Screening for Mutations in the Steroidogenic Acute Regulatory Protein and Steroidogenic Factor-1 Genes, and in CYP11A and Dosage-Sensitive Sex Reversal-Adrenal Hypoplasia Gene on the X Chromosome, Gene-1 (DAX-1), in Hyperandrogenic Hirsute Women*

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Abstract

Abnormalities in adrenal and/or ovarian steroidogenesis are found in most patients with hirsutism. The rate-limiting step in the synthesis of steroids in the ovary and the adrenal is the conversion of cholesterol into pregnenolone by cholesterol side-chain cleavage enzyme (P450scc), encoded by the gene *CYP11A*, after cholesterol is introduced into the mitochondria by the steroidogenic acute regulatory protein (StAR). DAX-1 is a repressor of StAR gene expression, and steroidogenic factor-1 (SF-1) is a regulator of *CYP11A*, DAX-1, and StAR gene. Mutations in any of these factors resulting in gain of function, or loss of repression, of StAR or P450scc might contribute to the steroidogenic abnormalities present in hirsute patients.

In the present study we have screened, using heteroduplex analysis, the genes encoding StAR and SF-1 as well as DAX-1 and *CYP11A* for mutations in genomic DNA from 19 women presenting with hirsutism and increased serum androgen levels. When variants were found, analysis was extended to a larger group of hyperandrogenic

patients and nonaffected women. Two variants were identified in the SF-1 gene. A G→C change in exon 6, resulting in an Arg³⁶⁵Pro mutation, was found in 1 of 45 patients, but not in controls. Also, a Gly¹⁴⁶Ala missense mutation, resulting from a G→C change in exon 4, was found in 2 of 48 patients and in 2 of 50 nonaffected individuals. We identified a C→T base pair change at position -33 of the StAR gene. Three of 48 patients and 3 of 43 controls presented this variant. No mutations were found in coding regions of the StAR gene. Analysis of *CYP11A*-coding regions identified a G→A change in exon 3, resulting in a Val¹⁷⁹Ile missense mutation. This mutation was found in 1 of 29 patients studied and was not present in 50 controls. Finally, analysis of DAX-1 showed no variant in any of the women studied.

In conclusion, mutations in StAR, SF-1, *CYP11A*, and DAX-1 are seldom found in hirsute patients and do not explain the steroidogenic abnormalities found in these women. (*J Clin Endocrinol Metab* 86: 1746–1749, 2001)

FAMILIAL AGGREGATION provides evidence pointing to a genetic basis of hyperandrogenism in women (1), especially in the polycystic ovary syndrome (PCOS), in which clinical and/or biochemical hyperandrogenism is associated with ovulatory dysfunction (2). However, there is also familial aggregation of cases of women presenting with hyperandrogenism who do not have ovulatory dysfunction (1).

Hirsutism results from androgen excess at the pilosebaceous unit and is considered a clinical marker of hyperandrogenism in women. In nearly all hirsute women, abnormalities in adrenal and/or ovarian steroidogenesis can be

demonstrated, suggesting that both glands contribute to some extent to the androgen excess responsible for hirsutism (3, 4).

The enzymes required for the synthesis of androgens are cholesterol side-chain cleavage enzyme (P450scc), encoded by the gene *CYP11A*; P450c17, encoded by *CYP17*; 3 β -hydroxysteroid dehydrogenase; and several isoenzymes of 17 β -hydroxysteroid dehydrogenase. The adrenal and the ovary share most of these enzymes. Moreover, the activities of P450scc, P450c17, 3 β -hydroxysteroid dehydrogenase, and 17 β -hydroxysteroid dehydrogenase type 5 are increased in thecal cells from PCOS patients maintained in primary culture, an abnormality that can be considered a primary event in the pathogenesis of this disorder (5, 6).

The genes encoding for the enzymes involved in androgen synthesis have been studied in women with hyperandrogenism, but no definite role for any of the variants found has been established to date. Polymorphisms in the regulatory regions of *CYP11A* and *CYP17* do not appear to be related to hyperandrogenism (7–10). The coding regions of *CYP11A* have not been studied, and the existence of nonclassical 3 β -hydroxysteroid dehydrogenase deficiency has never been demonstrated at the molecular genetic level (11). Furthermore, no association of variants

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in any of the genes encoding the 17 β -hydroxysteroid dehydrogenase isoenzymes with hyperandrogenism have been reported to date. Therefore, the genes encoding other factors involved in the regulation of adrenal and ovarian androgen synthesis should be considered candidates to explain the etiology of hyperandrogenism.

