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Mutations of the hexose-6-phosphate dehydrogenase gene rarely cause hyperandrogenemic polycystic ovary syndrome

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Abstract

Background/Aims—Hexose-6-phosphate dehydrogenase (H6PD) inactivating mutations cause cortisone reductase deficiency, which manifests with hyperandrogenism unexplained by commonly used tests and, thus, mimics polycystic ovary syndrome (PCOS). The aim of this study was to screen for mutations of H6PD gene in PCOS patients with biochemical hyperandrogenemia.

Methods—Direct DNA sequencing of the entire H6PD coding sequence was performed in 74 PCOS patients and 31 healthy controls. Results were confirmed by PCR-restriction fragment length polymorphism assay to determine the genotypic frequency of the variants.

Results—Multiple novel missense variants were detected in the study. Two exon 2 variants (accaggc deletion proximal to the start codon and D151A) and two exon 5 variants (R453Q and P554L) were common, occurring in 23.8%, 17.1%, 35.2%, and 16.1%, respectively. There was significant linkage disequilibrium between the exon 2 and exon 5 variants. No significant differences were observed in the genotype, allele distributions, or adrenal function tests of the variants between cases and control groups. We did not detect any reported inactivating mutations in our study.

Conclusion—Although the H6PD gene is very polymorphic and missense variants are common, coding variants rarely (<1.5%) are responsible for hyperandrogenemic PCOS. We suggest that genetic studies be reserved for patients with dexamethasone-suppressible adrenal hyperandrogenism who have a discrepancy between urinary 17 α -hydroxycorticoid and cortisol excretion.

Keywords

Cortisone reductase deficiency; hexose-6-phosphate gene variants; polycystic ovary syndrome

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1. INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common cause of anovulatory infertility, affecting about 10% of reproductive-age women [1]. There is broad agreement that the syndrome is definable by otherwise unexplained hyperandrogenism in the presence of anovulatory symptoms or a polycystic ovary, but controversy about defining it in the absence of hyperandrogenism, i.e., on the basis of the combination of only anovulatory symptoms and a polycystic ovary [2–4]. PCOS appears to arise as a complex trait that has genetic and environmental determinants related to insulin resistance that result in dysregulation of steroidogenesis [5–7]. However, PCOS is mimicked by monogenetic defects of steroidogenesis that cause hyperandrogenemia, such as congenital adrenal hyperplasia [8,9] and cortisone reductase deficiency (CRD) [10].

Cortisone reductase deficiency (CRD) is a disorder in which there is a failure to regenerate the active glucocorticoid cortisol from cortisone in peripheral tissues via the enzyme 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1). The pathogenic mechanism of CRD is that rapid cortisol turnover causes compensatory ACTH-mediated adrenal hyperandrogenism. The clinical features of CRD are precocious pseudopuberty and/or hirsutism, oligo-amenorrhea, and infertility. Circulating adrenal androgen levels are elevated and responsive to low-dose dexamethasone suppression, but ACTH testing yields non-specific results [11–15]. CRD has been diagnosed through the analysis of urinary corticoids, which are elevated, but consist of an abnormally low ratio of cortisol to cortisone metabolites [10].

Sequencing of the 11 β -HSD1 gene has not identified causative mutations in CRD patients [13,16]. Digenic inheritance of functional 11 β -HSD1 and H6PD variants implicated in the causation of CRD was not found in a series of PCOS patients who were not necessarily hyperandrogenic [17]. However, this and other studies failed to corroborate the earlier suggestion that these variants were causative of CRD [18–20]. Subsequently, it was demonstrated that homozygosity or compound heterozygosity for several novel H6PD mutants indeed caused CRD [10].

