BRIEF REPORT

Inactivating *PAPSS2* Mutations in a Patient with Premature Pubarche

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SUMMARY

Dehydroepiandrosterone (DHEA) sulfotransferase, known as SULT2A1, converts the androgen precursor DHEA to its inactive sulfate ester, DHEAS, thereby preventing the conversion of DHEA to an active androgen. SULT2A1 requires 3′-phosphoadenosine-5′-phosphosulfate (PAPS) for catalytic activity. We have identified compound heterozygous mutations in the gene encoding human PAPS synthase 2 (*PAPSS2*) in a girl with premature pubarche, hyperandrogenic anovulation, very low DHEAS levels, and increased androgen levels. In vitro coincubation of human SULT2A1 and wild-type or mutant PAPSS2 proteins confirmed the inactivating nature of the mutations. These observations indicate that PAPSS2 deficiency is a monogenic adrenocortical cause of androgen excess.

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Hyperandrogenic anovulation is a major clinical feature of the polycystic ovary syndrome,[1,2] which affects 5 to 15% of women and is associated with an increased incidence of the metabolic syndrome.[3-7] It has been suggested that premature pubarche, characterized by the growth of pubic hair in girls younger than 8 years of age, may be an early sign of the polycystic ovary syndrome.[8,9] Premature pubarche is most often the manifestation of premature adrenarche,[10] defined by an early increase in the levels of the adrenal androgen precursor DHEA and its sulfate ester, DHEAS, which is the most abundant steroid in human circulation.

DHEA is the principal precursor of androgen synthesis in women.[11,12] Unconjugated DHEA molecules can be converted directly to androgens, whereas the conversion of DHEAS molecules to androgens first requires cleavage of the sulfate group. Sulfation appears to be the predominant direction of the interconversion between DHEA and DHEAS,[13] which suggests that increased DHEA sulfation would limit the amount of DHEA available for androgen synthesis. Conversely, impaired sulfation would increase the amount of available DHEA and thus the levels of active androgens.

SULT2A1 is the major enzyme responsible for DHEA sulfation, converting DHEA to DHEAS mainly in the adrenal glands and the liver.[14] The sulfate donor PAPS is required by all sulfotransferases, including SULT2A1.[14] In humans, PAPS is synthesized by the two isoforms of PAPS synthase, PAPSS1 and PAPSS2.[14] A homozygous *PAPSS2* mutation that has been found in spondyloepimetaphyseal dysplasia, Pakistani type (Online Mendelian Inheritance in Man [OMIM] number +603005),[15]

is thought to be caused by impaired proteoglycan sulfation in growth-plate chondrocytes.[15-17]

Here we report on the case of a girl with androgen excess, premature pubarche, hyperandrogenic anovulation, and serum DHEAS levels below the limit of detection. We hypothesize that a defect in DHEA sulfation may explain the phenotype.

CASE REPORT

An 8-year-old girl, the daughter of nonconsanguineous parents of Turkish origin, was first referred for evaluation of pubic and axillary hair growth that had begun 2 years earlier. Pubic-hair development was classified as Tanner stage 4 and breast development as Tanner stage 2. Blood pressure was normal at 115/85 mm Hg. Bone age was accelerated (12 years, according to the classification of Greulich and Pyle). Her height was 125 cm (standard-deviation score for chronologic age, −1, and for bone age, −4.4). The ratio of her sitting height to her standing height was 0.54 (standard-deviation score for chronologic age, 0, and for bone age, +1.7), and her weight was 36.2 kg (standard-deviation score for weight according to height, +3). Target height (the patient's expected final height based on her parents' height and her sex) was 166.5 cm (standard-deviation score, −0.5). The father reported having had normal growth and pubertal development and the mother reported normal pubarche and menarche, but when she was in her early 30s, obesity, oligomenorrhea, and hirsutism had developed.

