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REVIEW ARTICLE

Congenital Adrenal Hyperplasia (CAH) due to 21-Hydroxylase Deficiency: A Comprehensive Focus on 233 Pathogenic Variants of *CYP21A2* Gene

Paola Concolino[1,2] · **Alessandra Costella**[1]

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Abstract Congenital adrenal hyperplasia (CAH) comprises a group of autosomal recessive disorders caused by complete or partial defects in one of the several steroidogenic enzymes involved in the synthesis of cortisol from cholesterol in the adrenal glands. More than 95–99% of all cases of CAH are caused by deficiency of steroid 21-hydroxylase, an enzyme encoded by the *CYP21A2* gene. Currently, *CYP21A2* genotyping is considered a valuable complement to biochemical investigations in the diagnosis of 21-hydroxylase deficiency. More than 200 mutations have been described in literature reports, and much energy is still focused on the clinical classification of new variants. In this review, we focus on molecular genetic features of 21-hydroxylase deficiency, performing an extensive survey of all clinical pathogenic variants modifying the whole sequence of the *CYP21A2* gene. Our aim is to offer a very useful tool for clinical and genetic specialists in order to ease clinical diagnosis and genetic counseling.

Key Points

An extensive effort was made in order to collect literature data reporting *CYP21A2* pathogenic variants.

We were able to provide 233 pathogenic *CYP21A2* variants and their clinical classification.

1 Introduction

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase (21-OH) deficiency represents 95–99% of all CAH cases [1–3]. The hallmark of the disease is the deficiency of enzyme activity, leading to poor cortisol production and the subsequent accumulation of precursor steroid hormones in the steroidogenic pathway, resulting in hyperandrogenism [4, 5]. This disorder has a broad spectrum of clinical forms, ranging from severe or classical (CL) to mild late-onset or non-classical (NC). CL CAH, affecting 1:13,000 to 1:15,000 live births, is represented by two phenotypes: simple virilizing (SV) and salt wasting (SW). SW CAH, the most severe among the two phenotypes, accounts for an estimated 75% of CL cases [4, 5]. The severe impairment of 21-hydroxylase enzyme (<2% enzyme activity) leads to an inadequate production of aldosterone and cortisol to sustain life. The lack of aldosterone, required for sodium homeostasis, if left untreated, will lead to vomiting, lethargy, and failure to gain weight. In fact, severely affected newborns usually present at 1–4 weeks of age with hyponatremia, hyperkalemia, hyperreninemia, and hypovolemic shock. These adrenal crises

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✉ Paola Concolino
paola.concolino@policlinicogemelli.it

[1] Polo Scienze per Immagini, di Laboratorio e Infettivologiche, Università Cattolica del Sacro Cuore, Fondazione Policlinico Universitario Agostino Gemelli, Largo Francesco Vito 1, 00168 Rome, Italy

[2] Institute of Biochemistry and Clinical Biochemistry, Catholic University of Rome, Largo A. Gemelli 8, 00168 Rome, Italy

may prove fatal if proper medical care is not delivered [1, 4, 5].

An increase of about 1–2% in 21-hydroxylase activity, compared to SW CAH, leads to SV CAH (25% of all CL CAH patients) and in this case, aldosterone is produced in an adequate amount, preventing an SW crisis. The typical result in severely affected girls is ambiguous or male-appearing external genitalia with perineal hypospadias, chordee, and undescended testes [4]. Finally, NC CAH refers to a partial 21-hydroxylase enzyme deficiency, typically around 20–50% of normal enzyme function. Though not requiring therapy for survival, cortisol production by the adrenal glands is insufficient to adequately suppress adrenocorticotropic hormone (ACTH) over-secretion and the shunting of precursor steroids leads to hyperandrogenemia [6, 7].

CAH due to 21-hydroxylase deficiency is inherited in an autosomal recessive manner. About 65–70% of CAH patients are compound heterozygous, and the milder of the two affected alleles is usually expressed phenotypically [8, 9]. Many studies have addressed a strong correlation between genotype and phenotype. However, there is well-documented evidence that divergence between genotype and phenotype can occur in some cases [10–13]. Over the last few years, much progress has been made in mutation detection and various screening strategies have been reported. This has led to the identification of a large number of variants and their classification [14–16].

Depending on residual activity of mutant enzyme, *CYP21A2* variants are classified in four groups (Null, A, B, and C). Variants of Null and A groups are both associated with the SW form of the disease. However, while Null variants show 0% enzyme activity during in vitro assay, group A variants, with the IVS2-13A/C>G mutation, preserve a minimal (< 1%) residual activity. Finally, group B (1–5% enzyme activity) and group C (20–50% enzyme activity) variants are related to the SV and the NC form, respectively [9].

In this review, we focus on molecular genetic features of the disease, performing an extensive survey of all clinically classified variants modifying the whole sequence of the *CYP21A2* gene. Our aim is to offer a very useful tool for clinical and genetic specialists in order to make clinical diagnosis and genetic counseling of 21-hydroxylase deficiency easier.

2 Genetics of 21-Hydroxylase Deficiency

2.1 Structure of the *CYP21A2* Gene and Gene Locus

The gene encoding 21-hydroxylase, *CYP21A2*, is located in the human leukocyte antigen (HLA) class III region on the short arm of chromosome 6p21.3 [17]. In this region, four tandemly arranged genes—serine/threonine kinase *RP*, complement *C4*, steroid 21-hydroxylase *CYP21*, and tenascin *TNX*—are organized as a genetic unit designated as an RCCX module. In an RCCX bimodular haplotype, duplication of the RCCX module occurs and the orientation of genes, from telomere to centromere, is *RP1-C4A-CYP21A1P-TNXA-RP2-C4B-CYP21A2-TNXB*. *RP1* encodes a putative nuclear protein similar to DNA helicase, *C4A* and *C4B* genes encode the fourth component of complement, and *TNXB* encodes an extracellular matrix protein, tenascin X, which overlaps the *CYP21A2* gene on the opposite strand. The three pseudogenes, *CYP21A1P-TNXA* and *RP2*, located between the two *C4* loci, do not encode functional proteins [18–20]. Both the *CYP21A2* gene and *CYP21A1P* pseudogene contain ten exons spaced over 3.1 kb. Their nucleotide sequences are 98% identical in exons and approximately 96% identical in introns [21]. In the Caucasian population, bimodular and monomodular RCCX organizations are present in about 69 and 17% of chromosome 6, respectively, while trimodular RCCX haplotypes have a frequency of about 14% [19]. A trimodular haplotype carrying one copy of the *CYP21A1P* pseudogene and two copies of the *CYP21A2* gene has been described in different nationalities [22–28].

2.2 Mutations Causing 21-Hydroxylase Deficiency

Due to the high degree of sequence homology and the tandem repeating order of the RCCX module sequence, the *CYP21A2* region seems to be the most likely area for misalignments to occur at meiosis, which would generate illegitimate genetic recombinations or unequal crossing over. In fact, intergenic recombinations are responsible for 95% of the mutations associated with 21-hydroxylase deficiency [5, 29, 30]. Mutations that are not apparently gene conversions account for 5–10% of 21-hydroxylase deficiency alleles in most populations [5]. Finally, about 1% of *CYP21A2*-inactivating mutations arise de novo [5].

2.2.1 Microconversion Events

Among the intergenic recombinations, approximately 75% are represented by mutations generally present in the *CYP21A1P* pseudogene and possibly transferred to the functional *CYP21A2* gene by microconversion events [31]. The deleterious mutations in *CYP21A1P* include the following: a nucleotide substitution (g.5774A/C>G; IVS2-13A/C>G) before the end of intron 2 that results in aberrant splicing of RNA, an 8-bp deletion in exon 3 (g.5826_5833 delGAGACTAC, p.G110Vfs?), an insertion of one nucleotide in exon 7 (g.6882_6883insT, p.L307Ffs?), and a nonsense mutation in exon 8 (g.7114

C>T, p.Q318X). We also reported that the 238-amino acid deletion is present in the pseudogene, with a frequency of 4.5% [28], and as described, this pathogenic pseudogene deriving mutation can also be transferred to the active gene by a microconversion event [32]. Lastly there are also six missense mutations in *CYP21A1P* (p.P30L, p.I172N, p.I136N, p.V237E, p.M239K, and p.R356W), transferred to *CYP21A2,* that have all been observed in patients suffering from 21-hydroxylase deficiency, and each of these is related to a different clinical form of CAH [33].

We would like to underline that not all pseudogenes harbor these mutations. In fact, in the last few years, some studies investigating the existence of *CYP21A2* wild-type loci in the *CYP21A1P* pseudogene reported specific allelic frequencies in the studied populations [28, 34–36]. In an Italian population, we reported frequencies of 3, 27, 16, and 57% for p.P30, p.V281, p.Q318, and p.R356 positions, respectively. No more wild-type loci have been detected [28]. This information should be considered during the planning of molecular testing for 21-hydroxylase deficiency [28, 37].

The remaining 20–25% of mutations are *CYP21A2* whole gene deletions or *CYP21A1P/CYP21A2* chimeric genes. Both events are the consequence of an unequal crossing over [5].

