

## 21-Hydroxylase Genotyping in Australasian Patients with Congenital Adrenal Hyperplasia

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### ABSTRACT

Mutations in *CYP21* (21-hydroxylase) lead to congenital adrenal hyperplasia (CAH). We genotyped 26 probands with CAH by PCR-sequencing the entire *CYP21* gene. 25/26 had homozygous or compound heterozygous mutations. The frequencies of mutations were similar to other populations with deletion/hybrid, I2 G splice and I172N the most common. Five patients with a I172N allele predicting simple-virilising CAH had a salt-wasting phenotype. Two other probands also had a more severe phenotype than predicted by genotype. Two families had both non-classic and salt-wasting phenotypes arising from combinations of three deleterious alleles. Two novel *CYP21* alleles were detected: D106N and a large deletion encompassing *CYP21* and adjacent pseudogene. Two rare *CYP21* alleles were also found. Three of these four novel/rare alleles were only detected as a result of sequencing the entire *CYP21* gene. Entire *CYP21* sequencing will increase the number of mutations detected in CAH, and in combination with functional studies should contribute a greater understanding of phenotype-genotype correlations.

### KEY WORDS

congenital adrenal hyperplasia, 21-hydroxylase deficiency, genotyping

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### INTRODUCTION

Congenital adrenal hyperplasia (CAH) caused by 21-hydroxylase deficiency is one of the most common autosomal recessive disorders in man. 21-Hydroxylase is encoded by *CYP21*, and the estimated carrier frequency of deleterious *CYP21* mutations is one in 50<sup>1,2</sup>. The CAH phenotype reflects the degree of 21-hydroxylase enzyme deficiency<sup>2</sup>. Complete enzyme deficiency with combined impairment of cortisol and aldosterone synthesis results in the classic salt-wasting (SW) form which is characterised by prenatal virilisation in females secondary to excess fetal androgen exposure and salt-wasting crises in the neonatal period. Partial enzyme deficiency leads to classic simple virilising (SV) CAH, which is characterized only by prenatal virilisation in females and pseudoprecocious puberty in males and females. Data from neonatal 17-hydroxyprogesterone (17OHP) screening of ~6.5 million newborn infants from USA, France, Italy, New Zealand, Japan, UK, Brazil, Switzerland, Sweden, Germany, Portugal, Canada, and Spain show an incidence of 1/15,000 live births for classic SW/SV CAH<sup>3,4</sup>. The non-classic (NC) form of CAH is associated with a milder degree of enzyme impairment, and is associated with pseudoprecocious puberty, hirsutism, acne, and/or subfertility<sup>2</sup>. The prevalence of NC CAH in the white population is estimated at ~1/1,000<sup>5,6</sup>.

Although the different clinical subgroups of CAH appear well defined there is in reality a spectrum of phenotypic severity ranging from carriers through to those with classic salt wasting. Males without evidence of salt wasting may be

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difficult to assign to either the simple virilising or the non-classic group. Likewise, a female without salt wasting can only be assigned to either the simple virilising or the non-classic group if the appearance of the external genitalia has been carefully documented at birth.

The 21-hydroxylase gene *CYP21* (*CYP21A2*, *CYP21B*, OMIM#201910) is located on chromosome 6p21.3 within the HLA histocompatibility complex in close proximity to a highly homologous inactive pseudogene *CYP21P* (*CYP21A1P*, *CYP21A*)<sup>7,8</sup>. Recombinations and conversions between *CYP21P* and *CYP21* result in the generation of dysfunctional *CYP21* alleles<sup>1,9</sup>. Approximately 90% of *CYP21* mutations are a result of *CYP21P*-derived conversions and recombinations<sup>1,2</sup>. In the case of compound heterozygous mutations the milder of the two mutated alleles determines the CAH phenotype.

*CYP21* genotyping can be useful in the diagnosis of CAH and can also predict the phenotype in 80-90% of cases<sup>10-13</sup>. Given the imprecision inherent in the clinical classification of CAH this degree of genotype-phenotype correlation could be expected. There is a good genotype-phenotype correlation for very severe and very mild *CYP21* mutations, whereas the correlation between genotype and phenotype is less precise when the underlying mutation results in an intermediate impairment in enzyme activity<sup>10,12-14</sup>.

There are a number of common haplotypes of the *CYP* locus<sup>15-20</sup>. The most common *CYP* haplotype is a single functional *CYP21* gene with one *CYP21P* gene upstream, haplotype A in Koppens *et al.*<sup>20</sup>. A large scale deletion event can lead to *CYP21* gene deletion, haplotype E, or the formation of a dysfunctional *CYP21P/CYP21* hybrid gene, haplotype E-Hyb<sup>20</sup>. The most common reported haplotypes in CAH are E, E-Hyb and A (with *CYP21* mutation). However, many other haplotypes have been reported in CAH and in normal individuals with varying numbers of *CYP21*, *CYP21P* and *CYP21P/CYP21* hybrid genes (haplotypes B, C, D, F, G, H and I<sup>20</sup>).

A common problem with any *CYP21* genotyping protocol is the high homology between *CYP21* and the pseudogene *CYP21P*. Some protocols achieve specificity for *CYP21* via puri-

fication of a 3.5 kb *Taq-I* digested genomic DNA fragment containing *CYP21* but not *CYP21P*<sup>13,21</sup>. This purified digestion product is then used for polymerase chain reaction (PCR) amplification and/or other methods (e.g. restriction fragment length polymorphism [RFLP], single strand conformational polymorphism [SSCP], denaturing gradient gel electrophoresis [DGGE]) to screen for mutations. Due to genetic variation, however, the 3.5 kb *Taq-I* fragment does not always contain the *CYP21* gene<sup>15</sup>.

Other assays involve direct PCR amplification from genomic DNA using allele specific PCR. A separate PCR reaction is performed for each of the common mutations screened<sup>14,22-25</sup>. These procedures are cumbersome due to the number of reactions required. Other assays involve direct amplification of *CYP21* from genomic DNA using specific primers, followed by a second round of PCR to screen for common mutations<sup>26-30</sup>. Any assay involving more than one round of PCR is subject to problems with specificity and sensitivity. Other assays screen for mutations via migration patterns of PCR products on gels (SSCP, DGGE)<sup>21,30,31</sup>, but can be associated with low sensitivity. Some studies describe first-round screening for common mutations using the methodologies described above, and if two mutations are not detected this is followed by DNA sequencing to detect rare or novel mutations<sup>12,13,21,25,26,31</sup>.

This paper describes a thorough genotyping methodology in which all mutations, common and rare, are detected by complete gene sequencing of *CYP21*-specific PCR products amplified directly from genomic DNA. We believe that our assay is simpler and more accurate than the other protocols discussed above. We performed genotyping analysis of the *CYP21* locus on a series of Australasian patients with CAH and family members as part of our diagnostic genotyping service at the Mater Children's Hospital. Phenotype-genotype correlations, family studies, and analysis of novel mutations and rearrangements are described.

