

**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:

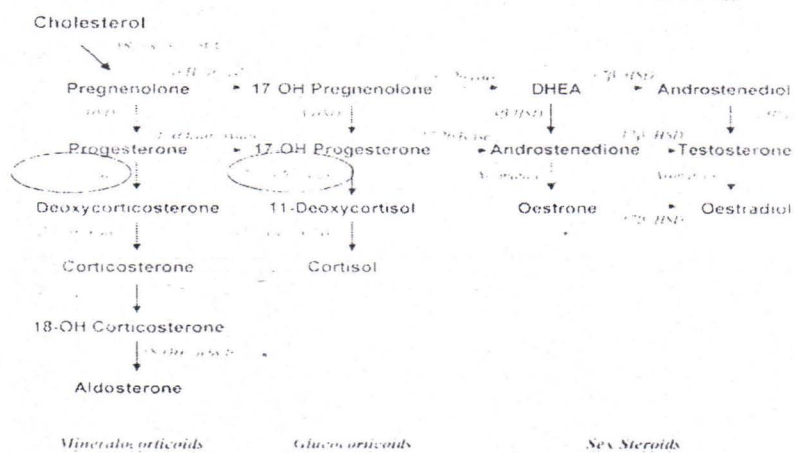
CAH due to 21-(OH) deficiency :

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

- | | | |
|--|---|-----------|
| <ul style="list-style-type: none"> i. Salt Wasting ii. Simple Virilizing iii. Non Classical | } | Classical |
|--|---|-----------|

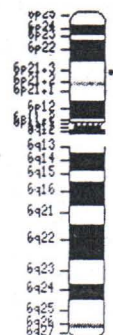
- ✦ Incidence 1: 10,000-17,000 in Western Europe & USA.
- World wide 1: 14,000 births.

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

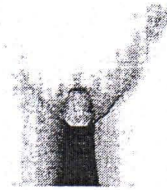
i. Salt Wasting

- 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.

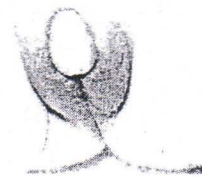


Introduction: Clinical Picture

iii. Non Classical

- The mild NC form of 21-OHD.
- Females - diagnosed at or after adolescence
- 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 | ▲ Salt wasting | ± 2 |
| Gln 318Stop | | |
| Ile1172Asn | Simple virilizing | 18±9 |
| Pro30Leu | | |
| Val281Leu | Non-classic Normal | 30-60 |

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

a. P30L

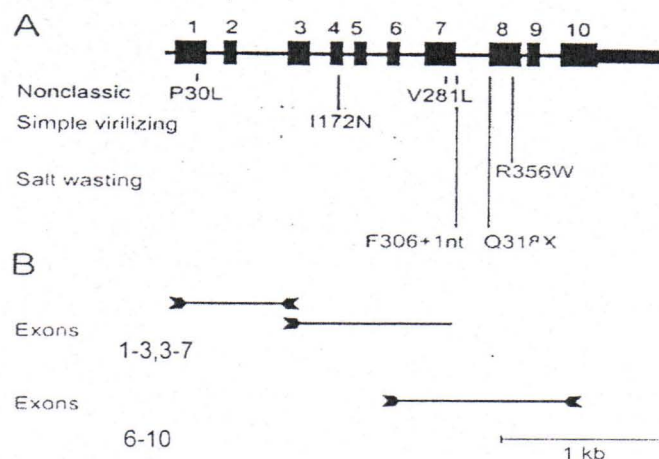
- ✦ The mutations in exon 1
- ✦ CCG (Proline) → to CIG (Leucine)
- ✦ Associated with nonclassical 21-hydroxylase deficiency

b. V281L

- ♣ The mutations in exon 7
- ♣ A change in codon 281 from GTG (Valine) → ITG (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) → IAG 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

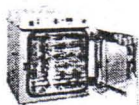
Blood samples were obtained from patients referred to Hospital Universiti Sains Malaysia, Kelantan, Malaysia during 1995-2002. A thorough clinical examination and hormonal analyses were performed. A total of 52 samples included patients with external genitalia or electrolyte imbalances.