2.2.2 *CYP21A2* Gene Deletion

Some reports have shown that complete deletion of *CYP21A2* in Caucasians appears as a *TNXA/TNXB* hybrid gene, resulting in a recessive form of Ehlers–Danlos syndrome (EDS) caused by tenascin-X deficiency [38–41]. A total of nine types of EDS were outlined, and the form related to TNXB deficiency involves features of marked skin laxity, pronounced joint hypermobility, and severe bruising [39]. The complete deletion of the *CYP21A2* gene changes the genomic organization in the RCCX module to the status of *C4A-CYP21A1P-TNXA/TNXB*. To date, at least nine kinds of chimeric *TNXA/TNXB* genes have been identified and associated with EDS as well as CAH [42].

2.2.3 *Chimeric CYP21A1P/CYP21A2 Genes*

A 26- or 32-kb deletion (depending on whether *C4B* is the short or long gene), involving the 3′ end of *CYP21A1P*, all of the *C4B* gene, and the 5′ end of *CYP21A2*, produces a single nonfunctional chimeric gene with its 5′ and 3′ ends corresponding to *CYP21A1P* and *CYP21A2*, respectively [43]. Several mutations within the *CYP21A1P* portion render such a gene incapable of encoding an active enzyme. To date, nine different chimeric *CYP21A1P/CYP21A2* genes have been found and characterized in different studies [44]. Chimeric *CYP21A1P/CYP21A2* genes have been classified into two categories, classic and attenuated, depending on the location of the junction sites relative to pseudogene mutation IVS2-13A/C>G within intron 2 [44]. Seven chimeras (CH-1, CH-2, CH-3, CH-5, CH-6, CH-7, and CH-8) carry the pseudogene specific mutation IVS2-13A/C>G in intron 2 and thus are associated with a severe SW phenotype [42–51]. This group of chimeras is common among CAH patients of Caucasian origin and has been referred to as the classic or common type of chimera [44].

In contrast, 21-hydroxylase enzyme activity is less severely impaired if the junction site occurs upstream of the IVS2-13A/C>G variant. By carrying a weak *CYP21A1P* promoter and an NC mutation, p.P30L, at exon 1 only, the chimera partially retains 21-hydroxylase activity, producing a milder clinical phenotype. In fact, CH-4 and CH-9, two uncommon chimeras, fall into the group of attenuated chimeras [44, 52].

2.2.4 *CYP21A2 Gene Duplication*

An unequal meiotic crossing-over event produces the duplication of *CYP21A2* gene. In fact, a trimodular haplotype carrying one copy of the *CYP21A1P* pseudogene and two copies of the *CYP21A2* gene has been described in different nationalities [22–28]. In most cases, the *CYP21A2* copy downstream of the *TNXA* gene showed a wild-type sequence or the IVS-13A/C>G mutation, while the *CYP21A2* gene next to *TNXB* carried the p.Q318X mutation [23–27]. Recently, the presence of this trimodular haplotype explained the lack of genotype–phenotype correlation in individuals of different families, suggesting the importance of trimodular haplotype assessment in CAH genetic diagnosis [53, 54]. In fact, the existence of rare trimodular haplotypes is a condition that should be strongly considered when CAH genetic analysis is offered at the prenatal level in order to define the real risk of the fetus and avoid further investigations at birth. It is very important to investigate this condition also when analysis is offered at the preconceptional level, in order to avoid unnecessary prenatal therapy.

We think that a robust assay for Copy Number Variation (CNV) assessment should be offered as an integral part of 21-hydroxylase deficiency genetic testing. Differently, most laboratories perform, as a sole investigation, *CYP21A2* gene sequencing. However, because individuals carrying the p.Q318X variant frequently have a duplication of the *CYP21A2* gene, when this mutation is detected, it is always recommended to established the correct number of copies. As reported above, providing this information is crucial in genetic counseling of couples as well as in prenatal diagnosis.

2.2.5 Novel *CYP21A2* Variants

Variants that are not apparently gene conversions account for 5–10% of 21-hydroxylase deficiency alleles in most populations [5]. These novel mutations are easy to detect using automated sequencing technologies in specialized laboratories and thus have been reported at an increased rate over the past few years. The functional effects of a new mutation are generally assessed in vitro by recreating it in *CYP21A2* complementary DNA (cDNA) and expressing the mutant cDNA using an appropriate expression vector in mammalian cells. A radioactive assay allows evaluation of the residual enzymatic activities of the mutant protein towards the two natural substrates of steroid 21-hydroxylase, 17-hydroxyprogesterone (17-OHP) and progesterone. Mutant enzyme activity is expressed as a percentage of the wild-type enzyme [55–58]. This approach allows correlation of each mutation to a different clinical form of CAH.

Over the last few years, much progress has been made towards predicting protein stabilities and correlating them to protein activities. Homology modeling and fast energetic calculations have emerged as useful tools to evaluate, through structure-based methods, the impairment of protein stability. Human 21-hydroxylase models have been built based on the available low homology CYP protein families. Structural features deduced from the models were in good correlation with clinical severity of mutations, confirming the applicability of a modeling approach in assessment of new *CYP21A2* mutations [59–67].

3 *CYP21A2* Variants Review

A list of some *CYP21A2* variants can be found in the *CYP21A2* database created by the Human Cytochrome P450 (*CYP*) Allele Nomenclature Committee (https://www.pharmvar.org/htdocs/archive/cyp21.htm) [68]. For each variant, the enzyme residual activity and the associated clinical phenotype is reported. The last update of this database was performed in 2011, after the release of our review [16]. However, over the last few years, many other novel *CYP21A2* mutations have been described.

The aim of this work is to provide a complete focus on all pathogenic *CYP21A2* variants reported to date in the literature. Our purpose is to offer very useful information for clinical and genetic specialists, making CAH diagnosis and genetic counseling easier. For this reason, we choose to consider only variants reported in literature papers where clinical and molecular information (genotype–phenotype correlation, functional characterization, structural study) are generally provided. So we exclude all those variants, reported in common databases, missing such data. We searched the National Center for Biotechnology Information (NCBI) PubMed literature database for articles in English, using the keywords "*CYP21A2* new mutation/variant" and "*CYP21A2* novel mutation/variant." More than 100 articles, published from January 2011 to December 2017, were read and evaluated for the real presence of new variants. In our update, we decided to report all detected variants according to Locus Reference Genomic (LRG) *CYP21A2* reference sequences: NG_007941.2 and NM_000500.6. Table 1 provides a list of all *CYP21A2* exonic variants with an assessed pathogenic role; to each variant we associate molecular and clinical information, reporting in the reference section the papers providing the greatest contribution in classifying that specific variant. Similarly, Table 2 shows all *CYP21A2* variants affecting the non-coding regions and their clinical significance. Finally, *CYP21A2* exonic polymorphisms were also assessed in the present review and are reported in Table 3.

3.1 *CYP21A2* Variants Distribution

A total of 212 *CYP21A2* disease-causing variants, affecting the coding region of the gene, are listed in Table 1, while 21 pathogenic variants within non-coding regions are reported in Table 2. According to our results, these 233 variants are scattered throughout the entire sequence of the *CYP21A2* gene and consist of (a) 65.2% missense mutations, (b) 9.4% nonsense mutations, (c) 7.7% splicing affecting variants, (d) 13.3% frameshift mutations, (e) about 3% small in-frame deletions/insertions (del/ins), and, finally, (f) 1.3% variants involving untranslated regions (promoter and 3′UTR) (Fig. 1a). The exons harboring the major number of mutations were the 10, 8, and 7, respectively. On the contrary, exon 5 was the less affected region of the gene (Fig. 2).

Codons 1 and 483, in exon 1 and 10, respectively, were affected by the highest number of mutations. In particular, codon 1 is affected by four missense mutations all related to the SW form (p.M1I, p.M1L, p.M1V, and p.M1T), while three missense (p.R483W, p.R483Q, and p.R483P) and one frameshift variant (p.R483P*fs*?) fall into codon 483 (Table 1).

The highest number of known variants affecting the splicing process is within intron 2 (27.8%) (Table 2); this is probably due to the fact that it is the most polymorphic region of the whole *CYP21A2* gene.

