A New Compound Heterozygous Mutation in the 11β-Hydroxysteroid Dehydrogenase Type 2 Gene in a Case of Apparent Mineralocorticoid Excess

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ABSTRACT

Apparent mineralocorticoid excess (AME) characterized by early-onset hypertension and hypokalemia is due to congenital deficiency of 11β-hydroxysteroid dehydrogenase (11βHSD). Two isoforms of human 11βHSD are known, and the type 2 isoform (11βHSD2) has been recently shown to be responsible for AME. In this study, we analyzed the 11βHSD2 gene of a Japanese patient with AME. PCR amplification and subsequent nucleotide sequencing of the 11βHSD2 gene from the patient and his family members revealed that the patient has a compound heterozygous mutation of this gene. In 1 allele, an undescribed single nucleotide transition in codon 208 in exon 3 resulted in a substitution of arginine to histidine (CGC to CAC: R208H). In the other allele, a deletion of 3 nucleotides in codons 337–338 in exon 5 resulted in a substitution of arginine to histidine and a deletion of tyrosine residue (CGTAT to CAT: Y338R), which has been previously shown to abolish 11βHSD2 enzyme activity. A chloramphenicol acetyltransferase assay-based expression study involving the mineralocorticoid receptor indicated that the novel R208H mutation eliminates the enzymatic activity of 11βHSD2. From the genetic analysis of 50 healthy subjects, the novel R208H mutation was unlikely to be due to polymorphism. Together, these results indicate that this patient is a compound heterozygote for the mutation in the 11βHSD2 gene (R208H and Y338R) and that these mutations inactivate the 11βHSD2 function and give rise to clinically manifest AME. (J Clin Endocrinol Metab 82: 4054–4058, 1997)

APPARENT mineralocorticoid excess (AME) is a rare disease characterized by early-onset low renin hypertension combined with hypokalemia despite subnormal levels of all known mineralocorticoids (1; for review, see Ref. 2). This disease is due to congenital deficiency of 11β-hydroxysteroid dehydrogenase (11βHSD), an enzyme that mediates the conversion of cortisol to cortisone.

The mineralocorticoid receptor (MR) is nonselective in vitro and does not distinguish between cortisol and aldosterone (3, 4). The mechanism by which MR is selectively occupied by aldosterone despite the presence of cortisol in the circulation at a 100–1000 times higher concentration than aldosterone is explained by inactivation of cortisol to cortisone by 11βHSD (5, 6). Accordingly, in the absence of 11βHSD activity, cortisol can bind to the MR and act as a mineralocorticoid.

To date, two isoforms of 11βHSD have been identified. The first isoform, 11βHSD type 1, is low affinity, NAD-dependent, and catalyzes both 11β-dehydrogenation and the reverse 11-oxoreduction reaction. It is found predominantly in liver, testis, lung, and central nervous systems. The human 11βHSD type 1 gene on chromosome 1 was cloned in 1991 (7), but analyses of this gene in patients with AME or 11-oxoreductase deficiency gave normal results (8). 11βHSD type 2 (11βHSD2) is high affinity, NAD-dependent, and catalyzes only 11β-dehydrogenation (9, 10). The human 11βHSD2 complementary DNA (cDNA) was cloned in 1994 and is expressed predominantly in kidney, placenta, colon, salivary gland, and fetal tissues (11–14). The 11βHSD2 gene is approximately 6.2 kilobases long, consists of five exons, and is located on chromosome 16q22 (15). After cloning of the 11βHSD2 gene, several missense mutations/deletions that reduce the enzymatic activity of 11βHSD2 were found in patients with AME (16–19), indicating that this 11βHSD2 gene is responsible for AME.

Due to the rare occurrence of AME, few mutations of the 11βHSD2 gene have been reported. We have analyzed the 11βHSD2 gene in a patient with typical AME and found that the patient has a novel compound heterozygous mutation in the 11βHSD2 gene that inactivates 11βHSD2 function.

Subjects and Methods

Subjects

The patient is a Japanese male with AME who has been previously reported (20). Briefly, he was born with intrauterine growth retardation and an elevated serum creatine phosphokinase level. He was studied at 2 yr of age because of polyuria and polydipsia lasting for 1 yr and was found to have hypokalemic alkalosis and sustained hypertension. His PRA and aldosterone levels were always low, and the ratio of urinary tetrahydrocortisol (THF) plus allotetrahydrocortisol (aTHF) to that of tetrahydrocortisone (THE) was very high (43.7 and 17.4 in two determinations). He has been successfully treated with a combination of spironolactone and nifedipine.

The patient was the second child of unrelated parents. His father
comes from Tochigi prefecture (in central Japan), and his mother comes from Gotou Island of Nagasaki prefecture (in southern Japan). His father, mother, and elder sister were normotensive, normokalemic, and had normal ratios of urinary THF plus aTHF/THE (2.1, 2.1, and 1.6, respectively).

