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Misregulation effect of a novel allelic variant in the Z promoter region found in *cis* with the *CYP21A2* p.P482S mutation: implications for 21-hydroxylase deficiency

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Abstract The aim of the current study was to search for the presence of genetic variants in the *CYP21A2 Z* promoter regulatory region in patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Screening of the 10 most frequent pseudogene-derived mutations was followed by direct sequencing of the entire coding sequence, the proximal promoter, and a distal regulatory region in DNA samples from patients with at least one non-determined allele. We report three non-classical patients that presented a novel genetic variant—g.15626A>G—within the Z promoter regulatory region. In all the patients, the novel variant was found in *cis* with the mild, less frequent, p.P482S mutation located in the exon 10 of the *CYP21A2* gene. The putative pathogenic implication of the novel variant was assessed by in silico analyses and in vitro assays. Topological analyses showed differences in the curvature and bendability of the DNA region bearing the novel variant. By performing functional studies, a significantly decreased activity of a reporter gene placed downstream from the regulatory region was found by the G transition. Our results may suggest that the activity of an allele bearing the p.P482S mutation may be influenced by the misregulated *CYP21A2* transcriptional activity exerted by the Z promoter A>G variation.

Keywords 21-hydroxylase deficiency · Molecular genetics · Z promoter novel variant · Misregulation transcriptional activity

Introduction

The 21-hydroxylase deficiency accounts for 90–95 % of congenital adrenal hyperplasia (CAH) cases. This autosomal recessive disorder has a broad spectrum of clinical forms, which includes the severe or classical salt-wasting and simple virilizing forms, and the mild late onset or non-classical (NCCAH) one [1].

The gene encoding 21-hydroxylase, *CYP21A2*, maps in 6p21.3 within the human leukocyte antigen complex (HLA), adjacent to the pseudogene *CYP21A1P* with which it shares 98 % nucleotide sequence identity. Due to the high degree of sequence identity, most of the disease-causing mutations described are likely to be the consequence of non-homologous recombination or gene conversion events between the *CYP21A2* gene and its pseudogene [2]. Nevertheless, an increasing number of

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naturally occurring mutations have been found in disease-causing alleles in the last years (http://www.hgmd.cf.ac.uk).

The basal transcriptional activity of the *CYP21* gene is mainly located in the first 176 nucleotides 5′ upstream of the first ATG codon [3]. Besides, a distal region—the Z promoter—located 4.6–5.6 Kb upstream from *CYP21A2* inside the intron 35 of the *C4* gene has been suggested to act as a transcriptional enhancer region [4, 5]. To date, only few works analyzed this distal regulatory region for the screening of putative disease-associated mutations [6–10]. Although no mutations were found, due to the limited number of 21-hydroxylase patients studied, the presence of still unidentified mutations in the Z promoter cannot be ruled out.

In the current study, we describe a novel genetic transition, g.15626A>G, in the Z promoter region adjacent to the active *CYP21A2* gene found in three NCCAH patients.

Materials and methods

Patients were included following the diagnostic criteria already described [11, 12]. Details on clinical manifestations in patients carrying the novel transition are presented in Online Resource 1.

Nucleotide numbering was performed using M13936.1 [13] as the genomic *CYP21A2* reference sequence, and U24578.1 as the genomic *C4* reference sequence [14]. All new data have been deposited in the GeneBank database.

DNA was isolated from peripheral blood leukocytes from 309 patients (74 classical, 235 NCCAH) and the 10 most frequent pseudogene-derived point mutations in the *CYP21A2* gene were screened [11]. In 66 samples (7 classical, 59 NCCAH) with at least one non-determined allele, presence of novel or less frequent disease-causing mutations was further analyzed by direct sequencing as previously described [8]. *CYP21A2/CYP21A1P* deletions/duplications and large gene conversion were screened by MLPA (SALSA P050-B3 CAH MLPA Mix, MRC-Holland BV, Amsterdam, Holland). When available, DNA from parents was analyzed to establish the segregation of the mutated alleles.