3.2 Variant–Phenotype Association

As the aim of this work is to provide a useful tool for clinical and genetic specialists, we focused on associating a clinical, specific phenotype to each variant. For this purpose, diverse papers reporting a specific variant were

Table 1 *CYP21A2* exonic variants and their clinical significance

Genomic reference sequence[≠]	cDNA reference sequence[&]	Exon	Codon	Protein change	Enzyme activity (17-OHP/prog)[Δ]	Structural evaluation[●]	Phenotype prediction	References[Ψ]
g.5119A>C	c.1A>C	E1	1	p.M1L	nd/nd	Yes[a]	SW	Tardy et al. (2007)
g.5119A>G	c.1A>G	E1	1	p.M1V	nd/nd	Yes[a]	SW	Tardy et al. 2007
g.5120T>C	c.2T>C	E1	1	p.M1T	nd/nd	No	SW	Toraman et al. 2013
g.5121G>A	c.3G>A	E1	1	p.M1I	nd/nd	Yes[a]	SW	Usui et al. (2004)
g.5164C>T	c.46C>T	E1	16	p.R16C	95 ± 3/81 ± 3	No	Very mild NC	de Paula Michelatto et al. (2016)
g.5174G>A	c.56G>A	E1	19	p.W19X	nd/nd	No	SW	Kharrat et al. (2004), Bidet et al. (2009)
g.5182_5183*ins*T	c.64_65*ins*T	E1	22	p.W22L*fs*?	nd/nd	No	SW	Ezquieta et al. (1999)
g.5183G>A	c.66G>A	E1	22	p.W22X	nd/nd	No	SW	Lajic et al. (1996), Di Pasquale et al. (2007)
g.5200_5212*del* CACCTCCCGCCTC	c.81_93*del* CACCTCCCGCCTC	E1	28	p.H28L*fs*?	nd/nd	No	SW	Kharrat et al. (2005)
g.5200_5201*ins*C	c.82_83*ins*C	E1	28	p.H28P*fs*?	nd/nd	No	SW	Lau et al. (2001)
g.5207C>T	c.89C>T	E1	30	p.P30L	34.1 ± 18.5/ 38.8 ± 22.2	Yes[a,b,c]	SV	Tardy et al. (2010)
g.5207C>A	c.89C>A	E1	30	p.P30Q	0.2 ± 0.2/0 ± 0	Yes[a,b,c]	SW	Lajic et al. (1999)
g.5231A>T	c.113A>T	E1	38	p.H38L	nd/nd	Yes[a,c]	SV	Oriola et al. (2011)
g.5239C>T	c.121C>T	E1	41	p.Q41X	nd/nd	No	SW	Marino et al. (2011)
g.5244*del*C	c.126_127*del*C	E1	42	p.P42P*fs*?	nd/nd	No	SW	Zeng et al. (2004)
g.5252C>T	c.134C>T	E1	45	p.P45L	105 ± 10.6/nd	Yes[c]	SV*	Nermoen et al. (2012), Brønstad et al. (2014)
g.5253_5254insC	c.135_136*ins*C	E1	46	p.I46N*fs*?	nd/nd	No	SV	Loidi et al. (2006)
g.5258A>G	c.140A>G	E1	47	p.Y47C	nd/nd	Yes[a,c]	NC	Tardy et al. (2007)
g.5261*del*T	c.141*del*T	E1	48	p.L48R*fs*?	nd/nd	No	SW	Krone et al. (1999)
g.5137_5146*del* CTGCTGCTGC	c.19_28*del* CTGCTGCTGC	E1	48	p.L48X	nd/nd	No	SW	Baradan-Heravi et al. (2007)
g.5278A>T	c.160A>T	E1	54	p.K54X	nd/nd	No	SW	Concolino et al. (2009)
g.5284G>A	c.166G>A	E1	56	p.G56R	0.7 ± 0.2/1.4 ± 0.5	Yes[a,c]	SV	Soardi et al. (2008)
g.5293T>A	c.175T>A	E1	59	p.Y59N	nd/nd	Yes[a,c]	SW	Tardy et al. (2007)
g.5303A>T	c.185A>T	E1	62	p.H62L	44.5 ± 28.0/20.7 ± 5	Yes[a]	NC	Soardi et al. (2008)
g.5309G>A	c.191G>A	E1	64	p.G64E	No activity	Yes[a]	SW	Ohlsson et al. (1999)
g.5417_5424*dup*	c.202_209*dup*	E2	68–70	p.V68_V70*dup*	nd/nd	Yes[d]	SW	Dubey et al. (2009)
g.5420G>C	c.205G>C	E2	69	p.V69L	nd/nd	Yes[c]	SV	Wang et al. (2016)
g.5435A>T	c.220A>T	E2	74	p.K74X	nd/nd	No	SW	Nunez et al. (1999)
g.5445T>C	c.230T>C	E2	77	p.I77T	3 ± 2/5 ± 3	Yes[a]	SV	Krone et al. (2005)

Table 1 continued

Genomic reference sequence[≠]	cDNA reference sequence[&]	Exon	Codon	Protein change	Enzyme activity (17-OHP/prog)[Δ]	Structural evaluation[●]	Phenotype prediction	References[Ψ]
g.5483G>T	c.268G>T	E2	90	p.G90V	No activity	Yes[a]	SW	Nunez et al. (1999)
g.5486A>G	c.271A>G	E2	91	p.R91G	nd/nd	No	SW	Wang et al. (2016)
g.5486A>T	c.271A>T	E2	91	p.R91X	nd/nd	No	SW	New et al. (2013)
g.5504*ins*T	c.289*ins*T	E2	97	p.Y97L*fs*?	nd/nd	No	SW	Balraj et al. (2013)
g.5788C>A	c.291C>A	E2	97	p.Y97X	nd/nd	No	SW	Krone et al. (1998)
g.5798_5799TC>AA	c.301_302TC>AA	E3	101	p.S101N	94 ± 3/74 ± 2	No	Very mild NC	de Paula Michelatto et al. (2016)
g.5811C>T	c.314C>T	E3	105	p.P105L	62 ± 9/64 ± 12	Yes[c]	NC	Nikoshkov et al. (1997)
g.5817T>G	c.320T>G	E3	107	p.L107R	0.4 ± 0.1/0.3 ± 0.1	Yes[a]	SW	Soardi et al. (2008)
g.5817T>G	c.320T>A	E3	107	p.L107Q	nd/nd	Yes[c]	SV	Bruque et al. (2016)
g.5826_5833*del* GAGACTAC	c.329_336*del* GAGACTAC	E3	110	p.G110V*fs*?	nd/nd	No	SW	Higashi et al. (1988)
g.5835C>A	c.338C>A	E3	113	p.S113Y	nd/nd	Yes[a,c]	NC	Haider et al. (2013), Bruque et al. (2016)
g.5835C>T	c.338C>T	E3	113	p.S113F	4 ± 1/4 ± 2	Yes[a,c]	NC	de Paula Michelatto et al. (2016)
g.5853A>G	c.356A>G	E3	119	p.H119R	31.6 ± 8/32.5 ± 7	Yes[a]	NC	Concolino et al. (2009)
g.5858A>C	c.361A>C	E3	121	p.K121Q	14±5/19.5±4	Yes[a]	NC	Riepe et al. (2008)
g.5862T>C	c.365T>C	E3	122	p.L122P	1.42 ± 2.13/− 1.86 ± 5.19	Yes[c]	SW	Massimi et al. (2014)
g.5862T>C	c.365T>G	E3	122	p.L122R	nd/nd	Yes[c]	NC	Bruque et al. (2016)
g.5868G>A	c.371G>A	E3	124	p.R124H	nd/nd	Yes[a]	NC	Usui et al. (2004)
g.5868G>A	c.371G>A	E3	124	p.R124C	nd/16 ± 0.6	No	SV	Krone et al. (2013)
g.5883T>C	c.386T>C	E3	129	p.L129P	nd/nd	No	SW	Milacic et al. (2015)
g.5891C>T	c.394C>T	E3	132	p.R132C	35.40 ± 7.4/ 15.5 ± 2.70	Yes[a]	NC	Taboas et al. (2014)
g.5892C>T	c.395G>A	E3	132	p.R132H	nd/nd	Yes[c]	NC	Bruque et al. (2016)
g.5913T>A	c.416T>A	E3	139	p.V139E	0.7 ± 1.3/0.5 ± 0.6	Yes[a,c]	SW	Barbaro et al. (2012)
g.5915G>A	c.418G>A	E3	140	p.E140K	11.30 ± 2.4/nd	Yes[d]	SW*	Brønstad et al. (2014)
g.5918C>T	c.421C>T	E3	141	p.Q141X	nd/nd	No	SW	Krone et al. (2013)
g.5922T>C	c.425T>C	E3	142	p.L142P	0.4 ± 0.2/0.4 ± 0.1	Yes[a,c]	SW	Soardi et al. (2008)
g.5928A>C	c.431A>C	E3	144	p.Q144P	nd/nd	Yes[c]	SW	Wang et al. (2016)
g.5936T>C	c.439T>C	E3	147	p.C147R	4.3 ± 0.9/3.6 ± 1.8	Yes[a,c]	SV	Barbaro et al. (2012)
g.6049C>T	c.445C>T	E4	149	p.R149C	35.8 ± 14.6/ 47.3 ± 12.9	Yes[a,c]	NC	Taboas et al. (2014)
g.6050G>C	c.446G>C	E4	149	p.R149P	23.4 ± 1.7/16.9 ± 2	Yes[c]	NC	Chu et al. (2014)
g.6053T>G	c.449T>G	E4	150	p.M150R	17.66 ± 1.87/ 4.57 ± 1.87	Yes[c]	NC	Massimi et al. (2014)