The initial step in adrenal and ovarian androgen synthesis is the conversion of cholesterol to pregnenolone. This conversion is catalyzed by the mitochondrial enzyme P450_{scc} and requires transport of cholesterol into the mitochondria, which is mediated by the steroidogenic acute regulatory protein (StAR). Dosage-sensitive sex reversal-adrenal hypoplasia gene on the X chromosome gene-1 (DAX-1) is a repressor of StAR gene expression, and steroidogenic factor-1 (SF-1) is a regulator of StAR gene, *CYP11A*, and DAX-1, among others.

Recently, it has been shown that inactivating mutations in DAX-1 and StAR genes are the cause of adrenal hypoplasia congenita and lipoid congenital adrenal hyperplasia, respectively (12, 13), and an inactivating mutation in SF-1 resulted in sex reversal and adrenal failure in a 46,XY subject (14). To date, inactivating *CYP11A* mutations have not been described.

Conversely, variants in the genes encoding for these factors, resulting in gain of function, or loss of repression, of StAR, P450_{scc}, or other steroidogenic enzymes, might contribute to androgen excess in hyperandrogenic women. To test this hypothesis, we have screened the genes encoding StAR, SF-1, DAX-1, and *CYP11A* for mutations in genomic DNA from patients presenting with hirsutism and increased androgen levels.

Subjects and Methods

Subjects

Nineteen hyperandrogenic hirsute women [mean $\pm$ SD: age, 22.3 $\pm$ 6.2 yr; body mass index, 26.7 $\pm$ 6.8 kg/m²; modified Ferriman-Gallwey score (15), 17.1 $\pm$ 5.3] were initially screened for mutations.

Serum samples were obtained during the follicular phase of the menstrual cycle. All of the patients presented hyperandrogenemia, as defined by increased serum concentrations of one or more of the following androgens: total testosterone above 2.30 nmol/L, calculated free testosterone (16) above 35 pmol/L, androstenedione above 16.0 nmol/L, and dehydroepiandrosterone sulfate above 9.9 μ mol/L. The mean $\pm$ SD circulating levels of these androgens were 2.9 $\pm$ 1.4 nmol/L, 61 $\pm$ 52 pmol/L, 12.8 $\pm$ 4.5 nmol/L, and 9.7 $\pm$ 4.7 μ mol/L, respectively. The assays employed for serum hormone measurements have been reported previously (17).

Nonclassical congenital adrenal hyperplasia and hyperprolactinemia were ruled out in all of the patients by ACTH-stimulated serum 17-hydroxyprogesterone levels below 30 nmol/L (18) and basal serum PRL concentrations below 24 μ g/L.

Nine of the 19 patients, who had oligomenorrhea defined by menstrual cycles longer than 36 days (19) fulfilled endocrine criteria for the diagnosis of PCOS (2).

When the screening of the 19 hirsute patients described above detected any variant, the analysis was extended to a larger group of hyperandrogenic hirsute patients with or without menstrual dysfunction (17) and to a group of 50 healthy women who did not have hirsutism, acne, alopecia, or menstrual disturbances (20). Data from these patients and controls regarding aspects of the pathophysiology of hirsutism and hyperandrogenism different from those studied here have been previously reported (17, 20).

The hospital ethics committee approved the study, and informed consent was obtained from every patient and control.

Molecular genetic analysis

Genomic DNA was extracted from peripheral leukocytes following the manufacturer's instructions (Wizard Genomic DNA Purification Kit, Promega Corp., Madison, WI). A broad screening was performed in the 19 patients for mutations in DAX-1, SF-1, and StAR genes, and in the coding regions of *CYP11A*. We have not evaluated the variable tandem repeat polymorphism in the promoter of *CYP11A*, as our previous study in a large series of hirsute patients showed no association either with hirsutism or hyperandrogenism (7).

DNA was used to amplify different portions of the genes described above. PCR amplifications were performed using an automated thermocycler (GeneAmp PCR System 2400, PE Applied Biosystems, Foster City, CA).

