Mutation screening in British 21-hydroxylase deficiency families and development of novel microsatellite based approaches to prenatal diagnosis

Majlinda Lako, Simon Ramsden, R Duncan Campbell, Tom Strachan

Abstract
21-hydroxylase deficiency is a recessively inherited disorder of steroidogenesis, resulting from mutations in the CYP21 gene. This 3.5 kb gene and a highly related CYP21P pseudogene reside on tandemly duplicated 30 kb segments of DNA in the class III HLA region, and the great majority of pathogenic mutations result from sequence exchanges involving the duplicated units. We now describe a comprehensive survey of CYP21 mutations in the British population, encompassing a screen for 17 different mutations in a total of 284 disease chromosomes. The most common mutations were as follows: large scale deletions/conversions (45% of the affected chromosomes), the intron 2 splice mutation (30.3%), R357W (9.8%), and I172N (7.0%). Mutations were detected in over 92% of the chromosomes examined, suggesting that accurate DNA based diagnosis is possible in most cases using the described strategy. In order to extend highly accurate prenatal diagnosis to all families where samples are available from a previously affected child, we have developed a linkage analysis approach using novel, highly informative microsatellite markers from the class III HLA region.

Keywords: 21-hydroxylase deficiency; CYP21; mutation screening

Steroid 21-hydroxylase deficiency is one of the more common recessive disorders; the severe classical congenital form, occurs with a frequency in white populations of about 1/10 000, while milder non-classical forms, including late onset development of symptoms, are much more common. The disorder manifests as a defect of adrenal steroidogenesis and is characterised by excessive virilisation. The biochemical basis is an abnormal accumulation of 17-hydroxyprogesterone (a major substrate for 21-hydroxylase) which leads to increased metabolic flux in a parallel pathway cumulating in androgen synthesis. Approximately 30% of classical cases show simple virilising features and the remaining 70% or so show additional "salt wasting", a potentially fatal inability to conserve dietary sodium owing to a parallel defect in aldosterone synthesis. Although both the salt wasting and excessive virilisation components are treatable, difficulties with management of the disease have fuelled a high demand for prenatal diagnosis and early termination of pregnancy.

The different phenotypic variants of the disorder result from mutations in a 3.5 kb gene, CYP21 (formerly called CYP21B), which maps within the HLA complex at 6p21.3. The gene resides on an about 30 kb segment which contains a complement C4 gene and other transcription units, and which has been duplicated in recent evolutionary history. As a result, there is a very high degree of sequence identity between the duplicated 30 kb segments. The duplication has resulted in an additional 3.5 kb long CYP21P-like sequence, formerly called CYP21A but more recently termed CYP21P to reflect its presumed status as a pseudogene. The two CYP21 genes show approximately 98% sequence homology, but the capacity of the CYP21P gene for encoding a functional 21-hydroxylase polypeptide has been eroded by the accumulation of a series of deleterious mutations spanning its length.

The very close similarity in sequence of the tandemly duplicated 30 kb segments which contain the CYP21P and CYP21P genes predisposes towards frequent unequal chromosome pairing in this region. Subsequent crossover events can lead to loss or gain of a 30 kb segment and to gene conversion-like sequence exchanges between the duplicated genes. As a result, the vast majority of CYP21 pathogenic mutations involve loss of some essential CYP21 gene sequence: either there is a 30 kb deletion which removes all, or more frequently a portion, of the original CYP21 gene sequence, or there is a gene conversion-like event whereby a small amount of the original CYP21 gene sequence is replaced by a similar but defective sequence copied from the CYP21P pseudogene. Direct evidence for 30 kb deletions has been obtained by pulsed field gel electrophoresis and such deletions are thought to arise as a result of meiotic unequal crossover, whereas the gene conversion-like events may arise more frequently in mitotic cells. Where examined, de novo gene conversion events giving rise to 21-hydroxylase deficiency appear to involve conversion of only a few hundred nucleotides. Disease chromosomes bearing a CYP21P pseudogene and a CYP21P gene which bears CYP21P specific sequences over a large component of its length, so called large scale "gene conversion" haplotypes, are also known. Large scale conversion at

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the 5’ end of the CYP21 gene appears to be particularly common and may often occur as a result of successive recombination events. Some other mutant alleles which may be uncommon in some populations show evidence of large scale conversion within the gene.

