

Genetic Alterations within the *DENND1A* Gene in Patients with Polycystic Ovary Syndrome (PCOS)

Mette B. Eriksen^{1,2,3*}, Michael F. B. Nielsen², Klaus Brusgaard⁴, Qihua Tan^{4,5}, Marianne S. Andersen¹, Dorte Glintborg¹, Michael Gaster^{1,2}

1 Department of Endocrinology, Odense University Hospital, Odense, Denmark, **2** Department of Clinical Pathology, Odense University Hospital, Odense, Denmark, **3** Department of Clinical Research, University of Southern Denmark, Odense, Denmark, **4** Department of Clinical Genetics, Odense University Hospital, Odense, Denmark, **5** Department of Epidemiology and Biostatistics, Institute of Public Health, University of Southern Denmark, Odense, Denmark

Abstract

Polycystic ovary syndrome (PCOS), the most common endocrine disease among premenopausal women, is caused by both genes and environment. We and others previously reported association between single nucleotide polymorphisms (SNPs) in the *DENND1A* gene and PCOS. We therefore sequenced the *DENND1A* gene in white patients with PCOS to identify possible alterations that may be implicated in the PCOS pathogenesis. Patients were referred with PCOS and/or hirsutism between 1998 and 2011 ($n = 261$). PCOS was diagnosed according to the Rotterdam criteria ($n = 165$). Sequence analysis was performed in 10 patients with PCOS. Additional patients ($n = 251$) and healthy female controls ($n = 248$) were included for SNP genotyping. Patients underwent clinical examination including Ferriman-Gallwey score (FG-score), biochemical analyses and transvaginal ultrasound. Mutation analysis was carried out by bidirectional sequencing. SNP genotyping was tested by allelic discrimination in real-time PCR in the additional patients and controls. Sequencing of the *DENND1A* gene identified eight SNPs; seven were not known to be associated with any diseases. One missense SNP was detected (rs189947178, A/C), potentially altering the structural conformation of the DENND1A protein. SNP genotyping of rs189947178 showed significantly more carriers among patients with PCOS and moderate hirsutism compared to controls. However, due to small sample size and lack of multiple regression analysis supporting an association between rs189947178 and FG-score or PCOS diagnosis, this could be a false positive finding. In conclusion, sequence analysis of the *DENND1A* gene of patients with PCOS did not identify alterations that alone could be responsible for the PCOS pathogenesis, but a missense SNP (rs189947178) was identified in one patient and significantly more carriers of rs189947178 were found among patients with PCOS and moderate hirsutism vs. controls. Additional studies with independent cohort are needed to confirm this due to the small sample size of this study.

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* E-mail: mette.eriksen@ouh.rsyd.dk

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in premenopausal women and affects up to 10% [1]. Based on the Rotterdam criteria, the PCOS diagnosis includes hyperandrogenemia (clinical/biochemical), chronic anovulation and/or polycystic ovaries when other causes have been excluded [2]. PCOS is associated with insulin resistance, resulting in a 5–8 times increased risk of type 2 diabetes (T2D) [3]. PCOS is often accompanied by hirsutism and more than 80% of hirsute patients are diagnosed with PCOS [4].

The pathogenesis of PCOS is thought to be caused by a combination of genetic and environmental factors [4,5]. Insulin resistance has been studied as a potential site of origin for the pathogenesis of PCOS [6,7] and the overlap between PCOS and T2D has led to candidate-gene based approaches focusing on T2D. A recent study found no significant associations between T2D susceptibility loci and PCOS [8]. In addition, no association was found between PCOS and single nucleotide polymorphisms (SNPs) representing susceptibility loci for T2D, metabolic or cardiovascular traits [9]. Association between SNPs in the T2D-related genes *FTO* and *MC4R* and

obesity was found in patients with PCOS, however the SNPs were not associated with PCOS itself [10]. The first genome wide association study (GWAS) with Chinese patients with PCOS found association between SNPs in three loci (2p16.3: rs13405728, 2p21: rs13429458, rs12478601, rs12468394, 9q33.3: rs10818854, rs2479106, rs10986105) and PCOS [11]. The three loci were located near the gene for a G protein-coupled receptor for luteinizing hormone and human chorionic gonadotropin (*LHCGR*) and within introns of the thyroid adenoma associated gene (*THADA*) and the DENN domain containing 1a (*DENND1A*) gene [11]. A second recent GWAS also conducted with Chinese patients with PCOS confirmed the three loci from the first GWAS, and additionally identified eight new loci associated with PCOS susceptibility [12].

