure 1: Presence of PCR product (873 bp) for CYP21 gene exon 1-3 using 2.0% agarose gel electrophoresis. Lane M : 100 bp DNA ladder, lane N : negative control, lane 1, 2 : normal samples, lane 3,4,5,6 : samples showing PCR amplification products for CYP21 gene.

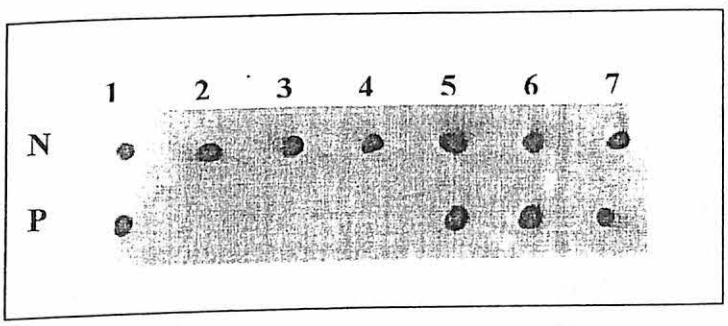

Figure 2: Dot blotting was performed using 1 µg genomic DNA. The blot was hybridized with 100 pmol/ml of the digoxigenin labeled antiphosphatase (DIG-AP) specific probe from samples of patient 1-7. ASOH of PCR product from CAH patients was performed with the probe corresponding to the site exon. The status N: normal, P: patient.

RESULTS

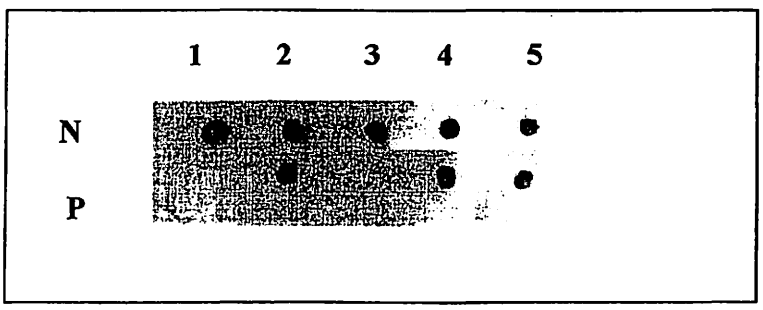

Figure 3: Pro30Leu hybridization DNA samples. Dot blotting was performed using 1 µg genomic DNA. The blot was hybridized with 100 pmol /ml of the digoxigenin labeled antiphosphatase (DIG-AP) specific probe Pro30Leu. ASOH of PCR product from CAH patients was performed with the probe (Pro30Leu) corresponding to the site exon 1. The status N: normal, P: patient.

RESULTS

Table 1

	Patient I	Patient II	Patient III	Patient IV	Patient V	Patient VI
Birth Weight (kg)	4.2	4.3	3.0	3.3	2.8	2.7
Age	32 days	81 days	2 months	At birth	10 weeks	6 days
Clinical Picture	Adrenal crisis Default follow up CPP Mental Retardation	Failure to thrive Normal male external genitalia Both testes descended Sepsis, meningitis & adrenal crisis	Haematuria Salt loss Normal male external genitalia Both testes descended	Normal male external genitalia Both testes descended Hypoglycae-mia	Failure to thrive Ambiguous genitalia Phallus 2 cm Single orifice at its base No palpable gonads	Neonatal jaundice Ambiguous genitalia Prominent phallus Single orifice at its base No palpable gonads Registered as male Reassigned as female
Sodium 135-150 mmol/L	120	110	119	131	92	118

RESULTS

	Patient I	Patient II	Patient III	Patient IV	Patient V	Patient VI
Potassium (3.5 – 5.0 mmol/L)	4.3	4.2	6.5	6.6	7.4	8.2
Blood urea (1.4 – 6.8 mmol/L)	28.8	23.0	NA	3.5	NA	NA
HCO3 (18 – 25 mmol/L)	1.6	4.4	NA	NA	NA	NA
17-OHP (ng/ml)	NA	NA	9.9 (0.07-1.7)	NA	>20 (up to 1.1)	33.2 (0.7-2.5)
Cortisol (nmol/L)	48 (139-501)	329 (138-690)	609 (140-500)	650 (138-690)	484 (139-501)	NA

RESULTS

	Patient I	Patient II	Patient III	Patient IV	Patient V	Patient VI
Testosterone (0.42-0.72 nmol/L)	1.4	NA	4.9	13.3	NA	NA
SRY gene	46,XY Present	46,XY Present	46,XY Present	46,XY Present	46,XX Not present	46,XX Not present
US pelvis / abdomen	-	-	-	-	Uterus & ovaries	Uterus & ovaries
Mutation in CYP21 gene	Val281Leu	Val281Leu	Pro30Leu	Pro30Leu	Val281Leu	Gln318Stop

NA: Not available, CPP: central precocious puberty

DISCUSSION

Majority of the mutations in the CYP21 gene in our patients are Val281Leu and Pro30Leu. These mutations were reported to result in 20-60% of normal enzyme activity and both are associated with the non-classical form of CAH[4,7].

In our study, we found that 3/52 patients (5.7%) have Val281Leu while 2/52 patients (3.8%) have Pro30Leu respectively. No mutations were observed in Ile172Asn. Patients with Val281Leu presented with adrenal crisis during infancy and were classified as salt wasting. Two patients with Pro30Leu mutations have normal male external genitalia and presented with hyponatraemia and hyperkalaemia. We did not identify any patient with I172N mutation which is known to result in clearly reduced enzymatic activity. About 1-2% of I172N mutation is usually associated with the SV form[8,9].

Our findings showed that patients with Pro30Leu mutations were associated with non-classical form of CAH whereas Val281Leu mutations were associated with salt wasting form of CAH.

CONCLUSION

This study suggested that phenotypes are not always concordant with the genotype in patients with Val281Leu mutations diagnosed with CAH.

ACKNOWLEDGMENT

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4TH HUGO PACIFIC MEETING AND 5TH ASIA-PACIFIC CONFERENCE ON HUMAN GENETICS

October 27-30, 2002

Program and Abstract

Genome and Health

Ambassador City Jomtien Pattaya, Chonburi
Thailand

peroxidase (6 blocks, no expression information at this time).

DY-16

Mutations of Pro30Leu and Val281Leu of the CYP21 Gene in Patients Diagnosed with Ambiguous Genitalia

M.N.Isa[1], Y.K.Muhamad[1], Fuziah MZ[2], Rus Anida A[3], M.Ros Sidek[1], S.F.Ramli[1]

[1]Human Genome Centre, [2]Department of Paediatrics, School of Medicine, USM, 16150 Kubang Kerian, [3]Department of Paediatrics, Hospital Kota Bharu, Kelantan, Malaysia.

Ambiguous Genitalia (AG) is one of the presentations of congenital adrenal hyperplasia (CAH). Congenital adrenal hyperplasia is an autosomal recessive disease caused by loss or severe decrease in the activity of 21-hydroxylase (21-OH). This enzyme is one of the five enzymes necessary for cortisol biosynthesis. Deficiency of the 21-OH is the most common form of CAH accounting for 90-95% of all cases of congenital adrenal hyperplasia. Congenital adrenal hyperplasia presents a wide spectrum of clinical manifestations and patients are divided into 3 groups: salt wasting (SW), simple virilizing (SV) and non-classical (NC). Salt Wasting patients manifest neonatal electrolyte disturbances together with virilization of external genitalia at birth in girls and early pseudoprecocious puberty in boys, while SV patients present the same manifestation as SW patients, but without electrolyte disturbances. Non-classical patients present with late onset symptoms of androgen excess, ranging from progressive virilization and pseudoprecocious puberty in childhood to menstrual disturbances, infertility and hirsutism in adult women. It was reported that more than 90% of cases of CAH are caused by mutation of the CYP21 gene. This steroid 21-OH (CYP21) gene is located in the HLA class III gene region on chromosome 6p21.3 and consist of 10 exons. Majority of the mutations on the CYP21 gene are Val281Leu and Pro30Leu. These mutations were reported to result in 20-60% of normal enzyme activity and both are associated with the non-classical form of CAH. Our aim of the study is to detect the presence of point mutations in Pro30Leu, Ile172Asn and Val281Leu of CYP21 gene. We have performed PCR-ASOH technique on 52 patients with ambiguous genitalia (AG). They presented with AG in Hospital Universiti Sains Malaysia & Hospital Kota Bharu, Kelantan Malaysia. We found that 3 out of 52 patients (5.7%) were having Val281Leu and 2 out of 52 patients (3.8%) of Pro30Leu respectively. No mutations were observed in Ile172Asn. Patients with Val281Leu presented with adrenal crisis during infancy and were classified as salt wasting. Two patients with Pro30Leu mutations have normal male external genitalia and presented with hyponatraemia and hyperkalaemia. Our findings showed that patients with Pro30Leu mutations were associated with non-classical form of CAH, whereas Val281Leu mutations were associated with salt wasting form of CAH. This study suggested that phenotypes are not always concordant with the genotype in patients with Val281Leu mutations diagnosed with CAH.