DNA amplification and sequence analysis of the 11βHSD2 gene

The genomic DNAs of all family members were extracted from peripheral white blood cells. PCR was performed to amplify the entire coding region of the 11βHSD2 gene (exons 1–5) using primer sets previously described (16). Reaction mixtures were modified and contained 200 ng genomic DNA, 25 pmol of each primer, 10 nmol of each deoxy-NTP, 2.5 U Taq DNA polymerase (Takara Shuzo, Kyoto, Japan), and its standard buffer in a total volume of 100 μL. Amplified gene fragments were purified by agarose gel electrophoresis, and each PCR product was sequenced directly using modified T7 DNA polymerase (Sequenase 7-deaza-deoxy-GTP Sequencing Kit, U.S. Biochemical Corp., Cleveland, OH) with the same primers as those used for PCR. Another internal primer in exon 5, 5′-AAGGACTACATCGGACCTTGCA-3′ (sense), was used to sequence the middle part of exon 5. Subcloning of the PCR products amplified from exons 3 and 5 of the patient was performed using the TA cloning kit (Invitrogen, San Diego, CA), and the sequencing products amplified from the patient was performed as described above.

Restriction analysis

The mutation in exon 3 found in the patient and his family members, which eliminates an FspI restriction site, was confirmed by digestion of PCR products with the restriction enzyme. The PCR products from 50 endocrinologically normal subjects were similarly analyzed to exclude polymorphism of the gene. Both normal and mutant alleles of the patient subcloned to a vector were separately analyzed in the same way.

Construction of plasmids

Full-length human 11βHSD2 cDNA (a gift from Dr. Z. S. Krozowski) was subcloned into the mammalian expression vector pcDNA3 (Invitrogen). An R208H mutation in 11βHSD2 cDNA was introduced by PCR using oligonucleotides containing the desired change (21). The cDNA of the rat MR encoding the DEF regions (nucleotides 2014–2946), which include the ligand-binding domain (E domain) and possess a ligand-induced transactivation function 2 (AF-2), was generated by reverse transcription-PCR from rat kidney messenger ribonucleic acid and subcloned to a vector were separately analyzed in the same way. The PCR-amplified 11βHSD2 gene from patient’s family was also analyzed by direct sequencing (Fig. 1). The results indicated that the father and the sister had a heterozygous mutation for R208H, whereas codons 337–338 of both subjects were normal. The patient’s mother had heterozygous mutation for R337H, ΔY338, whereas codon 208 was normal.

Restriction analysis

We confirmed the 1-bp transition in codon 208 (CGC to underlined CAC) using the restriction enzyme FspI, which recognizes the sequence TGGCGCA (codon 208 is underlined) in the normal allele. FspI digestion of the PCR products amplified from exons 3 and 4 of the normal allele should produce 2 fragments, 276 and 123 bp, whereas that of the mutated allele remains as an undigested 399-bp fragment. FspI digestion of the PCR products amplified from the patient, the father, and the sister produced 3 fragments of 399, 276, and 123 bp, whereas digestion of the PCR product amplified from the patient’s mother produced 2 fragments of 276 and 123 bp (Fig. 2). Thus, the patient and his unaffected father and sister are heterozygous, and his mother is normal homozygous for the mutation in codon 208. FspI digestion of exon 3–4 PCR products from 50 endocrinologically normal subjects invariably resulted in 2 fragments, 276 and 123 bp, indicating that the CGC to CAC transition in codon 208 is not a polymorphism.

11βHSD2 expression study

To examine whether the R208H mutation affects the enzymatic activity of 11βHSD2, wild-type and mutated 11βHSD2 were overexpressed in HeLa cells, and their in vivo enzymatic activity to convert cortisol into cortisone was assessed by examining the cortisol-induced transcriptional activation function by MR-AF-2. By using the GAL4 DNA-binding domain-MR ligand-binding domain fusion

Results

11βHSD2 gene sequence

To analyze the 11βHSD2 gene, we used a combination of primer sets and amplified the entire coding region from the genomic DNAs obtained from the patient and his family members as well as that from normal subjects. The PCR products amplified from the patient were sequenced directly, which revealed two heterozygous mutations (Fig. 1). One mutation was a G to A transition in the second nucleotide in codon 208 (exon 3) that resulted in a substitution of arginine to histidine (CGC to CAC: R208H). The other was a deletion of three nucleotides (GCT) in codons 337–338 (exon 5) that resulted in a substitution of arginine to histidine and a deletion of tyrosine (CCGCTAT to CAT: R337H, ΔY338). When the PCR fragments were sequenced after subcloning into a vector plasmid, the latter mutation in exon 5 was detected in five of the eight clones examined, whereas DNA sequence in the other three clones was normal. The possibility that the mutation was caused by PCR artifact was excluded by repeat PCR and direct sequencing in both directions as well as by the identical results obtained from subcloned PCR products. No other mutations were found in the exons of the 11βHSD2 gene amplified from the patient.