## PATIENTS AND METHODS

### Patients

Twenty-six unrelated probands with CAH and seven affected family members were genotyped. For clinical details see Tables 1 and 2. Detailed clinical information is only provided for those patients with unusual genotypes or family pedigrees (Table 1). The CAH phenotype was determined by clinicians according to defined clinical criteria<sup>2</sup>. Four CAH subtypes were used in order of increasing severity: carrier, NC, SV and SW (Tables 1, 2). The clinical phenotype of some patients can best be described as intermediate between categories, e.g. NC-SV for patient P1.

### CYP21 genotyping

#### DNA purification

Genomic DNA (gDNA) was extracted from peripheral leukocytes<sup>32</sup>. For prenatal testing, gDNA was extracted from chorionic villus samples using Wizard Genomic Purification Kit (CAT#A1120, Promega, Sydney, Australia).

#### Gene copy assay

The gene copy assay was used to determine the copy number of *CYP21* and *CYP21P* genes. PCR with non-gene specific primers was used to amplify a small region in exon 3 which contains an 8 base-pair (bp) deletion in *CYP21P* but not in *CYP21* (Fig. 1, 'Ex3- $\Delta$ 8bp' and 'Ex3-8bp intact'). The size and relative intensity of *CYP21*, *CYP21P* and control gene  $\beta$ -globin PCR products was determined by GeneMapper analysis (Fig. 1).

For the gene copy assay, gDNA was PCR amplified in 50  $\mu$ l containing 0.5  $\mu$ M each of primers 12/E3-665S and E3-824AS (see below for details of oligonucleotides), 0.2  $\mu$ M each of  $\beta$ -globinS and  $\beta$ -globinAS, 0.2  $\mu$ M dNTPs, 1.5 mM Mg<sup>++</sup>, 1 U Amplitaq Gold DNA polymerase and buffer (CAT# N8080161, Perkin Elmer, Sydney, Australia). PCR cycling conditions were: 95°C 3'; 35 x 95°C 30":58°C 30":72°C 30"; 72°C 3'. PCR products were visualised using agarose gel electrophoresis, purified using High Pure PCR product purification kit (CAT#1732668, Roche, Mannheim,

Germany) and subjected to GeneMapper analysis on a ABI 3130xl capillary DNA Sequencer (Griffith University Sequencing Facility, Nathan, QLD, Australia).

#### PCR sequencing of CYP21

Mutations in *CYP21* were detected by PCR-amplification of the entire *CYP21* gene using *CYP21* specific primers followed by sequencing using nested sequencing primers. Two PCR products were produced. The first PCR product spans the first six exons and proximal promoter region of the *CYP21* gene. The second PCR product covers the 5'-UTR, all ten coding exons, introns and partial 3'-UTR of *CYP21* (Fig. 1).

For the exon 1-6 PCR (1,574-bp product), gDNA was PCR amplified in 100  $\mu$ l containing 1  $\mu$ M each of 5'-m174S and E6-1400AS, 0.2  $\mu$ M dNTPs, 1.5 mM Mg<sup>++</sup>, 2 U TaqF1 DNA polymerase and buffer (CAT#TAQ-1, Fisher Biotec, Sydney, Australia). PCR cycling conditions were: 95°C 3'; 35 x 95°C 1':65°C 2':72°C 3'; 72°C 3'. For the exon 1-10 PCR (2,939-bp product), gDNA was PCR amplified in 100  $\mu$ l containing 1  $\mu$ M each 5'-m126S and 3'-2813AS, 0.2  $\mu$ M dNTPs, 1.25 mM Mg<sup>++</sup>, 3.75 U Expand Hi-Fidelity Taq polymerase with buffer (CAT# 04738250001. 4738250, Roche, Mannheim, Germany). PCR cycling conditions were: 95°C 1'; 14 x 95°C 1':65°C 1':72°C 6'; 20 x 95°C 1':65°C 1':72°C 6'+2" each cycle; 1 x 95°C 1':53°C 1':72°C 15'; 72°C 3'.

The PCR products were visualised on agarose gel, purified and sequenced using a variety of nested sequencing primers. Sequencing reactions utilised Big-Dye-Terminator-Cycle-Sequencing-Ready-Reaction (CAT#4303149, Applied Biosystems, Scoresby, Australia). Sequencing analysis was performed at Griffith University Sequencing Facility. All sequences were compared to consensus sequences for *CYP21* (Genbank RefSeq NC-000006).

Parents and family members were genotyped for the specific mutations detected in probands using PCR sequencing and gene copy assays.

### Nomenclature for *CYP21* mutations

All amino acids are referred to using the single letter code. Mutations are listed as amino acid substitutions in 21-hydroxylase protein, except where otherwise indicated. Amino acid numbering is as in the Human Gene Mutations Database ([www.hgmd.cf.ac.uk](http://www.hgmd.cf.ac.uk)) and [www.cypalleles.ki.se/cyp21](http://www.cypalleles.ki.se/cyp21); numbering starts from A in the initiation codon; the polymorphic insertion of amino acid L10 has been omitted. Nucleotides associated with amino acid substitutions are numbered relative to cDNA sequence Genbank RefSeq NM000500 where nucleotide 1 is the first A of the ATG start codon. All other nucleotides are numbered relative to gDNA sequence Genbank RefSeq NC000006, where the first A of the ATG start codon corresponds to nucleotide number 1.

### Details of oligonucleotides

***β-Globin primers:***  $\beta$ -GlobinS 5'ACACAAGTGTGTTCACTAGC3' and  $\beta$ -GlobinAS 5'CAACTCATCCACGTTACCC3'.

***CYP21 primers:*** Primer labels, e.g. E6-1400AS; exon or intron number - position of 5'-base of primer-antisense (AS) or sense (S). Primers E6-1400AS and 5'-m126S are specific for the *CYP21* gene. All other primer sequences are identical in both *CYP21* and *CYP21P*. Position of the 5'-base of the primer is numbered relative to Genbank RefSeq NC000006. PCR primers were 12/E3-665S 5'CCTGCAGACAAGCTGGTGTC3', E3-824AS 5'CACAGAACTCCTGGGTCAGCTGC3', 5'-m174S 5'CAAGGCCAATGAGACTGGTGT3', E6-1400AS 5'AGCTGCATCTCCACGATGTGA3', -5'-m126S 5'CGGGTCGGTGGGAGGGTA3' and 3'-2813AS 5'AGC GATCTCGCAGCACTGTGT3'.

Additional sequencing primers were:

5'-m152S 5'ATTCCAGAAAAGGGCCACTC3',  
E1-200AS 5'TGCAGCCCAAGGTGGAGC3',  
E2/I2-371S 5'AGACCTGAGCCACTTACCTG3',  
E3-826AS 5'CTCACAGAACTCC'TGGGTCA3',  
E4-1009S 5'TACCTCACCTTCGGAGACAA3',  
I5-1313AS 5'CATGAGAATGCAGCTGTGG3',  
I6-1449S 5'ACAGCCAGTGATGCTACC3',  
E7-1619AS 5'CCATCATGTCCCTCCACTG3',  
I7-1976AS 5'ATGATCGGGTGGAGTCCT3'.

