Apparent Mineralocorticoid Excess by a Novel Mutation and Epigenetic Modulation by *HSD11B2* Promoter Methylation

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Context: Apparent mineralocorticoid excess (AME) is a rare autosomal recessive disease resulting from mutations within the hydroxysteroid (11β-dehydrogenase2 [*HSD11B2*]) gene causing a prominent mineralocorticoid receptor activation by cortisol and hypokalemic low renin hypertension as the main clinical feature.

Objective: The objective of the study was to characterize AME for possible novel *HSD11B2* mutations and to define the role of *HSD11B2* promoter methylation in the phenotypic expression of the disease.

Subjects: Two proband brothers and 10 relatives participated in the study.

Methods: Peripheral blood mononuclear cell DNA was used for *HSD11B2* exon sequencing, and a new predicted structure of 11β-hydroxysteroid dehydrogenase type 2 was generated by an in silico three-dimensional modeling. Promoter methylation was determined by bisulfite pyrosequencing. Urinary tetrahydrocortisol plus allotetrahydrocortisol to tetrahydrocortisone ratio, a surrogate marker of 11β-hydroxysteroid dehydrogenase type 2 activity, was measured by gas chromatography-mass spectrometry.

Results: A novel homozygous variant at *HSD11B2* exon 3 site (*c.C662G*) resulting in an alanine-to-glycine change at position 221 was discovered by sequencing the DNA of the probands. A monoallelic mutation was found in the DNA of the parents and other four relatives. In silico three-dimensional modeling showed that the Ala221Gly substitution could perturb a hydrophobic interaction by reducing the enzymatic affinity for the substrate. The *HSD11B2* promoter methylation of normotensive heterozygous relatives was similar to that of wild types, whereas the hypertensive heterozygous subjects showed higher methylation than wild types, consistently with a transcriptional repressive effect of promoter hypermethylation.

Conclusions: A novel *HSD11B2* functional mutation accounting for an Ala221Gly substitution causes AME. The hypertension phenotype is also epigenetically modulated by *HSD11B2* methylation in subjects heterozygous for the mutation. **(*J Clin Endocrinol Metab* 100: E1234–E1241, 2015)**

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Received March 24, 2015. Accepted June 22, 2015.
First Published Online June 30, 2015

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Abbreviations: AME, apparent mineralocorticoid excess; BP, blood pressure; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2; LVH, left ventricular hypertrophy; MR, mineralocorticoid receptor; PBMC, peripheral blood mononuclear cell; THFs to THE, tetrahydrocortisol plus allotetrahydrocortisol to tetrahydrocortisone (THF+aTHF to THE) ratio.

Apparent mineralocorticoid excess (AME) is a hereditary autosomal recessive disorder first described by Ulick et al (1) in 1979, caused by the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) enzyme deficiency with consequent activation of the mineralocorticoid receptor (MR) not mediated by aldosterone (2). The 11β-HSD2 is a microsomal enzyme mainly expressed in mineralocorticoid target tissues (distal nephron, sweat and salivary glands, and colonic epithelium) that converts the active steroids cortisol and corticosterone into their inactive metabolites, cortisone and 11-dehydrocorticosterone, respectively. A deficient enzymatic function leads to an impaired conversion of cortisol into cortisone with a consequent accumulation of the former in the renal distal tubular cells. Both aldosterone and cortisol are ligands for the MR, but blood concentrations of cortisol are 100- to 1000-fold higher than those of aldosterone. If 11β-HSD2 is defective, more cortisol is therefore available to MR binding, resulting in a higher MR activation (3, 4).

The elevated intracellular cortisol brings to the MR activation, resulting in the typical clinical features of hypertension and hypokalemia in reason of the renal sodium reabsorption and potassium excretion. Although these features are typical of primary aldosteronism, the plasma aldosterone level is low as a consequence of hypokalemia and expanded plasma volume, and this is why the condition was called AME (1).

AME is a rare disease, with fewer than 100 cases described in the literature, associated with fewer than overall 50 mutations described at the *HSD11B2* gene site, most being missense single-point mutations (Figure 1) (5–11).

The diagnosis of AME disease is usually completed by documenting the presence of *HSD11B2* gene mutations along with the indirect demonstration of the enzymatic deficiency by means of measurement of the urinary steroid ratio. AME is characterized by a high plasma cortisol to cortisone ratio or by a high urinary steroid metabolite tetrahydrocortisol plus allotetrahydrocortisol to tetrahydrocortisone (THF+aTHF to THE alias THFs to THE) ratio, the 11β-HSD2 enzymatic activity being inversely related to the urinary THFs to THE ratio (12, 13).

In those patients carrying *HSD11B2* gene mutations in homozygosity, the hypokalemic hypertensive phenotype associated with the detection of low serum aldosterone and renin concentrations usually develops in childhood, whereas heterozygous patients may or may not become at all hypertensive or show a milder clinical phenotype, often indistinguishable from primary hypertension (14, 15).

Recent evidences suggest that epigenetic mechanisms, ie, promoter methylation of the *HSD11B2* gene, contribute to the modulation of 11β-HSD2 enzyme activity, possibly influencing hypertension incidence (16, 17).

