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A 9-bp Deletion Homoplasmy in Women with Polycystic Ovary Syndrome Revealed by Mitochondrial Genome-Mutation Screen

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Abstract Polycystic ovary syndrome (PCOS) is a complex and heterogeneous disorder presenting a challenge for clinical investigators. To investigate the association of a mitochondrial genetic basis with PCOS, we screened mutations of the whole mitochondrial genome in 57 women patients with PCOS and 38 healthy control individuals. Two-step PCR reactions were adopted to amplify and sequence the whole mitochondrial genome. A 9-bp deletion variant appeared in homoplasmy between PCOS patients and control individuals. In the 62 individuals with complete sequences, eight of 34 (23.5%) patients showed the 9-bp deletion, compared with only two of 28 (7.1%) in healthy controls. The 9-bp deletion variant in region V of mitochondrial DNA may be associated with the heterogeneous disorder PCOS.

Keywords Polycystic ovary syndrome · Mitochondrial genome · 9-bp deletion

Introduction

Polycystic ovary syndrome (PCOS) is a complex and heterogeneous disorder presenting a challenge for clinical investigators (Diamanti-Kandarakis and Piperi 2005). It is most common in women of reproductive age (Diamanti-Kandarakis

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et al. 1999). A meeting of experts in Rotterdam in 2003 suggested that the definition of PCOS should include two of the following three criteria: (1) oligo- and/or anovulation, (2) clinical and/or biochemical signs of hyperandrogenism, and (3) polycystic ovaries on ultrasonography and exclusion of related disorders (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004).

The spectrum of evidence for a genetic basis for PCOS is very broad, supporting the molecular basis of this abnormality, but it is not adequate to interpret the pathogenesis of the syndrome (Diamanti-Kandarakis and Piperi 2005). It indicates that PCOS is likely to represent a complex oligogenic trait with multiple genetic defects (Franks et al. 1997, 2001; Urbanek et al. 1999, 2003). Candidate genes cover a broad spectrum of an endless list of molecules in reproductive and metabolic pathways. Research on identification of these genes may provide valuable information on this complicated endocrine syndrome.

Mutations of the mitochondrial genome are widely recognized as important causes of disease (Schon et al. 1997). The mitochondrial genome is highly compact and exhibits little redundancy throughout its sequence of 16,569 bp, which encodes 37 genes: two genes encode ribosomal RNAs, 22 encode transfer RNAs (tRNA), and 13 encode essential proteins of the mitochondrial respiratory chain (Anderson et al. 1981). Since the association between mitochondrial mutations and PCOS was not clear, the aim of this study was to do the mutation screen on the whole mitochondrial genome of PCOS patients.

Materials and Methods

Samples

Fifty-seven patients with PCOS and 38 healthy controls, matched for age and body mass index, were enrolled in the present study. PCOS patients were recruited from the Hangzhou First People's Hospital, China. The diagnosis of PCOS was based on the presence of oligomenorrhea (six or fewer menses per year) or amenorrhea, elevated luteinizing hormone (LH) levels with normal follicle-stimulating hormone (FSH) levels, LH/FSH ratio >2, elevated testosterone (T) levels (>2.64 nmol/l), and the characteristic appearance of the ovaries on ultrasonography. Hyperprolactinemia, Cushing's syndrome, thyroid dysfunction, and nonclassic adrenal hyperplasia were excluded by appropriate tests before the diagnosis of PCOS was made. Control women had regular menstrual cycles with normal ovarian morphology, selected from those undergoing routine health examinations.

Mitochondrial DNA Sequencing

Two-step PCR reactions were adopted (Taylor et al. 2001). First, a series of overlapping primer pairs (previously described by Taylor et al. 2001) were used to amplify the human mitochondrial genome in nine fragments of ~2 kb from the cell lysate. Primary PCR amplifications were performed in 50 μl volume containing 1× PCR buffer, 0.2 mM dNTPs, 0.8 μM primers, 1 U DNA polymerase (Takara,

Japan), and 1 μl DNA template. Reaction conditions were 95°C for 1 min, 58°C for 30 s, and 72°C for 1.5 min.

In the second step, 28 pairs of primers that generate overlapping fragments of about 700 bp, spanning the entire sequence of the human mitochondrial genome, were used to investigate pathogenic mitochondrial DNA (mtDNA) mutations. For amplification of templates generated in the primary PCR, 2 μl of this initial PCR product was used as template in the secondary PCR amplification. Reaction conditions were exactly as described before (Taylor et al. 2001). PCR-amplified products were subsequently purified (Axygen PCR Purification Kit, USA) and sequenced directly using BigDye terminator cycle sequencing chemistry (PE Biosystems, USA) on an ABI 3730xl automated DNA sequencer. The sequence results were compared to the revised Cambridge Reference Sequence (rCRS, AC_000021.2) using the Phred/Phrap/Consed/Polyphred software suite obtained from the University of Washington, Seattle (Ewing and Green 1998; Ewing et al. 1998; Gordon et al. 1998).