18-2191AS 5'CTCACTCATCCCCAACCCCT3',  
18-2180S 5'GGGATGAGTGAGGAAAG3',  
3'-2776AS 5'AGGGGTTCGTACGGGAGCAA3'.

### *CYP* haplotypes and hybrid genes

*CYP21P/CYP21* hybrid genes can have breakpoints anywhere from just downstream of Ex3- $\Delta$ 8bp to exon 8 (exon 3 hybrid is the most common). *CYP21P/CYP21* hybrid genes give an identical result in the gene copy assay as intact *CYP21P* genes. *CYP21P/CYP21* hybrid genes with a breakpoint 5' of primer E6-1400AS will amplify in the exon 1-6 PCR assay but not in the longer exon 1-10 PCR assay. *CYP21P/CYP21* hybrid genes with a breakpoint 3' of primer E6-1400AS will not be amplified at all. For simplicity in presentation of data, *CYP21* deletion and *CYP21P/CYP21* hybrid alleles have been grouped together and listed simply as 'Deln/Hyb'.

It is possible to use gene copy results in combination with PCR sequencing data from probands and family members in order to estimate which of the known *CYP* haplotypes reported in the literature may be present (possible haplotypes have been listed using the nomenclature of Koppens *et al.*<sup>20</sup>).

### Ethics

All patients were genotyped by the Mater Molecular Genetics Laboratory, Mater Pathology, Mater Misericordia Hospital, South Brisbane, QLD. The Mater Molecular Genetics Laboratory is accredited by the National Australia testing authority (NATA). Patient genotyping by the Mater Molecular Genetics Laboratory has been approved by the Mater Health Services Human Research Ethics Committee (MHSHERC).

### RESULTS

Twenty-six probands (14 female, 12 male) with CAH as well as seven affected family members were referred for genotyping. All probands were from Australia or New Zealand. Two of the 26 probands (P6 and P7) were from families of Middle Eastern origin with consanguineous marriages.

**TABLE 1**  
Clinical details of probands and affected family members

ID#	Presentation and Notes	Meds*
<b>Probands:</b>		
P1	Unsure if NC- or SV-CAH. Diagnosed at birth, known family history (older sister diagnosed with SV-CAH at age 2 yr), 17OHP 125 mM. Adult height 162 cm, no incidence SW crisis. At age 29 yr compliant on treatment and CAH is well controlled.	HC, prednisone
P2	SV-CAH. Diagnosed at age 2.5 yr after presentation of sister at birth with SV-CAH. Rapid growth, large phallus, 17OHP 64 mM. At age 8 yr progressing well, growth p50-75.	HC
P3	SW-CAH. Presented at age 10 days with vomiting and hyponatremia (17OHP 1,747 mM, Na <sup>+</sup> 115 mM). No family history of CAH. At age 2.5 yr is progressing well. Growth p50-75, Na <sup>+</sup> /17OHP normal. Family pedigree in Figure 2.	FC, HC
P4	SW-CAH. Virilisation noted at birth. Presented age 14 days with SW crisis (Na <sup>+</sup> 130 mM, K <sup>+</sup> 5.7 mM, 17OHP 229 mM, cortisol 203 mM). Resuscitated with IV fluids. Urethrogram showed vaginal sinus only, no vaginal vault. From age 0-3 yr history of high 17OHP, SW crises and gastroesophageal reflux. At age 3 yr growth is satisfactory and clitoral enlargement less obvious.	FC, HC
P6	NC-CAH. P6 and his sister both have NC-CAH. Four other siblings of P6 are not affected. P6 and his wife are first cousins and are also related through a second line of descent. Consanguineous family, of Middle Eastern origin.	
P7	SW-CAH. Diagnosed at age 3 mo. At age 5 yr bone age is 12 yr and 17OHP/androgen levels are high. No family history of CAH. Parents normal stature. Older sister had normal genitalia, died at age 5 mo with pneumonia. Consanguineous family, of Middle Eastern origin	FC, HC
P13	SW-CAH. Presented age 16 days SW crisis, hyperkalemia, hyponatremia, high 17OHP/PRA, mild hyperpigmentation of genital skin. At age 5 yr is progressing well with normal growth/bone age. Family pedigree in Figure 3.	FC, HC, Na <sup>+</sup>
P14	SW-CAH. Also diagnosed with renal dysplasia. At age 8 yr is progressing well with normal bone age/stature, no virilisation. Family pedigree in Figure 3.	FC, HC Genotrop
P15	SW-CAH. Died of SIDS at age 5 mo, no history of vomiting, early sexual development or illness. Autopsy showed bilateral adrenal hyperplasia and biochemistry indicative of SW-CAH. A male sibling born after P15 was diagnosed with CAH prenatally using HLA typing. Family pedigree in Figure 3.	
<b>Affected family members:</b>		
A4	A4 diagnosed at birth with septo-optic dysplasia (SOD), panhypopituitarism, hypothyroidism, pituitary gland dysfunction. A4 was born 2 years before P4. When P4 presented with CAH diagnosis of A4 was amended to include SW-CAH. From age 0-4 yr history of hypoglycemia, hyponatremia, infections, irregular sleep, poor growth (age 4 yr height/weight <p5, bone age 2.75 yr. At age 5 yr progressing well; hypoglycemia less severe, good hearing, recurrent middle ear infections. Family history of type 2 diabetes mellitus but no history of CAH, SOD or consanguinity.	FC, HC, growth hormone, melatonin, thyroxine
A13	During a clinic visit for P13 the mother noted that older sister A13 at age 5 yr had clitoromegaly, body odour, acne. Examination showed height p75, weight p90, bone age 6.5 yr (17OHP 120 mM, renin 38 mU/l, Na <sup>+</sup> 138 mM, K <sup>+</sup> 4.7 mM). A13 diagnosed with NC-CAH. At age 6.5 yr progressing well, bone age 8 yr. Pedigree in Figure 3. Father had early onset puberty, cessation of growth at age 11-12 yr, adult height of 173 cm. Two of the father's siblings have NC-CAH features. No family history of consanguinity or miscarriages.	HC
A19	SW-CAH. Diagnosed in utero. Chorionic villus sample from mother genotyped at 10 weeks gestation.	

