Congenital Adrenal Hyperplasia Due to 21-Hydroxylase Deficiency: Genetic Characterization and the Genotype–Phenotype Correlation

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Introduction

Congenital adrenal hyperplasia (CAH) results from the insufficient synthesis of cortisol occurring as a consequence of a deficiency of one of the steroidogenic enzymes being inherited as an autosomal recessive disorder. Approximately 95% of the cases are due to 21-hydroxylase deficiency (21-OHd), an enzyme that is expressed mostly in the adrenal cortex [1]. It is one of the most common autosomal recessive diseases, but in spite of the progress in its genetic diagnosis, this is a complex process with significant risk of attaining incomplete results and consequently misunderstanding of the real situation [2].

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Congenital adrenal hyperplasia due to 21-hydroxylase deficiency (21-OHd) encompasses very important disorders with high morbidity (the classical forms) or high prevalence (the non-classical forms). They affect patients' life in many ways, ranging from salt-wasting and life-threatening crises to virilization with variable degrees of genital ambiguity and with all its consequences of gender determination and reconstructive surgeries, ultimately affecting normal sexuality and reproduction. Some researchers believe that this division of the classical forms is artificial as the simple virilizing forms may also have variable degrees of salt wasting [3–5]. Finally, there are non-classical forms (NCAH) in which the enzymatic activity is significantly retained, between 20% and 60%, and in consequence, there is normal production of mineralocorticoids and cortisol. The resulting phenotype is characterized mainly by androgen excess that is less severe or occurring later in life; nevertheless, it may have an impact on sexuality and reproduction or simply affect the patients' self-esteem and quality of life. One of the important features of NCAH forms is their much higher prevalence in comparison to the classical forms.

Genetic alterations of classical salt-wasting forms lead to complete absence of 21-OH enzyme and consequently to impairment of cortisol and aldosterone biosynthesis. In classical forms, there is also highly increased secretion of androgens, clinically translated in female virilization and precocious puberty in males. This is the result of increase in ACTH production (or action) due to enzymatic blockage causing loss of feedback control upon hypothalamus and pituitary, leading to diversion of steroidogenic precursors to the androgenic pathways.

Other pathogenic genetic variants cause less severe alterations that are compatible with the synthesis of 21-hydroxylase protein with some activity, some cortisol and aldosterone synthesis, in amounts that are compatible with life, without the need for their replacement. In those cases, the disease manifests itself later in life, mostly by excessive production of androgens. In these "non-classical," less severe forms, hyperandrogenemia also results from diversion of 17-hydroxyprogesterone and other precursors to alternative androgenic pathways in spite of the fact that cortisol is not significantly reduced, and ACTH not significantly elevated (altered enzymatic kinetics).

The Structure of the *CYP21A2* Gene

CYP21A2 is located in chromosome 6, in the area of the human histocompatibility complex (HLA), a region with a complex organization of genes with a great variability in their size and copy numbers [6–9].

At a distance of 30 kb from *CYP21A2*, there is a pseudogene (e.g., a nonfunctional gene) named *CYP21A1P*. Both the functional gene and the pseudogene have ten exons and share a high level of genetic homology (98% in their exons and 96% in their introns) [10, 11]. The pseudogene is inactive because it accumulates a great number of mutations along human development.

The neighbor localization and high homology of these two genes frequently lead to misalignments during meiosis that result in recombinations between the gene and the pseudogene (gene conversions) that are responsible for the majority of point mutations in the *CYP21A2* gene.

In close proximity to these two genes, there are other genes in the same region of chromosome 6 forming genetic units called RCCX. These genes are *RP1*, *C4*, *TNXB*, and two truncated pseudogenes, *RP2* and *TNXA* [12]. The genes *C4B* and *C4A* are translated into the fourth component of complement [13, 14], while *TNXB* encodes for an extracellular matrix protein called tenascin-X23 and the *RP1* gene gives origin to a serine/threonine nuclear protein kinase [12]. The proximity of *CYP21A2* and *TNXB* genes explains the existence of a syndrome with simultaneous characteristics of CAH and of the hypermobility type of Ehlers–Danlos syndrome, resulting from the simultaneous deletion of both genes the CAH-X syndrome [15–17].