The observations described above mean that the molecular pathology of 21-hydroxylase deficiency is characterised by a considerable degree of mutational homogeneity: gene deletions are of uniform size (around 30 kb), and there are only a limited number of point mutation classes (the CYP21P sequence is so similar to that of CYP21 that only a few types of pathogenic point mutation can be introduced by copying defective sequence from the pseudogene sequence). Molecular pathology studies have been conducted on a variety of different populations and while recording some differences in the frequency of certain mutation classes, such as gene deletions, they have established that there are a few common point mutations, although some minority alleles bear point mutations which do not appear to have been copied from the CYP21P pseudogene.

The comparative mutational homogeneity underlying 21-hydroxylase deficiency may suggest the possibility of using direct mutation based prenatal diagnosis as an alternative to indirect linkage analyses. In the present report we have conducted an extensive study of pathogenic mutations at the CYP21 locus in the UK population. After screening for 17 different mutations we were able to identify pathogenic mutations in 266 out of the 284 disease chromosomes. In order to extend accurate prenatal diagnosis of 21-hydroxylase deficiency to all families, therefore, we have sought to improve upon previous types of linkage analyses, which have often relied heavily on the use of closely linked class I and class II HLA gene markers. We describe the use of a variety of novel, highly polymorphic microsatellite repeats in the class III HLA region.

## Methods

### PATIENT AND SAMPLES

The patients in the current study were classical (congenital) 21-hydroxylase deficiency patients drawn from a variety of ethnic groups within the UK, but were predominantly white and with a substantial minority of Pakistani and Indian descent. Diagnosis was based on “salt wasting”, an inability to conserve dietary sodium, or by virilised features in female infants.

### Primer names are as described by Wedell and Luthman.10

*Mutations associated with non-classical 21-hydroxylase deficiency.

‡P48 or P55 were used as CYP21 specific primers.

§Allele specific primers are indicated in pairs with the normal sequence represented by bold lettering at the end of the primer name while the mutant sequence specific primer ends with bold and underlined lettering. Control primers are indicated in brackets (see Methods).