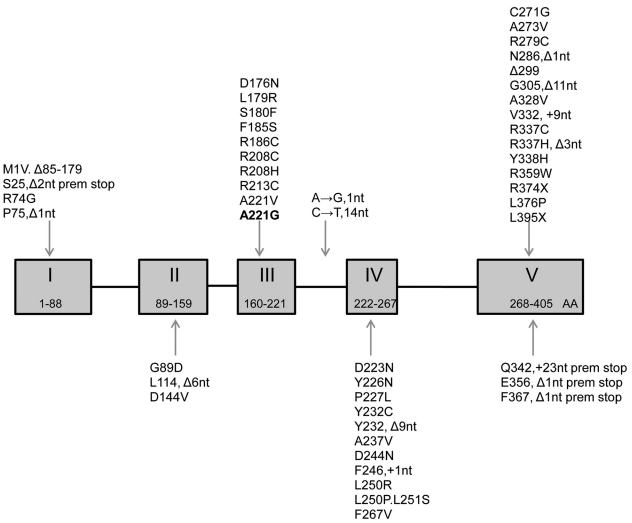

Figure 1. Location of *HSD11B2* gene mutations were found to be the molecular basis of AME syndrome. The *HSD11B2* gene is 6.2 kb long and has been mapped to chromosome 16q22 and consists of five exons. ~~Gray boxes~~ labeled I through V ~~represent exons~~ (numbers indicate the amino acid number), whereas introns are represented as connecting lines. Mutations are listed relative to their position in the coding sequence, with a number indicating the position of the mutated residue respect to the ATG codon.

Here we report the discovery of a novel functional missense mutation at the *HSD11B2* gene site and the role of *HSD11B2* promoter methylation in relation to AME disease clinical manifestations such as the degree of arterial hypertension by describing the family of two young probands affected by AME.

Subjects and Methods

Case presentations

Case 1

A 13-year-old male presented with a 3-month history of severe hypertension, with blood pressure (BP) values at different visits ranging from 160/110 to 180/120 mm Hg, first detected at a medical visit to assess physical suitability for competitive athletics. The physician who had visited the boy 1 year earlier reported that he had normal BP values. The boy was born after a full-term pregnancy, and his birth weight (3.85 kg) and childhood physical and psychomotor development were normal.

His physical examination was unremarkable, including normal height and weight, except for a significantly high BP (170/110 mm Hg). At echocardiography a mild left ventricular hypertrophy (LVH) was detected, whereas no alterations were found at renal ultrasonography.

The biochemical profile revealed hypokalemia (2.6 mmol/L), low renin (5.4 pg/mL), and undetectable aldosterone values. AME was promptly suspected and then confirmed by the finding of an abnormally high urinary THFs to THE ratio (equal to 8.91). Other causes of secondary hypertension were excluded. The boy was then treated with oral potassium chloride supplement and his BP normalized on amlodipine (5 mg once daily) and spironolactone (50 mg once daily), allowing the withdrawal of

potassium supplementation after about 2 weeks since he had started the MR antagonist treatment. After few months of therapy, because of the appearance of gynecomastia, spironolactone was substituted with eplerenone obtaining the regression of breast swelling while maintaining a good BP control. After a 1-year follow-up period, the boy is now doing well, having normal BP, normal serum potassium concentrations, regular growth rate, and partial regression of LVH at echocardiography.

Case 2

The younger brother of the proband is a 7-year-old boy. His birth weight (3.45 kg) and childhood milestones were normal. He was asymptomatic except for high BP (150/110 mm Hg) that was documented in a medical screening after his brother was diagnosed with hypertension. The clinical-biochemical screening showed hypokalemia, low renin, undetectable aldosterone serum concentrations, and THFs to THE ratio equal to 6.1, but, differently from his older brother, there was no evidence of organ damage such as LVH. Physical examination was normal except for BP values of 150/110 mm Hg. His BP normalized with oral amlodipine (5 mg/d) and eplerenone (100 mg/d). After a 1-year follow-up, the boy is doing well, presenting with normal BP and normal kalaemia.

Other family members

The parents of the probands were consanguineous (see family pedigree, Figure 2), the father being a first cousin of the maternal grandmother. The mother had normal BP, whereas the father was hypertensive since the age of 47 years, with a satisfactory BP value control obtained by using two antihypertensive drugs (an angiotensin-converting enzyme inhibitor associated with a β-blocker compound).

The study was approved by our local institutional review board, and all of the patients gave informed written consent.

Biochemical methods profile

Venous blood was collected after overnight fasting for routine analysis. Renin was measured as direct active renin by the LIAISON Direct Renin assay and aldosterone by RIA (both Sorin Biomedical Diagnostics). After a 24-hour urine collection, urinary tetrahydrocortisol, 5a-tetrahydrocortisol, and tetrahydrocortisone were analyzed by gas chromatography-mass spectrometry (18).