At presentation, the patient's plasma DHEAS level was below the detection limit, whereas the DHEA level was close to the upper limit of the normal range, and androstenedione and testosterone levels were about twice as high as normal levels for her sex and age (Table 1). Dexamethasone (0.5 mg administered every 6 hours for 48 hours) suppressed the plasma cortisol level to less than 0.02 nmol per liter (<0.0007 μg per deciliter), reduced the DHEA level from 15.0 to 1.6 nmol per liter (4.3 to 0.5 μg per liter), and reduced the androstenedione level from 4.1 to 2.9 nmol per liter (1.2 to 0.8 μg per liter), findings that ruled out autonomous steroid production by an adrenal or gonadal tumor. Congenital adrenal hyperplasia was also ruled out (for details see the Supplementary Appendix, available with the full text of this article at NEJM.org). Plasma gonadotropin levels were within the normal prepubertal range at baseline (luteinizing hormone, <0.2 U per liter; follicle-stimulating hormone, 0.7 U per liter) and after intravenous administration of 100 μg of gonadotropin-releasing hormone (luteinizing hormone, 4.5 U per liter; follicle-stimulating hormone, 8.5 U per liter). Levels of 17β-estradiol (36 pmol per liter [9.8 pg per milliliter]) were also in the normal range (<18 to 52 pmol per liter [<4.9 to 14.2 pg per milliliter]). The absence of a clearly pubertal response of levels of estradiol or luteinizing hormone to the administration of gonadotropin-releasing hormone, as well as the short history of breast budding, argued against the possibility that central precocious puberty was a major contributor to the patient's advanced bone age. The patient was closely followed but received no further treatment.

Menarche occurred at 11 years of age. At the age of 12 years, the patient's bone age was 16.5 years, her height was 139.0 cm (standard-deviation score, for chronologic age, −2.4, and for bone age, −4.6), the ratio of her sitting height to her standing height was 0.55 (standard-deviation score for chronologic age, +2.6, and for bone age, +2), and her weight was 58.3 kg (standard-deviation score for weight according to height, +3.0). Her body-mass index (the weight in kilograms divided by the square of the height in meters) was 30.2. Radiographs revealed mild lumbar scoliosis, flattened vertebrae (platyspondyly) with irregular vertebral end plates in the thoracolumbar region (see Fig. 1 in the Supplementary Appendix), and shortened tubular bones, but no epiphyseal or metaphyseal changes. The patient now had pubic-hair development at Tanner stage 5, breast development at Tanner stage 4, and clinically significant hirsutism and acne. At 13 years of age, secondary amenorrhea developed. The patient's height at 14.5 years remained 139.0 cm (standard-deviation score, −4.1).

Endocrine reassessment at 12 years of age (Table 1) mirrored the findings at 8 years, with androstenedione and testosterone levels that were twice as high as the upper limits of the normal ranges, a DHEA level at the upper limit of the normal range, and a DHEAS level below the limit of detection. Reassessment with a more sensitive assay (detection limit, 0.08 μmol per liter [29.5 ng per liter]) showed plasma DHEAS levels at 0.27 μmol per liter (99.5 ng per liter). DHEAS levels remained below 0.4 μmol per liter (147.4 ng per liter)

Table 1. Serum Hormone Concentrations in the Patient at Initial Presentation (8 Years of Age) and during Follow-up (12 Years of Age).*