Analysis of the distal regulatory region of *CYP21A2* gene

Initially, the Z promoter regions were amplified from genomic DNA with primers I35F and E36R (5′TGGTC CAGAAGCTAACCCTTA3′; 5′TCAAAGCCCACGCAC TC3′) and analyzed by direct sequencing. For all samples in which a sequence variation was observed, an amplification of a 5663 bp specific fragment of the 5′ upstream of each *CYP21A2* or *CYP21A1P* was performed using primers I35F-P48/P49 [15], respectively (Fig. 1a) and a Long PCR Enzyme Mix (Thermo Scientific, Waltham, Massachusetts, USA). The specificity of each fragment was achieved by a restriction enzyme assay after nested PCR comprising the 5′ TaqI sites of the *CYP21* genes. The Z promoter region was further amplified using each gene- or pseudogene-specific fragment as template and analyzed by direct sequencing as described above. Each fragment was sequenced in forward and reverse orientations, and the novel variation was tested again in two independent PCRs. In addition, the novel mutation was further analyzed by restriction enzyme digestion (see below).

Additionally, 101 subjects randomly selected from the general population were recruited, and DNA samples were screened for the novel mutation by a BstxI restriction enzyme assay (Online Resources 1). When a mutant sequence was detected, a specific amplification of the 5′ region upstream of the *CYP21A2* or *CYP21A1P* was performed as described above, and each of the Z promoter regions was analyzed by direct sequencing.

HLA typing

HLA haplotypes were assigned using the Olerup SSP® (OlerupSSP AB, Stockholm, Sweden) commercial kit.

Bioinformatic analyses

Sequence alignments

Similarities between regulatory Z promoters regions from different primates were assessed using genome sequences retrieved from different sources (Online Resource 1).