CRD was not found in two large series of hyperandrogenic women who were evaluated by examination of blood steroid levels [8,9] or in our sizable, but smaller, series in which serum steroid intermediates were evaluated in response to intensive dynamic testing [21]; however, these approaches would not be expected to diagnose CRD. Because the extent to which CRD may be under-diagnosed in hyperandrogenic women evaluated in this way is consequently unclear, we sequenced the entire H6PD coding region to search for mutations in our series of hyperandrogenic PCOS patients and nonhyperandrogenic controls. Our data indicate that the prevalence of H6PD deficiency in hyperandrogenic PCOS is <1.5%. In the course of these studies we discovered a common 8 bp deletion in the H6PD 5'-untranslated region and two new common missense variants that exhibit linkage disequilibrium, as well as two heterozygous missense mutations that were of no functional consequence.

2. EXPERIMENTAL

2.1. Subjects

The study was approved by the Institutional Review Board of the University of Chicago Hospitals and conducted within the University, Department of Pediatrics. PCOS patients and controls were recruited and written informed consent was obtained from all participants. We genotyped the 74 hyperandrogenic PCOS patients (95% had anovulatory symptoms, the others were eumenorrheic with a polycystic ovary) and 31 controls (eumenorrheic women

without evidence of either clinical or biochemical hyperandrogenism or anovulatory symptoms) whose DNA was available. Clinical and biochemical phenotyping of subjects was published previously [21]. This evaluation included demonstration of significantly elevated baseline dehydroepiandrosterone sulfate and post-ACTH dehydroepiandrosterone plasma levels, but no significant abnormality in simultaneous cortisol plasma levels in the PCOS cohort.

2.2. Genetic Analyses

DNA sequencing—We sequenced the entire coding region of H6PD, commencing with the ATG start codon and including intron-exon junctions, in all 74 PCOS and 31 control subjects. We used specific H6PD gene primers (primer sequences are available upon request) to PCR-amplify the gene's exons 2–5, including approximately 200 bp flanking sequences. The PCR products were sequenced by the University of Chicago Cancer Research Center DNA Sequencing Facility (<http://cancer-seqbase.bsd.uchicago.edu/>). Sequencing results were assembled and analyzed using the FinchTV software program (<http://www.geospiza.com>) and checked manually.

Restriction fragment length polymorphism assay (RFLP)—Twenty μ l of the PCR products were digested with specific enzymes at 37°C for 3 h and the digested products were separated by electrophoresis in a 2.5% agarose gel, and subsequently stained with ethidium bromide.

2.3. Statistical analysis

We used a web program (<http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl>) to test polymorphisms for deviation from Hardy-Weinberg equilibrium, to assess the relative association between patients and controls for genotype and allele frequencies, and to calculate the corresponding odds ratios and confidence intervals (95% CIs). Association (significance) was considered at $P < 0.01$, weak $P = 0.01$ to 0.05. Linkage disequilibrium LOD scores were computed from Haploview (<http://www.broadinstitute.org/haploview>). P-values were estimated by multiplying the lod score by 4.606 to obtain a chi-square with 1 degree of freedom.

3. RESULTS

3.1 Polymorphic deletion variant and missense variants of the H6PD gene

An eight bp (accagggc) deletion variant in the 5'-untranslated region (UTR) of the H6PD gene and three polymorphic missense variants of human H6PD gene were detected by sequencing (Fig. 1). The deletion destroys a Ban I restriction site, so was easily confirmed by RFLP (Fig. 1A). This deletion does not affect the 1st ATG (initial start codon) or the coding sequence of H6PD mRNA. Polymorphic missense variants were detected of D151A (c.452a>c) (Fig. 1B), R453Q (c.1358g>a) (Fig. 1C), and P554L (c.1661c>t) (Fig. 1D). Each of these nucleotide changes creates a specific enzyme restriction site: c.452a>c generates a Bbs I site, c.1358g>a generates a Bsg I site, and c.1661c>t generates a Ban II site. Therefore, the three polymorphic variants were re-confirmed by RFLP with specific restriction enzymes.

The genotype and allele frequencies of the polymorphisms in the PCOS and control groups were consistent with Hardy-Weinberg equilibrium distribution ($p > 0.05$). The frequencies of the deletion (23.8%) and the polymorphic missense variants (16.1–35.2%) were similar in PCOS and control subjects (Table 1).