Table 1 continued

Genomic reference sequence[≠]	cDNA reference sequence[&]	Exon	Codon	Protein change	Enzyme activity (17-OHP/prog)[Δ]	Structural evaluation[●]	Phenotype prediction	References[Ψ]
g.6061C>T	c.457C>T	E4	153	p.Q153X	nd/nd	No	SW	Wang et al. (2016)
g.6082_6083*ins*A	c.478_479*ins*A	E4	160	p.I160N*fs*?	nd/nd	No	SW	Janner et al. (2006)
g.6085G>T	c.481G>T	E4	161	p.E161X	0.29 ± 0.11/0.18 ± 0	No	SW	Massimi et al. (2014)
g.6093*del*A	c.489*del*A	E4	163	p.E163D*fs*?	nd/nd	No	SW	Hong et al. (2015)
g.6095T>C	c.491T>C	E4	164	p.F164S	nd/nd	Yes[c]	SV	Wang et al. (2016)
g.6097T>C	c.493T>C	E4	165	p.S165P	nd/nd	Yes[c]	NC	Milacic et al. (2015)
g.6101T>C	c.497T>C	E4	166	p.L166P	0.3 ± 0.06/0.4 ± 0.6	Yes[b,c]	SW	Robins et al. (2007)
g.6104T>C	c.500T>C	E4	167	p.L167P	0.7 ± nd/0.4 ± nd	Yes[a,c]	SW	Tardy et al. (2010)
g.6107C>A	c.503C>A	E4	168	p.T168N	nd/nd	Yes[a]	NC	Vrzalová et al. (2010)
g.6109T>C	c.505T>C	E4	169	p.C169R	0.10 ± 0.02/0 ± 2	Yes[a]	SV	Grischuk et al. (2006)
g.6109_6110*del* TG*ins*A	c.505_506*del*TG*ins*A	E4	169	p.C169T*fs*?	nd/nd	No	SW	Witchel et al. (1999)
g.6111C>A	c.507C>A	E4	169	p.C169X	nd/nd	No	SW	Vrzalová et al. (2010)
g.6112_6113*ins*A	c.508_509*ins*A	E4	170	p.S170L*fs*?	nd/nd	No	SW	Billerbeck et al. (2002)
g.6116T>A	c.512T>A	E4	171	p.I171N	0.7 ± 0.3/0.6 ± 0.03	Yes[a,b]	SV*	Barbaro et al. (2006)
g.6119T>A	c.515T>A	E4	172	p.I172N	4.3 ± 17/4.4 ± 1.8	Yes[a]	SV	Tardy et al. (2010)
g.6136G>A	c.532G>A	E4	178	p.G178R	0.4 ± 0.5/0 ± 0.6	Yes [a,c]	SW	Grischuk et al. (2006)
g.6137G>C	c.533G>C	E4	178	p.G178A	19% ± nd/0 ± nd	Yes[a,b,c]	SV*	Wang et al. (2016), Lobato et al. (1999)
g.6241*del*C	c.549*del*C	E5	183	p.D183E*fs*?	nd/nd	No	SW	Stikkelbroeck et al. (2003)
g.6263T>C	c.571T>C	E5	191	p.Y191H	37.1 ± 7/25.8 ± 9	Yes[c]	NC	Concolino et al. (2012)
g.6273T>A	c.581T>A	E5	194	p.I194N	33.2 ± 9/46.7 ± 10	Yes[a,c]	NC	Concolino et al. (2009)
g.6279_6281*del*AGG	c.587_589*del*AGG	E5	196	p.E196*del*	6.0 ± 4.5/23 ± 2.3	No	Severe NC	Nikoshkov et al. (1998)
g.6296A>G	c.604A>G	E5	202	p.S202G	85 ± 2/81 ± 3	Yes[c]	Very mild NC	de Paula Michelatto et al. (2016)
g.6328_6329*ins*T	c.636_637*ins*T	E5	213	p.P213S*fs*?	nd/nd	No	SW	Usui et al. (2004)
g.6451*del*A	c.659*del*A	E6	220	p.N220I*fs*?	nd/nd	No	SW	Girgis et al. (2013)
g.6463C>T	c.670C>T	E6	224	p.R224W	51.9 ± 9/45.6 ± 8	Yes[a]	NC	Concolino et al. (2009)
g.6466_6467*del*AG	c.673_674*del*AG	E6	225	p.R225A*fs*?	nd/nd	No	SW	New et al. (2013)
g.6473_6474*ins*A	c.680_681*ins*A	E6	227	p.K227K*fs*?	nd/nd	No	SW	Krone et al. (2006)
g.6475C>T	c.682C>T	E6	228	p.Q228X	nd/nd	No	SW	Ezquieta et al. (2002)
g.6482T>C	c.689T>C	E6	230	p.I230T	63.1 ± 22.3/70.6 ± 17	Yes[a,c]	NC	Tardy et al. (2010)
g.6490A>G	c.697A>G	E6	233	p.R233G	8.0 ± 2/2.0 ± 1	Yes[a,b]	Severe NC	Barbaro et al. (2015)
g.6491G>A	c.698G>A	E6	233	p.R233K	15 ± nd/8.1 ± nd	Yes[d]	SV	Tardy et al. (2010)
g.6500T>A	c.707T>A	E6	236	p.I236N	1 ± 0.1/2.4 ± 1.4	Yes[a,b]	SV	Robins et al. (2005)
g.6503T>A	c.710T>A	E6	237	p.V237E	0 ± 0/0.1 ± 0.3	Yes[a,b]	SW	Robins et al. (2005)

Table 1 continued

Genomic reference sequence[≠]	cDNA reference sequence[&]	Exon	Codon	Protein change	Enzyme activity (17-OHP/prog)[Δ]	Structural evaluation[●]	Phenotype prediction	References[Ψ]
g.6505_6507*del*GAG	c.712_714*del* GAG	E6	238	p.E238*del*	nd/nd	No	SW	Concolino et al. (2013)
g.6699*del*A	c.737*del*A	E7	246	p.E246G*fs*?	nd/nd	No	SW	Koyama et al. (2002)
g.6708T>C	c.746T>C	E7	249	p.V249A	nd/nd	Yes[a,c]	NC	Concolino et al. (2010)
g.6744T>C	c.782T>C	E7	261	p.L261P	nd/nd	Yes[a,c]	SW	Loke et al. (2001)
g.6746C>T	c.784C>T	E7	262	p.Q262X	nd/nd	No	SW	Ohlsson et al. (1999)
g.6749_6750*ins*C	c.787_788*ins*C	E7	263	p.G263A*fs*?	nd/nd	No	SW	Finkielstain et al. (2011)
g.6756C>T	c.794C>T	E7	265	p.A265V	92.0 ± 1.40/100 ± 4.3	Yes[a,c]	NC	Bleicken et al. (2009)
g.6761C>T	c.800C>T	E7	267	p.P267L	97 ± 1/87 ± 7	Yes[c]	Very mild NC	de Paula Michelatto et al. (2016)
g.6803 G>T	c.841G>T	E7	281	p.V281L	65.6 ± 10.9/ 63.4 ± 8.7	Yes[a]	NC	Tardy et al. (2010), Wu et al. (1991)
g.6803G>C	c.841G>C	E7	281	p.V281L	nd/nd	No	NC	Barbat et al. (1995)
g.6804T>G	c.842T>G	E7	281	p.V281G	3.9 ± 1.7/3.9 ± 2	Yes [a]	SV	Lajić et al. (2001)
g.6806C>A	c.844C>A	E7	282	p.H282N	1.6 ± 6/2.7 ± 5	No	SV	Concolino et al. (2012)
g.6809A>C	c.847A>C	E7	283	p.M283L	nd/nd	Yes[a,c]	NC	Ezquieta et al. (2002)
g.6809A>G	c.847A>G	E7	283	p.M283V	16.2 ± 9.3/19 ± 6.8	Yes[a,c]	NC	Taboas et al. (2014)
g.6833G>T	c.871G>T	E7	291	p.G291C	0 ± nd/0 ± nd	Yes[a]	SW	Nunez et al. (1999)
g.6833G>A	c.871G>A	E7	291	p.G291S	0.8 ± 0.4/0.8 ± 0.4	Yes[a]	SW	Stikkelbroeck et al. (2003)
g.6833G>C	c.871G>C	E7	291	p.G291R	0.5 ± 0.7/0.7 ± 0.2	No	SW	Barbaro et al. (2012)
g.6837G>A	c.875G>A	E7	292	p.G292D	0.5 ± 0.2/0.7 ± 0.4	Yes[a]	SW	Tardy et al. (2010)
g.6846C>A	c.884C>A	E7	295	p.T295N	5.0 ± 1.6/0.8 ± 0.4	Yes[a]	SW	Barbaro et al. (2012)
g.6860C>T	c.898C>T	E7	300	p.L300F	9.5±6.4/4.4±2.5	Yes[a]	SV	Lajić et al. (2001)
g.6864C>A	c.902C>A	E7	301	p.S301Y	nd/nd	Yes[a,c]	NC	Stikkelbroeck et al. (2003)
g.6866T>C	c.904T>C	E7	302	p.W302R	0.1 ± 0.2/0 ± 0.5	Yes[c]	SW	Grischuk et al. (2006)
g.6867G>C	c.905G>C	E7	302	p.W302S	3.0 ± 0.3/3 ± 0.5	No	SV	Bleicken et al. (2009)
g.6867G>A	c.906G>A	E7	302	p.W302X	nd/nd	No	SW	Levo et al. (1997)
g.6872G>A	c.910G>A	E7	304	p.V304M	46±18/26±10	Yes[a,c]	NC	Lajić et al. (2002)
g.6873T>A	c.911T>A	E7	304	p.V304E	nd/nd	Yes[c]	SW	Wang et al. (2016)
g.6876T>C	c.914T>C	E7	305	p.V305D	nd/nd	Yes[a,c]	SV	Haider et al. (2013), Bruque et al. (2016)
g.6878T>G	c.916T>G	E7	306	p.F306V	nd/nd	Yes[a,c]	SV	Haider et al. (2013), Bruque et al. (2016)
g.6881T>G	c.919T>G	E7	307	p.L307V	nd/nd	Yes[a,c]	NC	New et al. (2013)
g.6882_6883*ins*T	c.920_921*ins*T	E7	307	p.L307F*fs*?	nd/nd	No	SW	Higashi et al. (1988), Ezquieta et al. (2002)
g.6884C>T	c.922C>T	E7	308	p.L308F	0.2 ± 0.3/0.1 ± 0.3	Yes[a,c]	SV	Barbaro et al. (2012)