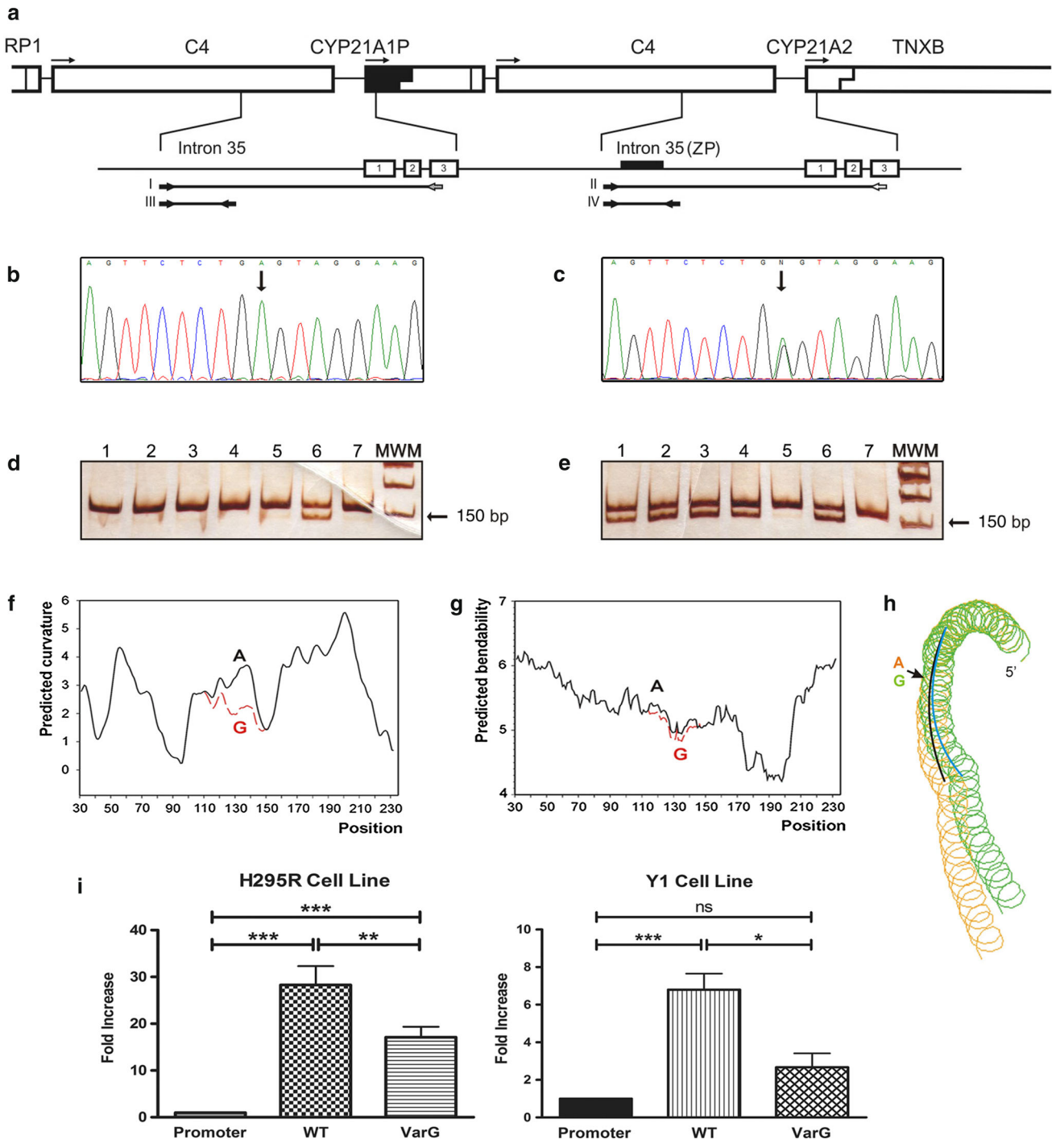
Bendability/curvature propensity

A DNA fragment of 263 residues including 131 nucleotides upstream and downstream to the mutated residue was analyzed using DNA tools [16] at the ICGEB server (http://hydra.icgeb.trieste.it/dna/).

The bendability/curvature propensity data were calculated with the bend.it program using the Consensus Scale (DNase I + nucleosome positioning data) and standard parameters [17, 18]. Three-dimensional models were obtained with the model.it program using consensus (trinucleotide) parameter [17, 18].

Functional assays

To assess the transcriptional activity of the Z promoter, the luciferase reporter plasmid pC21/−5.6 Kb/Luc, containing the entire DNA region from −5.6 Kb to the transcription


a
RP1
C4
CYP21A1P
C4
CYP21A2
TNXB
Intron 35
Intron 35 (ZP)
I
II
III
IV
b
c
d
1 2 3 4 5 6 7 MWM
150 bp
e
1 2 3 4 5 6 7 MWM
150 bp
f
Predicted curvature
A
G
Position
g
Predicted bendability
A
G
Position
h
A
G
5'
i
H295R Cell Line
Fold Increase
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Promoter
WT
VarG
Y1 Cell Line
Fold Increase
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Promoter
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◀**Fig. 1** Representative electropherograms, restriction enzyme assays, bioinformatic analyses, and functional studies of the novel g.15626A>G variant. **a** Diagram summarizing the strategy used for the screening of the novel variant in the Z promoter (ZP). A representative standard bimodular chromosome is shown. *I*, *II*: pseudogene- and gene-specific fragments, respectively. *White* and *gray arrows* denote gene and pseudogene specific primers, respectively. *III*, *IV:* ZP fragments. TNX: *Tenascine X* gene. RP1: *STK19* (serine/threonine kinase 19; formerly RP1). **b**, **c** Representative electropherograms of the *III* and *IV* ZP fragments, respectively. The g.15626A>G transition was found only in the ZP region adjacent to the active *CYP21A2* gene (*arrowheads*). **d**, **e** Silver stained 8 % acrylamide–bis acrylamide gel of a BstXI restriction enzyme assay for the g.15626A>G variant using ZP fragments *III* (**d**) and *IV* (**e**). The wild-type sequence remained uncut, while the mutant rendered fragments of 134 and 20 bp. *Lanes* 1–3: samples from patients bearing the A>G transition. *Lanes* 4–5: individuals C37 and C38 from the general population. *Lanes* 6–7: restriction enzyme assays from patient 1 and from the individual C38, respectively, using a ZP fragment amplified directly from genomic DNA (non-gene or pseudogene specific). *Lane* 7: 50 bp molecular weight marker. **f**–**h** Bioinformatic analyses. **f** Overlapping graphs of predicted curvature for the wild-type and G-mutant sequences. **g** Overlapping graphs of predicted bendability for the wild-type and G-mutant sequences. In both graphs, *solid black lines* correspond to the sequence of the wild-type and the *dashed lines* correspond to the G-mutant. Original and changed residues are shown. **h** Comparison of DNA models backbones: overimposed models of wild-type (*orange*) and G-mutant (*green*) DNA sequences. The considered nucleotides (*A* wild-type, *G*-mutant) lie in a curved region of the DNA. The replacement leads to changes in the roll, tilt, and twist angles in this position and the previous one (data not shown), generating a shift angle in the main axis of the structure that spreads the distortion to the rest of the molecule (*black* and *blue lines*). **i** Functional studies: enhancer activity of the wild-type Z promoter and the g.15626A>G transition. The pC21/-0,3 Kb/Luc (Promoter) vector containing the *CYP21A2* minimal promoter region, the pC21/-5,6 Kb/Luc luciferase vector (WT) containing the wild-type Z promoter region and the mutant pC21/-5,6 Kb/Luc, nucleotide G in the desired position (VarG), were assayed in 2 different adrenal cell lines: H295R and Y-1. The enhancer activity was expressed as the fold increase in the Luciferase/Renilla ratio of each construct in comparison to the activity of the basal promoter vector arbitrary designed as 1. Each *bar* represents the mean ± S.E.M. of triplicate data point from 2 to 4 different colonies for each construct assayed at least 4 times in independent experiments. ***$p < 0.001$, **$p < 0.05$, *$p < 0.01$. *ns* not significant