\* Meds = medications: FC = fludrocortisone; HC = hydrocortisone; Genotrop = Genotropin.  
CAH = congenital adrenal hyperplasia; NC = non-classic; SV = simple virilising; SW = salt-wasting.  
p = percentile

**TABLE 2**  
**CYP21 genotyping in probands and affected family members**

ID#	Sex	CYP21 genotype		Expected phenotypes		Observed phenotype	Gene copy			
		Allele 1	Allele 2	Phen. Allele 1	Phen. Allele 2		C21	C21P		
<b>Probands:</b>										
P1	M	I2 G Splice	WT	SW	Norm	NC/SV	2	2		
P2	M	I172N ( <i>M</i> )	H365Y <sup>N</sup> ( <i>P</i> )	SV	?	SV	2	2		
P3	M	3' Hybrid <sup>N</sup> ( <i>M</i> )	Deln/Hyb ( <i>P</i> )	SW	SW	SW	1	2		
P4	F	I172N	Large Δ <sup>N</sup>	SV	SW	SW	1	1		
P5 <sup>I</sup>	F	V281L	Ex3 Δ8bp	NC	SW	NC	1	4		
P6 <sup>H</sup>	M	R339H.P453S ( <i>M</i> )	R339H.P453S ( <i>P</i> )	NC	NC	NC	2	2		
P7 <sup>H</sup>	M	Q318X ( <i>M</i> )	Q318X ( <i>P</i> )	SW	SW	SW	2	2		
P8	F	P453S.Int2 ( <i>M</i> )	Q318X ( <i>P</i> )	SW	SW	SW	2	2		
P9 <sup>H</sup>	F	I2 G Splice ( <i>M</i> )	Deln/Hyb ( <i>P</i> )	SW	SW	SW	1	2		
P10 <sup>F</sup>	F	Deln/Hyb	I2 G Splice	SW	SW	SW	1	3		
P11	F	I2 G Splice ( <i>M</i> )	I172N ( <i>P</i> )	SW	SV	SV	2	3		
P12	F	P30L	I2 G Splice	NC	SW	NC	2	3		
P13	M	I2 G Splice ( <i>M</i> )	Deln/Hyb ( <i>P</i> )	SW	SW	SW	1	3		
P14	M	Deln/Hyb ( <i>M</i> )	Q318X ( <i>P</i> )	SW	SW	SW	1	2		
P15	M	Deln/Hyb ( <i>M</i> )	I172N ( <i>P</i> )	SW	SV	SW	1	3		
P16	M	I172N ( <i>M</i> )	I2 G Splice ( <i>P</i> )	SV	SW	SV	2	2		
P17	M	I172N	I172N or Δ??	SV	SV/SW	SV	-	-		
P18	F	I2 G Splice ( <i>M</i> )	Deln/Hyb ( <i>P</i> )	SW	SW	SW	1	2		
P19	F	Deln/Hyb ( <i>M</i> )	Deln/Hyb ( <i>P</i> )	SW	SW	SW	0	2		
P20	F	I172N ( <i>M</i> )	Deln/Hyb ( <i>P</i> )	SV	SW	SV	1	2		
P21	F	I172N ( <i>M</i> )	Deln/Hyb ( <i>P</i> )	SV	SW	SW	-	-		
P22	M	Deln/Hyb ( <i>M</i> )	I2 G Splice ( <i>P</i> )	SW	SW	SW	1	2		
P23 <sup>F</sup>	F	I172N	I2 G Splice	SV	SW	SW	2	2		
P24	F	I2 G Splice	I2 G Splice/Δ?	SW	SW	SW	-	-		
P25	M	P30L	I172N	NC	SV	SV	2	2		
P26	F	Prom. Cluster	I172N	Norm/NC	SV	NC	2	2		
<b>Affected family members:-</b>										
A4	M	I172N	Large Δ	SV	SW	SW	1	1	Brother	
A5 <sup>I</sup>	M	D106N <sup>N</sup>	WT	?	Norm	Norm	2	2	Partner	
A13	F	I2 G Splice ( <i>M</i> )	P30L ( <i>P</i> )	SW	NC	NC	2	2	Sister	
F14	M	V281L	Q318X	NC	SW	NC	2	3	Father	
A17	F	I172N	I172N or Δ??	SV	SV/SW	SV	-	-	Sister	
A19	-	Deln/Hyb ( <i>M</i> )	Deln/Hyb ( <i>P</i> )	SW	SW	SW	0	2	Sibling	
A20	F	I172N ( <i>M</i> )	Deln/Hyb ( <i>P</i> )	SV	SW	SV	1	2	Twin	

Phen. Allele 1, Phen. Allele 2 = phenotype associated with mutation on each allele, as listed in [www.cypalleles.ki.se](http://www.cypalleles.ki.se) (note that the expected phenotype is the milder of the two alleles).

Observed phenotype based on clinical information; Norm = normal; NC = non-classic; SV = simple virilising; SW = salt-wasting  
 Gene copy: C21 = copies of CYP21 gene (Ex3 8bp deletion absent); C21P = copies of CYP21P gene (Ex3 8bp deletion present). Gene copy assay for P17, A17, P21 and P24 technically unsatisfactory, re-collection declined.

(*M*) = maternal allele, (*P*) = paternal allele (if known, not all parents were genotyped).

<sup>^</sup> Novel mutation or interesting mutation.

**Table 2 continued**

<sup>U</sup> P5 was genotyped with her unaffected partner A5 prior to family planning (family pedigree in Figure 2). A5 is carrying a novel mutation. The novel mutation may be deleterious, thus A5 is included in the table even though unaffected.

<sup>H</sup> Family genotype/gene copy results are consistent with unusual haplotypes on the non-disease allele of unaffected subjects in families 6, 7 and 9 (data not shown).

<sup>F</sup> P10 and P23 have each been listed as index case probands even though they are distantly related.

*CYP21* genotype:

Deln/Hyb = entire deletion of *CYP21* gene, or *CYP21P/CYP21* hybrid gene.

3' Hybrid = *CYP21/CYP21P* hybrid gene.

Large Δ = novel large scale deletion encompassing *CYP21P* and *CYP21* genes.

Ex3 Δ 8bp = 8bp deletion in exon 3 (nucleotides 711-718).

I2 G Splice = intron 2 IVS-13A/C>G mutation (nucleotide 659).

R339H.P453S = double R339H and P453S mutations in *CYP21* gene.

P453S.Int2 = double P453S and I2 G Splice mutations in *CYP21* gene.

Prom. Cluster = promoter cluster, nucleotides minus -126C-T, -113G-A, -110T-C, -103 A-G.

WT = wildtype *CYP21* allele, i.e. no mutation detected.

Fourteen of the 15 probands (9 females, 5 males) with SW CAH presented at <3 months of age with virilisation and/or SW crises. One boy (P15) died at age 5 months and was diagnosed with SW CAH on autopsy. Seven probands (2 female, 5 male) presented with SV CAH during childhood while four probands had NC CAH (3 female, 1 male). See Tables 1 and 2 for more clinical details.