Table 1 continued

Genomic reference sequence[≠]	cDNA reference sequence[&]	Exon	Codon	Protein change	Enzyme activity (17-OHP/prog)[Δ]	Structural evaluation[●]	Phenotype prediction	References[Ψ]
g.7105C>T	c.943C>T	E8	315	p.Q315X	nd/nd	No	SV	Dolzan et al. (2005)
g.7108C>T	c.946C>T	E8	316	p.R316X	nd/nd	No	SW	Lee et al. (1998)
g.7109G>T	c.947G>T	E8	316	p.R316L	nd/nd	Yes[a,c]	SV	New et al. (2013)
g.7111C>A	c.949C>A	E8	317	p.L317M	nd/nd	Yes[a,c]	NC	Deneux et al. (2001)
g.7111C>G	c.949C>G	E8	317	p.L317V	nd/nd	Yes[a,c]	NC	Bojunga et al. (2005)
g.7114 C>T	c.952 C>T	E8	318	p.Q318X	nd/nd	No	SW	Globerman et al. (1988)
g.7120G>A	c.959G>A	E8	320	p.E320K	4.6 ± 1.8/4.5 ± 2.6	Yes[a]	SV	Tardy et al. (2010)
g.7124T>C	c.962T>C	E8	321	p.L321P	nd/nd	Yes[a,c]	SW	Haider et al. (2013), Bruque et al. (2016)
g.7127A>G	c.965A>G	E8	322	p.D322G	18.0 ± 1.2/27 ± 4.7	Yes[a]	NC	Bleicken et al. (2009)
g.7150_7159*del* TCCAGCTCCC	c.988_997*del* TCCAGCTCCC	E8	330	p.S330G*fs*?	nd/nd	No	SW	Lee et al. (1998)
g.7162*del*G	c.1000*del*G	E8	334	p.V334S*fs*?	nd/nd	No	SW	Krone et al. (2013)
g.7166C>T	c.1004C>T	E8	335	p.P335L	nd/nd	Yes[c]	NC	Bruque et al. (2016)
g.7170C>G	c.1008C>G	E8	336	p.Y336X	nd/nd	No	SW	Bernal González et al. (2006)
g.7178G>A	c.1016G>A	E8	339	p.R339H	nd/nd	Yes[a]	NC	Helmberg et al. (1992)
g.7183C>T	c.1021C>T	E8	341	p.R341W	5 ± 0.4/4 ± 3	Yes[a]	Severe NC	Barbaro et al. (2015)
g.7184G>C	c.1022G>C	E8	341	p.R341P	0.7 ± 0.3/0.7 ± 0.2	Yes[a]	SV	Barbaro et al. (2006), Pinto et al. (2003)
g.7213G>A	c.1051G>A	E8	351	p.E351K	1.1 ± 0.5/1.2 ± 0.3	Yes[a,b]	SV	Krone et al. (2005)
g.7214A>T	c.1052A>T	E8	351	p.E351V	nd/nd	No	SW	De Carvalho et al. (2016)
g.7220T>G	c.1058T>G	E8	353	p.L353R	nd/nd	Yes[a,d]	SW	Abid et al. (2008)
g.7222C>T	c.1060C>T	E8	354	p.R354C	nd/nd	Yes[a]	SW	Krone et al. (2000)
g.7223G>A	c.1061G>A	E8	354	p.R354H	10 ± 5/0 ± nd	Yes[a]	SW	Nunez et al. (1999)
g.7223G>C	c.1061G>C	E8	354	p.R354P	nd/nd	No	SW	Dubey et al. (2017)
g.7228 C>T	c.1066 C>T	E8	356	p.R356W	No activity/no activity	Yes[a]	SW	Chiou et al. (1990)
g.7229G>C	c.1067G>C	E8	356	p.R356P	0.15 ± 0.30/ 0.15 ± 0.30	Yes[a,b]	SW	Lajić et al. (1997)
g.7229G>A	c.1067G>A	E8	356	p.R356Q	0.65 ± 0.44/ 1.1 ± 0.94	Yes[a]	SV	Lajić et al. (1997)
g.7247C>T	c.1085C>T	E8	362	p.A362V	No activity/No activity	Yes[a]	SW	Ohlsson et al. (1999)
g.7250T>G	c.1088T>G	E8	363	p.L363W	nd/nd	Yes[a]	SV	Levo et al. (2001)
g.7255C>A	c.1093C>A	E8	365	p.H365N	nd/nd	No	NC	Khajuria et al. (2016)
g.7255C>T	c.1093C>T	E8	356	p.H365Y	Minimal activity/ minimal activity	Yes[a,b]	SW	Zeng et al. (2004), Gaffney et al. (2011)
g.7258C>T	c.1096C>T	E8	366	p.R366C	37 ± 7/28 ± 4	Yes[a,b]	NC	Barbaro et al. (2015)

Table 1 continued

Genomic reference sequence[≠]	cDNA reference sequence[&]	Exon	Codon	Protein change	Enzyme activity (17-OHP/prog)[Δ]	Structural evaluation[●]	Phenotype prediction	References[Ψ]
g.7259G>A	c.1097G>A	E8	366	p.R366H	nd/nd	Yes[a,d]	NC	Khattab et al. (2016)
g.7267C>T	c.1105C>T	E8	369	p.R369W	45.8 ± 1.8/ 48.5 ± 17.1	Yes[a,c]	NC	Tardy et al. (2010)
g.7368G>A	c.1123G>A	E9	375	p.G375S	1.6 ± 0.8/0.7 ± 0.7	Yes[a,c]	SW	Lajić et al. (2002)
g.7373C>A	c.1128C>A	E9	376	p.Y376X	nd/nd	No	SW	Stikkelbroeck et al. (2003)
g.7383G>C	c.1140G>C	E9	380	p.E380D	30 ± nd/nd	Yes[a]	SW*	Kirby-Keiser et al. (1997), Hsu et al. (1999)
g.7386G>A	c.1141G>A	E9	381	p.G381S	nd/nd	Yes[a,c]	SW	Haider et al. (2013), Bruque et al. (2016)
g.7402C>T	c.1157C>T	E9	386	p.P386L	nd/nd	Yes[a,c]	NC	Haider et al. (2013), Bruque et al. (2016)
g.7402C>G	c.1157C>G	E9	386	p.P386R	nd/nd	Yes[a,c]	SW	Vrzalová et al. (2010)
g.7406C>G	c.1161C>G	E9	387	p.N387K	nd/nd	Yes[a,c]	NC	Wasniewska et al. (2009)
g.7408T>G	c.1163T>G	E9	388	p.L388R	1.1 ± 0.6/nd	Yes[d]	SW	Brønstad et al. (2014)
g.7410_7418*del* CAAGGCGCC	c.1165_1173*del* CAAGGCGCC	E9	389–391	p.Q389_A391*del*	0/< 1	No	SW	de Paula Michelatto et al. (2016)
g.7416G>A	c.1171G>A	E9	391	p.A391T	38.7 ± 9.5/22.9 ± 4.7	Yes[a]	NC	Robins et al. (2007)
g.7421_7436*dup*16	c.1176_1191*dup*16	E9	398	p.W398P*fs*?	nd/nd	No	SW	Lee et al. (1998)
g.7455T>C	c.1210T>C	E9	404	p.F404L	nd/nd	Yes[a,c]	SW	New et al. (2013)
g.7456T>C	c.1211T>C	E9	404	p.F404S	nd/nd	Yes[a,c]	SW	Baradan-Heravi et al. (2007)
g.7459G>A	c.1214G>A	E9	405	p.W405X	nd/nd	No	SW	Wedell et al. (1993)
g.7464G>A	c.1219G>A	E9	407	p.D407N	72.7 ± 7/73.6 ± 10	Yes[a,c]	NC	Concolino et al. (2009)
g.7564C>T	c.1222C>T	E10	408	p.R408C	1.3 ± 0.5/0.6 ± 0.3	Yes[a]	SW	Soardi et al. (2008), Billerbeck et al. (2002)
g.7565G>T	c.1223G>T	E10	408	p.R408L	nd/nd	Yes[a]	SW	Yu et al. (2011)
g.7565G>A	c.1223G>A	E10	408	p.R408H	nd/nd	No	SV	Finkielstain et al. (2011)
g.7612G>A	c.1270G>A	E10	424	p.G424S	1.6 ± 0.4/2 ± 0.6	Yes[a]	SV	Tardy et al. (2010)
g.7612_7620*del* GGTGCCCGC	c.1270_1278*del* GGTGCCCGC	E10	424–426	p.G424_R426*del*	nd/nd	No	SW	New et al. (2013)
g.7614_7618*del* TGCCC	c.1272_1276*del* TGCCC	E10	424	p.G424G*fs*?	nd/nd	No	SW	Finkielstain et al. (2011)
g.7618C>T	c.1276C>T	E10	426	p.R426C	0 ± 0.5/0 ± 0.6	Yes[a]	SW	Grischuk et al. (2006)
g.7619G>C	c.1277G>C	E10	426	p.R426P	nd/nd	Yes[a]	SV	Finkielstain et al. (2011)
g.7619G>A	c.1277G>A	E10	426	p.R426H	0.5 ± 0.6/0.4 ± 0.2	Yes[b]	SW	Barbaro et al. (2006)
g.7624T>C	c.1282T>C	E10	428	p.C428R	nd/nd	No	SW	Wang et al. (2016)
g.7630_7631*del*GG	c.1288_1289*del*GG	E10	430	p.G430R*fs*?	nd/nd	No	SW	Minutolo et al. (2011)
g.7633G>A	c.1291G>A	E10	431	p.E431K	26.2 ± 3.8/24.2 ± 7.4	Yes[a]	NC	Taboas et al. (2014)
g.7637C>T	c.1295C>T	E10	432	p.P432L	nd/nd	Yes[a]	SV	Carvalho et al. (2010)