The primers used for PCR amplification of each exon and intron-exon boundaries of *CYP11A* and SF-1 genes and for amplification of the 5'-regulatory regions of SF-1 gene (from -246 to +55 in exon 1), DAX-1 (position -194 to +98 in exon 1), and StAR gene (position -950 to -753, which contains a putative SF-1-binding domain), were designed using OLIGO 4.0 Primer Analysis Software (Molecular Biology Insights, Inc., Plymouth, MN). Primer sequences and PCR conditions are available from the authors on request.

The primers used for the amplification of the two exons of DAX-1 and for the promoter and seven exons of the StAR gene have been previously described by others (21, 22).

Heteroduplex analysis (23) was used to detect variants, which were identified by direct sequencing of PCR products using an ABI 310 automated sequencer (PE Applied Biosystems). Digestion by *SphI* or *MspI* endonuclease restriction enzymes was used for the screening of mutations that cause changes in target sites for these enzymes. Mutations were confirmed by direct sequencing.

Variants present in more than 2% of the study population and in similar proportions in patients and healthy controls were considered common polymorphisms (24).

Results

The results are summarized in Table 1. We identified several novel variants in the SF-1 and StAR genes and in *CYP11A*. Analysis of SF-1 gene in the 19 hirsute patients initially screened detected 2 different heterozygous mutations. Two patients presented a G $\rightarrow$ C change in exon 4, resulting in a Gly¹⁴⁶Ala missense mutation. This mutation creates a restriction site for *SphI*, and digestion by this enzyme was used for further testing. The presence of the Gly¹⁴⁶Ala mutation in 2 of 48 hyperandrogenic patients and 2 of 50 controls suggested that this variant is a polymorphism (24).

A different patient, presenting with PCOS and increased serum androgens of adrenal and ovarian origin, showed a G $\rightarrow$ C change in exon 6, resulting in an Arg³⁶⁵Pro missense mutation. Using digestion by *MspI*, which is prevented by the Arg³⁶⁵Pro mutation, the latter was not found in any other patient or control.

No mutations were found in the promoter region of DAX-1 or in coding regions of the DAX-1 and StAR gene. On the contrary, a C $\rightarrow$ T bp change at position -33 from

TABLE 1. Observed frequencies of the mutations found in the SF-1 and StAR protein genes and in coding regions of *CYP11A* in hyperandrogenic hirsute patients and healthy controls.

Gene	Mutation	Observed frequencies [% (affected/total)]	
		Patients	Controls
SF-1 (exon 4)	Gly ¹⁴⁶ Ala	4.2 (2/48)	4.0 (2/50)
SF-1 (exon 6)	Arg ³⁶⁵ Pro	2.2 (1/45)	0.0 (0/50)
StAR (-33)	C $\rightarrow$ T	6.3 (3/48)	7.0 (3/43)
<i>CYP11A</i> (exon 3)	Val ¹⁷⁹ Ile	3.4 (1/29)	0.0 (0/50)

the transcription start site of the StAR gene was identified in 1 of the 19 patients initially screened. This mutation results in a target site for *MspI*, and digestion by this restriction enzyme was used to evaluate another 29 patients and 43 controls. A total of 3 patients and 3 controls presented this variant, which therefore was considered a polymorphism (24).

Finally, analysis of *CYP11A*-coding regions identified a G→A change in exon 3, resulting in a Val¹⁷⁹Ile missense mutation. The mutation was found in 1 of 29 patients and was not present in 50 nonaffected individuals. No other variants were identified in other coding regions of *CYP11A*.

None of the affected patients or controls had more than one of the variants described above.

Discussion

Recent data suggest that abnormalities in steroidogenesis are a primary event in the pathogenesis of PCOS, affecting nearly all of the enzymes involved in ovarian androgen synthesis. Nelson *et al.* (5) found increased messenger ribonucleic acid expression of the steroidogenic enzymes involved in androgen synthesis in ovarian thecal cells from patients with PCOS propagated in primary culture, including P450scc and *CYP17*, together with increased enzyme activity of P450scc, P450c17, 3 β -hydroxysteroid dehydrogenase, and 17 β -hydroxysteroid dehydrogenase type 5. On the contrary, the expression of StAR was comparable to that in controls (5). The increased activity of these enzymes resulted in increased synthesis of androgens and steroid precursors (5). The persistence of these findings after several passes in culture rules out a significant influence of nonovarian factors in the increased ovarian steroidogenesis of thecal cells from PCOS patients (5).