The most common organization of this region has two RCCX modules, one with the *CYP21A1P* pseudogene and the other with the *CYP21A2* active gene. The orientation of the genes from telomere to centromere is: *RP1-C4A-CYP21A1P-TNXARP2-C4B-CYP21A2-TNXB*. The haplotype, bearing two RCCX modules, is present in about 69% of the Caucasian population [18]. A mono-modular haplotype may also occur (in 17% of the population) while a "three modular" haplotype has been reported to be present in 14% [12, 19]. Most haplotypes with three modules have two copies of the *CYP21A1P* pseudogene and one copy of the *CYP21A2* gene. Two copies of the *CYP21A2* gene and only one of the *CYP21A1P* can also occur, and this situation has been described particularly in cases with the p.(Gln319∗) pathogenic variant and of chimeric *CYP21A1P*/*CYP21A2* genes [19–21].

Another important aspect of the structure of *CYP21A2* gene is the high prevalence of copy number variations, which in conjunction with the enormous amount of possible genetic variants makes the characterization of *CYP21A2* alleles a difficult task. Genetic variants have been identified in both the coding and non-coding regions of the gene inclusively in the 5′UTR and the 3′UTR regions. Consequently, it is recognized that the characterization of the gene must include the sequencing of every exon, and the intron–exon boundaries [2].

The Origin of *CYP21A2* Alterations

Due to gene and the pseudogene localization and genetic complexity of the region, recombination events are generally the cause of *CYP21A2* pathogenic modifications.

In fact, intergenic recombinations between the inactive and active genes (gene conversions) are responsible for more than 95% of the pathogenic variants causing 21-OHd [22, 23].

About 75% of the pathogenic variants are transferred by small conversions from the pseudogene during meiosis. These conversions can involve one or more pseudogene variants. They are called "microconversions."

In the other 20–25% of the cases, 21-OHd CAH results from gross misalignment (unequal crossing over) during meiosis. Gene deletions, gene duplications, and deletions involving CYP21A2 and other contiguous genes usually ensue [24, 25]. Rarely, 21-OHd CAH can also be the result of uniparental isodisomy [26].

More than 250 genetic variants of CYP21A2 are capable of causing human disease. The majority of these will result in classical forms of 21-OHd [27]. One hundred and fifty-three genetic variants have been demonstrated to be missense mutations and have been shown to be able to give rise to all forms of the disease. On the contrary, nonsense and frameshift mutations always result in classical 21-OHd forms [27].

Genetic variants have also been observed in non-translated regions of *CYP21A2* gene. Some of these affect the promoter, resulting in promoter conversion [28].

Sequencing of *CYP21A2* gene in suspected cases should be considered essential in modern medical practice. Only a complete genetic analysis can accurately determine the genotype as pathogenic variants are frequently complex [2].

The effort to genotype a patient with 21-OHd used to be simplified by focusing on a group of 10 pathogenic variants that account for the majority of affected alleles. However, a recent study has demonstrated that this targeted *CYP21A2* mutation analysis may fail to identify mutations on one allele in 10.4% of the cases [29].

In light of the present knowledge, familial segregation studies should always be done, as this is the only way to be sure if two detected pathogenic variants affect the two alleles (trans configuration) or are located in the same allele with the other one being normal (cis configuration). For this purpose, both parents have to be studied together with the proband.

CYP21A2 Genetic Modifications

There are large rearrangements and small conversions. Large recombinations result from unequal crossing over during meiosis. The other type of conversion occurs when a small segment of the functional *CYP21A2* gene is replaced by a segment from the *CYP21A1P* pseudogene (microconversions). The altered *CYP21A2* gene will carry a point mutation or a short sequence that may involve one or more exons [10, 22, 30, 31]. As these variants are pathologic, they will give rise to an inactive or at least significantly modified 21-hydroxylase protein.

Large Deletions and Conversions

Approximately 25% of alleles carry a deletion, a large gene conversion, or the formation of an inactive chimeric gene which is the result from a meiotic recombination in which the final product is a non-functional chimeric gene with its 5′ end belonging to *CYP21A1P* and the 3′ end to *CYP21A2* [32–34].