Table 1 continued

Genomic reference sequence[≠]	cDNA reference sequence[&]	Exon	Codon	Protein change	Enzyme activity (17-OHP/prog)[Δ]	Structural evaluation[●]	Phenotype prediction	References[Ψ]
g.7640T>C	c.1298T>C	E10	433	p.L433P	nd/nd	Yes[a]	SW	New et al. (2013)
g.7643C>T	c.1301C>T	E10	434	p.A434V	14.0 ± 2/12 ± 6	Yes[a]	SV	Krone et al. (2005)
g.7643C>A	c.1301C>A	E10	434	p.A434E	nd/nd	Yes[d]	SW	Carvalho et al. (2012)
g.7645T>G	c.1303C>T	E10	435	p.R435C	nd/6.5 ± 0.9	Yes[a]	NC	Krone et al. (2013)
g.7672C>T	c.1330C>T	E10	444	p.R444X	nd/nd	No	SW	Krone et al. (2006)
g.7673G>C	c.1331G>C	E10	444	p.R444P	nd/nd	Yes[a]	SW	Haider et al. (2013)
g.7679T>C	c.1337T>C	E10	446	p.L446P	0.5 ± 0.60/0 ± 0.10	Yes[a,c]	SW	Barbaro et al. (2006)
g.7690A>C	c.1348A>C	E10	450	p.T450P	< 1%/< 1%	Yes[a,c]	SW	de Paula Michelatto et al. (2016) Baradan-Heravi et al. (2007)
g.7691C>T	c.1349C>T	E10	450	p.T450M	78 ± 6/43 ± 5	No	Mild NC	de Paula Michelatto et al. (2016)
g.7699 C>T	c.1357C>T	E10	453	p.P453S	36.0 ± 5/44 ± 3	Yes[a,c]	NC	Riepe et al. (2008)
g.7717C>T	c.1375C>T	E10	459	p.P459S	nd/nd	Yes[c]	SV	Vrzalová et al. (2010)
g.7718C>T	c.1376C>T	E10	459	p.P459L	nd/nd	Yes[c]	SV	Wang et al. (2016)
g.7718C>A	c.1376C>A	E10	459	p.P459H	nd/6.8 ± 2.1	Yes[a,c]	NC	Jiang et al. (2012)
g.7720_7737 *del* TCGCTGCAG CCCCTGCCC	c.1378_1395*del* TCGCTGCAGC CCCTGCCC	E10	460–465	p.S460_P465*del*	nd/nd	No	NC	Bidet et al. (2009)
g.7729C>T	c.1388C>T	E10	463	p.P463L	2.6 ± 0.8/3 ± 0.5	Yes[a]	SV	Krone et al. (2006)
g.7732*del*C	c.1390*del*C	E10	464	p.L464C*fs*?	nd/nd	No	SW	Wang et al. (2016)
g.7738*dup*C	c.1396*dup*C	E10	466	p.H466P*fs*?	nd/nd	Yes[c]	SW	Bruque et al. (2016)
g.7759_7779*dup* ATGCAGCCTTT CCAAGTGGGG	c.1417_1437*dup* ATGCAGCCTT TCCAAGTGCGG	E10	473–479	p.M473_R479*dup*	nd/nd	No	SV	Loidi et al. (2006)
g.7761G>T	c.1419G>T	E10	473	p.M473I	85 ± 7/66 ± 12	Yes[d]	Mild NC	Barbaro et al. (2015)
g.7767*del*T	c.1426*del*T	E10	475	p.P475P*fs*?	nd/nd	No	SW	Ordoñez-Sánchez et al. (1998)
g.7778G>T	c.1436G>T	E10	479	p.R479L	75.5 ± 15.70/ 79.6 ± 12	Yes[a]	NC	Robins et al. (2007)
g.7783C>T	c.1441C>T	E10	481	p.Q481X	2.98 ± 4.08/ 0.07 ± 0.35	No	SW	Massimi et al. (2014)
g.7784A>C	c.1442A>C	E10	481	p.Q481P	nd/nd	Yes[a]	SW	Di Pasquale et al. (2005)
g.7786C>T	c.1444C>T	E10	482	p.P482S	91 ± 6/61 ± 6	Yes[a]	NC	de Paula Michelatto et al. (2016)
g.7789C>T	c.1447C>T	E10	483	p.R483W	nd/2.9 ± 1.5	Yes[a,b]	SW*	Carvalho et al. (2012)
g.7790_7791*del* GG*ins*C	c.1447_1448*del* GG*ins*C	E10	483	p.R483P*fs*?	nd/nd	No	SW	Wedell et al. (1992)

Table 1 continued

Genomic reference sequence[≠]	cDNA reference sequence[&]	Exon	Codon	Protein change	Enzyme activity (17-OHP/prog)[Δ]	Structural evaluation[●]	Phenotype prediction	References[Ψ]
g.7790G>A	c.1448G>A	E10	483	p.R483Q	2.00 ± 0.25/ 1.89 ± 0.30	Yes[a,b]	SV	Ono et al. (2008)
g.7790G>C	c.1448G>C	E10	483	p.R483P	1.0 ± 0.07/2.2 ± 0.9	Yes[a,b]	SV*	Nikoshkov et al. (1998)
g.7822_7823*ins*C	c.1480_1481*ins*C	E10	494	p.Q494P*fs*?	nd/nd	No	SW	Kirac et al. (2014)

17-OHP 17-hydroxyprogesterone, *cDNA* complementary DNA, *prog* progesterone, *NC* non-classical, *SV* simple virilizing, *SW* salt wasting, *nd* no data

[≠]Mutations are numbered in relation to the *CYP21A2* DNA reference sequence (GenBank accession number NG_007941.2)

[&]Mutations are numbered in relation to the *CYP21A2* cDNA reference sequence (GenBank accession number NM_000500.6), whereby nucleotide +1 corresponds to the A of the ATG-translation initiation codon

[Δ]Residual enzyme activity was evaluated towards the two natural substrates of steroid 21-hydroxylase, 17-OHP and progesterone, and was expressed as a percentage of the wild-type enzyme

[Ψ]We provide the complete list of references in the Electronic Supplementary Material

[●]If structural evaluation of the variant was performed, we report in apex specific references

*Variants with conflicting interpretation of significance

[a]Haider et al. Proc Natl Acad Sci USA. 2013;110:2605–10

[b]Robins et al. Mol Endocrinol. 2006;20:2946–64

[c]Bruque et al. Sci Rep. 2016;6:39082

[d]The structural evaluation was performed by the same authors indicated in the reference column

Table 2 *CYP21A2* variants affecting non coding regions and their clinical significance