Despite the differences in the genetic background between Chinese and white European patients with PCOS or patients of European ancestry, the association between PCOS and SNPs in the *DENND1A* gene has been replicated in several studies [13–16] and suggests a potentially important role for *DENND1A* in the PCOS pathogenesis. The linkage between underlying molecular defects or alterations in *DENND1A* and the PCOS pathogenesis is so far undetermined.

DENND1A, or *connecdenn1*, encodes a protein containing a domain differentially expressed in normal and neoplastic cells (DENN), localized in the N-terminus of the protein. Several proteins harbour this domain, which is conserved between species and throughout evolution [17]. The DENND1A protein was originally described in relation to synaptic clathrin-coated vesicles in neurons. The protein is present in high levels in brain and testis [18]. In addition to the N-terminal localized DENN domain, DENND1A contains binding motifs for clathrin, the clathrin adaptor protein-2 (AP-2) and Src homology 3 (SH3) domains [18]. The conserved DENN domain of DENND1A acts as a nucleotide exchange factor for the GTPase Rab35 [19]. Rab35 is found in the plasma membrane and endocytic compartments and functions by regulating endosomal recycling. Rab35 plays a major part in cytokinesis [20].

The aim of this study was to characterize the protein-coding sequence of the *DENND1A* gene in PCOS patients and to detect any genetic alterations possibly contributing to the PCOS pathogenesis. To our knowledge, the complete coding sequence of the *DENND1A* gene of patients with PCOS has not previously been described, despite the association between variation in *DENND1A* and PCOS, reported by us and others. The sequence analysis of *DENND1A* did not identify alterations that alone could be the PCOS pathogenesis; however a missense SNP was detected in one patient. Further analysis in additional patients and controls showed significantly more carriers of this SNP among patients with PCOS and moderate hirsutism compared to controls. The rs189947178 missense SNP may represent a part of a rare PCOS genotype or it may be a marker for PCOS related genetic alterations located apart from *DENND1A*.

Materials and Methods

Ethics statement

The study was approved by the local ethics committee (The Scientific Ethical Committee for Vejle and Funen Counties now referred to as The Scientific Ethical Committee of the Region of Southern Denmark) and all subjects gave written informed consent.

Patients and controls

In a previous study, the rs2479106 G allele was associated with decreased PCOS susceptibility in a cohort of white patients with PCOS [16]. The study cohort consisted of 261 white, female patients referred to Department of Endocrinology, Odense University Hospital, Denmark, with PCOS or hirsutism. The included patients were characterized by clinical hyperandrogenism only ($n = 96$) and/or fulfilled the Rotterdam criteria for PCOS ($n = 165$) [21].

Two hundred and forty eight healthy, white, pre-menopausal women were recruited as controls from the local area of Funen during the same period of time, and all with regular menstruations (cycle length of 26–34 days) and no complaints of hirsutism were included as controls [16,22]. Two hundred and nine controls were blood donors (initially, 345 women gave informed consent and completed a questionnaire concerning menstrual cycles and hirsutism. 136 female blood donors were excluded: 86 due to hysterectomy/menopause, 42 due to hirsutism and 8 blood donors due to irregular menstruations). Thirty nine controls were included from a study aimed to establish a reference interval for 17OHP responses during the ACTH test [22]. These women underwent clinical examination and were characterized by total testosterone < 1.8 nmol/l and maximum total Ferriman-Gallwey score ≤ 1 .

The patient- and control cohorts have been thoroughly described in previous studies [16,22]. Patients and controls paused oral contraceptives for at least three months before evaluation and did not use medicine known to affect hormonal or metabolic parameters.

We defined a Ferriman-Gallwey score ≥ 16 ($n = 47$) as the cut-off for moderate hirsutism, according to the 95th percentile in unselected populations of premenopausal white women [23–29]. PCOS ($n = 165$) was defined according to the Rotterdam criteria [2].