Hormone	Patient at 8 Years of Age	Sex- and Age-Specific Reference Range	Patient at 12 Years of Age	Sex- and Age-Specific Reference Range
DHEAS (μmol/liter)	<0.40	0.6–4.6	<0.40	1.4–10.4
DHEA (nmol/liter)				
Baseline	15.0	1.3–18.0	20.0	2.0–22.0
60 min after intravenous administration of 250 μg of cosyntropin			43.0	
Androstenedione (nmol/liter)	4.1	0.14–2.4	27.0	<12.0
Testosterone (nmol/liter)	1.2	0.03–0.65	2.1	0.51–1.26
Dihydrotestosterone (nmol/liter)			0.36	0.05–0.25
Luteinizing hormone (U/liter)				
Baseline	<0.2	<0.2–1.3	3.2	<0.05–20.2
30 min after intravenous administration of 100 μg of GnRH	4.5			
Follicle-stimulating hormone (U/liter)				
Baseline	2.0	<0.2–3.7	5.2	0.14–8.8
30 min after intravenous administration of 100 μg of GnRH	8.5			
17β-estradiol (pmol/liter)	36	<18–52	220	110–370
Estrone (pmol/liter)			260	65–220
17α-Hydroxyprogesterone (nmol/liter)				
Baseline	1.30	0.2–5.8		
60 min after intravenous administration of 250 μg of cosyntropin	3.1	(<20)		
17α-Hydroxypregnenolone (nmol/liter)				
Baseline			2.9	0.3–6.1
60 min after intravenous administration of 250 μg of cosyntropin			15.1	<40
11-Deoxycortisol (nmol/liter)				
Baseline			<0.17	<0.17
60 min after intravenous administration of 250 μg of cosyntropin			1.70	<7
Cortisol (nmol/liter)				
Baseline			140	100–500
60 min after intravenous administration of 250 μg of cosyntropin			650	>500

* To convert the values for DHEAS to nanograms per milliliter, divide by 0.002714. To convert the values for DHEA to micrograms per milliliter, divide by 3.467. To convert the values for androstenedione to micrograms per liter, divide by 3.492. To convert the values for testosterone to nanograms per milliliter, divide by 3.467. To convert the values for dihydrotestosterone to nanograms per milliliter, divide by 3.443. To convert values for 17β-estradiol to picograms per milliliter, divide by 3.671. To convert the values for estrone to picograms per milliliter, divide by 3.699. To convert the values for 17α-hydroxyprogesterone to micrograms per milliliter, divide by 3.026. To convert the values for 17-hydroxypregnenolone to nanograms per milliliter, divide by 3.008. To convert the values for 11-deoxycortisol to micrograms per deciliter, divide by 3.754. To convert the values for cortisol to micrograms per deciliter, divide by 27.59. GnRH denotes gonadotropin-releasing hormone.

throughout a cosyntropin stimulation test, whereas plasma DHEA levels increased during the test from 20.0 nmol per liter (5.8 μg per liter) at baseline to 38.0 nmol per liter at 30 minutes and 43.0 nmol per liter at 60 minutes (11.0 and 12.4 μg per liter, respectively). Analysis of urinary steroid metabolites over a 24-hour period showed increased excretion of the major androgen metabolite androsterone (5376 μg per 24 hours; normal range, 287 to 2215).

METHODS

HORMONE ASSAYS

Plasma levels of DHEA, DHEAS, androstenedione, and testosterone were determined by radioimmunoassay.[18] DHEAS levels were also measured with a luminescence immunoassay (Architect immunoanalyzer, Abbott), with a lower detection limit of 0.08 μmol per liter (29.5 ng per milliliter). Urinary excretion of steroid metabolites was analyzed by means of gas chromatography–mass spectrometry.[19]

DNA SEQUENCE ANALYSIS

Genetic studies were carried out with the written informed consent of the patient and her parents and were approved by the South Birmingham Research Ethics Committee. The coding sequences of the human genes *SULT2A1*, *PAPSS1*, and *PAPSS2* were amplified from genomic DNA obtained from the patient and her parents, and direct sequencing was performed (for details, see the Supplementary Appendix). Polymerase-chain-reaction–restriction-fragment–length polymorphism (PCR-RFLP) analysis was used to confirm the presence of mutations. Mutation numbering refers to the amino acid position in the human PAPSS2a protein (GenBank accession number, NP_004661). We analyzed both strands of all *PAPSS2* exons identified as harboring a mutation in 100 white persons of mixed European origin and 100 persons of Turkish origin, after obtaining their written informed consent.

ANALYSIS OF MESSENGER RNA EXPRESSION

Qualitative and quantitative analyses of messenger RNA (mRNA) expression were carried out for *SULT2A1*, *PAPSS1*, and *PAPSS2* (for details, see the Supplementary Appendix) with the use of human fetal adrenal tissue and cartilage, obtained according to the ethical and informed-consent guidelines of the Polkinghorne Committee,[20] and human adult liver, adrenal, testis, and ovary specimens (Clontech).