Intronic variant[a]	Intron	Predicted effect[b]	Phenotype prediction[c]	References[Ψ]
c.[-126C>T;-113G>A;-110T>C;-103A>G][d]	5′UTR	CYP21A1 promoter is 80% less active than the CYP21A2	NC	Araújo et al. (2007)
c.-126C>T	5′UTR	Decreased transcriptional activity to 52%	NC	Araújo et al. (2007)
g.5413A>G (IVS1-2A>G)	I1	Disrupted splice acceptor intron 1	SW	Lajic et al. (1996)
g.5505G>A (IVS2+1G>A)	I2	Disrupted splice donor intron 2	SW	Lee et al. (1998)
g.5509G>A (IVS2+5G>A)	I2	Reduces the consensus value for the intron 2 splice donor site from 70.9 to 59.3%	SW	Friães et al. (2006)
g.5774A/C>G (IVS2-13A/C>G)	I2	New splice acceptor site	SW	Rodrigues et al. (1987), Higashi et al. (1991)
g.5780C>G (IVS2-7C>G)	I2	The mutation impairs the usage of intron 2 acceptor splice site resulting in intron retention	SW	Rubtsov et al. (2011)
g.5785A>G (IVS2-2A>G)	I2	Disrupted splice acceptor intron 2	SW	Billerbeck et al. (2002)
g.5942G>A (IVS3+1G>A)	I3	Disrupted splice donor intron 3	SW	Raisingani et al. (2016)
g.6151G>C (IVS4+1G>C)	I4	Disrupted splice donor intron 4	SW	Wang et al. (2016)
g.6238G>A (IVS4-1G>A)	I4	Disrupted splice acceptor intron 4 Activation of an intronic cryptic acceptor site	SW	Concolino et al. (2017)
g.6428A>G (IVS5-2A>G	I5	Disrupted splice acceptor intron 5	SW	Taboas et al. (2014)
g.6434T>A (IVS5-8T>A)	I5	Alteration of the wild-type acceptor site Activation of an intronic cryptic acceptor site	CL*	Concolino et al. (2017)
g.6899G>C (IVS7+1G>C)	I7	Disrupted splice donor intron 7	SW	Wedell et al. (1993)
g.6900T>G (IVS7+2T>G)	I7	Disrupted splice donor intron 7	SW	Ordoñez-Sánchez et al. (1998)
g.7278G>A (IVS8+1G>A)	I8	Disrupted splice donor intron 8	SW	Finkielstain et al. (2011)
g.7359A>G (IVS8-2A>G)	I8	Disrupted splice acceptor intron 8	SW	Concolino et al. (2017)
g.7465G>C (IVS9+1G>C)	I9	Disrupted splice donor intron 9	SW	Krone et al. (2013)
g.7553C>A (IVS9-9C>A)	I9	New aberrant splice acceptor site at −7 position of intron 9	SW	Katsumata et al. (2010)
g.7561G>A (IVS9-1G>A)	I9	Disrupted splice acceptor intron 9	SW	Finkielstain et al. (2011)
c.*13G>A	3′UTR	Alteration of the RNA folding and expression	NC	Menabò et al. (2012)

CL classical, *NC* non-classical, *SV* simple virilizing, *SW* salt wasting, *UTR* untranslated region

[Ψ]We provide the complete list of references in the Electronic Supplementary Material

*The patient, carrying p.I172N/IVS5-8T>A genotype, was affected by SV form of disease. This phenotype could be determinate by the p.I172N mutation; in this case the IVS5-8T>A variant could be related to the SW form. Alternatively, also the IVS5-8T>A could be related to the SV phenotype

[a]Mutations are numbered in relation to the *CYP21A2* DNA reference sequence (GenBank accession number NG_007941.2)

[b]Predicted effect was determinate by in silico analysis or in vitro functional study

[c]Phenotype prediction was reported on the base of patient's phenotype and results of characterization studies

[d]The cluster of four variants producing promoter conversion was considered as a single variant

Table 3 *CYP21A2* exonic polymorphisms

Genomic reference sequence[≠]	cDNA reference sequence[&]	Exon	Codon	Protein change	Enzyme activity (17OHP/prog)[Δ]	Structural evaluation[●]	References[Ψ]
g.5143_5145*dup*CTG	c.25_27*dup*CTG	E1	9–10	p.L9_L10*dup*	96.5 ± 7.6/ 93 ± nd	No	Lajic et al. (1999)
g.5152C>A	c.34C>A	E1	12	p.L12M	99%/100%	Yes[a]	de Paula Michelatto et al. (2016)
g.5161G>A	c.43G>A	E1	15	p.A15T	100 ± 0/96 ± 6	Yes[b]	de Paula Michelatto et al. (2016), Dolzan et al. (2003)
g.5802G>A	c.305G>A	E3	102	p.K102R	119.7 ± 22.5/nd	Yes[b,c]	Brønstad et al. (2014)
g.6079G>A	c.475G>A	E4	159	p.A159T	126.6 ± 29.9/nd	Yes[b,c]	Brønstad et al. (2014)
g.6241C>G	c.549C>G	E5	183	p.D183E	100 ± nd/ 100 ± nd	Yes[b,c]	Higashi et al. (1991)
g.6323G>C	c.631G>C	E5	211	p.V211L	nd/nd	Yes[b,c]	Tardy et al. (2010)
g.6323G>A	c.631G>A	E5	211	p.V211M	99.5 ± 32.4/nd	Yes[c]	Brønstad et al. (2014)
g.6505G>A	c.712G>A	E6	238	p.E238K	nd/nd	Yes[c]	Kirac et al. (2014)
g.6509 T>A	c.716T>A	E6	239	p.M239K	95.4 ± 24.7/ 97.7 ± 7.7	Yes[b]	Robins et al. (2005)
g.6755G>T	c.793G>T	E7	265	p.A265S	90 ± 9/104 ± 15	Yes[c]	Barbaro et al. (2015)
g.6765G>C	c.803G>C	E7	268	p.S268T	103 ± 15/nd	Yes[b]	Wu et al. (1991)
g.7820G>A	c.1478G>A	E10	493	p.N493S	nd/nd	Yes[b]	Rodrigues et al. (1987)

17-OHP 17-hydroxyprogesterone, *cDNA* complementary DNA, *prog* progesterone, *nd* no data

[≠]Variants are numbered in relation to the *CYP21A2* DNA reference sequence (GenBank accession number NG_007941.2)

[&]Variants are numbered in relation to the *CYP21A2* cDNA reference sequence (GenBank accession number NM_000500.6), whereby nucleotide + 1 corresponds to the A of the ATG-translation initiation codon

[Δ]Residual enzyme activity was evaluated towards the two natural substrates of steroid 21-hydroxylase, 17-OHP and progesterone, and was expressed as a percentage of the wild-type enzyme

[●]If structural evaluation of variant was performed, we report in apex the specific references

[Ψ]We provide the complete list of references in the Supplemental material

[a]The structural evaluation was performed by the same authors indicated in the reference column

[b]Haider et al. [66]

[c]Brønstad et al. [73]

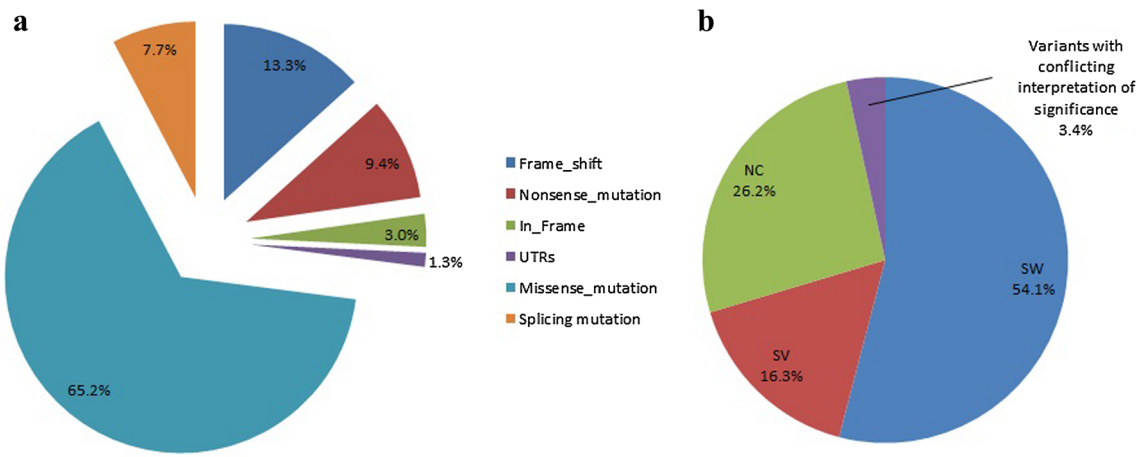

Fig. 1 **a** Percentages of missense, nonsense, frameshift, in-frame, splicing *CYP21A2* variants reported to date. Percentage of nucleotide substitutions affecting UTRs is also shown. **b** Percentages of SW, SV, and NC variants. Percentage of variants with conflicting interpretation of significance is also shown. *NC* non-classical, *SV* simple virilizing, *SW* salt wasting, *UTR* untranslated region

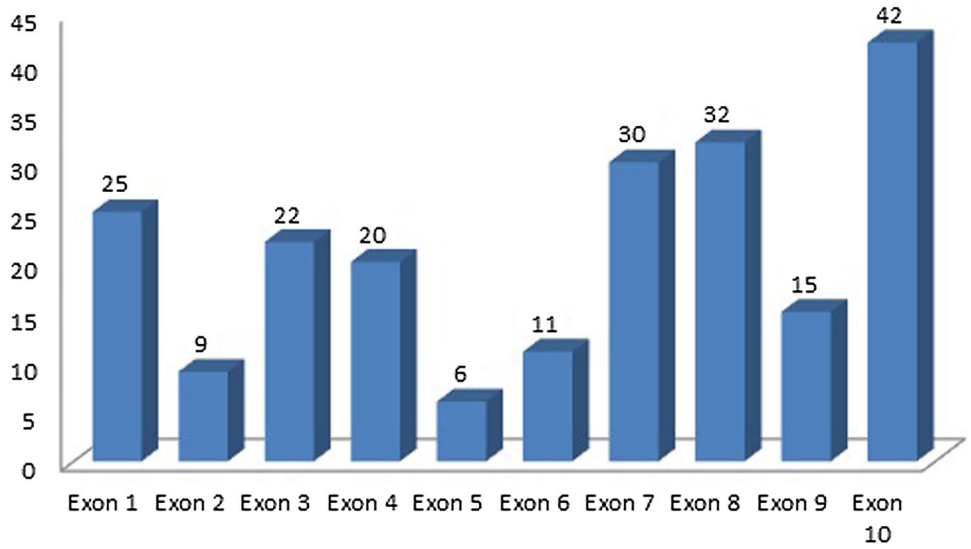

Fig. 2 Number of mutations affecting *CYP21A2* exons

carefully evaluated, considering the genotype and the phenotype of different described patients and analyzing all data from the performed structural and functional studies.