The genotyping results for the probands and affected family members are presented in Table 2 (refer to [www.cypalleles.ki.se/cyp21](http://www.cypalleles.ki.se/cyp21) and/or [www.hgmd.cf.ac.uk](http://www.hgmd.cf.ac.uk) for details on *CYP21* mutations listed in Table 2). The overall distribution of *CYP21* mutations in the Australasian CAH cohort is comparable to other studies (Table 3). The most common deleterious *CYP21* alleles were: 1) large scale rearrangements (*CYP21* deletion or hybrid genes) (33% of alleles); 2) I2 G splice (34%); and 3) I172N (11%). At least one of these three alleles was present in 85% of cases. The deletion/hybrid and I2 G splice mutations were associated with SW CAH. However, when I172N was the least severe mutation it was associated with a simple virilising phenotype in 55% of compound heterozygotes and a salt-wasting phenotype in 45% of compound heterozygotes.

Proband P1 was unique in having only one mutation of the *CYP21* gene detected. P4, A4 and A5 had novel mutations. P2 and P3 had rare mutations that have been previously reported in

CAH but which are discussed in detail herein as available information is limited.

Probands P6 to P12 and/or family members had unusual gene copy results suggesting the presence of unusual haplotypes<sup>20</sup>. The families of P13, P14 and P15 were each found to have three deleterious *CYP21* alleles, i.e. mixed pedigrees.

**Proband / Family 1**

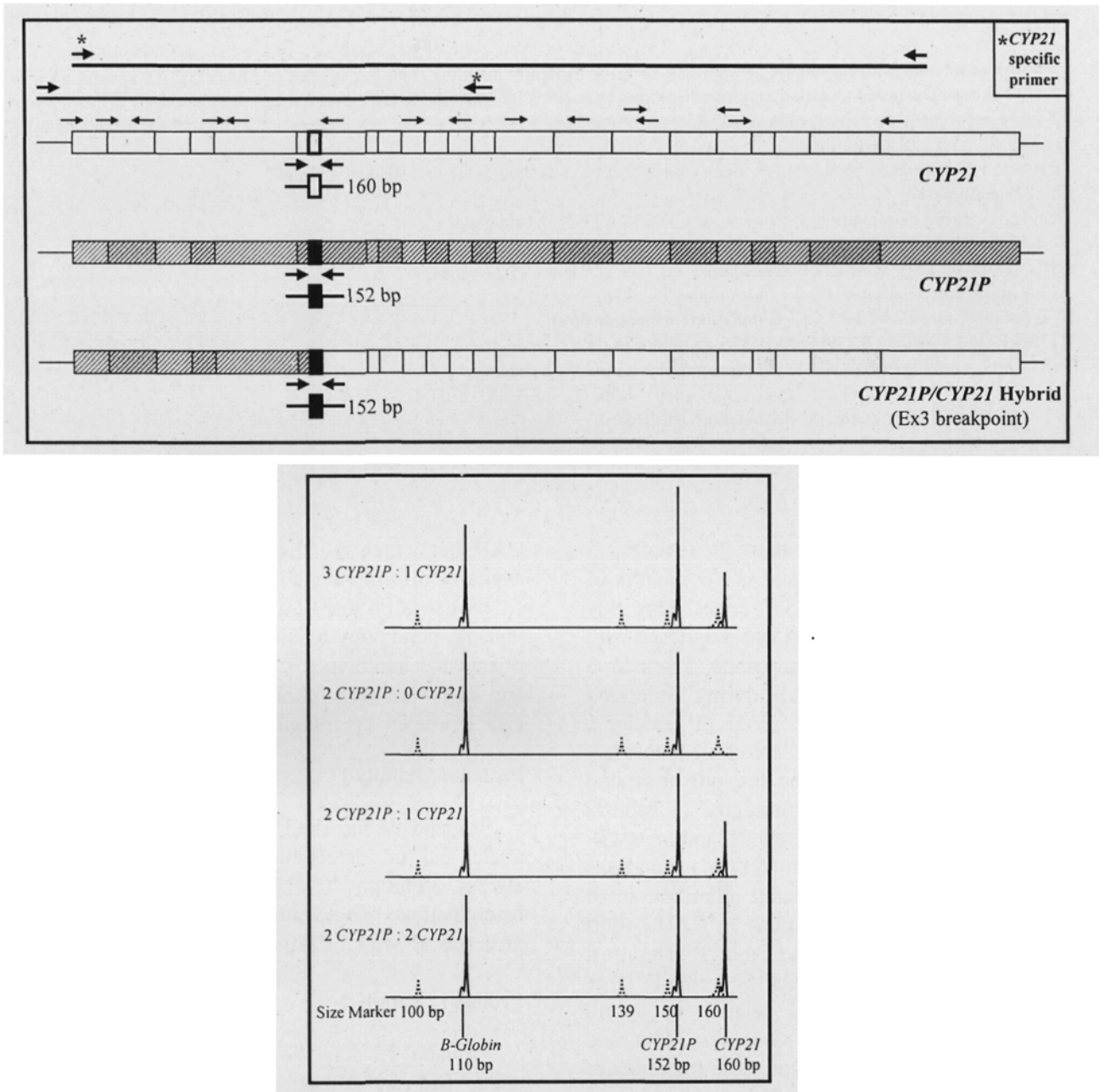
Proband P1 has the intron 2 G mutation on one allele and no detectable mutation on the other allele, although *CYP21* polymorphisms were heterozygous. Gene copy results indicate the presence of two *CYP21* genes.

**Proband / Family 2**

Proband P2 has the I172N mutation on one allele and an unusual amino acid substitution H365Y on the other allele. H365Y is a result of a nucleotide substitution in exon 8, n1096 CAC to TAC.

**Proband / Family 3**

P3 has the Deln/Hyb mutation on one allele and an unusual hybrid gene, 3' Hybrid, on the other allele consisting of *CYP21* sequences at the 5'-end and *CYP21P* sequences at the 3'-end. Putative haplotypes for Family 3 and a possible mechanism



**Fig. 1:** Upper panel: PCR assay of *CYP21* and *CYP21P*. *CYP21* and *CYP21P* genes are depicted, as is the common exon 3 hybrid gene. Shaded boxes represent *CYP21P* gene sequences, and the ten exons are depicted as grey boxes. Large arrows indicate PCR primers used for PCR sequencing and gene copy assays. Small arrows indicate sequencing primers. The two large *CYP21* PCR products used for *CYP21* gene sequencing are shown at the top. The three short gene copy PCR products are shown below each gene. The presence of the exon 3 8-base-pair (bp) deletion in *CYP21P* and the hybrid gene results in a product of smaller size than the *CYP21* PCR product. Lower panel: Gene copy assay. The short PCR products spanning the exon 3 8-bp deletion (152 bp for *CYP21P* and 160 bp for *CYP21*) are analysed quantitatively using capillary electrophoresis followed by GeneMapper analysis. Peak heights are compared to a  $\beta$ -globin gene PCR product to determine *CYP* copy numbers (assuming two  $\beta$ -globin gene copies).