FUNCTIONAL ANALYSIS OF *PAPSS2* MUTATIONS

Copies of mutant *PAPSS2* complementary DNA (cDNA) generated in vitro by means of site-directed mutagenesis were used for the bacterial expression of PAPSS2–glutathione s-transferase (GST) fusion proteins. Western blot analysis was performed[19] with the use of polyclonal human antibody to GST (Amersham). Wild-type and mutant PAPSS2 proteins were incubated with human SULT2A1 wild-type protein to measure the ability of PAPSS2 to facilitate sulfation of DHEA to DHEAS. Steroid extraction and quantification were performed as described previously.[21] All assays were performed in three independent triplicate experiments; data are reported as means (±SE). Further details on the functional assay are provided in the Supplementary Appendix.

RESULTS

DNA SEQUENCING

Hypothesizing that the concurrent excess of androgens and very low levels of DHEAS might be explained by a defect in DHEA sulfation, we sequenced the genes encoding the key enzymes of the DHEA–DHEAS sulfation system. No mutations were found in *SULT2A1* or *PAPSS1*. However, direct sequencing of the *PAPSS2* gene revealed a heterozygous substitution of cytosine for guanine at nucleotide position 143 (c.143C→G; g.49,330C→G) in exon 2 of *PAPSS2* in the patient and her father. This missense mutation substitutes an arginine for threonine at position 48 of the PAPSS2 protein (T48R). This residue is located in the adenosine 5′-phosphosulfate (APS) kinase domain of the enzyme and is highly conserved across species (Fig. 1). Furthermore, sequencing revealed a heterozygous substitution of cytosine for thymidine in complementary DNA (cDNA) position 985 (c.985C→T; g.67,422C→T) in exon 8 of *PAPSS2* in the patient and her mother, introducing a premature stop codon that is predicted to truncate the protein at position 329 (R329X), thereby disrupting the ATP sulfurylase domain of PAPSS2 (Fig. 1A).

PCR-RFLP analysis confirmed the two mutations identified by direct sequencing (Fig. 1C). No mutations were found on direct sequencing of *PAPSS2* exons 2 and 8 in 200 controls, including 100 Turkish persons, thereby excluding the possibility that the identified mutations represent population-specific sequence variants.

EXPRESSION OF mRNA

Qualitative mRNA-expression analysis of the major sites of DHEA sulfation — the adrenal glands and the liver — showed that both tissues expressed *SULT2A1* and *PAPSS2b*, whereas the shorter splice variant, *PAPSS2a*, which lacks exon 7B,[22] was expressed only in adrenal tissue (Fig. 2A). In contrast, fetal chondrocytes from three different locations in the skeletal system expressed *PAPSS2a*,