initiation site [5], was mutagenized using QuickChange II XL Site-Direct Mutagenesis Kit (Stratagene, La Jolla, CA, USA), following the manufacturer's instructions. The presence of the mutagenized base was checked by sequencing the whole 5.6 Kb insert.

The human NCI-H295R (H295R) and the mouse Y1 cells were transiently transfected with 0.5 μg of the pC21/−0.3 Kb/Luc (with only the minimal promoter region), pC21/−5.6 Kb/Luc, the mutagenized pC21/−5.6 Kb/Luc (G-mutant), or the empty vector (pGL3 Basic Vector) using Fugene HD transfection reagent (Promega, Wiscosin, USA). Transfection efficiencies were normalized by cotransfecting a *Renilla* luciferase plasmid (pRL-CMV, Promega). After 48 h, cells were lysed and luciferase assays were carried out using the Dual-Luciferase reporter assay system (Promega) in accordance with the manufacturer's instructions. Details on culture conditions are presented in Online Resource 1.

Triplicate samples were analyzed for each datum point, and 2–4 different mutagenized constructs were assayed at least 4 times in independent experiments. Differences between experimental groups were evaluated using ANOVA followed by a Tukey test between groups, or Kruskal–Wallis H test followed by Dunn's multiple comparison test.

Results

Following the screening of the 10 most frequent pseudogene-derived mutations, direct sequencing of the complete coding, proximal promoter, and a distal regulatory region were performed in those patients with at least one non-determined allele. This combined strategy revealed the presence of a novel sequence variation—g.15626A>G (accession number HQ665552)—in the regulatory region located in the intron 35 of the *C4* gene adjacent to the active *CYP21A2* gene, in three patients diagnosed with NCCAH 21-hydroxylase deficiency (Table 1). Representative electropherograms and restriction enzyme digestion assays are presented in Fig. 1b–e.

After sequencing, we also found that the three patients presented the less frequent g.2882C>T (p.P482S) mutation in exon 10 of the *CYP21A2* gene. Segregation studies performed in two of these patients (1 and 2), revealed that the g.15626A>G variant in the distal regulatory region and the p.P482S were located in *cis*. All the patients and the segregated parent presented the haplotype *HLA-A2*, *B41*, two of them the *HLA-DR3*, and one, the *HLA-DR4* variant (Table 1). None of the samples presented a duplication of the *CYP21A2* gene.

In addition, one individual from the general population (C37) presented the same transition in the distal Z promoter adjacent to the *CYP21A2* gene and the *HLA-A3*, *B40*, *DR8*, and *HLA-A2*, *B41*, *DR4* haplotypes. However, the p.P482S mutation was not found. A putative allele drop-out mechanism during PCR amplification was excluded due to the presence of the g.2908G>A and g.2916A>G allelic variants in heterozygosis. Moreover, none of the individuals from the general population or the other patients presented the p.P482S mutation (data not shown).

