Association Study of Four Key Folliculogenesis Genes in Polycystic Ovary Syndrome

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Abstract

Polycystic ovaries and impaired fertility are the result of abnormal folliculogenesis. Our objective was to determine the role of four candidate folliculogenesis genes in the development of polycystic ovary syndrome (PCOS). Women with and without PCOS (335 cases, 198 controls) were genotyped for single nucleotide polymorphisms in *GDF9, BMP15, AMH,* and *AMHR2.* Variants in these genes were not associated with PCOS. Certain *GDF9* variants were associated with hirsutism scores and parity in PCOS patients. *GDF9* may thus serve as a modifier gene. These results suggest that inherited defects in folliculogenesis are not major factors in the genetic susceptibility to PCOS.

Keywords

folliculogenesis; polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) affects 6–8% of reproductive age women.[1] PCOS is characterised by ovulatory dysfunction resulting in menstrual abnormalities and polycystic ovarian morphology that contribute to an increased risk of infertility.

Numerous factors affect the normal development of a follicle and subsequent ovulation. The transforming growth factor-beta (TGF-β) superfamily is a family of structurally related proteins that regulate basic biological processes including those having to do with growth and development. Several TGF-β superfamily members and their receptors are involved in folliculogenesis, including growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15), anti-müllerian hormone (AMH), and anti-müllerian hormone receptor type II (AMHR2).

During normal folliculogenesis, expression of GDF9, an early follicle growth promoter, begins in human oocytes at the primordial-primary follicle transition and increases with preantral follicle growth.[2] BMP15 is also expressed in the oocytes and is thought to be involved in oocyte maturation and follicular development.[2] Expression of AMH, an early follicle growth inhibitor, is normally low in primordial and primary follicles, increases to maximum levels in large preantral follicles and small antralstages, and then declines during final follicular maturation.[3] AMH appears to be the only natural ligand of AMHR2; *AMH* and *AMHR2* null mice both have the same phenotype.[4]

The current genetic study was inspired by studies that described dysregulation of several of these TGF-β proteins in the follicles of patients with PCOS. The first such study observed a decreased level of *GDF9* mRNA in developing PCOS and PCO oocytes compared with normal, with no differences in *BMP15* expression.[5] Another study found reduced AMH expression during the initial stages of folliculogenesis in PCOS.[3] Others found that AMH levels in follicular fluid and granulosa cell-conditioned media were higher for PCO compared to normal controls.[6] Lastly, *AMHR2* mRNA levels in granulosa cells of small follicles were elevated in PCOS.[7] While the clinical implications of these abnormalities are not completely understood, this paracrine dysregulation of growth factors in PCOS can potentially alter the intrafollicular environment, disturb granulosa cell interactions, and impair cytoplasmic and/or nuclear maturation of the oocyte.[3,6]

Given the critical role of disordered folliculogenesis in PCOS, our goal in this study was to examine the genes for *GDF9*, *BMP15*, *AMH* and *AMHR2* as candidate genes for PCOS and its component traits.

Methods

Subjects and Phenotyping

We studied 335 unrelated White PCOS patients and 198 White control women recruited at two centers, the University of Alabama at Birmingham (UAB; 287 PCOS and 187 controls) and Cedars-Sinai Medical Center (CSMC; 48 PCOS and 11 controls). Participation in research studies was offered to patients meeting inclusion criteria (premenopausal, non-pregnant, on no hormonal therapy for at least three months, and meeting diagnostic criteria for PCOS). Presence of PCOS was defined by the 1990 NIH consensus criteria.[8] The specific parameters for defining hirsutism, hyperandrogenemia, ovulatory dysfunction and exclusion of related disorders were previously reported.[1]

Controls were healthy women, with regular menstrual cycles, and without a family history of hirsutism. These women had no evidence of hirsutism, acne, or alopecia, or endocrine dysfunction and had not taken hormonal therapy (including oral contraceptives) for at least 3 months prior to testing. Controls were recruited by word of mouth and advertisements, through a call for "healthy women" without detailing further the nature of the studies.