However, the variants were clustered such that all occurred in 63% of subjects, alone or in combination. Linkage disequilibrium was highly significant between the exon 2 D151A and the exon 5 P554L SNPs (LOD score 8.3, $p=3.71^{-9}$) and nominally significant between the exon 2 accagc deletion variant and the exon 5 R453Q variant (LOD score 0.71, $p=0.03$). Two PCOS subjects had both linked pairs, which is not a significant difference in prevalence.

3.2. Rare variants of the H6PD gene

Heterozygous N484D was detected as the only variant in one control and one PCOS patient; it has been reported in Genbank. Heterozygous I402V was detected in one control; this is the first report of this variant. We did not detect the five inactivating mutations that have been reported to underlie CRD: c.325delc, p.R109AfsX3; c.948c>g, p.Y316X; c.1076g>a, p.G359D; and c.1860ins29bp/c.960g>a, p.D620fsX3 [10].

Alignment of amino acids around the residues of human H6PD missense variants that we identified (D151A, I402V, R453Q, N484D, and P554L) and the corresponding areas of other species demonstrated that the D151, P554 and I402 residues are highly conserved (75–92%), but R453 and N484 are not (Table 2).

3.3. Clinical significance of variants

No relationship could be ascertained between variant status, alone or associated, and the early morning plasma level of dehydroepiandrosterone sulfate, a marker of adrenal androgen production (Fig. 2). The two PCOS patients with both common variant pairs had mid-normal dehydroepiandrosterone sulfate levels (86 and 119 $\mu\text{g/dl}$). The responses of plasma cortisol, dehydroepiandrosterone, 17-hydroxypregnenolone, and androstenedione to a rapid low-dose cosyntropin test or of testosterone to dexamethasone were likewise unrelated to H6PD genotype (data not shown).

4. DISCUSSION

We did not identify any of the reported inactivating mutations in our hyperandrogenemic PCOS cohort after sequencing exons 2–5 of the H6PD gene, which contain the entire coding sequence, for mutations or polymorphisms in 74 cases and 31 normal controls. This is not unexpected, considering that to date only 11 cases of CRD have been diagnosed by urinary steroid analysis [10–15,22], and, among these, 4 cases carrying 5 deleterious H6PD gene mutations have been confirmed to have inactivating H6PD mutations by gene sequencing [10]. However, we did identify novel polymorphisms and apparent mutations in the H6PD gene in our PCOS patients and the healthy controls.

This is the first report detecting a polymorphic eight bp deletion in the 5'-URT of H6PD gene. Because a 5'-UTR deletion of BCS1L mRNA (a protein involved in the assembly of complex III of the mitochondrial respiratory chain) has been reported as a causative mutation in mitochondrial complex III deficiency by decreasing mRNA and protein levels [23], we analyzed the frequency of this deletion in our PCOS and control groups: this proved to be similar between patients and controls (Table 1).

In our study, we identified the H6PD missense variants D151A, I402V, R453Q, N484D, and P554L. Another missense mutation (G359D) was reported to cause a CRD case [10]. Alignment of the amino acids around these residues of human H6PD and the corresponding areas of other species demonstrated that the D151, I402, and P554 residues are highly conserved (75–92%), though not as much so as G359 (100%); but R453 and N484 are not (58% and 0%, respectively) (Table 2). Our study confirms extensive studies showing that H6PD R453Q is a polymorphism that does not cause CRD or hyperandrogenemia in PCOS

patients [18–20], although it was initially suspected of doing so [22]. Our study also showed that H6PD D151A and P554L are very common; and the frequencies of these two variants are not different between PCOS patients and controls (Table 1), although the wild-type residues are highly conserved among species (Table 2). Our study further identified a novel variant of I402V in a normal volunteer who was heterozygous; and detected heterozygous N484D in a PCOS patient and a normal control. Many other missense variants have been reported in GeneBank (Table 3). The rare variants are likely to be mutations, but their clinical relevance is unknown, and study of their function was beyond the scope of this project.