## Table 1: Point mutations in CYP21 and primers used for their genotyping

<table>
<thead>
<tr>
<th>Exon (Ex) or intron (Int)</th>
<th>Sequence changes (in bold; mutant underlined)</th>
<th>Protein change</th>
<th>Primers used in the first PCR†</th>
<th>Size of product (bp)</th>
<th>Primers used in the second PCR‡</th>
<th>Size of product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex 1</td>
<td>CCG→CTG</td>
<td>Pro31-Leu</td>
<td>PS4+P48</td>
<td>1009</td>
<td>P92CP92T+P48 (+P70)</td>
<td>625</td>
</tr>
<tr>
<td>Int 2</td>
<td>A/C→G</td>
<td>Splice mutation</td>
<td>PS5+P48</td>
<td>423</td>
<td>P659A/P659G+P48 (+P5)</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex 4</td>
<td>ATC→AATC</td>
<td>Ile73-Asn</td>
<td>PS55+P4</td>
<td>1385</td>
<td>P1004T/P1004A+P5 (+P19)</td>
<td>322</td>
</tr>
<tr>
<td>Ex 6</td>
<td>GTG→GAG</td>
<td>Val82-Cys</td>
<td>PS5+P4</td>
<td>2064</td>
<td>P1388T/P1388A+P5 (+P11)</td>
<td>706</td>
</tr>
<tr>
<td>Ex 7</td>
<td>GTG→TGT</td>
<td>Leu282-Val</td>
<td>PS5+P4</td>
<td>2064</td>
<td>P1688G/P1688T+P5 (+P11)</td>
<td>1005</td>
</tr>
<tr>
<td></td>
<td>GTG→AGT</td>
<td>Gly292-Ser</td>
<td>PS5+P4</td>
<td>2064</td>
<td>P1718G/P1718A+P5 (+P11)</td>
<td>1033</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>P1769G/P1769T+P5 (+P71)</td>
<td>1078</td>
</tr>
<tr>
<td>Int 7</td>
<td>GT→CT</td>
<td>Splice mutation</td>
<td>PS55+P4</td>
<td>2064</td>
<td>P1784G/P1784A+P5 (+P11)</td>
<td>1101</td>
</tr>
<tr>
<td>Ex 8</td>
<td>CAG→TAG</td>
<td>Gln319-Arg</td>
<td>PS55+P4</td>
<td>2064</td>
<td>P1990G/P1990T+P5 (+P11)</td>
<td>1317</td>
</tr>
<tr>
<td></td>
<td>CCG→TGG</td>
<td>Arg357-Trp</td>
<td>PS55+P4</td>
<td>2064</td>
<td>P2113G/P2113T (+P11)</td>
<td>1430</td>
</tr>
<tr>
<td>Ex 9</td>
<td>TGG→TAG</td>
<td>Trp406-Stop</td>
<td>PS55+P4</td>
<td>2064</td>
<td>P2344G/P2344A+P5 (+P11)</td>
<td>1660</td>
</tr>
<tr>
<td>Ex 10</td>
<td>CCC→CAG</td>
<td>Arg484-Pro</td>
<td>PS4+P4</td>
<td>619</td>
<td>P2584C/P2584T+P5 (+P11)</td>
<td>455</td>
</tr>
<tr>
<td></td>
<td>CCG→GG</td>
<td>Arg484-Pro</td>
<td>PS4+P4</td>
<td>237</td>
<td>P2675G/P2675C+P47 (+P11)</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>GG→CAG</td>
<td>Arg484-Pro</td>
<td>PS4+P4</td>
<td>237</td>
<td>P2675G/P2675C+P47 (+P11)</td>
<td>165</td>
</tr>
</tbody>
</table>

## Table 2: Novel microsatellite markers in the class III HLA region

<table>
<thead>
<tr>
<th>Microsatellite</th>
<th>Range (bp)</th>
<th>Repeat sequence</th>
<th>PCR primers (5’-3’)</th>
<th>Allele number</th>
<th>Heterozygosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>62</td>
<td>146–197</td>
<td>(TC),n/CCT,(T),n/(TC),n/TGCAA/(CA),,n(CA),,n</td>
<td>TCCAATGTCCCTGTGCACCTCTCTAGTTTATCGAGCACCCAGGGCAGAGCAGGCATTGTGATGAG</td>
<td>14</td>
<td>82.4</td>
</tr>
<tr>
<td>82-1</td>
<td>86–113</td>
<td>(CA),,n/GGAGCAGC,(CA),,n</td>
<td>13</td>
<td>81.3</td>
<td></td>
</tr>
<tr>
<td>9N-2</td>
<td>98–120</td>
<td>(CA),,n</td>
<td>7</td>
<td>72.3</td>
<td></td>
</tr>
<tr>
<td>D3A</td>
<td>113–144</td>
<td>(CA),,n/GAGA-(CA),,n</td>
<td>6</td>
<td>75.9</td>
<td></td>
</tr>
<tr>
<td>LH-1</td>
<td>79–103</td>
<td>(CA),,n</td>
<td>11</td>
<td>71.0</td>
<td></td>
</tr>
</tbody>
</table>
neonates and confirmed by standard hormone assays, notably of serum 17-
hydroxyprogesterone following intravenous administra-
tion of ACTH, as described previously.

Blood samples obtained with informed consent were used to prepare stand-
ard or high molecular genomic DNA, using conventional methods. In some cases genomic DNA samples were referred from UK labora-
tories who had transmitted requests for prena-
tal diagnosis for the condition.