Results

Two-step PCR Amplification of mtDNA

In the first step, nine fragments of about 2 kb that encompassed the entire mitochondrial genome were successfully amplified by PCR. Using 2 μl of the 50 μl first-round PCR product as template for the subsequent PCR reactions with the sequencing primers, products of the expected size (about 700 bp) were amplified from each of the single templates. No visible PCR products are evident in the negative control lanes.

First Whole Mitochondrial Genome of PCOS Patients

We randomly selected one of the PCOS patients and obtained the whole mitochondrial genome sequence data first. Sequence analyses revealed that more than 20 point mutations and one length variant were present in the 16 kb mitochondrial genome. To explore the involvement of mtDNA variants associated with the PCOS phenotypical impact, we screened the whole mitochondrial genome in the rest of the PCOS patients.

Mitochondrial Mutation Screen

Eighty point mutations and one-length variant located in at least two samples were determined with whole mtDNA genome sequencing (Table 1). The one-length variant was a deletion of one of the two copies of a 9-bp direct repeat sequence (CCCCCTCTA) between nt 8270 and nt 8289 in region V of mtDNA. Differentiating with all point mutations, in both PCOS patients and control individuals, the deletion variant appeared in homoplasmy with a *P*-value of 0.02. Thirty-four of 57 patients and 28 of 38 control individuals were sequenced successfully including the

Table 1 Mitochondrial DNA sequence changes from the rCRS in patients and control individuals

Nucleotide position	Gene feature	Nucleotide change	Nucleotide position	Gene feature	Nucleotide change
146 bp	D-loop	T → C	9101 bp	ATP6	T → G
150 bp	D-loop	C → T	9128 bp	ATP6	T → C
152 bp	D-loop	T → C	9180 bp	ATP6	A → G
195 bp	D-loop	T → C	9536 bp	COX3	C → T
200 bp	D-loop	A → G	9540 bp	COX3	T → C
207 bp	D-loop	G → A	9548 bp	COX3	G → A
235 bp	D-loop	A → G	9824 bp	COX3	T → C
263 bp	D-loop	A → G	10310 bp	ND3	G → A
489 bp	D-loop	T → C	10397 bp	ND3	A → G
663 bp	12S RNA	A → G	10398 bp	ND3	A → G
709 bp	12S RNA	G → A	10400 bp	ND3	C → T
752 bp	12S RNA	C → T	10609 bp	ND4L	T → C
827 bp	12S RNA	A → G	10873 bp	ND4	T → C
1438 bp	12S RNA	A → G	11084 bp	ND4	A → G
1736 bp	16S rRNA	A → G	11914 bp	ND4	G → A
3010 bp	16S rRNA	G → A	11944 bp	ND4	T → C
3316 bp	ND1	G → A	12026 bp	ND4	A → G
3394 bp	ND1	T → C	12406 bp	ND5	G → A
3497 bp	ND1	C → T	12705 bp	ND5	C → T
3970 bp	ND1	C → T	12882 bp	ND5	C → T
4086 bp	ND1	C → T	13759 bp	ND5	G → A
4248 bp	ND1	T → C	13824 bp	ND5	A → G
5147 bp	ND2	G → A	13928 bp	ND5	G → C
5178 bp	ND2	C → A	13942 bp	ND5	A → G
5301 bp	ND2	A → G	14067 bp	ND5	C → T
5417 bp	ND2	G → A	14668 bp	ND6	C → T
5460 bp	ND2	G → A	14693 bp	tRNA-Glu	A → G
6392 bp	COX1	T → C	14783 bp	CYTB	T → C
6962 bp	COX1	G → A	15038 bp	CYTB	A → G
7853 bp	COX2	G → A	15043 bp	CYTB	G → A
8281 bp	STS	CCCCCTCTA deletion	15244 bp	CYTB	A → G
			15301 bp	CYTB	G → A
8392 bp	ATP8	G → A	15346 bp	CYTB	G → A
8414 bp	ATP8	C → T	16129 bp	D-loop	G → A
8459 bp	ATP8	A → G	16172 bp	D-loop	T → C
8473 bp	ATP8	T → C	16189 bp	D-loop	T → C
8584 bp	ATP6	G → A	16223 bp	D-loop	C → T
8701 bp	ATP6	A → G	16290 bp	D-loop	C → T
8745 bp	ATP6	A → G	16319 bp	D-loop	G → A
8794 bp	ATP6	C → T	16362 bp	D-loop	T → C
9053 bp	ATP6	G → A	16519 bp	D-loop	T → C