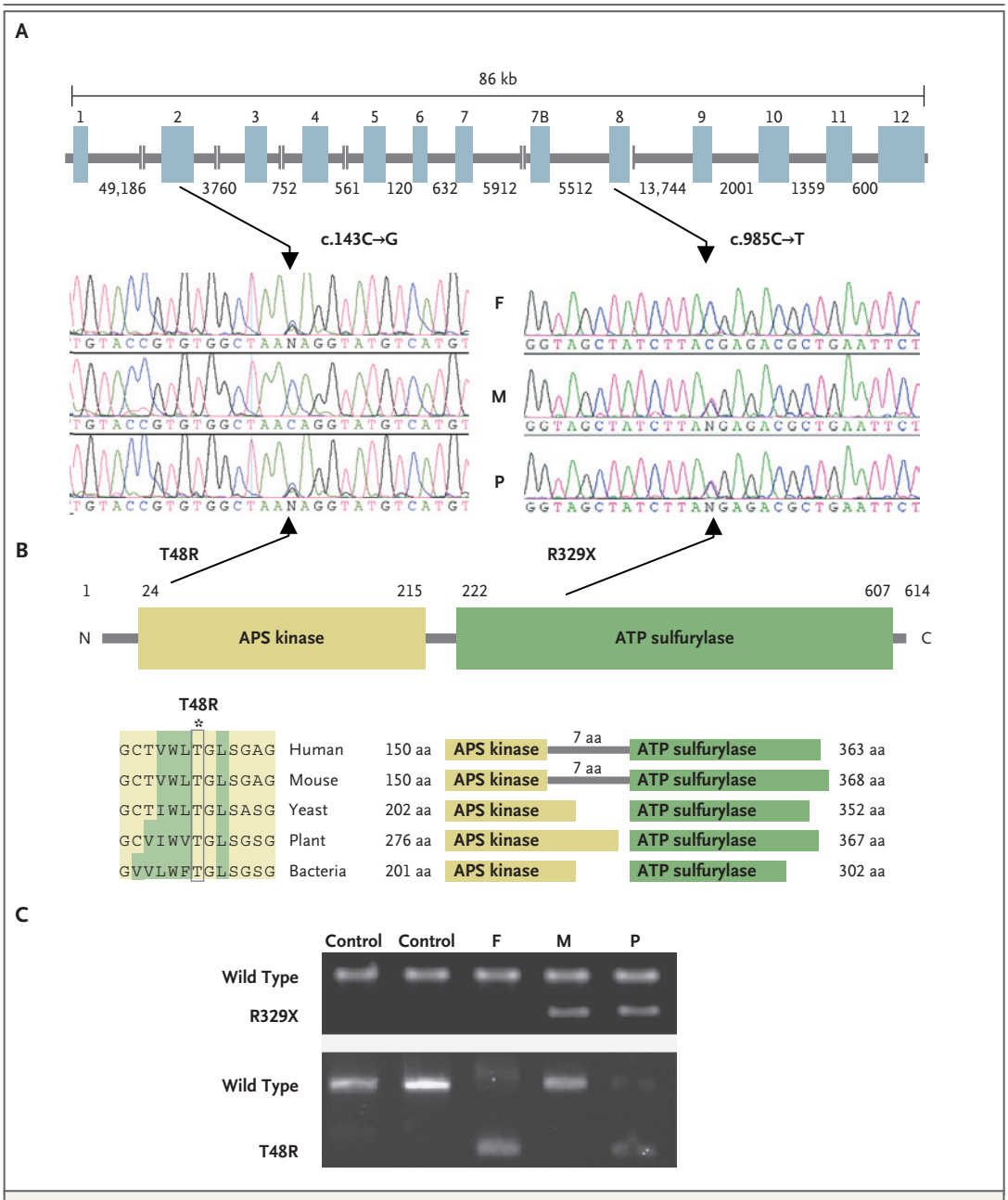

Figure 1. Location and Sequencing of the *PAPSS2* Mutations.

Panel A shows the location of the mutations identified in the gene encoding PAPS synthase 2 (*PAPSS2*) and the two functional domains of the PAPSS2 protein. The patient (P) was a compound heterozygote for the mutations, whereas the father (F) and mother (M) were heterozygous carriers. Panel B shows the cross-species alignment of the PAPSS2 region containing missense mutation T48R and provides a schematic depiction of their respective protein structures. Panel C shows the results of the polymerase-chain-reaction–restriction-fragment–length polymorphism (PCR-RFLP) analysis of *PAPSS2* mutations identified by direct sequencing, which confirm that the patient and her father were heterozygous carriers of the c.143C→G point mutation yielding T48R, and the patient and her mother both carried a heterozygous c.985C→T mutation resulting in the R329X nonsense mutation. Control DNA was confirmed as wild type through direct sequencing. (The protein-sequence alignments shown in Panel B were generated with the use of ClustalX2 [www.clustal.org] and of the deposited sequences for PAPSS2 in humans [GenBank accession number NP_004661], mice [NP_035994], yeast [CAA46252 and CAA60932], bacteria [A1AEU4 and AAG57859], and plants [CAA53426 and CAB78510].)