MUTATIONS OF Pro30Leu AND Val281Leu OF THE CYP21 GENE IN PATIENTS DIAGNOSED WITH AMBIGUOUS GENITALIA

M.N.Isa[4], Y.K.Muhamad[1], Fuziah MZ[2], Rus Anida A[3],M.Ros Sidek[1], S.F.Ramli[1]

[1]Human Genome Centre, [2]Department of Paediatrics, School of Medicine, USM, 16150 Kubang Kerian, [3]Department of Paediatrics, Hospital Kota Bharu, Kelantan, [4]International Medical University, Putrajaya, Malaysia.

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INTRODUCTION

Ambiguous genitalia (AG) are one of the clinical presentations of congenital adrenal hyperplasia (CAH). Congenital adrenal hyperplasia is an autosomal recessive disease caused by loss or severe decrease in the activity of 21-hydroxylase (21-OH). This enzyme is one of the five enzymes necessary for cortisol biosynthesis. Deficiency of the 21-OH is the most common form of CAH accounting for 90-95% of all cases of CAH[1,6].

Congenital adrenal hyperplasia presents a wide spectrum of clinical manifestations and patients are divided into 3 groups: salt wasting (SW), simple virilizing (SV) and non-classical (NC). Salt wasting patients manifest as neonatal electrolyte disturbances together with virilization of external genitalia at birth in girls and early pseudoprecocious puberty in boys, while SV patients present the same manifestation as SW patients, but without electrolyte disturbances. Non- classical patients present with late onset symptoms of androgen excess, ranging from progressive virilization and pseudoprecocious puberty in childhood to menstrual disturbances, infertility and hirsutism in adult women[2].

It was reported that more than 90% of cases of CAH are caused by mutation of the CYP21 gene. This 21-OH (CYP21) gene is located in the HLA class III gene region on chromosome 6p21.3 and consist of 10 exons[3]. Majority of the mutations on the CYP21 gene are Val281Leu and Pro30Leu. These mutations were reported to result in 20-60% of normal enzyme activity and both are associated with the non-classical form of CAH[4,5].

AIM OF THE STUDY

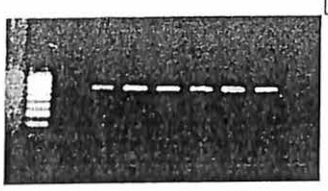
To detect the presence of point mutations in Pro30Leu, Ile172Asn & Val281Leu of CYP21 gene

MATERIALS AND METHODS

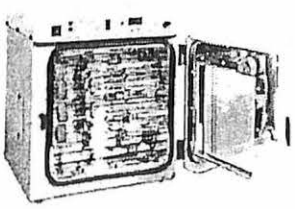
Blood samples were obtained from patients referred to Hospital Universiti Sains Malaysia, Kelantan, Malaysia during 1995-2002. A thorough clinical examination and hormonal analyses were performed. A total of 52 samples included were suspected to have CAH based on ambiguity of the external genitalia or electrolyte imbalances.

DNA extraction (non-phenol chloroform standard procedure)

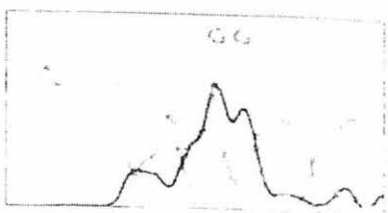
Polymerase Chain Reaction (PCR)



PCR-ASOH (Allele Specific Oligonucleotide Hybridization) mutational analysis using PCR-ASOH technique



DNA sequencing



RESULTS

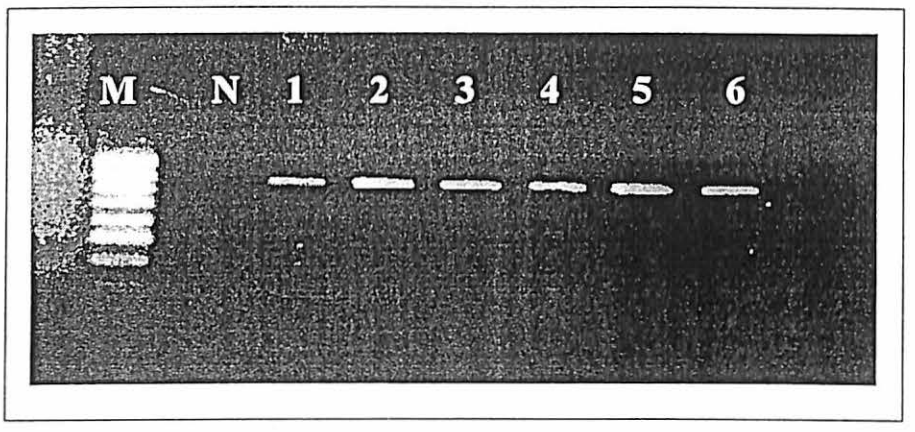


Figure 1: Presence of PCR product (873 bp) for CYP21 gene exon 1-3 using 2.0% agarose gel electrophoresis. Lane M : 100 bp DNA ladder, lane N : negative control, lane 1, 2 : normal samples, lane 3,4,5,6 : samples showing PCR amplification products for CYP21 gene.

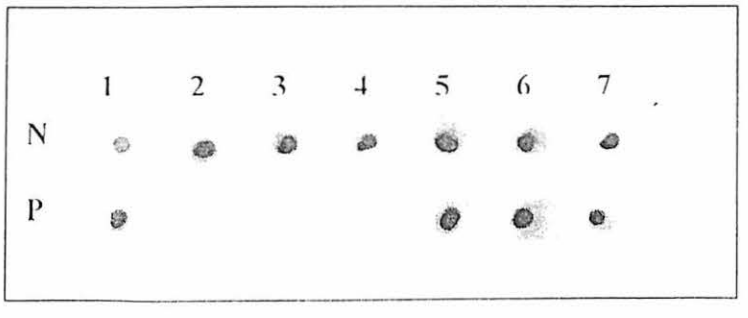


Figure 2: Dot blotting was performed using 1 µg genomic DNA. The blot was hybridized with 100 pmol/ml of the digoxigenin labeled antiphosphatase (DIG-AP) specific probe from samples of patient 1-7. ASOH of PCR product from CAH patients was performed with the probe corresponding to the site exon. The status N: normal, P: patient.

RESULTS

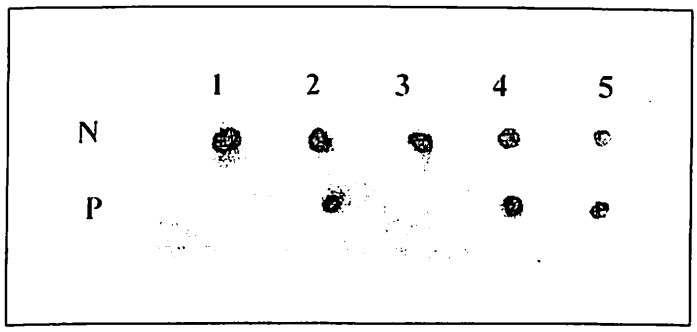


Figure 3: Pro30Leu hybridization DNA samples. Dot blotting was performed using 1 µg genomic DNA. The blot was hybridized with 100 pmol /ml of the digoxigenin labeled antiphosphatase (DIG-AP) specific probe Pro30Leu. ASOH of PCR product from CAH patients was performed with the probe (Pro30Leu) corresponding to the site exon 1. The status N: normal, P: patient.