Subjects underwent a physical examination and hormone measurements, per a previously described protocol.[1] In addition, we evaluated whether PCOS patients had severe

oligomenorrhea (defined as > 3 months between menses), infertility (yes/no), and parity (yes/no). Clinical characteristics of the study cohort are given in Supporting Information S1.

All subjects gave written informed consent, and the study was performed according to the guidelines of the Institutional Review Boards of UAB and CSMC.

Genetic Analysis

We selected five *GDF9* single nucleotide polymorphisms (SNPs) (rs10491279, rs254285, rs11739194, rs39830, rs30178), two *BMP15* SNPs (rs3810682, rs3897937), three *AMH* SNPs (rs4807216, rs10407022, rs3746158), and three *AMHR2* SNPs (rs11170547, rs2002555, rs11170553) using genotype data of the Caucasian population of the HapMap database (release 24, phase II, www.hapmap.org). These SNPs were selected because they are predicted to tag SNPs across the entirety of each gene, plus 5kb upstream and downstream. These SNPs capture 34 of 41 (83%) of the Caucasian HapMap SNP alleles at $r^2 > 0.8$ for all four genes. The thirteen SNPs were genotyped using Applied Biosystems Taqman Assays on Demand according to manufacturer's instructions.

The program Haploview v 4.1 (Broad Institute, Cambridge MA, USA) was used to calculate linkage disequilibrium (LD, the D′ statistic) between each pairwise combination of SNPs and determine haplotypes and their frequencies.[9] The solid spine of LD algorithm in Haploview was used to determine haplotype blocks. Only subjects whose haplotype assignment was >95% certain were used in analyses.

Statistical Analysis

Unpaired T-tests and chi-square tests were used to compare clinical characteristics between women with and without PCOS; quantitative trait values were log- or square root-transformed as appropriate to reduce non-normality.

Data are expressed as the median (interquartile range). Association of SNPs or haplotypes with presence/absence of PCOS and qualitative traits was evaluated using logistic regression, adjusting for recruitment site, age and body mass index (BMI). Within the PCOS women, association with quantitative phenotypic traits was evaluated using analysis of covariance (ANCOVA), again adjusting for recruitment site, age and BMI in all analyses except those in which BMI was the dependent variable, wherein analyses were adjusted for recruitment site and age only. To handle multiple testing, significance of each analysis was taken as P<0.003 (=0.05/16), considering that we analyzed four linkage disequilibrium groups of SNPs against four families of traits (PCOS diagnosis, androgens, metabolic traits, reproductive endpoints), yielding a Bonferroni correction factor of 16 (i.e. 16 independent comparisons).

The sample size of 335 cases and 198 controls has excellent power (≥90%) to detect association of risk alleles of frequency ≥0.2 with PCOS at odds ratio ≥1.75, and has fair-to-good power (40–80%) to detect association at odds ratio 1.5. Detailed power calculations given in Supporting Information S2 reveal lower power to detect association of rare risk alleles (frequency ≤0.1) with PCOS at odds ratios less than 1.75.

Results

GDF9

We genotyped five SNPs spanning *GDF9* (Fig. 1A). SNP frequencies are shown in Supporting Information S3. All markers were in Hardy-Weinberg equilibrium. Linkage

disequilibrium among these markers (D′) ranged from 0.40 to 1.0 (average pairwise D′ of 0.90). The common haplotypes are displayed in Supporting Information S4.

GDF9 SNPs and haplotypes were not associated with PCOS susceptibility. Among women with PCOS, minor allele carriers of SNPs rs11739194 or rs30178 had significantly higher modified Ferriman-Gallwey (mFG) hirsutism scores than non-carriers with values of 8.0 (6.5) for carriers versus 7.0 (5.0) for non-carriers of rs11739194 and values of 8.0 (5.0) in carriers versus 7.0 (6.0) for non-carriers of rs30178 (P=0.001 and 0.001, respectively). In PCOS women, haplotype 3 carriers had significantly increased mFG scores compared to non-carriers with a score of 8.0 (6.0) versus 7.0 (6.0) (P=0.0002). This haplotype result was consistent with the SNP results in that this haplotype carries the minor alleles of both SNPs that displayed similar trait associations. Additionally, in PCOS patients, minor allele carries of SNP rs254285 had reduced parity (OR=0.19, 95% CI=0.06–0.57, P=0.003).