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

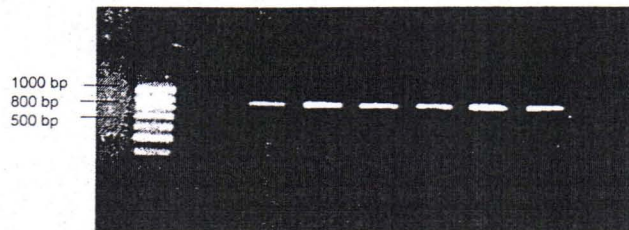


Figure 1: Presence of PCR product for CYP21 gene exon 1-3 using 2.0% agarose gel electrophoresis. Lane M : 100 bp. DNA ladder, lane N : negative control, lane 1: normal samples, lane 2-6 : samples showing PCR amplification products for CYP21 gene.

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:

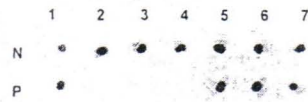


Figure 3: Dot blotting was performed using 1 µg genomic DNA. The blot was hybridized with 100 pmol/ml of the digoxigenin labeled antiphosphatase (DIG-AP) specific probe from samples of patient 1-7. ASOH of PCR product from CAH patients was performed with the probe V281L corresponding to the site exon. 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 | <u>C</u> CG→ <u>C</u> TG | Proline→Leusine | 2 |
| 7 | 281 | <u>C</u> GT→ <u>C</u> TT | Valine → Leusine | 3 |
| 8 | 318 | <u>G</u> CA→ <u>G</u> TA | Glutamin → Stop codon | 1 |

RESULTS

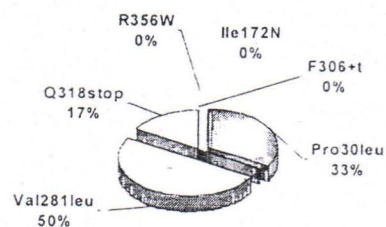


Figure 6 : A pie chart showing percentage of CYP21 gene mutation according to each exon.

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

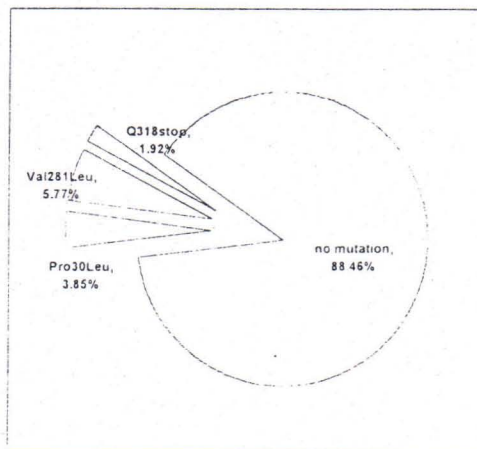
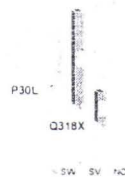


Figure 7 : A pie chart showing percentage of frequency for CYP21 gene mutation in patients diagnosed with CAH.

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 And A, M.R. Sidek, S.F., Ramli, N.A. Adam, M. N. Isa. Poster presentation at 13th National Biotechnology Seminar, 10-13th November 2001, Penang.

5) Molecular Analysis in Gender Assignment and Management of

Ambiguous Genitalia. Y.K. Muhamad, Fuziah MZ, Rus And 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.

Hormone Research

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Abstracts

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European Society for Paediatric Endocrinology (ESPE)

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KARGER

MOLECULAR ANALYSIS OF CYP21 GENE IN PATIENTS PRESENTING WITH AMBIGUOUS GENITALIA

F. Md. Zain¹; Y. KM²; R. A³; M. Sidek²; S. Ramli²; M. Isa²

¹Paediatrics, School of Medicine, Health Campus, Universiti Sains Malaysia, Kelantan, Malaysia; ²Human Genome Centre, School of Medicine, Health Campus, Universiti Sains Malaysia, Kelantan, Malaysia; ³Paediatrics, 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.

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