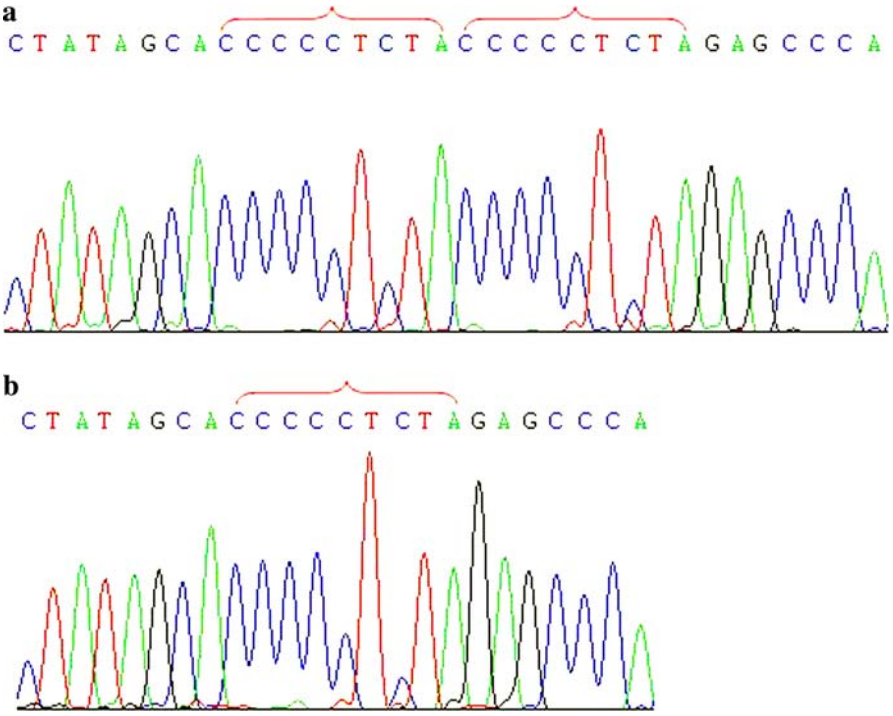

Fig. 1 Chromatograms of 9-bp deletion variant. **a** Two copies of 9-bp repeat; 26 of 34 (76.5%) patients, 26 of 28 (92.9%) healthy controls. **b** One copy of 9-bp repeat; eight of 34 (23.5%) patients, two of 28 (7.1%) healthy controls

9-bp direct repeat region; the others lacked the information of this region due to the bad sequence results. In the 62 individuals with complete sequences, eight of 34 (23.5%) patients and only two of 28 (7.1%) healthy controls showed the 9-bp deletion (Fig. 1).

Discussion

The candidate gene approach has already been useful in trying to understand the complex genetics of PCOS. A number of linkage and association studies of candidate genes in PCOS have yielded positive or mixed results (Diamanti-Kandarakis and Piperi 2005). For example, *CYP11a*, which encodes a P450 side-chain cleavage, appears to be a major susceptibility locus. Northern blot analysis revealed that *CYP11a* mRNAs were more abundant in PCOS theca cells than in normal ones (Wickenheisser et al. 2000). The expression of nuclear-encoded genes involved with mitochondria in PCOS patients was also studied. Reduced expression associated with mitochondrial oxidative metabolism in skeletal muscle of insulin-resistant women with PCOS was reported in previous research (Skov et al. 2007). To deal with the lack of a means for exploring mitochondrial mutations in PCOS

patients, we have applied a two-stage PCR amplification strategy to determine the complete sequence of the human mitochondrial genome.

More than 100 pathogenic point mutations and 200 deletions, insertions, and rearrangements have been identified since the first mitochondrial DNA mutations were described in 1988 (Naviaux 2000). The present study screened the mutations in the whole mitochondrial genome of PCOS patients. A statistical analysis with the χ^2 test showed that a 9-bp deletion variant was significantly different between PCOS patients and control individuals.

The 9-bp deletion involves one of the two copies of a direct-repeat sequence (CCCCCTCTA) at nt 8270–8289. Direct repeats are present at a high frequency in mtDNA, and large mtDNA deletions usually occur between flanking direct repeats because of homologous recombination or slipped mispairing (Schon et al. 1989). As slippage has been suggested as an explanation for the length changes detected in micro- and mini-satellites (Schlotterer and Tautz 1992), it is not unreasonable to postulate that it could also be responsible for mini-rearrangements of mtDNA. The two 9-bp copies of region V of the mtDNA represent a tandem direct repeat and could be a hot spot for deletions. There is evidence of a relationship between replication slippage of repetitive sequences and defective DNA repair, and it is well known that mtDNA repair systems are underdeveloped. Thus, the 9-bp deletion probably appeared as a result of an unrepaired error produced by slippage during replication.

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