BMP15, AMH and AMHR2

The genetic structure and linkage disequilibrium for *BMP15*, *AMH*, and *AMHR2* are displayed in Fig. 1B, 1C, and 1D. SNP frequencies (all in Hardy-Weinberg equilibrium) are shown in Supporting Information S3. The common haplotypes are displayed in Supporting Information S4. We did not observe any genotype-phenotype associations between *BMP15*, *AMH* or *AMHR2* variants and PCOS or any of the qualitative or quantitative traits.

Discussion and Conclusion

We hypothesized that polymorphisms in genes involved in folliculogenesis would influence susceptibility to PCOS, but found no association of four key genes, *GDF9*, *BMP15*, *AMH*, and *AMHR2*, with PCOS susceptibility.

While we are not the first to conduct genetic association studies of *BMP15* or *AMH* in PCOS, we are the first, to our knowledge, to evaluate *GDF9* and *AMHR2*. In addition to the molecular studies evaluating the TGF-β members in PCOS, there have been several genetic studies that yielded conflicting results. In one study, two polymorphisms in *BMP15* were associated with anovulation and infertility in PCOS.[10] In contrast, we did not find that polymorphisms in *BMP15* were associated with severe oligomenorrhea or infertility. Others found association of the *AMH* Ile49Ser variant (rs10407022) with reduced frequency of PCO and lower follicle numbers in PCOS women, concluding that genetic variation in *AMH* may contribute to abnormal ovarian morphology.[11] We could not replicate their findings because ultrasounds were not routinely performed in our subjects. However, our data does suggest that the potential association of *AMH* genetic variation with ovarian morphology seen previously does not translate into a significant effect on reproductive function as evaluated by clinical reproductive endpoints. Lastly, gene sequencing in 38 women with PCOS found no missense mutation in any exons of *GDF9* or *BMP15*.[12]

We found that variants in *GDF9* were associated with hirsutism scores, potentially as a result of increased theca cell growth in the ovary leading to elevated androgens. Even though we did not see an association between variants in *GDF9* and the serum androgen levels, androgens in women are difficult to measure, possibly obscuring this association. We also found that a variant in *GDF9* was associated with reduced parity. Association of genetic variants with parity may be confounded by factors such as the relatively young age of our patients (median 27 yr) and unavailable information such as number of partners or who had attempted to conceive. Nevertheless, we speculate that variants in *GDF9* may contribute to compromised oocyte development, leading to outcomes such as spontaneous abortion and consequently lower parity. With our current data, we cannot be sure of the functional role of these *GDF9* variants or the broader significance of these findings.

The value of our study is that it reports on a group of genes, TGF-β superfamily members involved in folliculogenesis, which have not been extensively evaluated in PCOS. Our data demonstrate that polymorphisms in major folliculogenesis genes, *GDF9*, *BMP15*, *AMH*, and *AMHR2*, are not associated with PCOS susceptibility. While these data suggest that inherited defects in folliculogenesis are not major contributors to the development of PCOS, examination of other genes in this superfamily would be needed to fully support this conclusion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Azziz R, Woods KS, Reyna R, Key TJ, Knochenhauer ES, Yildiz BO. The prevalence and features of the polycystic ovary syndrome in an unselected population. J Clin Endocrinol Metab. 2004; 89:2745–9. [PubMed: 15181052]

2. Yan C, Wang P, DeMayo J, DeMayo FJ, Elvin JA, Carino C, et al. Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. Mol Endocrinol. 2001; 15:854–66. [PubMed: 11376106]