To assess the putative pathogenic mechanism of the A>G variant, we performed multiple fragment alignments using DNA sequences from the Z promoter from different primates. As shown in Figure S1 (Online Resource 2), the Z promoter regions analyzed presented a high level of conservation within the primates. Moreover, the novel

Table 1 Phenotypes, genotypes, hormonal values, and *HLA* haplotypes of patients carrying the novel variation in the Z promoter regulatory region

Patient	Phenotype	Gender	Genotype[a]	Basal 17-OHP	Stimulated 17-OHP	ΔA_4	T	DHEA-S	HLA[a, b]
1	NC	F	[p.V281L]; [**g.15626>G**; p.P482S]	3.5	18.5	ND	ND	365.3	*A68*, *B14*, *DR13* and *A2*, *B41*, *DR3*
2[c]	NC	M	[p.V281L]; [**g.15626>G**; p.P482S]	4.8	24	0.9	0.7	458.1	*A2*, *B14*, *DR1* and *A2*, *B41*, *DR3*
3[d]	NC?[e]	F	[N]; [**g.15626>G**; p.P482S]	1.9	17	ND	ND	ND	*A2*, *B7*, *DR16* and *A2*, *B41*, *DR4*

The novel transition in the Z promoter is displayed in bold. Hormonal values are expressed in ng/mL

Cut off values: Basal 17-OHP: 2 ng/mL; Stimulated 17-OHP: 10 ng/mL. Reference values for ΔA_4, T, and DHEA (in ng/mL): Premenopausal women: 0.3–2.8, 0.3–0.9, and 350–3800, respectively; prepubertal: 0.3–2.0; <0.1, and 100–600, respectively

[a] Maternal; paternal alleles

[b] In patients 1 and 2, DNA from the parent carrying the novel variation and the p.P482S mutation was also analyzed; in patient 3, the haplotypes were assumed

[c] An Italian ancestry was ascertained for this patient during the interview

[d] This patient was adopted, thus segregation of the alleles was arbitrarily assigned

[e] The mutation on the second allele was not found for this patient. However, several studies have shown that heterozygous carriers of *CYP21A2* mutations may have an increased risk of hyperandrogenic symptoms and could exhibit ACTH-stimulated 17-OHP values similar to those described in patients with the 21-hydroxylase NCCAH form [27, 28]

17-OHP 17-hydroxyprogesterone, *Δ_4A* androstenedione, *T* testosterone, *DHEA-S* dehydroepiandrosterone sulfate, *ND* not determined, *N* Normal wild-type allele

g.15626A>G variant is located in a fully conserved position throughout the different lineages.

We also analyzed the predicted curvature and bendability of DNA region comprising the novel variant. As shown in Fig. 1f and g, the A>G variation introduces distortions in the local DNA conformation. Consistently, the overlapped backbones of DNA fragments corresponding to the wild-type (WT) and G-mutant showed significant differences in the 3D space (Fig. 1h).

We next assessed the influence of the A>G substitution on the in vitro transcriptional activity of the Z promoter. Two adrenal cells lines—the human H295R and the murine Y1 cells—were transiently transfected with a WT or a mutant luciferase reporter vector containing the entire DNA region from −5.6 Kb up to the transcription initiation site. In accordance to previous reports [5], Fig. 1i shows an enhanced luciferase activity in the presence of the Z promoter WT sequence. In our conditions, the 5.6 Kb insert exerted in average a 28.3 ± 11.4 (S.E.M.) and 6.8 ± 1.7 fold increase of the basal activity in both human and mouse adrenal cells, respectively. Changing the wild-type A nucleotide by a G in the desired position significantly reduced the enhanced gene expression exhibited to 17.1 ± 6.4 and 2.7 ± 1.5 in the H295R and Y1 cell lines, respectively.

Discussion

In this work, we report a novel heterozygous g.15626A>G transition found in the Z promoter region in 3 NCCAH 21-hydroxylase-deficient patients. The Z promoter region has been suggested to act as a transcriptional enhancer region of the *CYP21A2* gene [4, 5].