**TABLE 3**  
Allelic distribution of mutations in CAH

Study	% of total alleles													
	Australasia	United Kingdom	Germany	Netherlands	Sweden	Denmark	Middle Europe	Italy	Greece	Finland	USA	Japan	China	Brazil
# Alleles	134	284	310	370	400	136	696 <sup>E</sup>	146	222	156	394	68	130	228
Year	2007	1999	2000	2003	1998	1999	2005	1996	2001	1997	1995	1999	1998	1998
Ref. #		22	13	26	25	21	31	27	23	12	14	29	30	24
<b>Mutation</b>														
<i>Deln/hybrid</i>	33.5	45	27.4	31.9	32.2	36.8	30.6	20	13.5	46.8	25.6	11.8	10.8	10.9
<i>I2 G splice</i>	34.4	30.3	30.3	28.1	26.6	32.4	31.2	19.9	29.3	9.6	29.9	26.5	41.5	18.5
<i>I172N</i>	11.3	7	19.7	12.4	19.8	9.6	14.5	6.2	13.5	31.4	9.6	11.8	22.3	13.2
<i>V281L</i>	3.7	0	2.9	2.2	5.7	2.2	3.4	11	10.8	2.6	9.4	0.0	0.0	16.6
<i>P30L</i>	2.2	0	2.6	0.3	1.6	2.2	3.7	2.7	11.3	0.0	2.5	1.5	0.0	1.7
<i>Ex 3 8 bp</i>	1.5	nt	1.6	4.3	1.1	0.0	1.0	1.4	2.3	0.0	2.8	0.0	1.5	0.9
<i>Q318X</i>	3.0	0	4.8	3.5	2.4	7.4	2.6	8.2	8.6	1.3	4.1	8.8	0.8	4.9
<i>R356W</i>	0	9.8	4.5	8.4	3	0.7	2.4	0	0.5	0.0	3.8	17.6	15.4	7.2
<i>Ex6 cluster</i>	0	nt	1	3	1.1	0.7	0.3	0	0.0	0.0	nt	1.5	0.0	0.4
<i>P453S</i>	0	0	0.3	0.5	0.5	0.0	0.7	nt	3.6	0.0	nt	nt	0.0	1.3
<i>PG Mutn *</i>	3.7	0	0.3	2.2	0.5	2.9	7.2	0	0.5	3.2	8.1	2.9	2.3	4.6
<i>Other **</i>	6.7	7.9	4.6	3.2	5.5	5.1	2.4	30.6	6.3	5.1	4.1	17.6	5.4	19.9
<b>Total</b>	<b>100</b>													
<b>Number of mutations screened</b>	all	17	all	all	all	all	all	9	12	all	10	10	11	16

Compiled results from extensive literature search of worldwide cohort studies, including analysis of methods used in each study.

The % allele frequency for each study was calculated from raw data cited in the literature.

The table includes data on a total of 67 unrelated Australasian CAH probands (patients from Australia and New Zealand; 134 alleles). Only 26 of these 67 probands are presented in detail in this paper (Table 2). Forty-one of the 67 CAH probands were genotyped in our laboratory from de-identified blood samples in the process of optimising the *CYP21* assay and generating positive control samples for quality control; no clinical data on these 41 subjects are available.

\* PG Mutn = other pseudogene mutations tested for. Note that not all pseudogene mutations were tested for in each study (includes combinations of pseudogene mutations, i.e. those tested for).

\*\* Other - includes: 1. rare non-pseudogene mutations; 2. novel mutations; 3. no mutation; 4. 'undefined genotype', e.g. other known mutations not tested for.

nt = not tested for.

<sup>E</sup> Middle Europe: MESPE database subjects = Austrian, Czech, Hungarian, Slovakian, Slovenian (individual populations had similar allele frequencies).

for generation of the 3'-Hybrid are depicted in Figure 2. A crossover between haplotypes F and D may have resulted in generation of the 3' Hybrid allele. The breakpoint of the 3' Hybrid gene is somewhere between the exon 3 8-bp deletion and I172N mutations. It is impossible to determine the precise position of the breakpoint due to the high sequence identity between *CYP21* and *CYP21P*. Chi-like sequences may be recognition sites for the recombinase complex responsible for gene recombination<sup>33-35</sup>. There is a chi-like sequence GCTGGGC at the beginning of intron 3 suggesting that this may be the 3' Hybrid breakpoint.

#### Proband / Family 4

P4 and her affected brother A4 both have the I172N mutation on one allele and a large novel deletion on the other allele. The extent of the large deletion was not determined, but includes both *CYP21* and *CYP21P*.

#### Proband / Family 5

P5 has NC CAH and was referred for pre-conception genotyping with her unaffected partner A5 (Fig. 2). The *CYP21* mutations in P5 are previously described and consistent with her phenotype. The partner A5 was found to carry a novel amino acid substitution D106N on one allele. D106N is a result of a nucleotide substitution in exon 3, n319 GAC to AAC. *CYP* genotypes and haplotypes for Family 5 are depicted in Figure 2.

#### Probands / Families 6 to 12

Genotyping and gene copy results for P6, P7, P8, P9, P10, P11, P12 and available family members indicate the presence of unusual haplotypes B (*CYP21P* deletion), C (one copy *CYP21*, two copies *CYP21P*) and D (*CYP21* deletion, two copies *CYP21P*).

#### Proband / Family 13

Three different deleterious *CYP21* alleles, Deln/Hyb, intron 2 G and P30L were found in Family 13, consistent with the observed phenotypes of both SW and NC CAH (Fig. 3). The Deln/Hyb allele in P13 has an extra copy of *CYP21P*

(haplotype D) and the non-disease allele in the mother has a *CYP21P* deletion (haplotype B).

#### Proband / Family 14

Three deleterious *CYP21* alleles were found in Family 14, consistent with the SW and NC CAH phenotypes observed (Fig. 3). The V281L allele found in patient F14 has a *CYP21P* duplication (haplotype C).