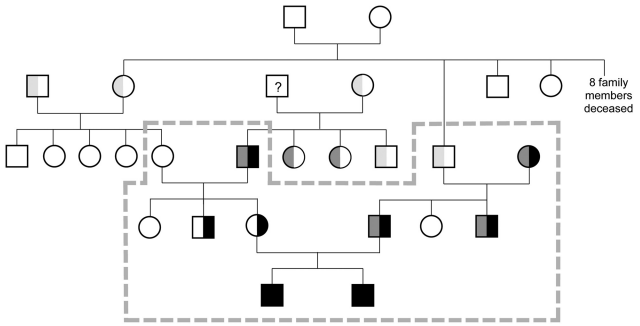

Figure 2. Family pedigree showing genotype (inheritance of mutation A221G) and phenotype (onset of hypertension) of the investigated subjects. The black color indicate the presence of the mutated allele: black square represents homozygous affected subject, whereas half-black square indicates the heterozygous subject. Gray color indicates hypertension onset: dark gray shapes represents young hypertensive subject, whereas light gray shapes are old hypertensive individuals. White shapes represent wild-type normotensive subjects. Dotted line highlights those family members screened for the presence of the mutation A221G.

Exons sequencing of *HSD11B2* gene

See Supplemental Methods and Supplemental Table 1 for exon sequencing.

Sequence alignment and protein modeling

Sequence retrieval of the entire family was performed by using the program PsiBlast (19) on the UNIPROT database (http://www.uniprot.org/). The family of curated sequences was then used to build up the multiple sequence alignment by using the PROMALS program (20). The alignment was then funneled through the HHPred program (21) for template search. We have used a multitemplate approach for the building of the model. In particular, among the chosen templates, we considered the structure of the 11β-HSD1 from *Homo sapiens* (PDB accession code 2BEL) that was cocrystalized with the cofactor nicotinamide adenine dinucleotide phosphate and its inhibitor carbenoxolone. The target and template share a sequence identity of 30%.

The structure modeling of 11β-HSD2 was performed using Modeler 9 version 2 software (22). Cortisol structure was obtained from the PDB database (accession code 4P6X). It was optimally superimposed onto the structure of carbenoxolone by using the Chimera program (23) to maintain the chemical features in the structural alignment. We thus obtained the structure of the 11β-HSD1 with cortisol in the binding cavity. The substrate was positioned in the binding cavity of 11β-HSD2 by optimally superimposing the modeled structures of 11β-HSD2 and 11β-HSD1 and then transferring the coordinates of cortisol.

Bisufite pyrosequencing of the *HSD11B2* gene promoter

Pyrosequencing analysis was performed on two *HSD11B2* promoter regions, namely region 1 and 2 (bases −419 to −177 and bases −692 to −595, respectively, relative to the transcription starting site). Genomic DNA (20 ng) was modified by sodium bisulfite using the EZ DNA methylation kit (ZYMO Research) according to the manufacturer's instructions. Region 1 was amplified using a forward and a biotinylated reverse primer designed by PSQ assay design (Biotage AB), and region 2 was amplified according to a previously reported method (16).

To avoid the possible influence of methylation status on the amplification reaction, primers were designed in CpG-free regions. The amplification reactions were performed in a 25-μL reaction volume with the primer sets and 5 U of Taq polymerase (Solgent Co). PCR products were visualized on a 2% agarose gel by ethidium bromide staining for verification. Pyrosequencing reactions were done with sequencing primers on the PSQ HS 96A system (Biotage AB) according to the manufacturer's specifications. For each region, the methylation status of four CpG sites was evaluated and expressed as a percentage of methylation: mCyt/(mCyt + Cyt). The average DNA methylation (percentage) was then calculated for each region.

Statistical analysis

Statistical analysis was performed using the IBM SPSS 20 statistical software (IBM Inc). Distribution of continuous vari-

ables is expressed as mean ± SD. Nonparametric tests were adopted according to the small sample size: analysis of comparisons between two groups (hypertensives vs normotensives) was undertaken by a Mann-Whitney test, and among the three groups with different mutation status by a Krusker-Wallis test. Values of $P < .05$ were considered statistically significant.

Results

Molecular studies

Sequence analysis, of the DNA of the two probands, revealed a novel missense homozygous mutation in exon 3 of the *HSD11B2* gene, which caused a base transition from cytosine to guanine at position 662 (*c.C662G*), leading to a change in the codon at position 221 from GCG to GGG (Supplemental Figure 1). This change results in an amino acid conversion from alanine 221 to glycine 221 (p.A221G).

Both parents were found to have a monoallelic heterozygous substitution at the same position. Moreover, four of eight family members who were screened for the possible presence of the novel genetic variant were found to be heterozygous carriers (maternal and paternal uncle, maternal grandfather, and paternal grandmother), whereas no mutations were observed in the other family members (Figure 2 and Table 1).

Neither one of the two consanguineous family members (maternal grandmother and paternal grandfather) hosted the mutated allele (Figure 2 and Table 1).