Wijesuriya et al. [5] described three possible regions within the human Z promoter named F1, F2, and F3, implicated in the binding of different transcription factors [19]. The novel A>G substitution is located 20 bp 5′ upstream from the F3 site, in a fully conserved position throughout different lineages of primates.

It is well known that in different biological systems, DNA recognition sequences for regulatory proteins are non-contiguous sites and the protein–protein interaction between components of the basal transcription machinery and bounded regulatory proteins may require distortion of the DNA helix by DNA looping, or by DNA bending. Indeed, we found that the curvature and bendability of the region change upon the substitution. The asymmetry in the local conformations could lead to limit the possible angles of distortion. These changes could affect proper assembly of the transcription machinery in the regulatory region, and/or could facilitate or hinder the protein–protein contact between a transcription activator or repressor and the transcription machinery.

Accordingly, our functional assays showed that the substitution of the g.15626A>G significantly reduced (approximately 35 %) the enhanced activity exhibited by the WT construct. These results might suggest that the change in the Z promoter region, albeit not specifically in the described binding sites for known transcription factors, could lead to alter the rate of the *CYP21A2* gene transcription modulating the final amount of the enzyme. Of

note, the in vitro approach used in this work relies on the expression of a reporter gene downstream from a large insert containing the whole −5.6 Kb region of the transcription starting site, and may better represent the native DNA structure in the region and the (patho) physiological condition found in vivo.

We also observed that the three patients having the novel variation in the Z promoter presented in *cis* the less frequent p.P482S mutation in the *CYP21A2* gene. In vitro studies have demonstrated that the p.P482S is related to the NCCAH, exerting a residual enzymatic activity of 60–70 % [20, 21]. In addition, this missense mutation has been described in Italian patients [20, 22–25], suggesting an apparent founder effect. Indeed, by analyzing the HLA haplotypes, Barbaro et al. [20] found that 4 out of 6 patients with the p.P482S mutation had the *HLA-A2*, *B41*, *DR4* haplotype. Likewise, all the individuals from our cohort who presented the Z promoter variant—including the control individual—had the *HLA-A2*, *B41* haplotype. Two of them presented the *HLA-DR3* variant and two the *HLA-DR4*. These results, together with the observation that the p.P482S mutation was absent in the individual from the general population carrying the g.15626A>G substitution, might suggest a putative founder effect for the p.P482S mutation on a preexisting allele with the Z promoter A>G variant, followed by probable recombination events in the *HLA* locus. Despite the fact that an Italian origin was ascertained only for one of our patients, it is noteworthy that individuals with an Italian ancestry are largely present in the Argentinean population.

Interestingly, some authors argued that due to its high residual enzymatic activity, the p.P482S mutation may only result in a clinical phenotype when presented in combination with other genetic factors rather than being the single cause for a NCCAH phenotype [26]. In line with this concept, we postulate that the final in vivo activity of an allele bearing the p.P482S might be the consequence of the residual activity of the missense mutation plus the decrease gene transcription exerted by the A>G transition in the Z promoter. Notably, one female patient described by Barbaro et al. [20] with the severe c.290-13A/C>G mutation in the homologous allele, disclosed hormonal values compatible with a heterozygous status and little signs of impaired adrenocortical function. Additionally, she was the only one who exhibited a different *HLA-B39* allele. We speculate that recombination events in the *HLA* locus, or an independent mutation event, could render an allele bearing the p.P482S mutation but without the Z promoter g.15626G variant. Such an allele may exert a higher enzymatic activity than those with the *HLA-B41* background. It would be interesting to analyze the presence of the g.15626A>G transition in those patients described up to date having the p.P482S mutation to elucidate the putative contribution of both variants in the patients' phenotypes. Moreover, screening of more patients is necessary to further evaluate the possibility that this novel variant could be related to the clinical manifestation of the disease by its own or in combination with other mutations.

In conclusion, we described for the first time an allelic g.15626A>G variant in the distal Z promoter regulatory region of the *CYP21A2* gene. The results shown in the current study may suggest that the A>G variation in the Z promoter is involved in misregulating the transcriptional activity of the *CYP21A2* gene.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Ethical Approval All the procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all patients and parents involved in this work. The study was approved by the ethics committee of the Instituto de Biología y Medicina Experimental, and the Centro Nacional de Genética Médica, Buenos Aires, Argentina.

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