RESULTS

Table 1

	Patient I	Patient II	Patient III	Patient IV	Patient V	Patient VI
Birth Weight (kg)	4.2	4.3	3.0	3.3	2.8	2.7
Age	32 days	81 days	2 months	At birth	10 weeks	6 days
Clinical Picture	Adrenal crisis Default follow up CPP Mental Retardation	Failure to thrive Normal male external genitalia Both testes descended Sepsis, meningitis & adrenal crisis	Haematuria Salt loss Normal male external genitalia Both testes descended	Normal male external genitalia Both testes descended Hypoglycae-mia	Failure to thrive Ambiguous genitalia Phallus 2 cm Single orifice at its base No palpable gonads	Neonatal jaundice Ambiguous genitalia Prominent phallus Single orifice at its base No palpable gonads Registered as male Reassigned as female
Sodium 135-150 mmol/L	120	110	119	131	92	118

RESULTS

	Patient I	Patient II	Patient III	Patient IV	Patient V	Patient VI
Potassium (3.5 – 5.0 mmol/L)	4.3	4.2	6.5	6.6	7.4	8.2
Blood urea (1.4 – 6.8 mmol/L)	28.8	23.0	NA	3.5	NA	NA
HCO3 (18 – 25 mmol/L)	1.6	4.4	NA	NA	NA	NA
17-OHP (ng/ml)	NA	NA	9.9 (0.07-1.7)	NA	>20 (up to 1.1)	33.2 (0.7-2.5)
Cortisol (nmol/L)	48 (139-501)	329 (138-690)	609 (140-500)	650 (138-690)	484 (139-501)	NA

DISCUSSION

Majority of the mutations in the CYP21 gene in our patients are Val281Leu and Pro30Leu. These mutations were reported to result in 20-60% of normal enzyme activity and both are associated with the non-classical form of CAH[4,7].

In our study, we found that 3/52 patients (5.7%) have Val281Leu while 2/52 patients (3.8%) have Pro30Leu respectively. No mutations were observed in Ile172Asn. Patients with Val281Leu presented with adrenal crisis during infancy and were classified as salt wasting. Two patients with Pro30Leu mutations have normal male external genitalia and presented with hyponatraemia and hyperkalaemia. We did not identify any patient with I172N mutation which is known to result in clearly reduced enzymatic activity. About 1-2% of I172N mutation is usually associated with the SV form[8, 9].

Our findings showed that patients with Pro30Leu mutations were associated with non-classical form of CAH whereas Val281Leu mutations were associated with salt wasting form of CAH.

CONCLUSION

This study suggested that phenotypes are not always concordant with the genotype in patients with Val281Leu mutations diagnosed with CAH.

RESULTS

	Patient I	Patient II	Patient III	Patient IV	Patient V	Patient VI
Testosterone (0.42-0.72 nmol/L)	1.4	NA	4.9	13.3	NA	NA
SRY gene	46,XY Present	46,XY Present	46,XY Present	46,XY Present	46,XX Not present	46,XX Not present
US pelvis / abdomen	-	-	-	-	Uterus & ovaries	Uterus & ovaries
Mutation in CYP21 gene	Val281Leu	Val281Leu	Pro30Leu	Pro30Leu	Val281Leu	Gln318Stop

NA: Not available, CPP: central precocious puberty

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Original Articles

Molecular Analysis in the management of Congenital Adrenal Hyperplasia (CAH) and Ambiguous Genitalia

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Patients presenting with ambiguous genitalia (AG) often pose a dilemma to the attending clinicians with regard to sex assignment. To avoid gender confusion in later life, it is important to recognize the underlying cause, initiate treatment and appropriate sex assignment as soon as possible. We present 3 case reports to illustrate the use of fluorescence in-situ hybridization (FISH), chromosome karyotyping and SRY gene identification in the evaluation of patients with AG. Current hormonal and imaging studies for sex determination could be complemented by the application of chromosomal and molecular genetic studies which provide rapid and reliable genetic diagnosis. Delay in diagnosis and treatment may result in death due to undiagnosed adrenal crisis in case of congenital adrenal hyperplasia (CAH) and wrong sex assignment with its tragic consequences. Molecular study and determination of the SRY gene are additional tools for the investigations of patients with AG and intersex disorders.

Key words: congenital adrenal hyperplasia (CAH), ambiguous genitalia (AG), sex determination region-Y (SRY).

INTRODUCTION

The issue of gender assignment causes anxiety and the problem of acceptance on the part of the parents. Patients with ambiguous genitalia should be investigated in order to understand the underlying pathology and to provide a rational approach to management and sex assignment. Diagnosis is aided by hormonal, imaging studies and genetic information. Current clinical and hormonal investigation protocols for sex determination can be enhanced by the application of chromosomal and molecular genetic studies using SRY gene.

The word ambiguous means indeterminate, doubtful or uncertain. The term ambiguous genitalia (AG) describe external genitalia that do not conform to that of male or female. This would include severe hypospadias with or without palpable gonads or micropenis[1]. Other description for such abnormalities include enlarged phallus/clitoris, scrotalisation of the labioscrotal folds, partial or complete fusion of the labioscrotal folds, chordee, urogenital sinus and blind vaginal pouch.

The problem in the management of a baby with AG is not only sex determination and appropriate assignment, it is also prompt recognition of underlying

medical emergencies that may be associated. The clinician must must be quick to detect life–threatening adrenal crisis in case of salt losing congenital adrenal hyperplasia (CAH)[2], and initiate immediate investigations and management[3,7]

The initial treatment goals include firstly, determination of the appropriate sex of rearing as quickly as possible. Secondly, establishment of definitive diagnosis, which may take longer to achieve. To avoid gender identification crisis, social, personality and religious problems during adulthood, follow up care and management is needed to ensure physical and psychological development concordant with the assigned sex[4].

Availability of specific hormonal investigation is limited. Conventionally, genetic sex is determined by chromosome karyotyping. The result is generally available within one month but a preliminary report may be available within a week. Currently new methods are available using molecular technology such as fluorescence in-situ hybridization (FISH) using the X (Figure 7) and Y (Figure 8) probes to identify the types of chromatin present. Identification of SRY gene (Figure 9) is another of these new molecular techniques [4].

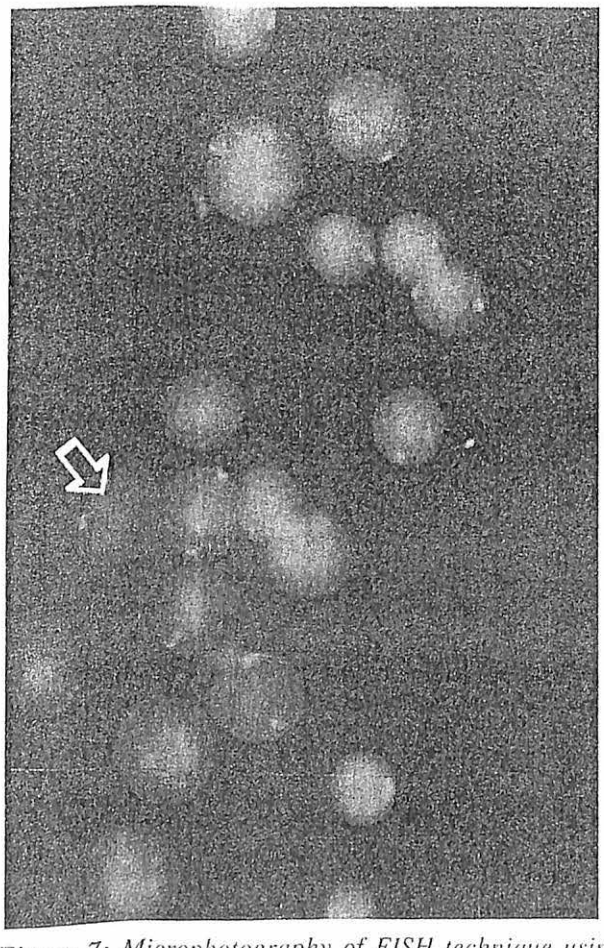

Figure 7: Microphotography of FISH technique using the chromosome X probe (pDMX1) on interphase cell, arrow shows 2 signals of the chromosome X

The SRY gene is the testis-determining gene located on the short arm of the Y chromosome. It initiates a cascade of events that ultimately lead to testis differentiation. Studies showed that SRY gene in an individual indicates the presence of testicular tissues. The use of the SRY gene testing as a tool in complementing hormonal, imaging studies in the evaluation of children with AG is helpful. The result can be obtained in less than a week [4]. Decision on gender assignment and registration of birth of baby can be made without much delay.

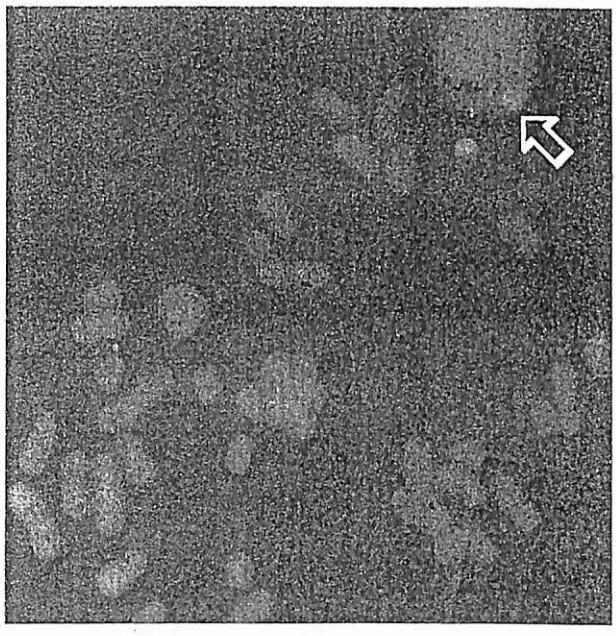

Figure 8: Microphotography of FISH technique using pHY.1 probe. Arrow shows the specific Y chromosome.