Protein modeling

To investigate the functional consequences of the substitution of an alanine with a glycine residue at amino acid position 221, we followed a two-step strategy: 1) transferring the structure of cortisol into the binding cavity of the crystal structure of the 11β-HSD1 to assess the role of each of the residues in the binding cavity, in particular the role of Ala172 (that corresponds to Ala221 in 11β-HSD2); and 2) modeling the structure of the 11β-HSD2 and transferring the coordinates of cortisol into its binding cavity by using a multitemplate protocol to study the putative role of Ala221 in the interaction with cortisol.

By analyzing the 11β-HSD1 complexed with cortisol, it can be clearly appreciated that Ala172 lies in a hydrophobic pocket that is crucial for the interaction with the substrate (Figure 3): the presence of a glycine at position 172 will affect the ligand binding by avoiding an important hydrophobic interaction with the substrate, namely with cortisol (Figure 3B). The same features can be observed in the case of the 11β-HSD2 model as shown in Figure 3C in which it can be strikingly appreciated that Ala221 most likely participates in the binding of cortisol by offering a hydrophobic side chain to stabilize the configuration of the ligand. The lack of such a side chain (Figure 3D), ie, in the case of the substitution with a glycine due to the mutation, may affect the affinity of the protein for cortisol. The loss or gain of a methyl group in pairwise interactions between hydrophobic side chains is related with an approximately 0.7-kcal/mol energy change, as measured in systematic studies (24), thus implying that the loss of the methyl group of alanine will result in a less stable interaction with cortisol.

Although the cofactor was also transferred to the model, it was found not to be close to the Ala221 residue.

Clinical studies

The data reported in the family pedigree (Figure 2) and in Table 1 show that, apart from the two probands who were observed to be homozygous for the mutation (221GG), 4 of the 10 screened family members became hypertensive at a young age, and all four of them were heterozygous for the mutation (221AG). The mother of

Table 1. Clinical and Biochemical Features of the Family Members

	Age, y	K^+, mmol/L	THFs/THE Ratio (Normal if < 1.5)	P-Renin, pg/mL (Range 2–24)	P-Aldosterone, pg/mL (Range 35–300)	Diagnosis of Hypertension	Presence of A221G Variant
Proband 1-boy 1	13	2.60	8.91	3.24	Undetectable	Yes	Homozygous
Proband 2-boy 2	7	3.28	6.41	2.46	Undetectable	Yes	Homozygous
Mother	46	4.28	1.91	6.24	134	No	Heterozygous
Father	50	4.33	1.79	5.34	Undetectable	Yes	Heterozygous
Maternal grandfather	81	3.12	2.49	8.46	134	Yes	Heterozygous
Maternal grandmother	74	4.80	1.80	8.90	128	No	Absent
Paternal grandfather	79	3.85	1.63	40.40	117	Yes	Absent
Paternal grandmother	75	3.42	1.74	13.86	134	Yes	Heterozygous
Paternal aunt	48	3.94	0.87	7.56	185	No	Absent
Paternal uncle	52	4.03	2.54	0.84	Undetectable	Yes	Heterozygous
Maternal uncle	51	3.84	2.43	11.40	106	No	Heterozygous
Maternal aunt	49	3.64	0.99	6.06	132	No	Absent

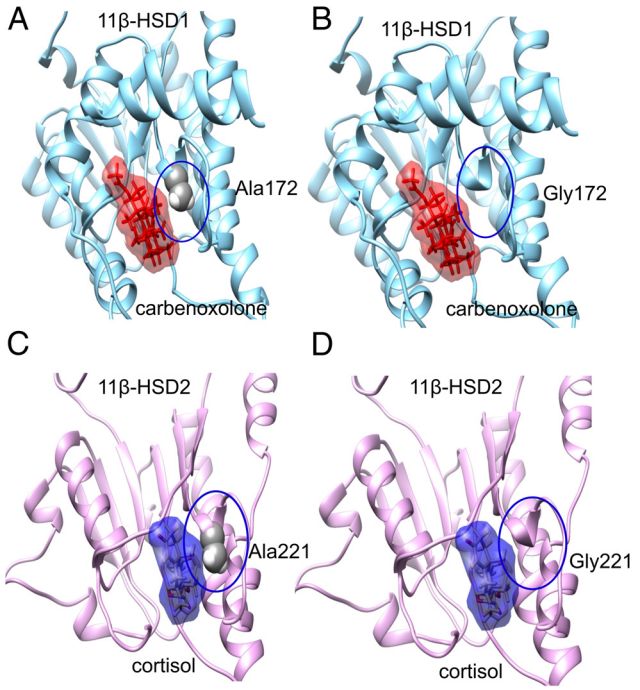

Figure 3. Structural assessment of the variant in the template structure (human 11β-HSD1, PDB database accession code 2BEL) and in the modeled structure of human 11β-HSD2. 11β-HSD1 wild type (A) and A172G mutated 11β-HSD1 (B) complexed with carbenoxolone. 11β-HSD2 wild type (C) and A221G-mutated 11β-HSD2 complexed with cortisol (D).