Furthermore, using promoter constructs containing successive truncations of the 5'-flanking sequence of the human *CYP17* gene, these researchers have recently shown that the region between -235/-109 bp of the transcription site is critical to the increased *CYP17* messenger ribonucleic acid expression and activity in PCOS thecal cells (25). Cotransfection with SF-1 was required for the detection of basal and forskolin-stimulated *CYP17* promoter activity, in agreement with the presence of SF-1 response elements in that region (25).

Therefore, variants in the genes encoding the enzymes involved in androgen synthesis and in those of their regulatory factors might be related to the up-regulation of steroidogenic enzymes found in hyperandrogenism and hirsutism. As these factors are present in both the adrenal and the ovary, their abnormalities might explain not only the increased synthesis of androgens in the ovary, but also the frequent finding of adrenal hyperandrogenism in hirsute and PCOS patients (26).

The first and critical step in ovarian and adrenal steroidogenesis is the conversion of cholesterol into pregnenolone. This step is mediated by the actions of StAR and P450scc. The genes encoding both proteins are influenced by several factors, including DAX-1 and SF-1, which are believed to play a crucial role in the development and function of the adrenal

and gonads. The interaction between DAX-1 and SF-1 is poorly understood. DAX-1 is a potent repressor of StAR gene expression, blocking steroidogenesis (12) and also repressing the transcriptional activity of SF-1 (27). SF-1 is generally considered to be a transcriptional activator mediated by recruiting coactivators; however, binding of DAX-1 to SF-1 turns SF-1 into transcriptional repressor, because DAX-1 is able to recruit corepressors (27). The influence of DAX-1 and SF-1 is not limited to the initial steps of adrenal and ovarian steroidogenesis; it also modulates the activity of enzymes such as P450c17 and 3 β -hydroxysteroid dehydrogenase (28, 29).

In the present study we screened the genes encoding these factors and enzymes in women presenting with hirsutism and increased serum androgen levels. The novel variants found in our patients were not frequent enough to explain the hyperandrogenism of these women.

The G¹⁴⁶A variant in SF-1 gene and the C→T change at -33 of the StAR protein gene can be considered polymorphisms using the standard definition, as more than 2% of our population is heterozygous for that locus (24). The small proportion of patients affected and the similar proportion of controls carrying the variant suggest that these are common polymorphisms (30) and that the contribution of these variants to the pathogenesis of hyperandrogenism, if any, is minor.

On the contrary, the variants found in exon 6 of the SF-1 gene and in exon 3 of *CYP11A* were not present in controls. Comparison of the human and murine *CYP11A* sequences revealed that mice present Ile at the equivalent position, suggesting that the Val¹⁷⁹Ile missense mutation found in one patient probably has no important consequences for the function of this enzyme. Moreover, Val and Ile are similar amino acids in terms of composition, polarity, and molecular volume, properties that correlate with the evolutionary exchangeability of protein residues (31, 32).

The Arg³⁶⁵Pro mutation found in one patient may have a relevant impact on the function of SF-1, as an Arg is present at equivalent positions of the murine, rat, and bovine SF-1 genes, and the introduction of a proline can affect the secondary structure of the SF-1 molecule.

Further work is needed to properly describe the Arg³⁶⁵Pro missense mutation in exon 6 of SF-1. Examination of parental genotypes is needed to evaluate the Mendelian inheritance of this variant or the possibility of a *de novo* mutation. Moreover, functional studies using transcription assays after mutagenesis in appropriate vectors will be required to evaluate the impact of the Arg³⁶⁵Pro missense mutation on SF-1 activity in different cell systems.

In conclusion, variants in DAX-1, SF-1, and StAR genes and in coding regions of *CYP11A* are seldom found in women with hyperandrogenic hirsutism. Moreover, the variants identified in our present study are either equally frequent in patients and controls or are rare or conservative changes and thus not likely to be significant contributors to hyperandrogenism. Therefore, these genes do not appear to play a major role in the pathogenesis of hirsutism and hyperandrogenism.

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