Regarding the 5′ and 3′ UTRs, only two disease-causing variants have been reported in addition to the cluster of four variants producing promoter conversion: the c.-126C>T (from the cluster that produces the promoter conversion c.-126C>T; c.-113G>A; -110T>C; -103A>G) and the c.*13C>T, both related to the NC form of the disease (Table 2) [69, 70]. Differently, 17 out of 18 splicing affecting variants are plausibly related to the most severe form of disease, the SW syndrome (Table 2). The IVS5-8T>A variant was detected in trans with the p.I172N in a patient affected by SV CAH. In this case, it is not possible to establish if the intronic variant is related to the SW or SV phenotype [71].

Overall, 126 (54.1%) out of 233 *CYP21A2* mutations are associated with the SW form of the disease, 38 are associated with the SV form (16.3%), and 61 (26.2%) are related to the NC form (Fig. 1b). Seven exonic variants and one intronic variant (3.4%, variants marked with an asterisk in Tables 1, 2) are not definitively associated with a specific phenotype (SW, SV, NC) as there is missing or conflicting information in the literature (see next section) (Fig. 1b).

3.2.1 *Variants with Conflicting Interpretation of Significance*

We were not able to assign a definitive clinical phenotype to seven exonic variants. In the literature, we found several patients carrying the same variant, but showing different phenotypes; in most cases, the results of functional studies were not in agreement with the patient's phenotype or with structural analysis. In addition, some variants have only been reported once by inconclusive papers.

3.2.1.1 p.P45L This variant was detected in an SV CAH Norwegian patient carrying a complete *CYP21A2* gene deletion on the second allele [72]. Subsequently, a functional study was performed; in vitro enzyme activity was normal, but the proline-to-leucine shift in position 45 was predicted as pathologic by computer modeling [73]. The p.P45 residue is located in the N-terminal region of the enzyme close to the hydrophobic domain that anchors the protein to the endoplasmic reticulum (ER) membrane [66]. As the functional characterization of the p.P45L mutant was carried out in the absence of membranes, the severely reduced *in vivo* activity compared to wild-type protein could be missed in the system used. In fact, the authors affirm that future studies should be performed using cell-free expression of 21-hydroxylase with the traditional use of transiently transfected COS7 cells including a wide range of known and well-characterized *CYP21A2* variants [73]. Recently, Bruque et al. reported that, contrary to the in vitro assay results, the in silico-predicted p.P45L activity may correlate better with the SV form of the disease [67].

In our opinion, there is sufficient information for a definitive classification of this variant as SV. In fact, the discordance could be due to results obtained by functional studies performed using a different system from the conventional procedures.

3.2.1.2 E140K The variant was detected in an SW patient carrying a complete *CYP21A2* gene deletion on the second allele [73]. The residual enzyme activity was estimated by quantifying 11-deoxycortisol using 17-OHP as the substrate, and an in vitro assay showed a significant

reduction in enzyme activity (11.3% ± 2.4). However, it was still much higher than expected for the SW phenotype. Structural analysis showed that the p.E140 residue forms a salt bridge with the R444 residue, and the shift from negatively charged glutamic acid to positively charged lysine breaks the salt bridge. This prediction could be in agreement with a severe form of disease, explaining the SW phenotype of the patient [73]. To date, there are no other papers in the literature reporting this variant in CAH patients.

We think that, despite the data obtained from the functional study, this rare variant is most likely related to the SW form. We recommend considering this possibility in genetic and prenatal counseling.

3.2.1.3 p.I171N This variant was reported by Barbaro et al. [74] in an NC CAH Italian patient carrying the p.V281L mutation on the other allele. As it was impossible to classify this novel variant according to the clinical findings of this patient, a functional study was performed [74]. The results obtained showed that the variant was associated with the CL CAH phenotype [74]. However, 6 years later, a structural study classified this kind of variant as resulting in NC CAH [66]. The p.I171 residue is located in a predicted alpha helix structure (helix E) and the substitution of a polar asparagine to a hydrophobic non-polar isoleucine affects the hydrophobic property of this region [66]. To date, there are no other papers in the literature reporting this variant in CAH patients.

We think that further studies are needed in order to provide a definitive classification of this variant. To date, based on results from functional study, the possibility of a severe form of disease should be considered.

3.2.1.4 p.G178A A Spanish patient, diagnosed as having SV CAH, carried both the p.G178A and the NC p.V281L mutation on the paternal allele and the IVS2-13A/C>G mutation on the maternal allele. The functional study was performed, and the p.G178A mutant was not able to metabolize progesterone at significant levels, while it retained significant activity (19%) for 17a-hydroxyprogesterone [75]. According to the authors, these results correlated well with the identification of the p.G178A in a patient with the SV form [75]. Subsequently, structural models showed that this variant could be related to a severe form of the disease (SW) [59, 66]. In fact, according to the authors, CYP21A2 protein inactivation can occur when conformational flexibility is impaired, mostly because of the introduction of bulkier residues. For example, the sharp fold between the E and F helices, where G178 is located, can only accommodate a small residue. The mutation of glycine to alanine will hinder the flexibility of this fold, impairing stability [66]. To date, there are no other papers in the literature reporting this variant in CAH patients. In our opinion, further studies would be needed for a final classification of this variant, because of the discrepancies in the currently available data.

3.2.1.5 p.E380D The p.E380D variant was found in a homozygous patient suffering from the severe SW form of the disease [76]. A functional study, performed by Hsu et al. [77], indicated a 30% residual activity from the p.D380 mutant protein (17-OHP used as substrate) that does not correlate with the phenotypic presentation of the disease [77]. The structural study performed by Robins et al. [59] showed that there are only minor difference between a glutamate and an aspartate residue at the 380 position, while the structural model of Haider et al. classified the variant as related to the SW form [66]. To the best of our knowledge, there are no other reports in the literature of this variant in CAH patients.

The conflicting results do not allow a definitive classification of this variant. While waiting for further studies, we recommend considering the possibility of the SW form also in this case.

3.2.1.6 p.R483 The 483 codon harbors three rare variants that all seem to be related to the CL form of the disease. While data in the literature have definitively correlated p.R483Q to SV disease, contrasting information is reported for p.R483W and p.R483P variants [59, 66, 78–82]. We believe, based on the information reported above, that both these variants are reasonably related to the CL form of disease, with phenotypic manifestations associated with the SV or SW form.

3.2.1.7 p.R483W This variant was first detected, in homozygous status, in a Tunisian patient who was diagnosed as having the SW phenotype [78]. Subsequently, p.R483W was reported in a Chinese girl carrying the E6 cluster mutation on the second allele. She was affected with the SV form [79]. The results obtained from the functional study were in accordance with the phenotype of the patient. So, authors classified the variant as related to the SV disease [79]. However, structural studies reported the p.R483W variant as being associated with SW [59, 66], showing that p.R483 residue makes a salt-bridge with p.D322, maintaining the structural elements in the correct position. The mutation interrupts this interaction, causing SW CAH [66].

3.2.1.8 p.R483P This rare variant was reported in 1998 in two compound heterozygote subjects, with the unique p.R483P mutation on one allele and the well-known p.I172N mutation on the other [80]. They had a moderate

form of the disease, with early clitoral enlargement in the female sibling, but without SW. The R483P mutant retained 1–2% of the activity (both substrates) of the native enzyme, and this was in agreement with the clinical phenotypes of the patients [80]. Subsequently, Finkielstain et al. described an SW patient carrying the p.R483P variant in the hemizygous condition [81], while a few years later Krone et al. associated the mutation with SV CAH [82]. Finally, a structural study in 2006 reported the variant as associated with the CL form (SV/SW) of the disease [59], while a more recent paper classified it as an NC variant [66].

4 Conclusion

In the diagnosis of 21-hydroxylase deficiency, *CYP21A2* genotyping is a valuable complement to biochemical investigations. Genotyping can confirm the diagnosis (or carrier state) and, at the same time, give prognostic information on disease severity. This is especially important in male newborns detected in neonatal screening, since they do not display genital malformations as a sign of CL disease [4]. In addition, the use of genetic testing is also helpful in prenatal counseling and prenatal diagnosis with the goal of preventing genital virilization of the female fetus [83]. To date, more than 200 mutations of the *CYP21A2* gene have been reported in different studies, and although it is well-known that some exceptions exist, there is good agreement between clinical phenotype and patient genotype [1–4, 9]. For this reason, much effort is spent in the classification of new variants. Clinical classification of new variants should be clarified with clinical investigations on groups of patients carrying the same variant with a different genotype (being the phenotype defined by the milder of the two mutations present). In addition, in vitro enzyme activity assays and structural studies, although they are artificial systems that cannot provide absolute indications reflecting the in vivo situation, offer a means to investigate rare variants, comparing these to the more common mutations for which abundant clinical data are available.

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Compliance with Ethical Standards

Conflict of interest The authors (PC and AC) declare that they have no conflict of interest.

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