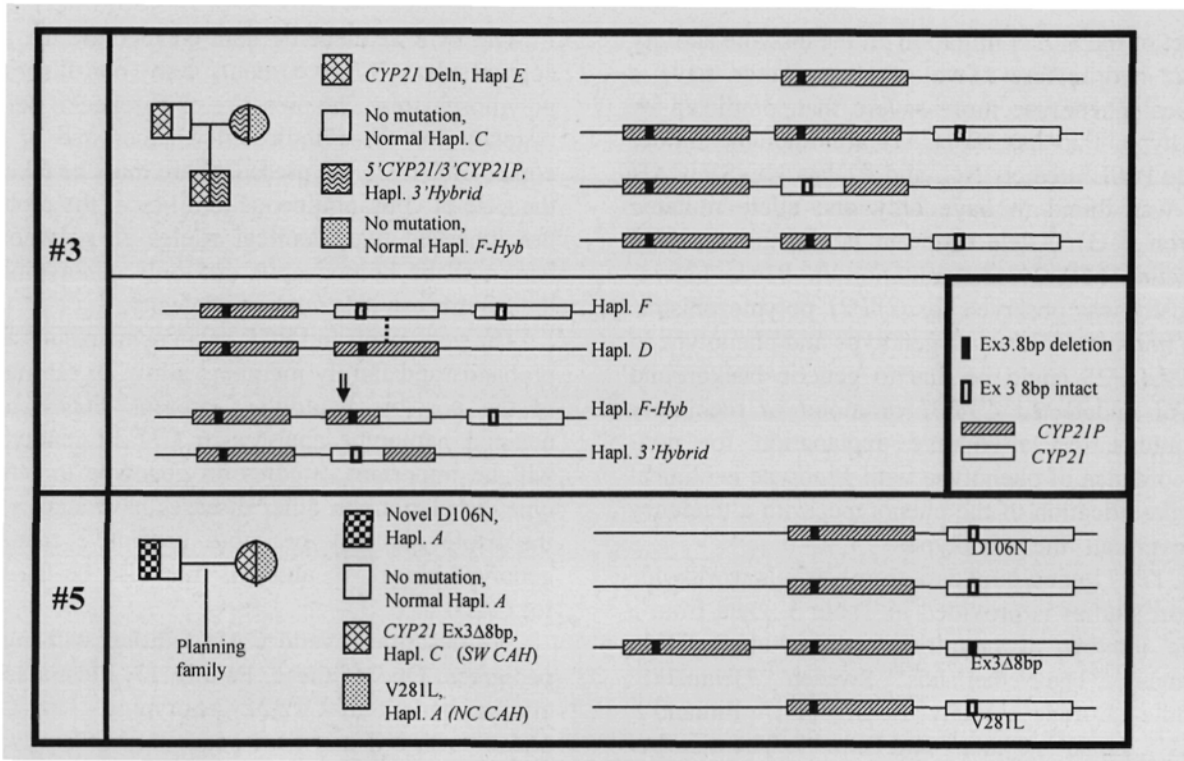
#### Proband / Family 15

P15 has the I172N mutation on one allele and the Deln/Hyb mutation on the other allele. The index case P15 and an affected sibling have been previously described in a case report<sup>36</sup>. Additional genotyping data on family members is reported herein. The maternal aunt is a carrier of the Deln/Hyb allele. Her husband was also genotyped and found to carry the mutation P453S (Fig. 3). The Deln/Hyb allele found in P15 has an extra copy of *CYP21P* (haplotype D).

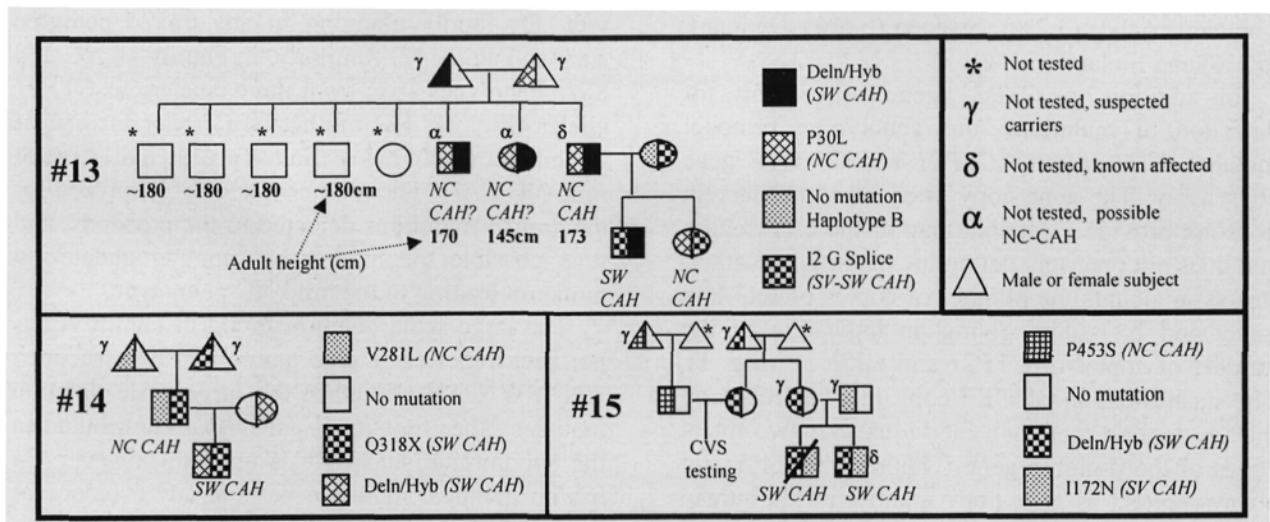
## DISCUSSION

Unlike many *CYP21* genotyping protocols the methodology used in this study involves sequencing of the entire *CYP21* gene including exons, splice sites and the proximal promoter, therefore allowing for the detection of less common mutations. Three of the 26 probands had novel or rare mutations which would have remained undetected if only common mutations had been screened for. The phenotypes predicted from the genotyping results generally agree with the clinical phenotype (76% of cases). However, we found the I172N mutation was associated with either a SV or SW phenotype, although generally reported in the literature as being associated with SV CAH (Table 2). The high sensitivity in detecting mutations and the good genotype/phenotype correlation illustrates the value of *CYP21* genotyping by sequencing.

As mentioned, five patients (P4, A4, P15, P21 and P23) have SW CAH although the milder allele I172N predicts a SV phenotype. Other studies have also indicated phenotypic variability for the I172N mutation<sup>10,12,13,26,31</sup>. It is possible that the genetic background may play a role in modulating the



**Fig. 2:** *CYP21* genotypes and haplotypes. Upper panel: Pedigree and genotyping results for Family 3. The gene copy results are consistent with known haplotypes E, F-Hyb and C. A possible mechanism for the generation of the unusual '3'Hybrid' allele is indicated in the lower part of the figure. Lower panel: Pedigree, genotypes and possible haplotypes (based on gene copy results) for Family 5.



**Fig. 3:** Families with three *CYP21* mutations.

effect of the I172N mutation on the enzyme activity of 21-hydroxylase. Two other probands have a clinical phenotype more severe than predicted by genotype: P25 has SV CAH although the milder allele P30L predicts NC, and P1 has NC-SV CAH but was found to have only one allele mutated (intron 2 G). Allele drop-out is not the cause of detection of only one mutation in P1 as heterozygosity was observed for *CYP21* polymorphisms. The non-concordance of genotype and phenotype in P1 and P25 could be due to genetic background and/or undetected *CYP21* promoter or regulatory mutations. An alternative explanation for non-concordance of phenotype with genotype is clinical misclassification of the phenotype, with a tendency to 'over-call' the phenotype.