From the patient cohort, we selected ten patients with PCOS for direct sequencing of the *DENND1A* gene (table 1). The ten sequenced patients were randomly selected from 29 patients that fulfilled the following criteria: the patients fulfilled all three of the Rotterdam criteria [2], had free testosterone > 0.034 nmol/l and a Ferriman-Gallwey score ≥ 7 [30].

Recruiting family members was avoided.

Clinical and biochemical analyses

Routine evaluation included clinical evaluation, blood samples, and transvaginal ultrasound. Waist circumference was determined as the minimum circumference between the iliac crest and lower costae, whereas the hip circumference was determined as the maximum circumference over the gluteal region.

Table 1. Clinical and biochemical characteristics of patients with PCOS and controls.

	Controls (n = 39)	Patients (n = 261)	Sequenced	
			Unsequenced PCOS (n = 155)	PCOS (n = 10)
Age (years)	25 (23-27)	31 (25-36)**	29 (24-34)	30 (25-32)
Weight (kg)	65.0 (60.0-73.0)	72.8 (64.1-86.8)**	72.8 (64.1-87.6)	88.8 (76.8-101.0)†
BMI (kg/m²)	23.3 (21.6-24.8)	26.0 (22.6-30.6)**	26.1 (22.7-30.6)	30.5 (27.0-38.4)†
WHR	0.73 (0.70-0.77)	0.82 (0.76-0.86)**	0.82 (0.77-0.87)	0.86 (0.79-0.93)
Ferriman-Gallwey score	0 (0-1)	10 (6-14)**	10 (6-13)	15 (10-21)†
Free testosterone (nmol/l)	0.019 (0.013-0.024)	0.033 (0.021-0.048)**	0.034 (0.023-0.053)	0.049 (0.039-0.066) †
SHBG (nmol/l)	65 (51-84)	54 (37-74)*	51 (35-72)	33 (18-38)†
Fasting glucose (mmol/l)	4.9 (4.6-5.2)	4.6 (4.3-5.0)*	4.8 (4.3-5.2)	4.7 (4.1-4.8)
Fasting insulin (pmol/l)	31 (20-40)	52 (36-82)**	52 (38-83)	82 (57-98)
Triglyceride (mmol/l)	0.8 (0.6-1.0)	1.1 (0.8-1.5)**	0.9 (0.7-1.4)	1.4 (1.3-2.3)†
HDL (mmol/l)	1.5 (1.3-1.8)	1.3 (1.1-1.6)*	1.3 (1.2-1.6)	1.4 (1.1-1.6)
LDL (mmol/l)	2.3 (1.9-2.6)	2.9 (2.4-3.4)**	2.8 (2.3-3.3)	3.3 (2.5-4.3)

Data are presented as median (25-75% quartiles). P-values were calculated by Mann-Whitney test. These results have partly been published before [16,21,22].

*. P < 0.05 - patients vs. controls.

**.. P < 0.001 - patients vs. controls.

†. P < 0.05 - sequenced PCOS vs. PCOS.

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Sex hormones levels were determined as total testosterone, free testosterone and sex hormone-binding globulin (SHBG). The applied assays have previously been described [21,31]. In brief: Total testosterone was determined at Statens Serum Institute (Copenhagen, Denmark) by liquid chromatography tandem mass spectrometry. Free testosterone levels were calculated from total testosterone and SHBG levels according to Vermeulen et al. [32]. Free testosterone levels > 0.034 nmol/l were considered elevated levels. In Denmark, the normal range of free testosterone levels in fertile aged women is [0.006–0.034] nmol/l, which is used nationally. Insulin was analyzed by time-resolved fluoroimmunoassay with a commercial kit (AutoDELFIA; Wallac Oy, Turku, Finland). Plasma total cholesterol, high-density lipoprotein (HDL), cholesterol and triglyceride (TG) levels were analyzed by enzymatic colorimetric reactions (Modular P, Roche, Hvidovre, Denmark), and low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation. Blood glucose was

measured on capillary ear blood using Hemo Cue (Quest Diagnostics, Denmark).

DNA purification and mutation analysis

Genomic DNA was isolated from peripheral leukocytes using a Maxwell®16 robot (Promega, Denmark). All exons and exon-intron boundaries of *DENND1A* (accession no. NM_020946.1) was analyzed by bi-directional sequencing using the Big Dye® Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Denmark) and an ABI3730XL capillary sequencer (Applied Biosystems, Denmark). Specific forward and reverse primers were designed to align with exons and exon-intron boundaries of *DENND1A* (table S1).