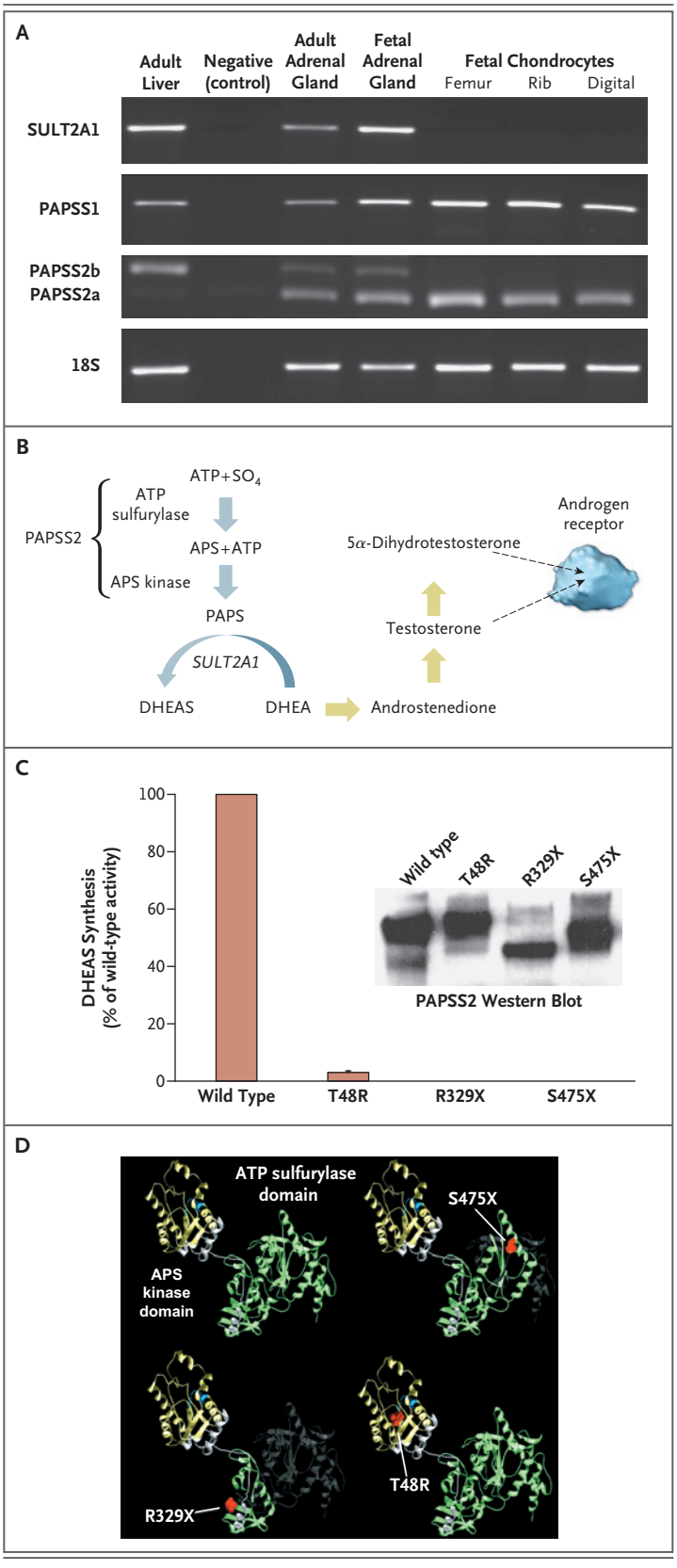

Figure 2. Tissue-Specific Expression and Functional Assessment of the DHEA Sulfation System.