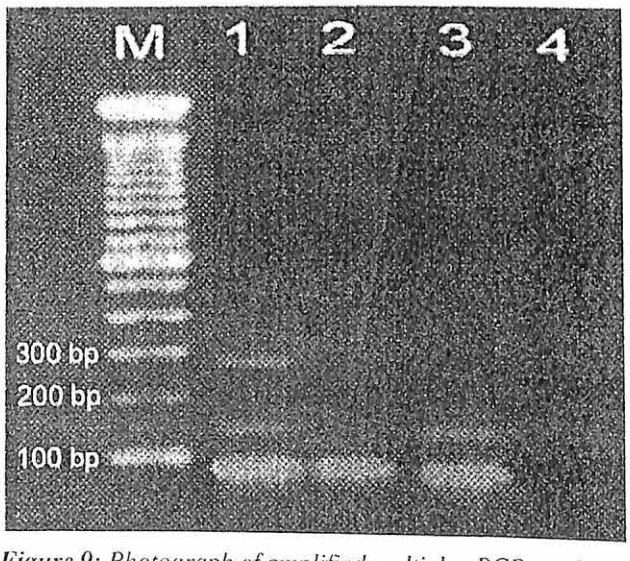

Figure 9: Photograph of amplified multiplex PCR products using X, Y chromosome and SRY gene specific PCR primers.
M- 100 bp DNA ladder (marker)
1 - DNA sample from 46 XY with the presence of SRY gene (273 bp)
2 - DNA sample from 46 XX in the absence of the SRY gene
3 - DNA sample from 46 XY with the absence of SRY gene
4 - Negative control (without DNA)

OBJECTIVE

The aim of the study is to apply the molecular studies to complement the investigations for the genetic assignment of patients with ambiguous genitalia by presenting 3 case reports.

METHODS

Three patients were referred to Hospital Kota Bharu (HKB) and Hospital Universiti Sains Malaysia (HUSM) for further management of ambiguous genitalia. Blood specimen of 3-5 ml from the patients with ambiguous genitalia was collected in sodium EDTA or lithium heparin container. Blood was cultured for metaphase cells for cytogenetic and FISH according to the standard protocol. Genomic DNA was extracted and subjected to the PCR analysis of SRY gene method[5, 6].

RESULTS

Patient 1, presented to Hospital Kota Bharu (HKB) at 10 weeks of age with intermittent vomiting and failure to thrive since birth. The patient, a product ofa non-consanguineous marriage was born full term by spontaneous vaginal delivery at a district hospital with a birth weight of 2.8 kg (25-50th percentile), length 56 cm (>97th percentile) and head circumference 33 cm (10-25th percentile). She is the youngest of 5 siblings and the eldest sibling who was a boy died at 17 days old of an unknown cause. On examination she was mildly dehydrated with a weight of 2.8 kg (<3rd percentile). She was afebrile and the blood pressure was 60/40 mmHg. No other abnormalities were detected apart from AG with a prominent phallus measuring 2 cm. No gonads or rugae were noted at the labioscrotal folds, which was not hyperpigmented. There was a single orifice at the base of the phallus. The baby was registered as a girl by the parents as they were not aware of the ambiguity of the external genitalia at that particular time.

The initial electrolytes showed hyponatraemia, hyperkalaemia, increased blood urea and metabolic acidosis (Table 1). A diagnosis of salt-losing CAH was made and she was started on glucocorticoids and mineralocorticoids. The hormonal investigations showed that 17-hydroxyprogesterone (17-OHP) and aldosterone were elevated with normal cortisol level (Table 1).

The results of hormonal investigation of the 3 patients are tabulated as follows:

Tests	Normal Range	Patient 1	Patient 2	Patient 3
Sodium	135-150 mmol/L	92	118	138*
Potassium	3.5 – 5.0 mmol/L	7.4	8.2	5.5*
Blood urea	1.4 – 6.8 mmol/L	11.0	NA	5.7*
Standard bicarbonate (HCO_3)	18 – 25 mmol/L	12.0	NA	NA
17-OHP		>20 ng/ml 3 mth-5 yr: up to 1.1 ng/ml	33.2 ng/ml (<0.7-2.5)	2.8 nmol/L** (normal for age)
Cortisol		484 nmol/L (am : 139-501)	NA	67 nmol/L* (am: 221-690 pm : 110-345)
ACTH		ND	50.7 pg/ml (N: <37)	ND
Testosterone		NA	<20 ng/dl (no reference)	3.0 nmol/L adult female: 0.9-2.8
Aldosterone		>3,300 pmol/L (111-860 pmol/L)	NA	1,400 pmol/L* erect: 110-860 supine : 30-440
Plasma renin activity		NA	NA	72.0 ng/ml/h* erect: 1.3-4.0 supine: 0.1-2.4
Karyotyping		46,XX	46,XX	46,XX
SRY gene		Not present	Not present	Not present
Ultrasound		Uterus and ovaries	Uterus	Uterus. Bilateral pelvi-calyceal system dilatation. Mildly distended bladder.
Genitogram		Figure 3	Figure 5	ND

*Table 1: **Results obtained prior to hormonal therapy * Patient was on hormonal therapy NA: not available ND: not done*

On follow-up she had normal weight gain and serum electrolytes. At 2 years of age there was evidence of under treatment as there was an acceleration in growth velocity. Bone age was reported as between 6 to 9 months old. The dose of cortisone acetate was increased to 10 mg/m²/day and 5 months later, it was further increased to 30 mg/m²/day. Serum 17-OHP was monitored. Her bone age at 6 years old was reported as 5 years and 10 months. The blood pressure was normal except for a borderline reading of 120/70 mm Hg at 4 6/12 and 5 6/12 years old.

At 6 5/12 years old, she was admitted to our centre for fever, vomiting and 3 episodes of fits. Patient had hypotension and needed assisted ventilation. She was managed as adrenal crisis and meningitis[1] (Figure 1). Her height was 110 cm (25-50th percentile), weight 20 kg (75-90th percentile). She appeared muscular with hypertrophied clitoris measuring 3.2 cm (Figure 2). During the course of admission she was noted to have hypertension that was initially attributed to the high dose of intravenous hydrocortisone.

An echocardiography performed revealed a thickened left ventricular wall with reduced contractility. Patient was thought to have a long-standing hypertension. She was started on antihypertensives and digoxin. Fludrocortisone was discontinued. The results of molecular studies showed a genotype of 46, XX and SRY gene was not present. Ultrasound of the abdomen and pelvis showed female internal reproductive system.

On follow-up, the serum electrolytes without mineralocorticoid therapy were normal. Genitogram of patient 1 (Figure 3) showed opacification of both the bladder and vagina. The perineal opening continued into a short segment urogenital sinus that bifurcated just at the level of inferior pubic rami. Clitoroplasty and vaginoplasty were performed. Patient was maintained on glucocorticoids at a dose of 16 mg/m²/day and her blood pressure readings were normal.

Patient 2, was born full term by spontaneous vaginal delivery at a district hospital with a birth weight of 2.7 kg (25th percentile). He was registered as a boy at birth. On day 6 of life, he was referred to HKB for severe neonatal jaundice that required intensive phototherapy. Patient is the younger of 2 siblings and a product of a consanguineous marriage.

During hospitalization, it was noted that patient had an AG described as a prominent phallus, hyperpigmented labioscrotal folds with a single orifice at the base of the phallus and gonads were not palpable. Initial serum electrolytes showed severe hyponatraemia and hyperkalaemia, Based on the clinical picture and electrolyte abnormality a diagnosis of CAH was made. Patient was started on glucocorticoid and mineralocorticoid. Hormonal

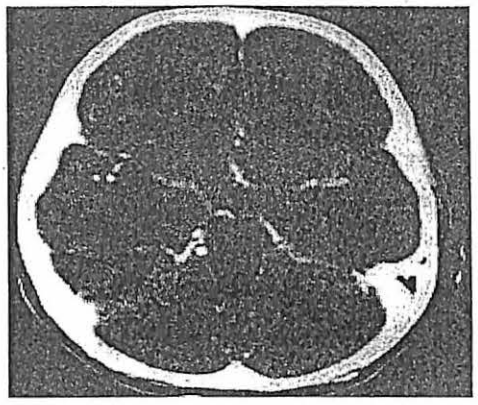

Figure 1: CT scan of patient 1: Presence of meningeal enhancement on the right temporo-parietal region suggestive of meningitis.