*CYP21* genotyping data from various worldwide cohort studies is provided in Table 3. Data from a large number of countries are included: UK<sup>22</sup>, Germany<sup>13</sup>, The Netherlands<sup>26</sup>, Sweden<sup>25</sup>, Denmark<sup>21</sup>, Middle Europe<sup>31</sup>, Italy<sup>27</sup>, Greece<sup>23</sup>, Finland<sup>12</sup>, USA<sup>14</sup>, Japan<sup>29</sup>, China<sup>30</sup> and Brazil<sup>24</sup>. The distribution of *CYP21* mutations in the Australasian CAH cohort is similar to that in other populations. As would be expected, studies that screened for only the common *CYP21* mutations often report a higher incidence of 'undefined' (novel/rare) mutations, for example Italy (30.6%) and Japan (17.6%). It is of note that the incidence of rare/novel mutations in our Australasian cohort (6.7%) is slightly higher than in the other studies that screened for all *CYP21* mutations, for example Germany (4.6%), The Netherlands (3.2%), Sweden (5.5%), Denmark (5.1%) and Finland (5.1%).

In addition to *CYP21* gene sequencing for detection of mutations, our genotyping protocol includes a quantitative *CYP21* and *CYP21P* gene copy assay. The gene copy assay described herein does not provide a detailed map of the *CYP* region and does not precisely determine haplotype. Rather, this assay counts the number of copies of Ex3-8bp intact and Ex3-Δ8bp giving an indication of the number of copies of *CYP21* and *CYP21P* (Fig. 1). The quantification of *CYP* copy number relies on the assumption that each individual has two intact copies of the β-globin gene. This assumption is not always correct as gene copy number in the human genome is variable<sup>37</sup>.

The PCR sequencing data corroborate the gene copy results. *CYP21* contains many non-functional polymorphisms. The presence or absence of heterozygosity for these indicates whether one or two copies of *CYP21* are present. Care must be taken in the case of consanguineous families; if the proband has inherited two identical alleles all polymorphisms will be homozygous and only genotyping of the parents can rule out hemizyosity.

The gene copy and PCR sequencing results from probands and family members allow an estimation of the possible haplotypes present. Future more detailed haplotype analysis in *CYP21* genotyping will be important. Studies on genotype to phenotype correlations in other diseases have shown that the haplotype may be more predictive than the genotype alone<sup>37-39</sup>, and this may also be the case for CAH.

We describe several CAH families with mixed pedigrees. One of these, Family 13, illustrates the clinical benefit of *CYP21* genotyping. Two CAH phenotypes, NC and SW, occur in this family due to the presence of three deleterious *CYP21* alleles (Fig. 3). The members of this family with NC CAH (father, sister) were only detected as a consequence of the presentation of the proband with SW CAH. The father had early puberty and diminished adult height which has been reported in NC CAH<sup>40-44</sup>. The results for Family 13 suggest that NC CAH may go undetected in the general population and genotyping should help those patients who may benefit from treatment. In addition, genotyping is vital for family planning in any mixed pedigree such as Family 13. Similarly, in Family 14 NC and SW phenotypes arise from three deleterious *CYP21* alleles (Fig. 3). The mother is a carrier for one of the mutations but has a clinical profile indicative of mild NC CAH. The mother was only genotyped for the known mutations detected in the probands, and it is possible the mother has another undetected mutation leading to the mild NC phenotype.

The large scale deletion found in Family 4 has not been reported to date and would be associated with SW CAH. Whether the large scale deletion includes other functional genes that contributed to the hypopituitarism in A4 is unclear; however, P4 has an identical mutation without any evidence of hypopituitarism.

An unusual hybrid gene, 3' Hybrid, was detected in Family 3 consisting of *CYP21* sequences at the 5'-end and *CYP21P* sequences at the 3'-end (Fig. 3). A similar rearrangement has been described in the literature<sup>45</sup>. The pseudogene sequences at the 3'-end of the 3' Hybrid gene include many deleterious mutations which render the gene incapable of encoding active enzyme, resulting in SW CAH.

One novel amino acid substitution D106N is reported herein but it is unclear based on clinical data whether this is associated with CAH. The rare mutation H365Y has been reported previously in CAH<sup>46</sup> but no phenotypic information was provided. Expression studies on D106N and H365Y have not been performed. Cotton *et al.*<sup>47</sup> outline criteria for predicting the effect of missense mutations. If an amino acid change is non-conservative (i.e. results in a change in chemical properties of the side chain), if the residue is conserved amongst various species, and if the change is observed in affected but not in control individuals, then it is likely to have an effect on protein function. All criteria outlined by Cotton *et al.*<sup>47</sup> are fulfilled by the two missense mutations.

Robins *et al.*<sup>48</sup> have provided a structural model of human 21-hydroxylase and analysed the effect of many mutations associated with CAH including H365Y. The active site of 21-hydroxylase includes a cysteine binding pocket which binds covalently to a haem molecule and the H365 residue is directly involved in haem binding<sup>48,49</sup>. Robins *et al.*<sup>48</sup> predict that H365 is most likely associated with SV or SW CAH. Proband P2 in our patient cohort has SV CAH and has the H365Y mutation on one allele and I172N on the other allele. We have found that I172N is associated with both SV and SW CAH and it is thus impossible to predict based on the phenotype of P2 which of H365Y or I172N is the milder allele.

The novel substitution D106N was identified in a normal individual A5 in the process of carrier testing prior to family planning. The effect of conversion of the small negatively charged aspartic acid residue at position 106 to the small, polar, uncharged asparagine residue is unknown. The detection of a novel mutation in the partner of a patient affected with CAH provides problems with

prenatal testing. If the novel mutation is detected in the offspring in addition to a second mutation, then it is impossible to predict CAH phenotype. The difficulty in prediction of CAH phenotype for the novel mutation D106N illustrates the importance of *in vitro* expression analysis and modelling studies as well as detailed descriptions of the phenotype when novel mutations are reported.

The assay described in this paper is simple and thorough, a fact illustrated by the detection of three novel/rare mutations that would have been missed using other protocols. *CYP21* genotyping using entire gene sequencing has a number of potential benefits that have been illustrated in this paper. Detection of novel mutations not only confirms the genetic basis of CAH in individual probands but also contributes to our understanding of genotype-phenotype correlations when combined with functional studies. Moreover, the application of accurate and sensitive genotyping methods should improve neonatal screening strategies and therefore facilitate genetic counselling.

#### ACKNOWLEDGEMENTS

Dr. C.S.Y. Choong is a recipient of a Telethon Trust Award.

The authors wish to acknowledge collaboration with the following clinicians and scientists: D. Anderson, J. Batch, D. Bouchier, C. Bragg, J. Carter, S. Chitturi, A. Colley, J. Couper, P. Crock, B. Culling, A. Cupitt, M. Edwards, M. Epstein, R. Gardener, A. Hackett, W. Harris, R. Heazlewood, B. Holland, G. Leong, F. Mackenzie, M. Marsh, D. McDonald, S. McGrath, R. Messer, L. Morgan, R. Ogle, A. Pena, R. Cherry, T. Roscioli, F. Thatcher, M. Thomsett, G. Warne, T. Warnock, J. Williams, M. Wilson.

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