Panel A shows tissue-specific mRNA expression of the DHEA sulfation system, including DHEA sulfotransferase (SULT2A1), PAPS synthase 1 (PAPSS1), and PAPS synthase 2 (PAPSS2), with splice variants PAPSS2a and PAPSS2b. In Panel B, a schematic representation of the DHEA sulfation system, PAPSS2 generates the sulfate donor PAPS through the successive activities of ATP sulfurylase and APS kinase. DHEA sulfotransferase (SULT2A1) requires PAPS for efficient DHEA sulfation. Unconjugated DHEA molecules are ultimately converted to active androgens. In Panel C, the inactivating nature of the *PAPSS2* mutations is clearly depicted in a graphic representation of the results of an in vitro DHEAS generation assay involving coincubation of human DHEA sulfotransferase (SULT2A1) and wild-type and mutant PAPSS2a proteins. The Western blot analysis shows that the wild-type and mutant PAPSS2 preparations are equal in terms of protein content. In Panel D, three-dimensional modeling of wild-type and mutant PAPSS2 shows that PAPSS2 contains two domains, ATP sulfurylase and APS kinase. Mutant S475X (as previously reported[15]) and maternally inherited R329X result in early truncation of the ATP sulfurylase domain, whereas paternally inherited T48R affects the APS kinase P loop (blue), an area that is crucial for enzymatic activity. Mutation locations in the S475X, R329X, and T48R proteins are indicated in red. (The radiographic structure of human PAPSS1[23] served as the template for modeling wild-type and mutant PAPSS2 with the use of Swiss-Pdb-Viewer [Swiss Institute of Bioinformatics] and POV-Ray 3.6 [POV-Team].)

but not *PAPSS2b* and also lacked expression of *SULT2A1*. *PAPSS1* was expressed in all tissues examined (Fig. 2A).

Quantitative real-time mRNA-expression analysis confirmed the adrenal glands and the liver as the major sites of DHEA sulfation, with high levels of expression of *SULT2A1* and *PAPSS2* but relatively low levels of expression of *PAPSS1* (see Fig. 2 in the Supplementary Appendix). Only very low levels of expression of *SULT2A1* were found in tissues from the ovary and testis, a finding that rules out the gonads as major contributors to DHEA sulfation.

PAPSS2 MUTATIONS

Impaired proteoglycan sulfation in chondrocytes has previously been implicated in the pathogenesis of spondyloepimetaphyseal dysplasia, Pakistani type, because of an S475X mutation in *PAPSS2*.[15] Given our finding that fetal chondrocytes expressed only the *PAPSS2a* variant (Fig. 2A), we used wild-

type and mutant PAPSS2a protein for coupled assays with SULT2A1. The functional assay represents an in vitro reconstruction of the DHEA sulfation system (Fig. 2B), measuring the amount of DHEAS generated from DHEA by SULT2A1, a catalytic process in which PAPS, generated by PAPSS2, is used by SULT2A1 for the sulfation of DHEA to DHEAS. Assay results confirmed the inactivating nature of the mutations, with no detectable activity for R329X or S475X and only minor residual activity for T48R (6.0±0.6% of wild-type PAPSS2 activity) (Fig. 2C). Structural analysis revealed that both nonsense mutations result in considerable truncation of the ATP sulfurylase domain, whereas the missense mutation T48R affects the APS kinase domain of the PAPSS2 protein (Fig. 2D).

DISCUSSION

We identified compound heterozygous mutations in *PAPSS2* that are highly likely to be a novel monogenic adrenocortical cause of androgen excess. *PAPSS2* encodes human PAPS synthase 2, which generates PAPS, the sulfate donor required for the sulfation of DHEA to DHEAS by DHEA sulfotransferase (SULT2A1).[14] In humans, DHEAS is the most abundant steroid in circulation. However, only unconjugated DHEA can feed directly into androgen synthesis, which occurs by means of rapid conversion to androstenedione through 3β-hydroxysteroid dehydrogenase activity, followed by further conversion to the active androgens testosterone and 5α-dihydrotestosterone. Our observations highlight the crucial role of DHEA sulfation as a gatekeeper to human androgen synthesis by showing that impaired DHEA sulfation increases the DHEA pool available for conversion to active androgens, thus causing androgen excess. These findings raise questions about the practice of using high levels of androstenedione to define ovarian hyperandrogenism and high levels of DHEAS to define adrenal hyperandrogenism[2-6] — a practice that would have incorrectly classified our patient as having androgen excess of ovarian origin, even though the underlying disorder involves a defect in adrenal sulfation.