### Table 3 Distribution of point mutations and deletions in 284 disease chromosomes in British 21-hydroxylase deficiency families

<table>
<thead>
<tr>
<th>Deletion Type</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large scale deletions/5' conversions</td>
<td>45.0 %</td>
</tr>
<tr>
<td>Intron 2 splice mutation</td>
<td>30.3 %</td>
</tr>
<tr>
<td>R357W</td>
<td>9.8 %</td>
</tr>
<tr>
<td>H172N</td>
<td>7.0 %</td>
</tr>
<tr>
<td>Unidentified mutations</td>
<td>7.8 %</td>
</tr>
</tbody>
</table>

**DETECTION OF LARGE SCALE DELETION AND LARGE SCALE 5' CONVERSION EVENTS**

The duplicated CYP21 and CYP21P genes and the adjacent complement C4B and C4A genes were tracked by hybridising CYP21P and
C4 specific probes (p21A-1.8 and C4B550 respectively) to Southern blots of TaqI digested genomic DNA samples. Diagnostic
3.7 kb and 3.2 kb TaqI restriction fragment length variants (RFLVs) were associated with the CYP21 and CYP21P genes respectively,
while the complement C4A and C4B genes were identifiable by the presence of a 7.0 kb TaqI RFLV and a 6.0/5.4 kb TaqI RFLV respectively.

**DETECTION OF POINT MUTATIONS**

Detection of CYP21 point mutations was carried out essentially as described by Wedell and Luthman.
Specific amplification of the CYP21 gene was achieved in the first round of PCR using primers that make use of a major sequence difference between the CYP21 and
CYP21P genes, an 8 bp sequence which is present in exon 3 of the CYP21 sequence but which is absent in the CYP21P sequence.
Primers P55 and P48 were designed from sense or antisense sequence from the region which included this 8 bp sequence, as initially advocated by Wedell and Luthman;
where required CYP21P specific amplification was possible using primers P49 and P56 mapping to the corresponding part of CYP21P.

In the first round of amplification, the primer P1, which was derived from sense sequence in the 5' UTR, was used with the CYP21 specific primer P48 in order to amplify a segment of the CYP21 gene spanning the 5' UTR and exons 1-3, of the CYP21 gene. To amplify the remainder of the CYP21 gene, the CYP21 spe-
cific primer P55 was used with the primer P4, which derived from antisense sequence in the 3' UTR.

Genotypings for point mutations were performed on the CYP21 specific amplification products by an ARMS based approach in a sec-
dond allele specific round of PCR from 1 µl of the initial reaction. First round P1/P48 amplification products were used to detect mutations upstream of the CYP21 specific 8 base pair sequence in exon 3; mutations down-
stream of this sequence were screened using P55/P4 amplification products (for a list of point mutations and primers used for genoty-
ping see table 1). Positive controls containing amplified P1/P48 and P55/P4 fragments for each of the 16 different point mutations were supplied by A Wedell (personal communica-
tion). For all mutations, except those in exon 10, primer P48 or P55 was used as a conserved sequence primer in the second round of amplifi-
cation, together with a normal or mutant allele specific primer. In addition, a primer generating a longer fragment than the allele specific fragment was used as an internal con-
trol of the PCR reaction. In cases where a sub-
ject appeared to type as homozygous for the

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**Figure 1** Genotyping of a 21-hydroxylase deficiency family for the intron 2 splice site mutation and the R357W mutation. (A) ARMS based typing of the intron 2 splice
mutation. Normal alleles carry either an A or a C at the mutation site; mutant alleles have a G. For each individual the types of allele which have been detected are shown at the top,
with the mutant allele underlined. 1=father, 2=mother, 3=homozygous control, 4=child. The 2064 base pair sequence in exon 3; mutations down-
stream of this sequence were screened using P55/P4 amplification products (for a list of point mutations and primers used for genoty-
ping see table 1). Positive controls containing amplified P1/P48 and P55/P4 fragments for each of the 16 different point mutations were supplied by A Wedell (personal communica-
tion). For all mutations, except those in exon 10, primer P48 or P55 was used as a conserved sequence primer in the second round of amplifi-
cation, together with a normal or mutant allele specific primer. In addition, a primer generating a longer fragment than the allele specific fragment was used as an internal con-
trol of the PCR reaction. In cases where a sub-
ject appeared to type as homozygous for the
Normal alleles carry a T at the mutation site, mutant alleles carry an A. For each person, the allele types which have been detected are shown at the top.