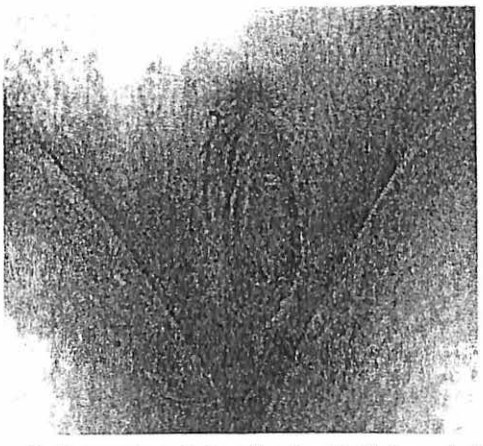

Figure 2: External genitalia of patient 1: Enlarged clitoris of 3.2 cm.

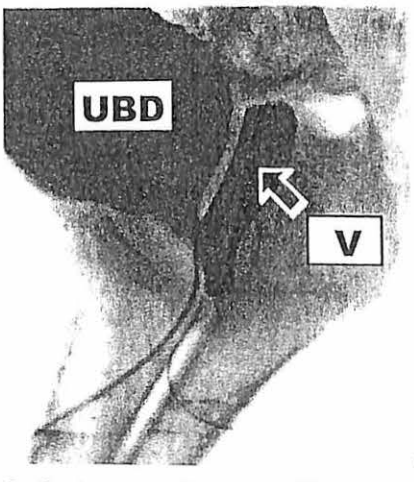

Figure 3: Genitogram of patient 1: The urinary bladder (UBD) and vagina (V) are opacified with a single perineal opening.

investigations showed elevated 17-OHP and ACTH level. The testosterone was <20 ng/dl. Chromosome analysis showed a 46, XX karyotype and SRY gene was not present. The ultrasound of abdomen and pelvis revealed the presence of a uterus (Figure 4) and the genitogram demonstrated a normal urinary bladder (Figure 5). Patient was re-assigned as female at the age of 6 months. Patient was first seen in our centre at the age of 9 months. Weight was 6.4 kg (10-25th percentile), height 64.3 cm (50th percentile) and head circumference 42.5cm (25-50th percentile). The external genitalia was hyperpigmented over the labia majora. Gonads were not palpable. The clitoris measured 2.5 cm with a single orifice at its base. Subsequent 17-OHP and testosterone level was suppressed while on treatment. Further surgical intervention was planned.

Patient 3 was referred to our centre on Day 13 of life for further management of AG. Patient was born full term by spontaneous vaginal delivery at a private hospital with a birth weight of 3.1 kg (75th percentile). The parents are non-consanguineous and patient has an elder sister who is healthy. Mother had a miscarriage for the first pregnancy. On Day 5 of life patient had hyponatraemia and hyperkalaemia. Based on the clinical finding of ambiguous genitalia and typical electrolyte changes, diagnosis of CAH was made. Treatment was started with glucocorticoids and mineralocorticoids after performing some hormonal investigations. Ultrasound of the abdomen revealed bilateral large adrenal glands and presence of a uterus.

On examination, the weight was 3.3 kg (75-90th percentile) and length 52 cm (>97th percentile). Baby had oral thrush and greenish discharge from the umbilicus. The external genitalia appeared feminine with a clitoris measuring 0.9 cm in length. There was slight hyperpigmentation and scrotalisation of the medial aspect of the labia majora. Gonads were not palpable. There was no fusion of the labia minora and there were 2 slit-like orifices seen representing the urethra and vaginal orifices. The electrolytes while patient was on hormonal therapy showed normal serum sodium and blood urea level with slight hyperkalaemia (Table 1).

Patient was maintained on glucocorticoids and mineralocorticoids pending results of hormonal investigations. A repeat ultrasound examination at our centre showed presence of uterus, normal suprarenal glands, dilatation of the pelvicalyceal system of both kidneys (Figure 6) and mildly distended urinary bladder. The urine examination was normal and no organisms were cultured. Umbilical swab showed a mixed growth of Gram negative organism and the full blood picture showed reactive thrombocytosis. The

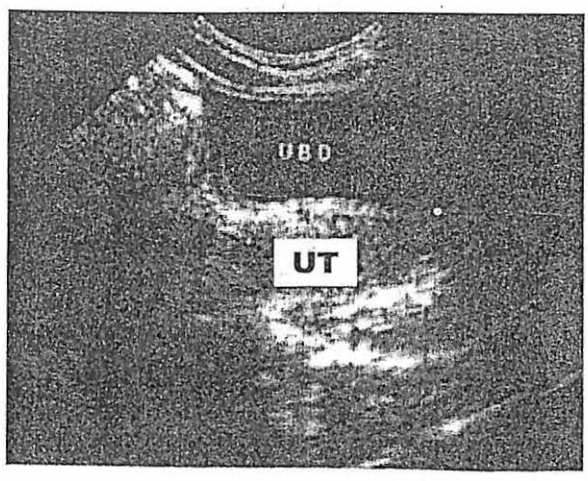

Figure 4, US pelvis of patient 2 showing the uterus (UT), urinary bladder (UBD)

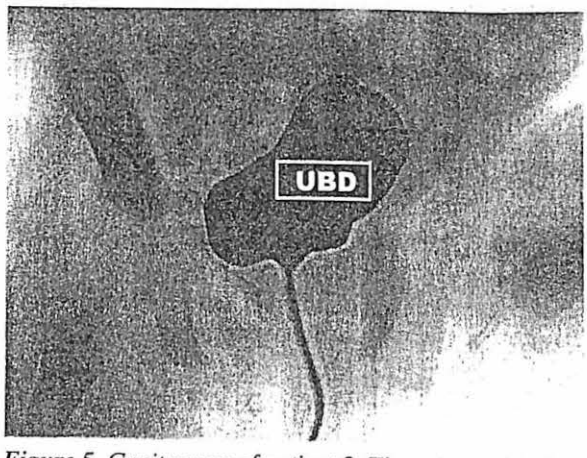

Figure 5, Genitogram of patient 2, The urinary bladder (UBD) was filled with contrast and normal in outline.

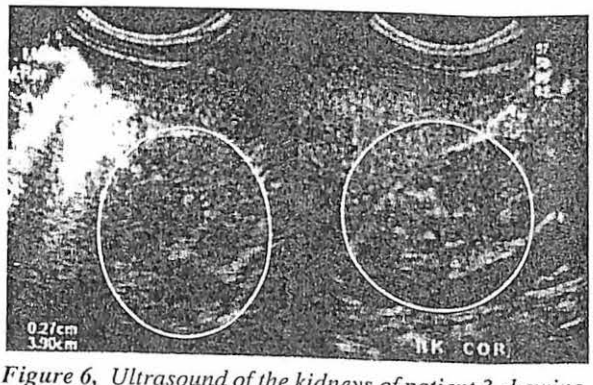

Figure 6, Ultrasound of the kidneys of patient 3 showing bilateral pelvicalyceal system dilatation (circle).

serum testosterone level was slightly elevated according to the adult female range. Serum 17-OHP level before commencement of hormonal therapy was normal. Aldosterone and plasma renin activity obtained when patient was on mineralocorticoid therapy were still elevated for age. Molecular studies confirmed that patient's karyotype was 46 XX, SRY gene was not present.

Based on the results of investigations, the glucocorticoids and mineralocorticoids were gradually tapered and discontinued respectively. On follow-up, the clitoris size normalized and the rugation of the medial aspect of the labia majora disappeared. Subsequent electrolytes values were within the normal range. A repeat ultrasound of the urinary system one month later was reported as normal.

DISCUSSION

The presentation of the 3 patients illustrates the problem encountered in managing AG/CAH. The diagnosis of CAH has to be suspected from the physical appearance, symptoms or positive family history. Investigations have to be done to confirm the diagnosis of CAH and the genetic sex of the child. Delay in diagnosis and treatment of CAH lead to the development of life threatening adrenal crisis as in patients 1 and 2. Wrong gender assignment has tragic consequences[8]. In a newborn baby with AG urgent investigations are warranted. Sex assignment should not be attempted based on appearance of external genitalia. Patient 2 was wrongly assigned as male and family had to suffer the psychological trauma of re-assignment as female at the age of 6 months. Genetic sex of the 3 patients were subsequently established using karyotyping and determination of SRY gene[9].