Several different chimeric *CYP21A1P/CYP21A2* genes have been identified as a result of variable length deletions [35–38].
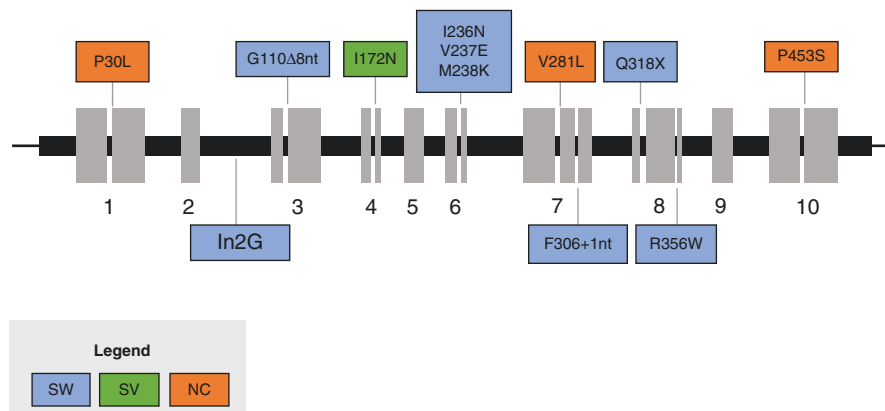
Fig. 1 Location of the most common *CYP21A2* mutations that are transferred by gene conversion and their association with the clinical outcome. Numbered boxes represent *CYP21A2* exons. *SW* salt wasting, *SV* simple virilizing, *NC* non-classical

Point Mutations and Small Deletions/Insertions

Most of the intergenic recombinations (75%) correspond to abnormal nucleotides normally present in the pseudogene that are transferred to the functional gene by microconversion [30] (Fig. 1). Other rearrangements have been reported including a deletion of 10 nucleotides in exon 8 or a duplication of 16 nucleotides in exon 9.

Other Pathogenic Variants

Many different pathogenic variants have been described, and these will certainly increase with the widespread utilization of molecular diagnosis. Most of these are rare, having been described in only one case or one family (see http://www.cypal-leles.ki.se/cyp21.htm and http://www.hgmd.cf.ac.uk). Less than 5% of the pathogenic variants are not caused by gene conversions and so possibly not being presented in the pseudogene [39, 40].

Polymorphisms

Some alterations that are found in *CYP21A2* gene do not affect 21-hydroxylase production and are considered normal polymorphisms [6]. It is possible that at least some of these variants are also present in the *CYP21A1P* gene and represent gene conversions not affecting the enzyme. However, others have been described only in the *CYP21A2* gene.

Genotyping and Pregnancy

Genotyping of *CYP21A2* gene as completely as possible is very important in couples who wish to conceive. It allows confirmation of the diagnosis, and it is the only way to do a correct genetic counseling.

The parents want to learn about the risk of having a child with salt-wasting form or with severe virilization in case of a female fetus.

It is well established that in a woman with CAH, the progeny will or will not have CAH according to the partner's genotype. If he does not have any pathogenic variant in *CYP21A2*, the children will be carriers but will not have the disease. If the woman's genotype consists of two mild pathogenic variants, for instance being homozygotic for V281L (p.(Val282Leu)) and her partner is a carrier of a *CYP21A2* pathogenic variant, whatever it may be, 50% of her children will have NCAH (50% being merely a mathematical probability). However, in general population, the probability of being a carrier for a severe pathogenic variant is 1.7% (1 in 60) [41], and consequently the risk for having a child with classical 21-OHd CAH would be expected to be 1:600 since it has been reported that the probability of a patient with NCAH having a severe pathogenic variant together with a mild one is approximately 2/3 [29, 42]. The real frequency is however closer to 2.5% [43], and this made researchers believe that the carrier frequency is probably higher than is suspected, at least in some populations.

The actual recommendation is that both parents should be genotyped whenever possible as part of the prenatal study protocol if one potential progenitor has any form of 21-OHd CAH [43].

Genotype–Phenotype Correlations

There is a good correlation between genotype and the phenotype generally reported as reaching 90% or even 95% [33, 44–47]. However, it must be acknowledged that the clinical picture resulting from 21-hydroxylase deficiency is in fact a continuum of reductions in enzyme activity, and the three degrees of severity, in which the disease is generally classified, are only a simplification aiming to facilitate the clinical practice [48]. It is also recognized that the phenotypes can change with time and consequently a perfect correlation between genotype and phenotype is virtually impossible.