3. Stubbs SA, Hardy K, Da Silva-Buttkus P, Stark J, Webber LJ, Flanagan AM, et al. Anti-mullerian hormone protein expression is reduced during the initial stages of follicle development in human polycystic ovaries. J Clin Endocrinol Metab. 2005; 90:5536–43. [PubMed: 16030171]

4. Mishina Y, Rey R, Finegold MJ, Matzuk MM, Josso N, Cate RL, et al. Genetic analysis of the Mullerian-inhibiting substance signal transduction pathway in mammalian sexual differentiation. Genes Dev. 1996; 10:2577–87. [PubMed: 8895659]

5. Teixeira Filho FL, Baracat EC, Lee TH, Suh CS, Matsui M, Chang RJ, et al. Aberrant expression of growth differentiation factor-9 in oocytes of women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2002; 87:1337–44. [PubMed: 11889206]

6. Pellatt L, Hanna L, Brincat M, Galea R, Brain H, Whitehead S, et al. Granulosa cell production of anti-Mullerian hormone is increased in polycystic ovaries. J Clin Endocrinol Metab. 2007; 92:240–5. [PubMed: 17062765]

7. Catteau-Jonard S, Jamin SP, Leclerc A, Gonzales J, Dewailly D, di Clemente N. Anti-Mullerian hormone, its receptor, FSH receptor, and androgen receptor genes are overexpressed by granulosa cells from stimulated follicles in women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2008; 93:4456–61. [PubMed: 18697861]

8. Zawadzki, JK.; Dunaif, A. Diagnostic criteria for polycystic ovary syndrome: towards a rational approach. In: Dunaif, AJG.; Haseltine, F.; Merriam, GR., editors. Polycystic Ovary Syndrome. Boston: Blackwell Scientific; 1992. p. 377-84.

9. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 2005; 21:263–5. [PubMed: 15297300]

10. Gonzalez A, Ramirez-Lorca R, Calatayud C, Mendoza N, Ruiz A, Saez ME, et al. Association of genetic markers within the BMP15 gene with anovulation and infertility in women with polycystic ovary syndrome. Fertil Steril. 2008; 90:447–9. [PubMed: 17905236]

11. Kevenaar ME, Laven JS, Fong SL, Uitterlinden AG, de Jong FH, Themmen AP, et al. A functional anti-mullerian hormone gene polymorphism is associated with follicle number and androgen levels

in polycystic ovary syndrome patients. J Clin Endocrinol Metab. 2008; 93:1310–6. [PubMed: 18230658]

12. Takebayashi K, Takakura K, Wang H, Kimura F, Kasahara K, Noda Y. Mutation analysis of the growth differentiation factor-9 and -9B genes in patients with premature ovarian failure and polycystic ovary syndrome. Fertil Steril. 2000; 74:976–9. [PubMed: 11056243]
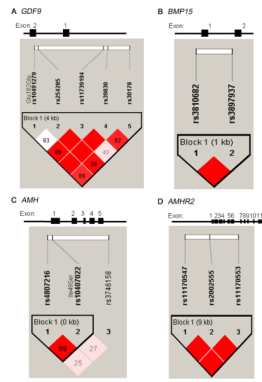
Figure 1.
Gene structure and linkage disequilibrium plot for A. *GDF9*, B. *BMP15*, C. *AMH*, and D. *AMHR2*. Gene structure is shown at top; *GDF9* has 2 exons, is located on chromosome 5q31.1, and is 5.46 kb in size. *BMP15* has 2 exons, is located on chromosome Xp11.2, and is 5.91 kb in size. *AMH* has 5 exons, is located on chromosome 19p13.3, and is 2.95 kb in size. *AMHR2* has 11 exons, is located on chromosome 12q13.13, and is 7.67 kb in size. The locations of the genotyped SNPs relative to the exons are indicated. The linkage disequilibrium plot beneath each gene displays D′ values (%) for each pair of SNPs in the box at the intersection of the diagonals from each SNP. The solid blocks indicate D′ = 1 (100%) for the corresponding pair of variants with a logarithm of the odds (LOD) score of ≥2. For each gene, the SNPs were considered together in haplotype blocks as indicated.