Single-nucleotide polymorphism assay

All patients and controls were tested for presence of the missense SNP rs189947178 (c.2351C>A p.Ala 784 Asp) using allelic discrimination by real time PCR (StepOnePlus™ Real-time PCR Systems, Naerum, Denmark) and probe-based allelic discrimination assay (Pentabase, Odense, Denmark). The method takes advantage of the single base change C/A that discriminates between wild type and mutation. The real time PCR were carried out with probes designed specifically for either the wild type sequence (HEX-CCC TGC TCG CCC TCC-BHQ-1) or the mutation sequence (FAM-CCC TGC TCG ACC TCC T-BHQ-1), and specific primers flanking the region of interest (forward primer: 5'- TGC AGG CAC GAG CAG TGA - `3, reverse primer: 5'- AGG CTG TGC TGA GCG GGT - `3). Reactions were prepared in 96 wells plates as follows: 3.5 µl TaqMan Genotyping Master Mix (Applied Biosystems, Denmark), 1.26 µl forward primer (5 µM), 1.26 µl reverse primer (5 µM), 0.14 µl wild-type probe (5 µM), and 0.14 µl mutation probe (5 µM). The PCR conditions were: step 1: 10 min at 95 °C; step 2 (repeated 40 times): 5 s at 95 °C, 30 s at 60 °C; step 3: 30 s at 60 °C, step 4: 4 °C.

Pathogenicity analysis of mutation

Database of Single Nucleotide Polymorphisms (dbSNP) (Bethesda, MD: National Center for Biotechnology Information, National Library of Medicine; dbSNP Build ID: 131, Genome Build 37.4) was used to retrieve information on minor allele frequencies (MAF) [33]. Pathogenicity analyses were performed with PolyPhen [34], Mutationtaster [35] and SIFT [36–40]. Polyphen, Mutationtaster and SIFT mainly do predictions based on the presence or absence of conservation of highly related sequences. The Alamut software (Alamut version 2.1, Interactive Biosoftware, Rouen, France) ESE and Splice site prediction tools were used for predicting protein function effects of nucleotide changes. The Research Collaboratory for Structural Bioinformatics PDB (<http://www.pdb.org/pdb/home/home.do>) was used to obtain protein crystal structures.

Statistical analyses

The Mann-Whitney U test was used to compare clinical and subclinical characteristics and the data were presented as median (25–75 quartiles) using SPSS (SPSS version 19).

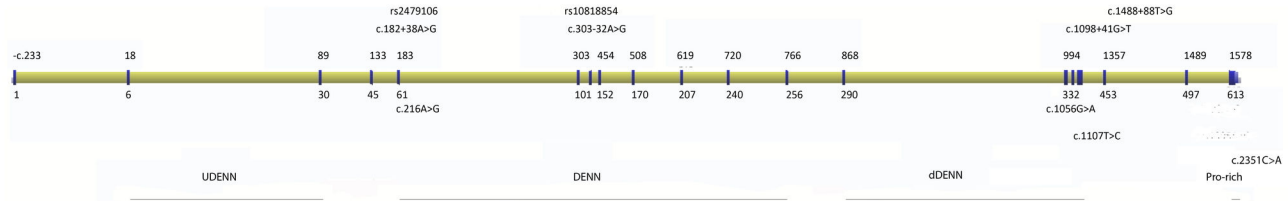


Figure 1. Distribution of the eight detected SNPs in *DENND1A*. The distribution of the eight SNPs detected by sequencing of *DENND1A* in patients with PCOS: rs1778890, rs9785285, rs12377595, rs116974312, rs61736953, rs3829851, rs10739633, rs189947178.

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Multiple regression analysis was applied to detect association of rs189947178 genotype distribution with PCOS status and with clinical variables, while controlling for age effects using the generalized linear model with a link function of binomial family. Model fitting was done using the *glm* function in the free R package *stats*. Adjustment for multiple comparisons was done using Bonferroni correction. After correction, the type I error rate was set at 0.004 (type I error at 0.05 divided by 13 sub-tests).