There are genetic variants that are considered to cause the most severe forms of the disease (100% enzymatic deficiency), resulting in salt-wasting forms of the classical 21-OHd CAH. These variants are called *severe pathogenic variants* (Fig. 2).

The missense pathogenic variant I172N (p.(Ile173Asn)) confers around 1–2% of the normal 21-hydroxylase activity, and even this small enzymatic activity is sufficient for a near normal aldosterone production, reducing almost completely the risk of salt-wasting crises. Thus, it is associated with the simple virilizing form of 21-OHd CAH. Pathogenic variants, as this one, are called the *intermediate pathogenic variants* (Fig. 2).
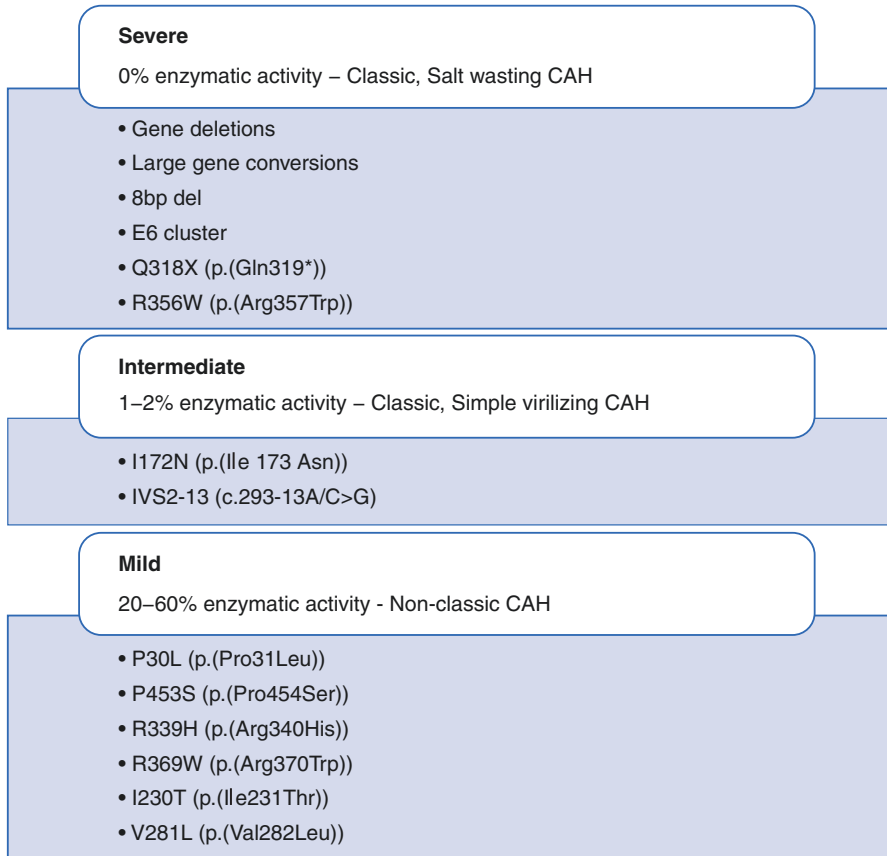
Severe

0% enzymatic activity – Classic, Salt wasting CAH

- Gene deletions
- Large gene conversions
- 8bp del
- E6 cluster
- Q318X (p.(Gln319*))
- R356W (p.(Arg357Trp))

Intermediate

1–2% enzymatic activity – Classic, Simple virilizing CAH

- I172N (p.(Ile 173 Asn))
- IVS2-13 (c.293-13A/C>G)

Mild

20–60% enzymatic activity - Non-classic CAH

- P30L (p.(Pro31Leu))
- P453S (p.(Pro454Ser))
- R339H (p.(Arg340His))
- R369W (p.(Arg370Trp))
- I230T (p.(Ile231Thr))
- V281L (p.(Val282Leu))

Fig. 2 *CYP21A2* pathogenic variants and their correlation with phenotype

Other pathogenic variants including P30L, P453S, R339H, R369W, I230T [48], and V281L (clearly the most frequent pathogenic variant in NCAH cases in most series) result in a conservation of enzymatic activity of 20–60% and consequently in less severe symptoms which are associated with the non-classical form (*mild pathogenic variants*) (Fig. 2).