**Figure 2** Microsatellite typing and mutation screening for a 21-hydroxylase deficiency family. (A) ARMS based genotyping of the I172N mutation. Lako, Ramsden, Campbell, et al.

**Results**

Molecular Characterisation of Pathogenic Mutations in British 21-Hydroxylase Deficiency Patients

Southern blot analysis was used to detect CYP21 gene deletions or large 5' CYP21 "gene conversions" and ARMS based allele specific amplification was used to screen for 16 known point mutations in 284 disease chromosomes in British 21-hydroxylase deficiency families (see Methods). Based on these two methods, the disease mutation was established in 92.3% of the disease chromosomes (table 3). As established by a combination of pulsed field gel electrophoresis and conventional Southern analyses in a previous smaller study, large scale deletions and large scale "gene conversions" are very common in the UK population, and the present study endorsed this finding. The diagnostic 3.7 kb Taq restriction fragment associated with the CYP21 gene was absent in 45% of the affected chromosomes (table 3). Of the various point mutation classes observed, there was a very frequent representation of the established intron 2 splice mutation, positive identification of both parental disease alleles was established in the carrier parents in order to avoid possible problems of misinterpretation owing to haplotype dropout during PCR amplification.

Microsatellite Typing

Genomic DNA was amplified with the oligonucleotide pairs shown in table 2. The forward primer was end labelled with 32P-γATP (Amersham International plc) and T4 polynucleotide kinase (New England Biolabs). PCR was carried out in a thermocycler in a 10 µl volume containing 50-100 ng of genomic DNA template, 0.5 µmol/l of each primer, 50 µmol/l of each dATP, dCTP, dGTP, dTTP, 1 unit of Taq polymerase, and 1 × Taq polymerase buffer (Promega). Samples were overlaid with 20 µl mineral oil. The (CA) repeat microsatellites were amplified through 30 cycles, each consisting of 30 seconds at 96°C, 15 seconds at 62°C, and 30 seconds at 72°C. The last elongation step was carried out for a further three minutes at 72°C. Three µl of the amplified products were mixed with 3 µl of dye and electrophoresed in denaturing polyacrylamide gel containing 6% acrylamide and 8 mol/l urea at room temperature at 50 W for two to three hours. Gels were fixed, dried, and exposed at -70°C for 10 hours.

**Use of Novel Microsatellite Markers in the Class III HLA Region in Prenatal Diagnosis of 21-Hydroxylase Deficiency Patients**

The above mutation screening protocol identified pathogenic mutations in over 92% of the disease chromosomes sampled. In order to extend accurate prenatal diagnosis to all 21-hydroxylase deficiency families where samples are available from a previously affected child, indirect linkage analyses using extremely closely linked markers are required. Ten highly polymorphic (CA)n(TG)n microsatellite markers have very recently been identified in the class III HLA region and in the current study we have tested five of the new microsatellite markers, LH-1, D3A, 9N-2, 62, and 82-1 (table 2), by typing 50 unrelated subjects and 26 subjects belonging to six families with a previously affected child. Three of the markers are located distal to the CYP21 gene and two are proximal, with the distance between the marker and CYP21 varying from 100 to 400 kb (fig 3). The distribution and frequencies of different alleles calculated from the study of 50 unrelated subjects are presented in table 2.

The segregation of alleles defined by microsatellite typing was always found to correspond with segregation of mutations defined by direct
analysis of the CYP21B gene. Fig 2 shows one example. In this case ARMS screening identified both parents to be heterozygous carriers of the I172N mutation, while the CVS sample and a sample from the previously affected child were shown to be homozygous for this mutation, confirming the result obtained by microsatellite typing. The use of such highly polymorphic very closely linked markers should permit accurate prenatal diagnosis in essentially all 21-hydroxylase deficiency families where a sample is available from a previously affected child.