We also found many common coding region single nucleotide polymorphisms (SNP) that do not alter the residues and have been reported in the GeneBank (<http://www.ncbi.nlm.nih.gov>), such as c.636 g>a, c.740t>c, c.2019t>c c.2118a>g, for which the SNP numbers are rs7524046, rs11121350, rs9434742, and rs9434743, respectively. These SNPs are unlikely to significantly impact H6PD enzymatic activity.

In conclusion, polymorphic variants of the H6PD gene are common, but are similar in PCOS patients and controls, suggesting that these genetic variations are rare factors (<1.5%) in the pathogenesis of hyperandrogenism. We suggest that screening studies for adrenal hyperandrogenism include baseline urinary 17 α -hydroxycorticoids and that genetic studies for CRD be reserved for patients with elevated urinary 17 α -hydroxycorticoids, but normal cortisol excretion and dexamethasone-suppressible adrenal hyperandrogenism.

Nonstandard abbreviations used

5'-UTR	5'-untranslated sequences
CRD	cortisone reductase deficiency
H6PD	hexose-6-phosphate dehydrogenase
PCOS	polycystic ovary syndrome

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Fig. 1.

Direct sequencing and restriction fragment length polymorphism assay (RFLP) results for the H6PD gene. The polymorphisms were detected by direct PCR sequencing and confirmed by RFLP: **(A)** An eight bp (accaggc) deletion confirmed by Ban I digestion (The 68-bp fragment is not seen because it was too low on the gel). **(B)** Bbs I digestion confirms the c.452 genotype a/c, which encodes the D151A missense variant. **(C)** The Bsg I digestion confirms the c.1358 genotype g/a, which encodes the R453Q missense variant (the 179-bp fragment is not seen because it was too low on the gel). **(D)** The Ban II digestion confirms the c.1661 genotype c/t, which encodes the P554L missense variant (the 53 and 6-bp fragments are not seen because they were too low on the gel). Double and single underlines locate the homozygous and heterozygous accaggc sequences, respectively; and arrows indicate the variants.

Fig. 2.

Scattergram showing lack of relationship of common H6PD gene variants to blood levels of dehydroepiandrosterone sulfate (DHEAS), an indicator of adrenal androgenic function. WT =wild-type, UTRdel+R453Q = exon 2 acccagc deletion+exon 5 R453Q pair, D151A +P554L = SNP pair; two PCOS with both variant pairs are included in the former group. "Other" includes singletons or other combinations of the aforementioned variants or the rare variants that are designated by asterisks.

Table 1

Genotype distribution of H6PDH gene in PCOS and healthy controls.

Genotype	Controls (n=31)	Cases (n=74)	Odds ratio (C.I.)	P
acccaggc				
+/+	23 (74.2%)	57 (77.6%)		
+/-	8 (25.8%)	17 (22.4%)		
-/-	0 (0%)	0 (0%)		
Allele frequency difference			0.88 (0.36–2.15)	0.77
Risk of deletion			0.86 (0.33–2.26)	0.76
c.452a/c; p.D151A				
a/a	27 (87.1%)	60 (81.6%)		
a/c	4 (12.9%)	13 (17.1%)		
c/c	0 (0%)	1 (1.3%)		
Allele frequency difference			1.64 (0.52–5.14)	0.40
Risk of p.151A			1.58 (0.47–5.23)	0.46
c.1358g/a; p.R453Q				
g/g	21 (67.7%)	45 (61.8%)		
g/a	7 (22.6%)	24 (31.6%)		
a/a	3 (9.7%)	5 (6.6%)		
Allele frequency difference			1.12 (0.55–2.31)	0.75
Risk of p.453Q			1.35 (0.56–3.28)	0.50
c.1661c/t; p.P554L				
c/c	27 (87.1%)	58 (78.9%)		
c/t	4 (12.9%)	15 (19.7%)		
t/t	0 (0%)	1 (1.3%)		
Allele frequency difference			1.88 (0.61–5.84)	0.27
Risk of p.554L			1.86 (0.57–6.1)	0.30