Importantly, most of the 21-hydroxylase-deficient patients are compound heterozygotes instead of homozygotes [3, 29], and this implies that the phenotype is generally affected by the residual 21-hydroxylase activity and consequently by the less severely mutated allele [33]:

1. The most severe phenotypes (the classical forms) must have severe pathogenic variants in both alleles and none of the mild pathogenic variants.
2. The NCAH patients may have two mild mutations (a situation that occurs in approximately 35% of the cases) or one mild and one severe ones (in the other 65%).

A mild pathogenic variant present in one of the alleles allows the synthesis of 21-hydroxylase up to 50–60% of the normal activity, in spite of the fact that the severe pathogenic variant, present in the other allele, does not contribute to any synthesis.

Although the correlation between genotype and phenotype is high, it has been observed that some of these pathogenic variants confer different phenotypes depending on if they are isolated or associated with another pathogenic variant.

In fact, there is some diversity of the phenotypes in patients with less severe pathogenic variants [5, 33, 49, 50]. It has been reported that in spite of being predictable that the phenotype will correspond to the less severely affected allele, the presence of a second allele with a more severe pathogenic variant can result in a more serious clinical phenotype [51–55] with higher degrees of hirsutism and also of higher 17OHP levels than cases with two mild genetic alterations [42, 56].

The pathogenic variants IVS2-13 (c.293-13A/C>G) and I172N (p.(Ile173Asn)) can result in variable degrees of 21-hydroxylase activity (possibly through alternative splicing). Patients with these mutations are generally expected to be simple-virilizing cases but may sometimes present as salt-wasting forms, and the others may stand closer to NCAH [34, 57]. Similarly, the pathogenic variant P30L (p.(Pro31Leu)) which is considered to be a cause of NCAH has been reported to be responsible for cases of SV-CAH, as well [58, 59]. Considering about the residual 21-hydroxylase activity which is expected to present with P30L mutation, this is unexpected. The NCAH patients with P30L mutation can exhibit stronger virilization with clitoromegaly and advanced bone maturation [60]. Different mechanisms have been proposed for the increased androgenization of these patients, including the influence of other residues, accompanying promotor variability or mutations and finally variations in individual androgen sensitivity [59]. In fact, some concomitant factors capable of modifying the phenotype have been suggested such as the number of CAG repeats of the androgen receptor, other genes encoding proteins with 21-hydroxylase activity and alternative pathways of androgen biosynthesis capable of causing fetal virilization in females [59].

In a multi-national study of 1507 families with CAH, SV form of CAH was found in 17/74 patients having P30L mutation (23%) [46].

Even when it does not clearly result in a SV form, the clinical manifestations in patients with P30L will include stronger signs of virilization, earlier adrenarche, clitoromegaly, and some patients require treatment with glucocorticoids compared to other patients with NCAH form [39, 44, 61].

Moreover, genotypes P30L/I2 splice, P30L/Q318X, and P30L/8Δbp are especially associated with SV form of CAH [46]. If not treated with glucocorticoids, SV progresses steadily during childhood causing early puberty, short adult stature, and fertility problems in both genders including testicular adrenal rest tumors (TARTs) in men.

Incomplete correlation between genotype and phenotype may also result from not sequencing the whole gene and hence not having a full picture of the genetic alterations.

Genetic Sequencing

Sequencing of the entire gene by PCR mutation-detection methods together with multiplex ligation-dependent probe amplification are the golden standard for studying the *CYP 21A2* gene.

General Considerations

Specific gene amplification by PCR has dramatically improved the sensitivity of different techniques to detect *CYP21A2* pathogenic variants. Modernly, PCR conditions have been identified that allow the amplification of *CYP21A2* without amplifying the very homologous *CYP21A1P* pseudogene. These conditions result from amplifying CYP21A2 in two segments (Fig. 3).

PCR-based diagnosis may be complicated by the failure of amplifying one haplotype which may result in misdiagnosis. Examination of flanking microsatellite markers in all family members can minimize this problem.