**Discussion**

The molecular pathology of 21-OH deficiency is, in very large measure, the result of sequence exchanges between the duplicated CYP21 and CYP21P loci. As a result of this, and the very high extent of sequence homology between these genes, there are comparatively few types of pathogenic mutation,11 12 prompting the diagnostic use of direct mutation typing. In addition to allele specific oligonucleotide hybridisation approaches, rapid PCR based methods have been used to identify point mutations in the CYP21 gene by selective amplification of CYP21 sequences using locus specific primers, followed by ARMS based allele specific amplification13 35 37 or amplification created restriction site approaches.36 39 The current study, in addition to testing for deletions, was designed to type for a total of 16 pathogenic point mutations. Some of these were known to be associated with the non-classical form of 21-hydroxylase deficiency11 12 (table 1) and would not have been expected in classical disease chromosomes, and some represented rare point mutations which have not been introduced from the CYP21P.29 30 The single largest mutation class was the large scale deletion and large scale 5' CYP21 gene conversion category which accounted for over 40% of the disease chromosomes sampled. Although Southern blotting of conventional TaqI digests cannot readily distinguish large scale deletions from large scale conversions, direct quantitation of deletions is possible using PFGE based BssHII restriction mapping and has suggested a ratio of 3:1 for large scale gene deletion to large scale gene conversion events in the British population.13 European populations often show broadly similar frequencies of gene deletion/large scale 5' CYP21 gene conversion, but in other populations the reported figure may be quite low.27 The high frequency of the intron 2 splice site mutation described in the present report also parallels that observed in several other population surveys, but the R357W mutation appears particularly frequent in the British population when referenced against other reports.

Although in the present study deletions and large scale conversions were sampled by Southern blot hybridisation typing, rapid PCR based alternatives for screening for such mutations could also be used. In this case primers can be designed to amplify simultaneously equivalent segments of the CYP21 and CYP21P genes at a position immediately upstream of the coding sequence where the presence (in CYP21P) or absence (in CYP21) of a TaqI site provides the basis for the diagnostic 3.2 kb and 3.7 kb TaqI RFLPs. Subsequent TaqI digestion of the CYP21/CYP21P amplification product will yield additional small digestion products in the presence of the CYP21 gene (false negative results can be discounted by adding an internal control of a DNA fragment known to possess a TaqI site resulting in additional diagnostic band sizes after TaqI digestion). Because of the ability to PCR type for deletions and large scale conversions and because these two mutation classes plus only three other point mutations (the intron 2 splice mutation, R357W, and I172N) account for the pathogenesis in over 92% of disease chromosomes in British 21-hydroxylase deficiency families, accurate DNA based diagnosis can be carried out simply and rapidly in the great majority of such cases. The pathogenic mutations in a significant proportion (7.8%) of disease chromosomes in the present study could not be identified, however, and this may suggest that there are several other classes of rare mutation, in addition to
the known ones that were typed in the present study. Therefore, while direct mutation typing may be useful in prenatal diagnosis in families where the pathogenesis has previously been determined, the prospect of prenatal diagnosis by rapid multiplex mutation screening without previous knowledge of the pathogenesis is not an attractive one. In addition, such multiplex typing may not always be straightforward and some PCR artefacts can lead to difficulty in interpretation, as in the case of the intron 2 splice mutation.34

The usefulness of alternative linkage based approaches for prenatal diagnosis is determined by the number of markers available for testing and their heterozygosity indices, and the accuracy of the tests depend on how closely linked the markers are, and on whether flanking markers are available. Traditionally, linkage analyses have relied heavily on class I and class II HLA gene markers,35 which are often disadvantaged by not being straightforward to use, and although located close to the CYP21 locus, a significant frequency of recombination with the disease locus is expected. During this study, however, 10 new highly polymorphic microsatellite markers were identified within 100-400 kb from the CYP21 gene36 (fig 3, table 1) and we typed five of these in 50 unrelated subjects and 26 subjects from six families with at least one previously affected child with CAH. These highly informative markers have proved very simple and easy to apply, resulting in a novel, rapid approach to prenatal diagnosis of 21-hydroxylase deficiency which requires minimal amounts of DNA from the parents and a previously affected child.

We are grateful to the Wellcome Trust for financial support of this work and to A Wedell for providing positive control samples for screening of some point mutations.


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