The presence of PAPS is a prerequisite for catalytic efficiency in all sulfation reactions, including those involving proteoglycans, for which sulfation is a key process in the formation of extracellular matrix and therefore in bone development and growth. A homozygous *PAPSS2* mutation (S475X) was identified in a large Pakistani kindred affected by spondyloepimetaphyseal dysplasia, Pakistani type,[15] manifested as disproportionately short stature associated with short, bowed lower limbs, enlarged knee joints, kyphoscoliosis, and generalized brachydactyly.[24] Chondroitin 6-*O*-sulfotransferase requires PAPS for catalytic activity; mutations in *CHST3*, the gene that encodes chondroitin 6-*O*-sulfotransferase, result in spondyloepiphyseal dysplasia, Omani type.[25] Some features of the bone phenotype in our patient resemble those observed in spondyloepimetaphyseal dysplasia, Pakistani type,[15] although the bone changes were milder in our patient and there were no long-bone epiphyseal or metaphyseal changes. The androgen excess in our patient was probably involved in the acceleration of bone age. However, the absence of a pubertal growth spurt, the compromised final height, and the increased ratio of sitting height to standing height more likely reflect the bone dysplasia.

Affected patients in the Pakistani kindred[15] were homozygous for the inactivating S475X mutation. The maternally inherited R329X mutation in our patient results in premature truncation of the ATP sulfurylase domain, rendering PAPSS2 void of activity, as shown in vitro. The paternally inherited T48R mutation is located in immediate proximity to the P-loop structure in the APS kinase domain, a region identified as critical for enzymatic activity.[26] We detected residual activity of approximately 6% for this mutant, which might be sufficient to explain the difference in the severity of the bone phenotype between our patient and the Pakistani kindred.

There is no information available about bone-age advancement or a potential androgen-excess phenotype in the Pakistani kindred, since the affected patients live at the Pakistani–Afghan border, an area difficult to access; the research team[15] was allowed only limited access to female family members (Cohn DH, University of California at Los Angeles: personal communication). Therefore, in affected women, the clinical phenotype regarding signs of androgen excess could not be ascertained. The mother of our patient, a heterozygous carrier of the completely inactivating R329X mutation, reported a history suggestive of mild polycystic ovary syndrome, although no formal endocrine studies were performed in either parent. Consequently, information on the biochem-

ical phenotype of heterozygous carriers of *PAPSS2* mutations is lacking.

Why the ubiquitously expressed PAPSS1 cannot compensate for loss of PAPSS2 activity is unknown. However, since previous in vitro studies have shown that the catalytic efficiency of PAPSS2 is 10 to 15 times as great as that of PAPSS1,[22] we speculate that PAPSS1 alone might not provide sufficient quantities of PAPS in tissues with a high sulfation rate, such as the adrenal glands and liver. Indeed, we have shown that *PAPSS2* mRNA expression in the adrenal glands and the liver is considerably higher than that of *PAPSS1* expression. In addition, it has been reported that PAPSS1 is predominantly nuclear, whereas PAPSS2 appears to be predominantly cytosolic[27]; this difference in subcellular compartmentalization might explain the differential effects of PAPSS1 and PAPSS2 on cytosolic SULT2A1.

In conclusion, we have uncovered a molecular defect associated with androgen excess — inactivating mutations in the sulfate donor enzyme PAPSS2 — that highlights the critical role of DHEA sulfation in regulating the synthesis of androgens in humans. In our patient, androgen excess was manifested as premature pubarche and later as hirsutism, acne, and secondary amenorrhea, thus apparently fulfilling the current diagnostic criteria for the polycystic ovary syndrome.[1,2] However, this diagnosis is one of exclusion; our data show that PAPSS2 deficiency should be added to the list of defined causes of androgen excess that must be ruled out before diagnosing the polycystic ovary syndrome. Future studies will be needed to determine the frequency and functional consequences of *PAPSS2* sequence variants[28] in well-characterized cohorts of patients with premature pubarche and the polycystic ovary syndrome in order to further define the role of PAPSS2 and DHEA sulfation in these androgen-excess disorders.

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No potential conflict of interest relevant to this article was reported.

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