Another important aspect is that, if only a DNA sample from the patient is analyzed, it is impossible to distinguish compound heterozygosity for different pathogenic variants occurring in *trans* and the presence of two pathogenic variants in the same allele (*cis*). Therefore, ideally both parents should also be analyzed, so as to most reliably determine the phase of different pathogenic variants (i.e., whether they lie on the same or opposite alleles). Analysis of parental alleles also permits differentiating homozygotes and hemizygotes (i.e., individuals who have a pathogenic variant on one chromosome and a deletion on the other).
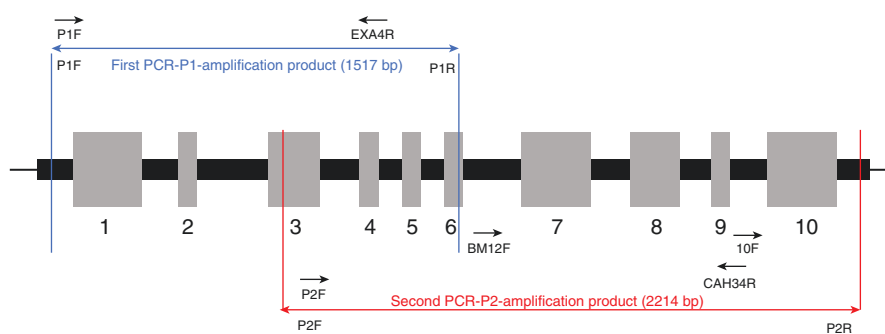

Fig. 3 Proposed strategy to whole-gene sequence of the *CYP21A2* gene. Numbered boxes represent *CYP21A2* exons and arrows represent the primers. Adapted from [62]

DNA Sanger Sequencing

The whole-gene sequencing (together with MLPA) has become the standard procedure in cases of 21-OHd. It usually covers the coding regions and the flanking intron–exon regions of the gene. This method not only detects the more common genetic variants but can also detect the novel sequence variants helping to explain some cases in which there has not been a correlation between genotype and phenotype. Main difficulty results from the homology between the gene and the pseudogene. To avoid the co-amplification of pseudogene *CYP21A2,* whole-genomic sequencing may be performed selecting the functional *CYP21A2* gene and amplifying it by PCR into two partially overlapping fragments, P1 and P2 respectively with one 517 and two 214 base-pairs (bp). After selective amplification of the targeted gene and subsequent purification, the PCR product is sequenced with internal primers that cover the entire *CYP21A2* gene [62].

MLPA

After sequencing the entire *CYP21A2* gene, one should also look for deletions and duplications [63, 64]. This is currently done using multiplex ligation-dependent probe amplification (MLPA) [65].

The MLPA assay is a technique that enables the detection of variations in the copy number of several human genes. Due to the large number of genes or genetic sequences that can be simultaneously analyzed, MLPA assay has become the gold standard for molecular analysis of all pathologies derived from the presence of gene copy number variation [66]. Besides, MLPA can be used to confirm the point mutations identified by sequencing analysis.

Detection of deletions and duplications of *CYP21A2* gene and the *CYP21A1P* pseudogene by MLPA is currently performed, using the P050-CAH Kit (MRC-Holland). This high-resolution method uses only a single pair of PCR primers, and the specificity relies on the use of progressively longer oligonucleotide probes, in order to generate locus-specific amplicons of increasing size that can be resolved electrophoretically. Comparing the peak pattern obtained to that of the reference samples, it is possible to determine which probes/locus show abnormal copy numbers [67, 68].

Final Considerations

Genotyping can also be used for disease prevention. Preimplantation genetic diagnosis is increasingly being performed to limit the transmission of several diseases, including CAH, being used in conjunction with in vitro fertilization.

Another aspect of prenatal diagnosis consists of early gender determination, by the detection of SRY in circulating fetal DNA that is present in maternal blood at very early stages of pregnancy. This allows the identification of male fetuses that do not need to be treated prenatally. In case of a female fetus, obstetricians and pediatricians may treat these cases during early stages of pregnancy to prevent genital ambiguity. It is also possible to perform sequencing methods of the *CYP21A2* gene in circulating fetal DNA, but this is complex and still carries a significant possibility of false positives or false negatives. Chorionic villus sampling and amniocentesis can still be used for prenatal treatment to prevent the masculinization of external genitalia of the female fetus with classical 21-OHd; however, they are performed rather late [2, 29, 69].

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