Congenital adrenal hyperplasia was suspected in patient 3 based on some ambiguity of the external genitalia and electrolyte imbalances at the initial presentation. After the relevant hormonal investigations were taken, patient was started on glucocorticoids and mineralocorticoids by the attending paediatrician so as to prevent possible adrenal crisis. There was elevated aldosterone level and plasma renin activity despite treatment. The full blood picture showed thrombocytosis and an umbilical swab showed a mixed growth of gram negative bacilli suggestive of possible infection. Urosepsis was ruled out from the urine examination and culture. In view of bilateral pelvicalyceal system dilatation and mildly distended urinary bladder, a vesicoureteric reflux and urinary tract infection were considered as possible causes. However we could not confirm vesicoureteric reflux on repeat ultrasound a month from presentation. Infection probably causes unresponsiveness of the distal renal tubules to aldosterone, a condition known as pseudohypoaldosteronism[10, 11].

Imaging studies as ultrasound (US), computed tomography (CT) scan and magnetic resonance image (MRI) of the abdomen/pelvis can aid in identifying the uterus (Figure 4) and gonads. The latter however may not be easily visualised. Genitogram needs to be done to identify the anatomy of the genitourinary system (Figure 3 and 5) that is important for future surgical correction of the external genitalia. Clitoroplasty is usually performed in infancy but not performed in patient 1 due to poor understanding of the condition by the parents. Vaginoplasty with division of the fused labial folds can be delayed until puberty.

Close regular evaluation and dosage adjustment of hormonal treatment especially glucocorticoids is needed for a successful treatment. Under dosage of glucocorticoids cause virilisation while over dosage causes hypertension (patient 1) and short stature as an adult.

CONCLUSION

Molecular studies for SRY gene is one of the tools for sex confirmation in addition to hormonal and imaging studies in the evaluation of AG. Determination of SRY gene may be helpful in sex determination and gender assignment. It is a rapid and reliable technique and will help the clinicians in making the right management decision. Regular medical assessment and monitoring of treatment is the key to the successful management of CAH/AG.

Molecular studies have enabled us to confirm the sex of the 3 patients presenting with AG. It has aided us in family counseling and further management including the surgical correction of the external genitalia as in patient 1 and 2. Furthermore we could counsel the parents with regards to sex reassignment in our second patient and prevent future virilisation and gender crisis. As for patient 3, we were able to counsel the parents who are anxious about the true gender of their baby and the fact that she need not receive life-long hormonal therapy. In the 3 patients that we managed, the parents were content to have the problem rectified, better understanding of the condition and to accept the appropriate management.

In our experience with the cases of AG, we noted that hormonal investigations should be done to confirm CAH and to include the molecular analyses as a complementary tool in the battery of investigations.

ACKNOWLEDGMENT

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DETECTION OF POINT MUTATION (Pro30Leu) in EXON 1 OF THE 21-HYDROXYLASE GENE (CYP21) IN PATIENT with CONGENITAL ADRENAL HYPERPLASIA USING DIGOXYGENIN SYSTEM

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ABSTRACT

Congenital adrenal hyperplasia is an autosomal recessive disease with a wide range of clinical manifestations. Deficiency of the 21-hydroxylase is the most common form of congenital adrenal hyperplasia, accounting for over 90% of cases. The aim of the study is to detect the presence of point mutations using specific probe P30L in exon 1 of CYP21 gene. Point mutations were studied using the PCR-ASOH Allele Specific Oligonucleotide Hybridization) technique with Digoxigenin (DIG) system. The Pro30Leu(P30L) mutation is associated with electrolyte disturbances was found in 2 patients out of 30 patients. The P30L mutation might cause electrolyte imbalances during neonatal periods that subsequently normalize without hormonal replacement therapy.

Key Words: Congenital adrenal hyperplasia, ASOH (Allele Specific Oligonucleotide Hybridization).

INTRODUCTION

Deficiency of the 21- hydroxylase (21-OH) is the most common form of congenital adrenal hyperplasia (CAH), accounting for over 90% of cases. This enzyme deficiency results in a reduced ability to synthesize cortisol and aldosterone, leading to increase secretion of adrenocorticotrophin (ACTH), which causes hyperplasia of the adrenals and an increase in androgens[2]. Three different clinical phenotypes is described: which includes the salt wasting (SW), simple virilizing (SV) and the non classical (NC) form. The 21-hydroxylase gene (CYP21) is located in chromosome 6p 21.3[1]. Molecular diagnostic techniques rely on the assumption that specific mutation of CYP21 gene give rise to the same form of CAH expression. PCR–Allele Specific Oligonucleotide Hybridization (PCR-ASOH) method can be applied subsequently to identify point mutations in the amplified CYP21 gene[3], with using specific probe Pro30Leu (P30L) to detect in exon 1 of CYP21 gene. P30L mutations are associated with the simple virilizing or non-classical form of CAH.

AIM

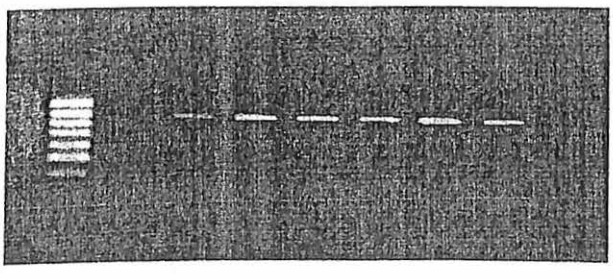
To detect the presence of point mutations using specific probe P 30 L in exon 1 of CYP21 gene.

METHODOLOGY

73 blood samples were obtained from patients and their families referred to Hospital Universiti Sains Malaysia, Kelantan. A thorough clinical examination and hormonal analyses were performed. A total of 30 samples included were suspected to have CAH based on ambiguity of the external genitalia or electrolyte imbalances. The other samples were obtained from parents and patients siblings whenever possible.

DNA extraction
(non-phenol chloroform standard procedure)

Polymerase Chain Reaction(PCR)



PCR-ASOH (Allele Specific Oligonucleotide Hybridization) technique with Digoxigenin (DIG) system.

RESULT

Presence of PCR product (873 bp) for CYP21 gene exon 1-3 using 2.0 % agarose gel electrophoresis (see figure 1).

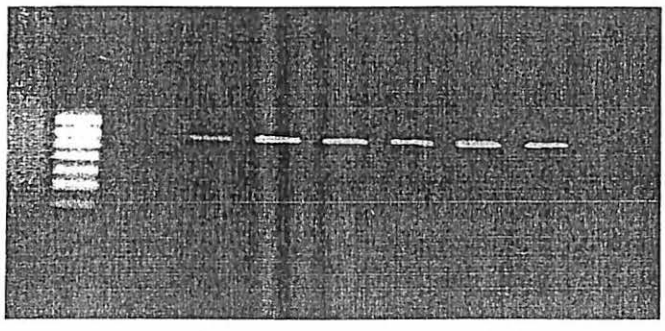


Figure 1: Lane M is a 100 bp DNA ladder, lane N is negative control,lane 1,2, are normal sample and lane 3,4,5,6 sample showed PCR amplification products for CYP21 gene.

The presence of P30L mutations, which have been established to be the cause of complete or partial 21- hydroxylase enzyme inactivation, was analyzed by PCR – ASOH using DIG system in 30 patients with ambiguous genitalia as well as in 43 family members.

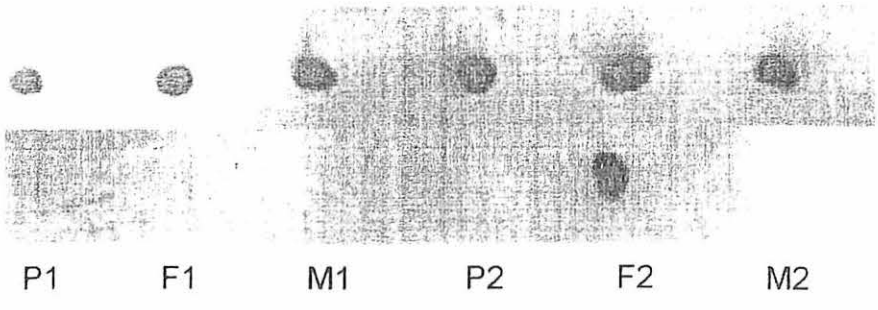


Figure 2:
Dot blotting was performed using 1µg genomic DNA. The blot was hybridized with 100 pmol/ml of the digoxigenin labeled antiphosphatase (DIG-AP) specific probe P30L. ASOH of PCR product from CAH patients or parents, with P30 L corresponding to the site exon 1 .The status P: Patient, M: Mother, F: father.