Results

Clinical and biochemical characteristics of sequenced patients with PCOS (n = 10) compared to unsequenced patients with PCOS (n = 155) are listed in table 1. Sequenced patients with PCOS was characterized by significantly higher weight, BMI, Ferriman-Gallwey score, free testosterone and triglyceride levels and lower SHBG levels compared to the additional patients with PCOS. Clinical and biochemical characteristics of the patients (n = 261) and controls are also listed in table 1. These results have partly been published before [16,21,22].

Sequencing of *DENND1A*

Eight SNPs were detected in the *DENND1A* exons and exon-intron boundary sequences (Figure 1). As depicted in Table 2, a number of genetic variants were observed in the sequenced PCOS patients. Seven of the eight SNPs detected were all synonymous or intron variants (Table 3). Further analysis using Alamut, predicted none of these seven synonymous/intron variants to influence *DENND1A* function. One patient harboured a missense SNP (c.2351C>A, p.Ala784Asp, rs189947178), replacing Alanine (Ala) with Aspartic acid (Asp). All of the eight SNPs detected are described in the dbSNP, hence we did not detect any new SNPs and thus the sequences of *DENND1A* were not uploaded. Figure 2 shows the chromatogram of the detected missense SNP, rs189947178. The MAF of patients with PCOS and moderate hirsutism (n = 27) was 0.056 (Table 3). According to dbSNP, the MAF of rs189947178 is 0.007.

Pathogenicity analysis conducted with PolyPhen predicted the rs189947178 variant to be “Probably Damaging” with a

Table 2. SNPs detected in *DENND1A* sequences of patients.

cDNA position	Protein level	dbSNP	SNP	MAF	Patients MAF
c.182+38A>G.	Intron	rs1778890	Intron	0.237	0.250
c.216A>G	p.Thr72Thr.	rs9785285	Exon 5	0.410	0.250
c.303-32A>G	Intron	rs12377595	Intron	0.271	0.250
c.1098+41C>A	Intron	rs116974312	Intron	0.033	0.100
c.1056G>A	p.Arg352Arg.	rs61736953	Exon 14	0.064	0.200
c.1107T>C	p.Asp369Asp	rs3829851	Exon 15	0.167	0.100
c.1488+88T>G	Intron	rs10739633	Intron	0.318	0.550
c.2351C>A	p.Ala784Asp	rs189947178	Exon 22	0.007	0.050

dbSNP: reference SNP number is indicated. Protein level: the result of nucleotide change on protein sequence is indicated when information was available. The allele frequency distributions were obtained from dbSNP (Genome Build 37.4).

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Table 3. Genotype distribution and allele frequencies of rs189947178.

Genotype (n = 248)	PCOS		Clinical hyperandrogenism		Patients PCOS with moderate hirsutism		PCOS with moderate hirsutism	
	Controls (n = 248)	Patients (n = 261)	Patients (n = 165)	(n = 96)	(n = 47)	(n = 27)	(n = 27)	
Carriers	2 (0.8)	5 (1.9)	4 (2.4)	1 (1.0)	3 (6.4)	3 (11.1)		
Non-carriers	246 (99.2)	256 (98.1)	161 (97.5)	95 (99.0)	44 (93.6)	24 (88.9)		
MAF	0.004	0.010	0.012	0.005	0.032	0.056		
P-value	-	0.61	0.37	0.98	0.04	0.003*		

Carriers: Subjects with the A allele (AC genotype).

Non-carriers: Subjects without the A allele (CC genotype).

PCOS was defined according to the Rotterdam criteria [2].

*. P-values ≤ 0.004.

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score of 0.926. Mutationtaster and SIFT predicted the variant to be a polymorphism.