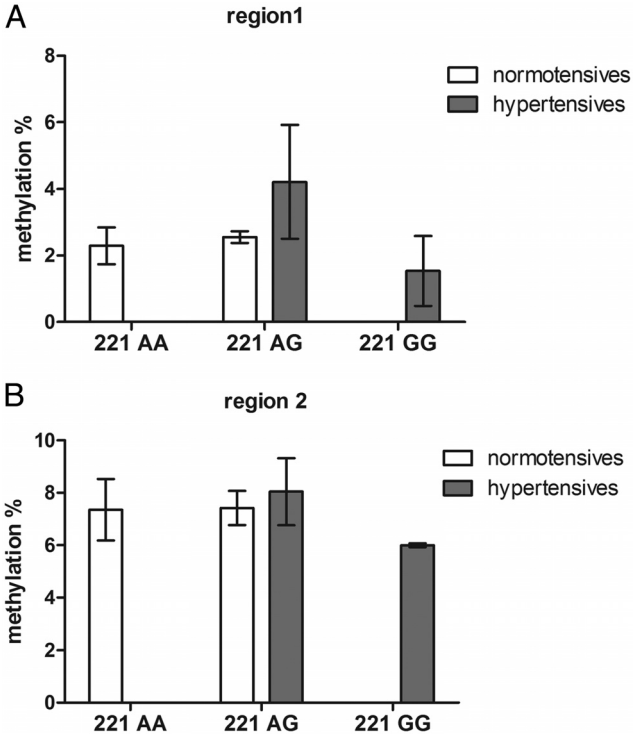

Figure 4. : Methylation index in region 1 (panel A) and region 2 (panel B) according to hypertensive and C662G mutation status in the family members. AA, wild type; AG, heterozygous; GG, homozygous.

the probands and their maternal uncle were normotensive, despite the presence of the mutated allele.

Subjects bearing the mutated allele tended to have lower aldosterone and renin values as compared with subjects carrying the ancestral allele (221AA), but only one of the heterozygous subjects was hypokalemic (Table 1 and Supplemental Table 2). Of note, except for the two probands who were studied when not undertaking drugs interfering on the renin angiotensin aldosterone system, the other hypertensive family members were, instead, studied during treatment with hypertensive drugs.

All subjects harboring the mutation displayed a THFs to THE ratio higher than 1.5. When the four wild-type subjects were analyzed, the two youngest had a normal ratio, whereas the two oldest had a ratio between 1.5 and 2 (Table 1). In the two subjects with THFs to THE ratio higher than 1.5 and not harboring the mutation, as for the probands, all exons and intron-exons boundaries of the *HSD11B2* gene were sequenced, but no other mutations in the gene were detected. The ratio increased in parallel with the number of mutated alleles (Supplemental Table 2).

HSD11B2 promoter methylation

In all of the screened family members, the mean methylation index at *HSD11B2* promoter was 2.85% ± 1.4% at region 1 and 7.37% ± 1.2% at region 2. The comparison of the methylation levels for each person according to the genotype (presence-absence of the mutated allele) is detailed in Figure 4: the two proband boys were characterized by the lowest methylation value in both regions and normotensive heterozygous had a methylation percentage similar to that observed for the wild-type subjects, whereas hypertensive heterozygous subjects were more heavily methylated. Statistical analysis within subgroups was not allowed because of the small number of subjects.

Discussion

The present work allowed the identification and characterization of a novel functional mutation within exon 3 of the *HSD11B2* gene, associated, in the homozygous status, with AME disease in two affected children. Moreover, the role of epigenetic features, namely DNA methylation at the *HSD11B2* promoter site, is for the first time described here as a possible further key mechanism, beyond genetic defects, to explain the different phenotypic expression of the disease.

Most of the previously described mutations within the *HSD11B2* gene related to AME disease, resides within exons 3, 4, or 5 and are mostly missense mutations, therefore resulting in the expression of a protein with a completely abolished or a severely impaired enzymatic activity (7). Other genetic aberrancies including insertions, deletions, and point mutations potentially causing aberrant

splicing have been also described (15, 25, 26). Loss of the enzyme catalytic activity seems to be the main responsible factor for the development of the disease but also a loss of affinity toward the substrate-cofactor, an impaired protein stability, are thought to be important mechanisms leading to a loss of enzymatic function and onset of disease (26).

In the present study, the sequencing approach demonstrated the presence of a C-to-G substitution with a nucleotide transition at codon 221 that results in the change of the amino acid sequence by the substitution of an alanine with a glycine. The presence of the amino acid alanine at position 221 appears to be highly conserved, suggesting a crucial role for this residue [(see multiple sequence alignment containing 11β-HSD2s and their close relatives 17β-HSD2s [26]). To gain deeper insights into the enzyme function and to analyze the putative effects of the 221A>G substitution, we have thus predicted the structure of 11β-HSD2 by generating a model of the protein complexed with its substrate, ie, cortisol. In silico structure analysis showed that Ala221 lies in a hydrophobic pocket involved precisely in the cortisol binding. In fact, Ala221 lies within a chain of residues already shown to form the substrate-binding pocket (http://www.ebi.ac.uk/pdbe-srv/view/entry/2bel/summary.html). The alanine 221 substitution into a glycine by lacking a side chain, in this position, is therefore likely to alter the local hydrophobic environment, thus impairing the effective binding of cortisol and ultimately inducing a mild impairment of the enzyme activity.