C.I.: 95% confidence interval

Table 2

Alignments of H6PD missense variants and their vicinity amino acids from 12 species

	I402V	D151A	G359D*	R453Q	N484D	P554L	Accession No.
Human	FHIGHG	FAYEDIA	SGKALD	PVRERDA	SWNFWTP	SPLVSAW	NP_004276
Rhesus monkeys	FHIGHG	FAYADIA	SGKALD	PVQERDA	SWGFWTP	SPLVSAW	XP_001099298
Horse	FYIGHG	FAYADIA	SGKALD	PVREQDA	SWVFWTP	SPLISAW	XP_001490983
Dog	FYIGHG	FAYADIA	SGKALD	PVREQDA	SWVFWTP	SPLISAW	XP_546762
Cow	FYIGHG	FAYVDIA	SGKALD	PVREQDA	SWVFWTP	SPLISAW	XP_615112
Rat	FYIGHG	FAYADIA	SGKALD	PVREQDA	SWVFWTP	SPLITAW	NP_001100168
Mouse	FYIGHG	FAYADIA	SGKALD	PVREQDA	SWVFWTP	SPLITAW	NP_775547
Rabbit	FYIGHG	FAYADIA	SGKALD	PVKERDA	SWVFWTP	SPLISAW	A47563
Frog	FHIGHG	FTYTDIA	SGKALD	PVQEMDA	SWKFWTP	ADMVSAW	NP_001106505
Opossum	FYIGHG	FAYAGIA	SGKALD	PRQERDA	SWDVWTP	SPLVSAW	XP_001362573
Chicken	FYIGHG	FAYTEIA	SGKALD	PVKERDA	SWAFWTP	SPLVSAW	XP_425746
Zebra Finch	FYFGHG	FAYTEIA	SGKALD	PVRERDA	SWAFWTP	NSMVSNW	XP_002187325

The locations of human H6PD variants are bold and underlined. Bold letters refer to the amino acids of each species that different from the human variant at the corresponding site.

* A missense mutation causes CRD [10].

Table 3

Known human H6PD gene deletion and missense variants

Region	dbSNP allele	Function	Clinically association	Accession No.
Exon 2	Del -1 to -8(accaggc)	5' UTR	polymorphism	Present study
	c.325delc; p.R109AfsX3	frameshift	CRD	[10]
	c.452a>c; p.D151A	missense	polymorphism	Present study NM_004285
	c.525c>t; p.R169W	missense	N/A	NM_004285
Exon 3	c.653g>a; p.R218Q	missense	N/A	NM_004285
	c.948c>g; p.Y316X	nonsense	CRD	[10]
	c.692g>a; p.R231Q	missense	N/A	NM_004285
Exon 4	c.857c>t; p.A286V	missense	N/A	NM_004285
	c.960g>a;	Splicing	CRD	[10]
Exon 5	c.1076g>a; p.G359D	missense	CRD	[10]
	c.1187g>a; p.R396Q	missense	N/A	NM_004285
	c.1204a>g; p.I402V	missense	N/A	Present study
	c.1301c>a; p.P434H	missense	N/A	NM_004285
	c.1318g>a; p.G440S	missense	N/A	NM_004285
	c.1442g>a; p.A448T	missense	N/A	NM_004285
	c.1358g>a; p.R453Q	missense	polymorphism	Present study NM_004285 [10,17,18,20,22]
	c.1450a>g; p.N484D	missense	N/A	Present study NM_004285
	c.1555c>t; p.R519W	missense	N/A	NM_004285
	c.1661c>t; p.P554L	missense	polymorphism	Present study NM_004285
	c.1820a>c; p.Y607S	missense	N/A	NM_004285
	c.1860ins29bp;p.D620fsX3	frameshift	CRD	[10]

Bold indicates causative mutants that have been reported in CRD.