The PCR-amplified 11βHSD2 gene from patient’s family was also analyzed by direct sequencing (Fig. 1). The results indicated that the father and the sister had a heterozygous mutation for R208H, whereas codons 337–338 of both subjects were normal. The patient’s mother had heterozygous mutation for R337H, ΔY338, whereas codon 208 was normal.
construct [pGAL-MR(DEF)], we avoided potential interference by endogenous factors targeting the cognate DNA-binding site of MR. The enzymatic function of 11βHSD2 was analyzed essentially as described by Warriar et al., who studied 11βHSD1 (25). In the absence of overexpressed 11βHSD2, the CAT activity representing the ligand-induced transactivation of cortisol (Fig. 3) and aldosterone (data not shown), and a cortisol concentration as low as $10^{-8}$ mol/L could stimulate MR-AF-2 transcriptional activation. However, when wild-type 11βHSD2 was coexpressed in HeLa cells, efficient cortisol-induced transcriptional activation was observed only at concentrations higher than $10^{-6}$ mol/L. This indicates that the overexpressed 11βHSD2 effectively converted cortisol to inactive cortisone. In contrast, no enzymatic inactivation of cortisol was seen with 11βHSD2 R208H mutant, as transcriptional activation by MR-AF-2 was induced even at $10^{-8}$ mol/L cortisol concentration (Fig. 3, A and B). We could reproduce similar results using COS-1 cells (data not shown). These results suggest that R208H mutation abolishes the enzymatic activity of 11βHSD2.

**Discussion**

To date, 10 mutations of 11βHSD2 gene abnormalities have been reported in 15 families (18 patients) with AME (16–19) (Fig. 4). In the present study we demonstrated a new compound heterozygous mutation of 11βHSD2 gene in a Japanese patient affected with AME. Interestingly, all mutations, including ours, are clustered in exons 3–5, suggesting that this region contains residues important for 11βHSD2 enzyme function or that this region has an increased tendency to develop mutations.

The G to A transition in the second nucleotide of codon 208 resulted in a substitution of arginine to histidine (R208H) in one allele identified by direct sequencing (middle upper panel) and a codon 337–338 mutation (CGCTAT to CAT; R337H, ΔY338) in the other allele identified by both direct sequencing (middle lower panel) and sequencing after subcloning (lower panel). The nonaffected father and sister are carriers of the codon 208 mutation, and the mother is a carrier of the codon 337–338 mutation. *Stars* indicate mutated nucleotides/amino acids. The *arrow* indicates the proband.
North American (16) and the other from Oman (17); both were homozygous for arginine (CGC) to cysteine (TGC) substitution (R208C). Considering that this arginine residue is highly conserved within human, rat, mouse, and sheep 11\(\beta\)HSD2 (14, 26–28), these findings underscores the critical role of this arginine residue in 11\(\beta\)HSD2 function.

The other mutation found in our patient was a three-nucleotide deletion in codons 337–338, resulting in a substitution of arginine to histidine and a deletion of tyrosine (R337H, D\text{Y}338). Exactly the same homozygous mutation has been found in three other families, two from India and one from Iran (16, 17). In addition, the results of expression study indicated that 11\(\beta\)HSD2 harboring this mutation has no detectable enzyme activity (16). Thus, it is highly likely that this mutation as well as the R208H mutation is the cause of the enzyme defect in this patient.

Among the nonaffected family members, the patient’s father and sister carried only the R208H mutation, whereas the mother carried only the R337H, D\text{Y}338 mutation. These heterozygous carriers were totally normal for blood pressure, serum potassium, and urinary THF plus aTHF/THIE ratio. This indicates that the heterozygotes of 11\(\beta\)HSD2 gene mutation are clinically indistinguishable from normal subjects, which is consistent with the autosomal recessive inheritance of this disease.

The affected AME patients in 14 of 15 families were all homozygotes for their 11\(\beta\)HSD2 mutations (16–19). The only compound heterozygote was an Irish-American, whose 11\(\beta\)HSD2 gene had an 11-bp deletion giving rise to a premature termination codon as a result of a frame shift and a 9-bp deletion eliminating the catalytic site (16). On the other hand, most of the homozygous mutations were missense mutations or 1- to 3-bp deletions, some of which do not totally abolish the enzyme activity. Accordingly, it was suggested that an identical mutation on both alleles is required for clinical manifestation of the disease and that a subject who is a compound heterozygote may have a very mild disease that goes undetected (17, 29). Our patient is a typical case of AME with early presentation of hypertension, hypokalemia, and high THF plus aTHF/THIE ratio, together with intrauterine growth retardation, which proved to be a common feature of this disease (19, 20). The finding that this
patient had a compound heterozygous mutation, one missense mutation and one 3-bp deletion, verified that a compound heterozygous mutation in the 11βHSD2 gene can be the cause of AME. It is also clear that patients with homozygous and compound heterozygous mutations are indistinguishable by clinical studies.

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References

10. Stewart PM, Murrey BA, Mason JJ. 1994 Human kidney 11β-hydroxysteroid dehydrogenase is a high affinity nicotinamide adenine dinucleotide-dependent enzyme and differs from the cloned type I isomerase. J Clin Endocrinol Metab. 79:480–484.