Moreover, the novel mutation described here lies into two base pairs from the intron 3 donor splice site and could thus compromise the pre-mRNA splicing, as already speculated by Quinkler et al (25) when reporting a different mutation in the same codon (A221V). The 221A>G mutation is situated only two codons upstream to another previously described amino acid substitution (D223N) that is associated with an almost completely abolished dehydrogenase activity (5). The structure modeling of the mutant enzyme revealed that this substitution (D223N) changes the enzyme surface electrostatic potential, therefore affecting the cofactor enzyme binding (5).

The 221A>G substitution is therefore very likely to have a negative impact on the ligand binding and it is consequently predicted to be causative of AME by attenuating the 11β-HSD2 enzyme activity. The attenuation but not complete loss of function of the enzyme gives reason for the relatively late onset of the disease in the two probands, especially considering that in most of the other AME cases, the disease usually develops in newborns.

The degree of 11β-HSD2 loss of function, as revealed by the THFs to THE ratio used as a surrogate marker of the enzymatic function, appears to correlate with the severity of clinical manifestations of the disease in AME (7, 14, 15). Interestingly, individuals pertaining to the same family and carrying the same mutation in heterozygosity display a different phenotype, with altered urinary THFs to THE ratio but different severity of hypertension that developed only in some but not in all the family members (7, 14, 15, 27). Despite the presence of an identical genetic defect, the reason for such a differential phenotypic expression of the disease has not been deeply investigated thus far.

Considering that hypertension is a multifactorial disease, it is reasonable to hypothesize that in heterozygous subjects several environmental factors, such as obesity, age (28), and sodium intake (29), may play an important function in regulating the differential degree of BP elevation (7). Because epigenetic phenomena can be modified through those kinds of lifestyle factors, the epigenetic regulation by DNA methylation, which is known to be functional at *HSD11B2* promoter site from previous studies using a rodent model and in cultured human cell lines (17, 30), may be among the responsible mechanisms for the differential phenotypic expression of the disease notwithstanding a similar genetic background.

Methylation of DNA is the main epigenetic feature in mammalian cells and is deputed to gene transcriptional regulation, usually by leading to transcriptional repression when the gene promoter site is hypermethylated (31). To date, the role of DNA methylation has been largely addressed in cancer but most recently has been proposed also in the pathogenesis of several other major chronic diseases including arterial hypertension (32).

In the present study, among the six heterozygous family members (221AG), we documented a higher methylation index in the four hypertensive subjects as compared with the two normotensive subjects, therefore confirming the transcriptional repression effect induced by methylation at *HSD11B2* promoter region (31), leading to a reduced 11β-HSD2 synthesis that eventually facilitate the hypertensive phenotype. In this regard, a possible limitation of the study is the relatively small number of subjects that can, however, be considered relevant due to the fact that all subjects harbored the same missense mutation.

Remarkably, in the two probands, homozygous AME brothers, the promoter methylation appeared reduced, rising the hypothesis of an epigenetic mechanism of compensation inducing an increase in gene expression in which the activity of the enzyme is more severely compromised because of the effect of the missense mutation. As we previously demonstrated, it could also be considered that the genetic effect may be prevalent in terms of gene expression regulation rather that an epigenetic effect due to methyl-

ation (33). A similar process, even if not demonstrated, would not be surprising, taking into account recent evidences that DNA methylation is dynamically regulated (34). According to previous findings, we also observed an overall higher methylation status at *HSD11B2* promoter region 2 as compared with region 1 (16). Whether there is a prevalent effect on gene expression regulation of region 1 or 2 cannot be established from the present findings, and only ad hoc-designed studies may be helpful for this purpose.

The absence of the mutation in the two consanguineous offspring (the maternal grandmother and paternal grandfather) was an unexpected finding, but it is reasonable to hypothesize an unidentified consanguinity in preceding generations along the family tree. Of note, both parents and their ancestors were living in the same native little town. It is therefore highly conceivable that other consanguineous marriages had occurred in the past in the same lineage.

Looking at individual characteristics of all family members, it is worth noting that the two relatives lacking the mutated allele had a relatively high THFs to THE ratio (between 1.5 and 2). However, even if not foreseen, this finding is not surprising if one considers that in some hypertensive patients it was already reported that a mild increment of the ratio is not always explained by the presence of genetic mutations (7, 35). Moreover, those two relatives showing high THFs to THE ratios were older than 70 years, and it was previously demonstrated that age positively correlates with the THFs to THE ratio, suggesting that the reduced 11β-HSD2 activity could contribute to the high prevalence of arterial hypertension in the elderly population (28).

In this study the methylation status was analyzed on peripheral blood mononuclear cell (PBMC) DNA. Epigenetic mechanisms are considered tissue specific; therefore, there is room for speculation on how to interpret such data related to the epigenetic regulation by methylation of an enzyme that, although gene expression has been demonstrated also in PBMC-derived RNA (36), is considered functional and expressed mainly in mineralocorticoid target tissues such as renal tubular tissue, colon, small intestine, and placenta as well as other tissues (37). It could be hypothesized, as for other systemic diseases including hypertension (17, 32), that methylation status in PBMC DNA may reflect a systemic situation rather than a tissue-specific condition and may be a potentially useful molecular biomarker for clinical purposes (32).