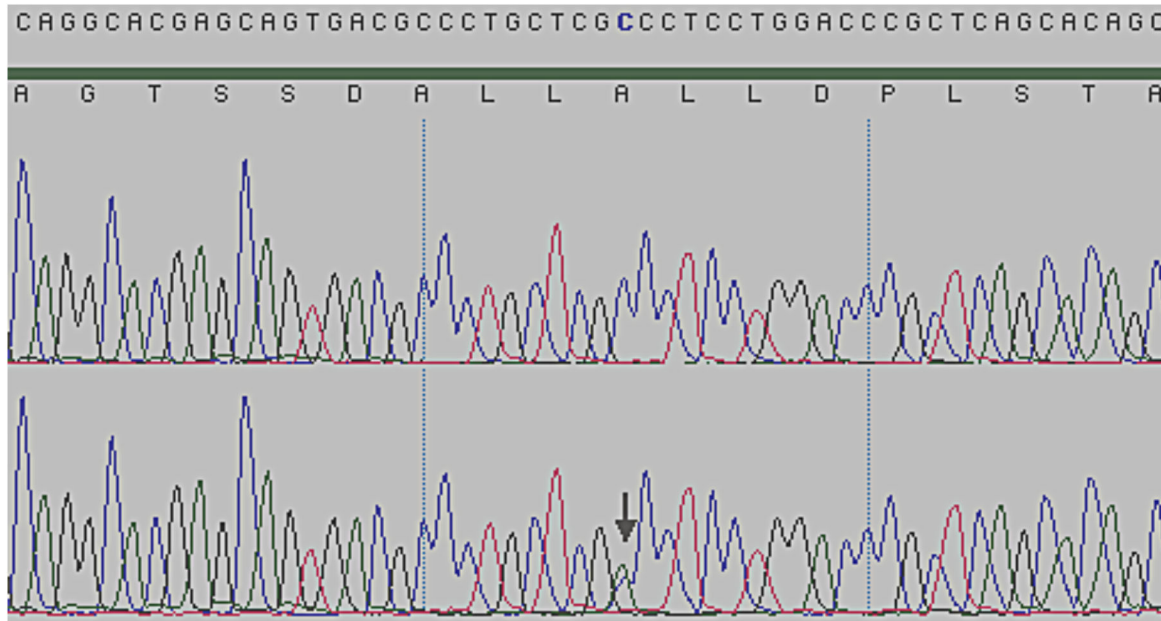


Figure 2. Chromatogram showing the rs189947178 variation (c.2351C>A, p.Ala784Asp) in exon 22 of *DENND1A*. Representation of the partial sequence of *DENND1A* exon 22 displaying in the upper pane a wild type sequence and in the lower pane the c.2351C>A allele (depicted by an arrow). Above the chromatograms the consensus protein and cDNA sequences are shown.

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The classification made by Polyphen additionally includes information on tertiary structure and structural parameters. So far, no crystal structure of *DENND1A* has been published, but a crystal structure of the homologous *DENND1B* has been published as PDB: 3TW8 [41]. Despite the great homology, *DENND1A* and *DENND1B* varies in the C-terminal portion, thus an assessment of functional consequence could not be drawn from structural comparison. Garnier et al. [42] and Chou-Fasman's [43] secondary structure evaluation predicted Ala784 to be part of an alpha helical amphipathic internal structure (data not shown). Thus, a change of the hydrophobic Ala784 for a polar amino acid might be damaging to protein function. Contrary to the highly conserved N-terminal domain DENN (uDENN), DENN and downstream DENN (dDENN) modules, the C-terminal tail shows large deviation between different DENN-containing molecules. In contrast to this, the C-terminal region of *DENND1A* shows strong phylogenetic conservation (data not shown), pointing to a distinct important function.

Carrier status and allele frequencies of rs189947178

The AA genotype (homozygote mutation genotype) was not found in any patients or controls and table 3 shows carriers (AC genotype) and non-carriers (CC genotype) only.

Results for the all patients (n = 261) including the sequenced patients with PCOS (n = 10) and unsequenced patients with PCOS (n = 155) compared to controls (n = 248) are shown in table 3. There were no significant differences in the distribution of carriers versus non-carriers in: patients (n = 261) vs. controls (n = 248), patients with PCOS (n = 165) vs. controls (n = 248),

patients with clinical hyperandrogenism (n = 96) or patients with moderate hirsutism (n = 47) vs. controls. There were significantly more carriers among patients with PCOS and moderate hirsutism (n = 27) compared to controls (P = 0.003). Multiple regression analysis was applied to evaluate carrier status of rs189947178. Ferriman-Gallwey score and PCOS status was not associated with carrier status (AC genotype, data not shown). In addition, no other clinical and biochemical parameters (testosterone levels, HDL, LDL, weight, fasting glucose, fasting insulin levels) were associated with carrier status of rs189947178 missense SNP.

The SNP rs2479106 previously found to be associated with PCOS and the missense SNP rs189947178 was not in linkage disequilibrium ($D' = 0.201$, $r^2 = 0$).