DISCUSSION

The Pro30Leu mutation is associated with electrolyte disturbances was found in 2 patients out of 30 patients. Patient I, fullterm delivered by emergency Caesarean section due to bleeding placenta praevia. Baby was admitted to the neonatal intensive care unit for transient tachypnoea of the newborn. Examination of the genitalia was normal male with both testes descended in the scrotum. Initial electrolytes showed hyponatraemia and hiperkalaemia with normal serum cortisol level. On follow-up patient was gaining weight with normal electrolytes. Patient II presented at the age of 2 months with hyponatraemia and hyperkalaemia. There was a normal male external genitalia with both testes descended. He was treated with sodium chloride and fludrocortisone. In both patients, there was no ambiguity of the external genitalia and both have salt loss. However the salt loss only require short term replacement therapy with sodium chloride and fludrocortisone in patient II and no treatment in patient I. We would like to hypothesize that P30L mutation might cause electrolyte imbalances during neonatal period that subsequently normalize without hormonal replacement therapy. From the parents' results, we observe that P30L is present in the mother whereas P30L is present in one of the fathers of both patients. We need to study more patients to be able to conclude a similar finding.

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MOLECULAR ANALYSIS IN GENDER ASSIGNMENT AND MANAGEMENT OF AMBIGUOUS GENITALIA

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ABSTRACT

Patients presenting with ambiguous genitalia (AG) often pose a dilemma to the attending clinicians as to definite sex assignment. To avoid any doubt about gender of rearing it is necessary that correct diagnosis is made within a short period of birth. Current hormonal and imaging studies for sex determination could be supported by the application of chromosomal and molecular genetic studies using the SRY gene. Delay in diagnosis and treatment of congenital adrenal hyperplasia (CAH) lead to adrenal crisis. Moreover inappropriate gender assignment has tragic consequences. Clinicians must be aware as well that pseudohypoaldosteronism can masquerade as CAH and causes inappropriate hormonal therapy. The SRY gene complements the investigations and has aided us in patient management and counseling of parents. In the near future we are proceeding to the analysis of the CYP21 gene to confirm diagnosis of CAH due to 21- hydroxylase deficiency.

INTRODUCTION

The issue of correct gender assignment causes some amount of anxiety and the problem of acceptance on the part of the parents. Patients with ambiguous genitalia should be investigated for definite sex assignment. Diagnosis will be aided by hormonal, imaging studies and genetics information. Current clinical and hormonal investigation protocols for sex determination could be supported by the application of chromosomal and molecular genetic studies using SRY gene as one of the tools for gender assignment.

AIM

To apply molecular studies to complement investigations for the genetic assignment of patient with ambiguous genitalia.

METHODS

Three patients were referred to Hospital Kota Bharu (HKB) and Hospital Universiti Sains Malaysia (HUSM) for further management of ambiguous genitalia. 3 - 5 ml blood specimen from the patients with ambiguous genitalia was collected in sodium EDTA or lithium heparin container. Blood was cultured for metaphase cells for cytogenetic and FISH (Fluorescence in situ hybridization) according to the standard protocol. Genomic DNA was extracted and subjected to the PCR analysis of SRY gene using modified method described earlier [1,2].

RESULTS

Table 1

Tests/Patient	I	II	III
Sodium 135-150 mmol/L	92	118	138**
Potassium 3.5 – 5.0 mmol/L	7.4	8.2	5.5**
17-OHP	>20 3 mths-5 yrs : up to 1.1 ng/ml	33.2 (<0.7-2.5)	2.8 nmol/L* (normal for age)
Cortisol	484 am :139-501	NA	67 nmol/L (am: 221-690)
ACTH	ND	50.7 pg/ml (N: <37)	ND
Testosterone	NA	<20 (ng/dl)	3.0 nmol/L adult female: 0.9-2.8
Aldosterone	>3,300 (111-860 pmol/L)	NA	1,400 pmol/L* erect: 110-860 supine : 30-440
PRA	NA	NA	72.0 ng/ml/h erect: 1.3-4.0 supine:0.1-2.4
Karyotype	46,XX	46,XX	46,XX
SRY gene	Not present	Not present	Not present
US pelvis/abdomen	Uterus and ovaries	Uterus	Uterus. Bilateral pelvi-calyceal system dilatation. Mildly distended bladder.
Genitogram	Figure 2	Figure 3	Not done

* results from overseas

** patient was on glucocorticoids and mineralocorticoids

NA: not available; ND : not done

RESULTS

PT	AGE	SEX	CLINICAL FEATURES	DIAGNOSIS	THERAPY	COMPLICATION / PROGRESS
I	10 w	F	Failure to thrive. Adrenal crisis. AG: phallus 2 cm, no gonads palpable, no rugae, no hyperpigmentation, single orifice at base of the phallus.	CAH, salt loser	G and M	**Undertreatment and poor compliance caused virilisation, Patient was put on high dose of glucocorticoids to suppress virilisation. At 6 years old, she had fits and treated as meningitis. Had hypertension and left ventricular hypertrophy.**
II	6 d 9 m	M F	Severe neonatal jaundice. AG: prominent phallus, hyperpigmented labio-scrotal folds, single orifice at the base of phallus, no gonads palpable. Reassigned as female. Clitoris measures 2.5 cm	CAH, salt loser	G and M	**Sex reassignment at 6 months old.**
III	13 d	F	AG: clitoris 0.9 cm Slight hyperpigmentation and scrotalisation of the medial aspect of the labia majora. No palpable gonads, no fusion of labia minora, 2 slit–like orifices seen. Mild enlargement of adrenal glands.	Pseudohypoal-dosteronism	G and M	**Bilateral pelvi-calyceal system dilatation. Mildly distended urinary bladder. Both abnormalities resolved after one month. Normal electrolytes after discontinuation of hormonal treatment. Normal repeat US of genitourinary system. Normal MCU.**

PT: patient, d: day, w: week, m: months, F: female, M: male, G: Glucocorticoids, M: mineralocorticoids, US: ultrasound, MCU : micturating cystourethrogram

RESULTS

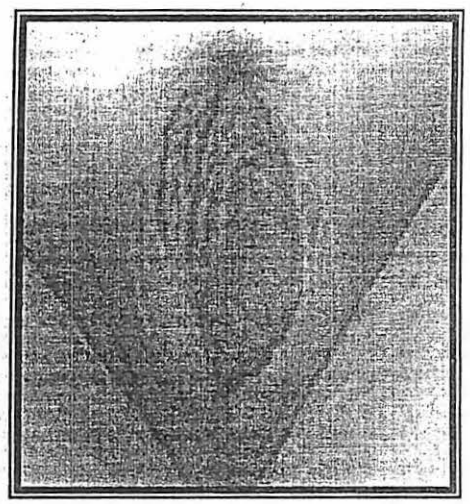

Fig. 1 : External genitalia of patient enlarged clitoris of 3.2 cm.

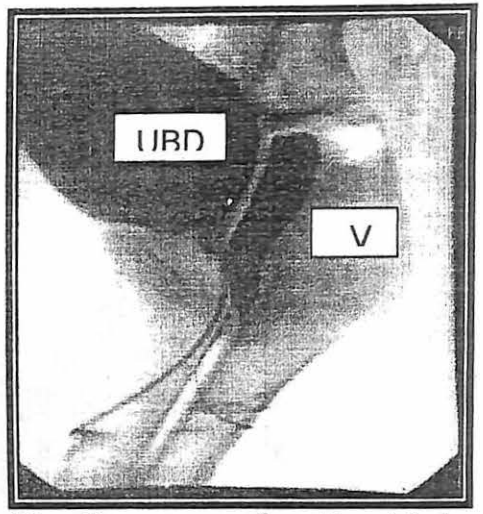

Fig. 2 : Genitogram of patient I, both the bladder and vagina (V) are opacified. Single perineal opening with short segment urogenital sinus.

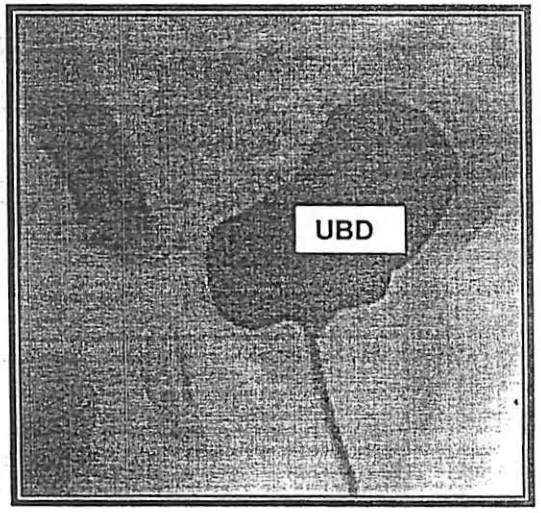

Fig. 3 : Genitogram of patient II, the urinary bladder (UBD) was filled with contrast and normal in outline

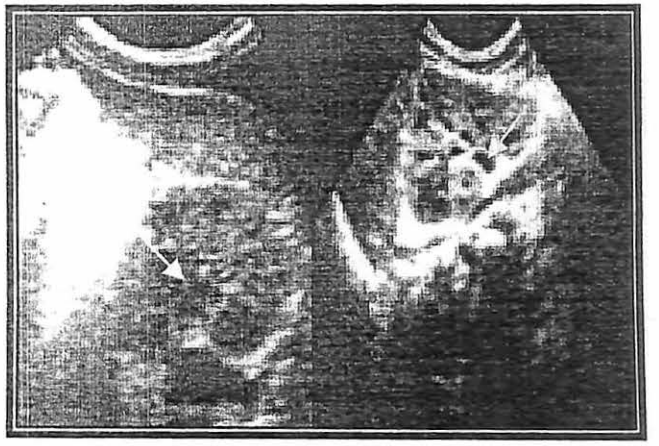

Fig. 4 : Ultrasound of the kidneys of patient III : Dilated pelvicalyceal system of both kidneys (arrow). Mild dilatation of urinary bladder. No adrenal mass seen.