The novel observation of a potential role of promoter methylation in regulating *HSD11B2* gene expression in this family with AME-affected members strengthens the hypothesis of a possible role of a mildly impairment of 11β-HSD2 activity due to differential modulation by epigenetic mechanisms in a number of cases of essential hypertension that appears to mimic AME, although in a less expressed form. Classical AME induced by *HSD11B2* gene mutations is a rare disease; however, a milder reduction of 11β-HSD2 activity was already hypothesized to play some role in a subset of patients with essential hypertension as in patients with corticosteroid-induced hypertension (7, 35, 38), although studies in this regard show some inconsistencies mostly due to the setting of patients and the study design (39, 40).

An epigenetic modulation of *HSD11B2* gene expression could certainly explain a mild reduction in enzyme activity as documented by an altered THFs to THE ratio but not associated with *HSD11B2* gene mutations as seen in some hypertensive patients (7, 35), even if such hypothesis should be confirmed in a large sample set of essential hypertensive subjects. This is in line with the emerging evidence that several epigenetic modifications have major functions within the pathways related to arterial hypertension, with the fascinating challenge of identifying the epigenetic patterns possibly modifiable by environmental factors (32).

Acknowledgments

The work was performed in part in the Laboratorio Universitario di Ricerca Medica (University Laboratory for Medical Research), University of Verona.

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This work was supported by research grants from the Ministero dell'Istruzione dell'Università e della Ricerca (to F.P., S.F., and O.O.).

Disclosure Summary: The authors have nothing to disclose.

References

1. **Ulick S, Levine LS, Gunczler P, et al.** A syndrome of apparent mineralocorticoid excess associated with defects in the peripheral metabolism of cortisol. *J Clin Endocrinol Metab*. 1979;49:757–764.
2. **Mune T, Rogerson FM, Nikkila H, Agarwal AK, White PC.** Human hypertension caused by mutations in the kidney isozyme of 11β-hydroxysteroid dehydrogenase. *Nat Genet*. 1995;10:394–399.
3. **Arriza JL, Weinberger C, Cerelli G, et al.** Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science*. 1987;237:268–275.
4. **Funder JW, Pearce PT, Smith R, Smith AI.** Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. *Science*. 1988;242:583–585.
5. **Carvajal CA, Gonzalez AA, Romero DG, et al.** Two homozygous