Discussion

In the present study, we sequenced the PCOS candidate gene, *DENND1A* to further investigate the association between PCOS and *DENND1A*, reported by us and others (11-15). Sequencing of the *DENND1A* gene from patients with PCOS identified eight SNPs within the coding region of *DENND1A*. One of these SNPs is a missense SNP (rs189947178) that gives rise to an amino acid change (alanine to aspartic acid) and therefore potentially could be associated with the PCOS pathogenesis.

The rs189947178 mutation variant leads to a change of a hydrophobic amino acid to a polar amino acid and therefore has a possibly damaging effect on the *DENND1A* protein. The

involvement of *DENND1A* in endosomal membrane traffic could affect several organ systems as proposed by Goodarzi [14]. We therefore investigated if this mutation was only present in patients with PCOS and genotyped well characterized patients with PCOS or hirsutism and healthy controls. We found significantly more carriers among patients with PCOS and moderate hirsutism compared to controls. Due to the small sample size of patients with PCOS and moderate hirsutism, it is possible that the significant more carriers among patients with PCOS and moderate hirsutism was a false positive association – hence, the multiple regression analysis did not support an association between this SNP and Ferriman-Gallwey score or PCOS diagnosis.

A recent study of genotype-phenotype correlations of the GWAS-identified PCOS susceptibility SNP rs2479106, found an association between the GG + AG group of patients and elevated serum insulin after a glucose load [44]. The *DENND1A* protein is in part involved in endosomal recycling [19,20] and changes in the conformation could alter protein function, which hypothetically could affect insulin secretion. Our data did however not indicate any association of the rs189947178 missense carrier status and insulin levels.

Our findings regarding rs189947178 carrier status, along with previous findings of association between SNPs found in *DENND1A* [11,13–16], could indicate that these SNPs serves as genomic markers for alterations in genes separate from *DENND1A*. The SNP rs2479106 was associated with PCOS in several studies. rs2479106 is located in an intron of the *DENND1A* gene, thus not within a coding region. A study by Goodarzi found an association between rs10818854 located in *DENND1A*, but not rs2479106 (13). The association between PCOS and SNPs in *DENND1A* could instead relate to these SNPs being in linkage disequilibrium with variation in a co-localized gene encoding a microRNA (miR601), as suggested by Goodarzi (13). miR601 is involved in expression of actin cytoskeleton and down regulation of the Fas induced apoptosis pathway and expression of nuclear factor-kappaB transcription factor dependent reporter [45]. The fact that SNPs in *DENND1A* instead could relate to functional variation in other genes are a limitation to this study. However, since the association between SNPs in *DENND1A* has been replicated in several studies [12–16], we considered *DENND1A* a possible candidate gene for PCOS.

Further studies in investigating the prevalence of rs189947178 in additional, independent larger cohorts could be

relevant to conduct. If these studies replicate our finding, conducting functional studies determining the precise molecular effect of the missense variation rs189947178 could be relevant. It is important to mention that only the coding regions of *DENND1A* were sequenced in this study. This is a limitation to the study since we may have missed functional non-coding regulatory variation within the gene.

In conclusion, sequences of the *DENND1A* gene from patients with PCOS did not reveal alterations that alone could be causing the PCOS pathogenesis. We found significantly more carriers of the missense SNP rs18994717 among patients with PCOS and moderate hirsutism. However, because of the small sample size and the lack of association between this SNP and Ferriman-Gallwey score or PCOS diagnosis, this may be a false positive association. The PCOS pathogenesis may be heterogenic and caused by low penetrant common variants in different genes. The rs189947178 missense SNP may represent a part of a rare PCOS genotype or it may be a marker for PCOS related genetic alterations located apart from *DENND1A*. Further studies are needed to clarify this.

Supporting Information

Table S1. Sequences of oligonucleotide forward and reverse primers for sequencing of the *DENND1A* gene. (DOC)

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Author Contributions

Conceived and designed the experiments: MBE MFN KB QT MSA DG MG. Performed the experiments: MBE MFN KB QT DG MG. Analyzed the data: MBE MFN KB QT MSA DG MG. Contributed reagents/materials/analysis tools: MBE MFN KB QT MSA DG MG. Wrote the manuscript: MBE MFN KB QT MSA DG MG.

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