RESULTS

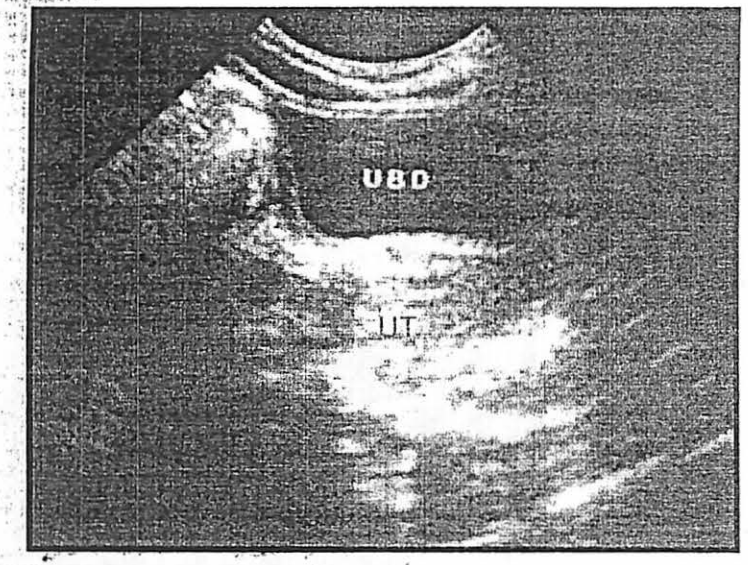

Fig. 5 : US pelvis showing the uterus (UT) & urinary bladder (UBD).

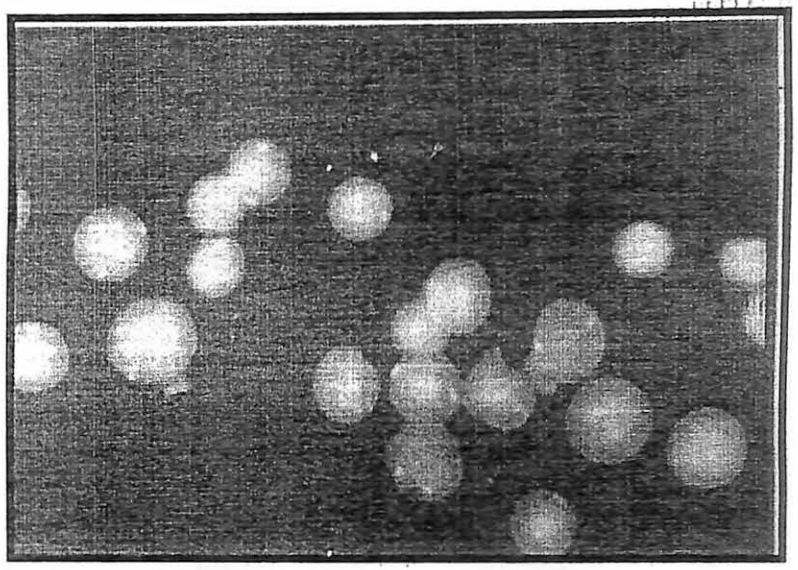

Fig. 6:FISH with X chromosome.

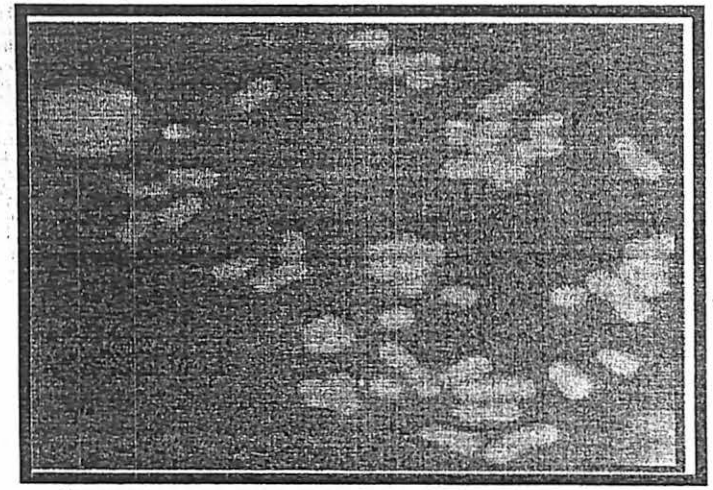

Fig. 7:FISH with Y chromosome

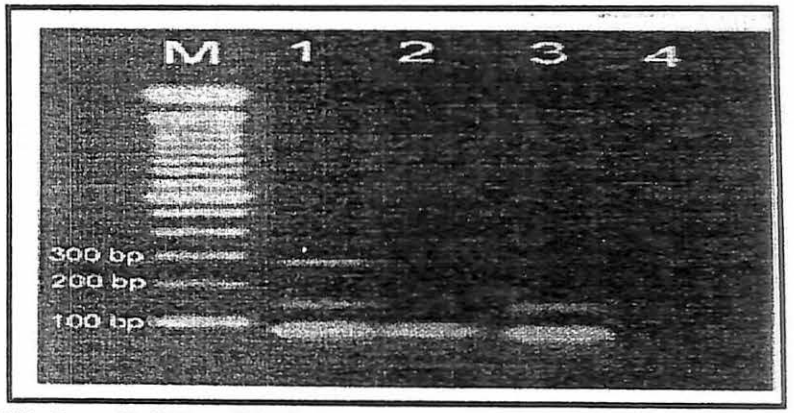

Fig. 8 : Multiplex PCR of X,Y chromosome and SRY gene specific regions.
M-100 bp DNA ladder
1-DNA sample from 46 XY with the presence of SRY gene
2- DNA sample from 46 XX
3- DNA sample from 46 XY with the absence of SRY gene
4-negative control (without DNA)

DISCUSSION

The presentation of the 3 patients illustrated the problem encountered in managing AG/CAH. The diagnosis of CAH has to be suspected and confirmed through investigations. Delay in diagnosis and treatment of CAH lead to development of adrenal crisis which can be life threatening **(pt.1 & 2)**.

Inappropriate gender assignment has tragic consequences. In a newborn baby with AG, sex assignment should not be attempted until the results of appropriate investigations are available. Patient 2 was assigned as male at birth but after the AG was noted and investigated, patient was reassigned as female. Sex confirmation through genetic studies includes karyotyping and SRY gene.

Patient 3 was treated as CAH and relevant hormonal investigations performed. From the results of the investigations **(table 1)**, pseudohypoaldosteronism was suspected. Urosepsis was ruled out from the urine examination. However, the full blood picture showed reactive lymphocytes, thrombocytosis and an umbilical swab showed a mixed growth of Gram negative bacilli suggestive of a possible infection. Subsequent ultrasound of the genitourinary system and electrolytes was normal after cessation of hormonal medications. Pseudohypoaldosteronism can masquarade as the salt losing type of CAH.

Imaging studies can aid in identifying the uterus **(fig.5)** and gonads. Genitogram will identify the anatomy of the genitourinary system **(fig. 2&3)**, which is important for future surgical correction. Clitoroplasty was not performed in patient I due to parents' poor understanding of the importance of surgical correction.

Close monitoring of the patient is needed to ensure that virilisation or hypertension did not occur due to under or overdosage of hormonal therapy respectively.

CONCLUSION

Molecular studies complement hormonal and imaging studies for sex confirmation and appropriate sex assignment or reassignment. Regular medical assessment and monitoring of treatment is the key to the successful management of CAH. Pseudohypoaldosteronism is another differential diagnosis to be thought of in cases of electrolyte imbalances that causes inappropriate hormonal therapy. Counseling of the parents is important to avoid mismanagement, complication and psychosocial issues. We intend to study the CYP21 gene rearrangement to confirm diagnosis of CAH in those patients presenting with AG.

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