mutations in the 11β-hydroxysteroid dehydrogenase type 2 gene in a case of apparent mineralocorticoid excess. *J Clin Endocrinol Metab*. 2003;88:2501–2507.
6. **Lin-Su K, Zhou P, Arora N, Betensky BP, New MI, Wilson RC.** In vitro expression studies of a novel mutation δ299 in a patient affected with apparent mineralocorticoid excess. *J Clin Endocrinol Metab*. 2004;89:2024–2027.
7. **Ferrari P.** The role of 11β-hydroxysteroid dehydrogenase type 2 in human hypertension. *Biochim Biophys Acta*. 2010;1802:1178–1187.
8. **Parsa AA, New MI.** Low-renin hypertension of childhood. *Endocrinol Metab Clin North Am*. 2011;40:369–377, viii.
9. **Parvez Y, Sayed OE.** Apparent mineralocorticoid excess (AME) syndrome. *Indian Pediatr*. 2013;50:416–418.
10. **Alzahrani AS, Aljuhani N, Qasem E, et al.** Apparent mineralocorticoid excess caused by a novel mutation in 11β-hydroxysteroid dehydrogenase type 2 enzyme: its genetics and response to therapy. *Endocr Pract*. 2014;20:e151–e156.
11. **Zahraldin K, Janahi IA, Ben-Omran T, Alsulaiman R, Hamad B, Imam A.** Two Qatari siblings with cystic fibrosis and apparent mineralocorticoid excess. *Ann Thorac Med*. 2015;10:69–72.
12. **Ferrari P, Sansonnens A, Dick B, Frey FJ.** In vivo 11beta-HSD-2 activity: variability, salt-sensitivity, and effect of licorice. *Hypertension*. 2001;38:1330–1336.
13. **Wilson RC, Dave-Sharma S, Wei JQ, et al.** A genetic defect resulting in mild low-renin hypertension. *Proc Natl Acad Sci USA*. 1998;95:10200–10205.
14. **Lavery GG, Ronconi V, Draper N, et al.** Late-onset apparent mineralocorticoid excess caused by novel compound heterozygous mutations in the HSD11B2 gene. *Hypertension*. 2003;42:123–129.
15. **Morineau G, Sulmont V, Salomon R, et al.** Apparent mineralocorticoid excess: report of six new cases and extensive personal experience. *J Am Soc Nephrol*. 2006;17:3176–3184.
16. **Zhao Y, Gong X, Chen L, et al.** Site-specific methylation of placental HSD11B2 gene promoter is related to intrauterine growth restriction. *Eur J Hum Genet*. 2014;22:734–740.
17. **Friso S, Pizzolo F, Choi SW, et al.** Epigenetic control of 11β-hydroxysteroid dehydrogenase 2 gene promoter is related to human hypertension. *Atherosclerosis*. 2008;199:323–327.
18. **Campino C, Carvajal CA, Cornejo J, et al.** 11β-Hydroxysteroid dehydrogenase type-2 and type-1 (11β-HSD2 and 11β-HSD1) and 5beta-reductase activities in the pathogenia of essential hypertension. *Endocrine*. 2010;37:106–114.
19. **Altschul SF, Madden TL, Schaffer AA, et al.** Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. 1997;25:3389–3402.
20. **Pei J, Kim BH, Tang M, Grishin NV.** PROMALS web server for accurate multiple protein sequence alignments. *Nucleic Acids Res*. 2007;35:W649–652.
21. **Soding J, Biegert A, Lupas AN.** The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Res*. 2005;33:W244–W248.
22. **Eswar N, Webb B, Marti-Renom MA, et al.** Comparative protein structure modeling using MODELLER. In Coligan JE, et al. *Current Protocols in Protein Science*. 2007: Chapter 2:Unit 29.
23. **Pettersen EF, Goddard TD, Huang CC, et al.** UCSF Chimera—a visualization system for exploratory research and analysis. *J Computat Chem*. 2004;25:1605–1612.
24. **Zhu BY, Zhou NE, Kay CM, Hodges RS.** Packing and hydrophobicity effects on protein folding and stability: effects of beta-branched amino acids, valine and isoleucine, on the formation and stability of two-stranded alpha-helical coiled coils/leucine zippers. *Protein Sci*. 1993;2:383–394.
25. **Quinkler M, Bappal B, Draper N, et al.** Molecular basis for the apparent mineralocorticoid excess syndrome in the Oman population. *Mol Cell Endocrinol*. 2004;217:143–149.
26. **Manning JR, Bailey MA, Soares DC, Dunbar DR, Mullins JJ.** In silico structure-function analysis of pathological variation in the HSD11B2 gene sequence. *Physiol Genomics*. 2010;42:319–330.
27. **Li A, Li KX, Marui S, et al.** Apparent mineralocorticoid excess in a Brazilian kindred: hypertension in the heterozygote state. *J Hypertens*. 1997;15:1397–1402.
28. **Henschkowski J, Stuck AE, Frey BM, et al.** Age-dependent decrease in 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) activity in hypertensive patients. *Am J Hypertens*. 2008;21:644–649.
29. **Lovati E, Ferrari P, Dick B, et al.** Molecular basis of human salt sensitivity: the role of the 11β-hydroxysteroid dehydrogenase type 2. *J Clin Endocrinol Metab*. 1999;84:3745–3749.
30. **Alikhani-Koopaei R, Fouladkou F, Frey FJ, Frey BM.** Epigenetic regulation of 11β-hydroxysteroid dehydrogenase type 2 expression. *J Clin Invest*. 2004;114:1146–1157.
31. **Jones PA.** The DNA methylation paradox. *Trends Genet*. 1999;15:34–37.
32. **Friso S, Carvajal CA, Fardella CE, Olivieri O.** Epigenetics and arterial hypertension: the challenge of emerging evidence. *Translat Res*. 2015;165:154–165.
33. **Friso S, Lotto V, Choi SW, et al.** Promoter methylation in coagulation F7 gene influences plasma FVII concentrations and relates to coronary artery disease. *J Med Genet*. 2012;49:192–199.
34. **Smith ZD, Meissner A.** DNA methylation: roles in mammalian development. *Nat Rev Genet*. 2013;14:204–220.
35. **Williams TA, Mulatero P, Filigheddu F, et al.** Role of HSD11B2 polymorphisms in essential hypertension and the diuretic response to thiazides. *Kidney Int*. 2005;67:631–637.
36. **Agalou S, Butt AN, Chowienczyk P, Swaminathan R.** Hypertension and circulating mRNA for 11β-hydroxysteroid dehydrogenase type II. *Ann NY Acad Sci*. 2008;1137:290–295.
37. **Draper N, Stewart PM.** 11β-hydroxysteroid dehydrogenase and the pre-receptor regulation of corticosteroid hormone action. *J Endocrinol*. 2005;186:251–271.
38. **Olivieri O, Pizzolo F, Ravagnani V, et al.** Urinary cortisol to cortisone metabolites ratio in prednisone-treated and spontaneously hypertensive patients. *J Hypertens*. 2008;26:486–493.
39. **Iki K, Miyamori I, Hatakeyama H, et al.** The activities of 5β-reductase and 11β-hydroxysteroid dehydrogenase in essential hypertension. *Steroids*. 1994;59:656–660.
40. **Santini DL, Lorenzo BJ, Koufis T, Reidenberg MM.** Cortisol metabolism in hypertensive patients who do and do not develop hypokalemia from diuretics. *Am J Hypertens*. 1995;8:516–519.