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Further Delineation of Familial Polycystic Ovary Syndrome Via Whole-Exome Sequencing: PCOS-related rare *FBN3* and *FN1* gene variants are identified

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Abstract

Aim: To identify pathogenic rare coding Mendelian/high-effect size variant(s) by whole-exome sequencing in familial PCOS patients to elucidate PCOS related pathways.

Methods: Twenty women and their affected available relatives diagnosed with polycystic ovary syndrome according to Rotterdam Criteria were recruited. Whole-exome sequencing on germ-line DNA from 31 polycystic ovary syndrome probands and their affected relatives were performed. Whole-exome sequencing data was further evaluated by pathway and chemogenomics analyses.

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Disclosure

The authors declare that they have no conflict of interest.

In-slico analysis of candidate variants were done by VarCards for functional predictions and VarSite for impact on 3D structures in the candidate proteins.

Results: Two heterozygous rare *FBN3* missense variants in three patients, and one *FN1* missense variant in one patient from three different PCOS families were identified.

Conclusions: We identified three novel *FBN3* and *FN1* variants for the first time in the literature and linked with polycystic ovary syndrome. Further functional studies may identify causality of these newly discovered PCOS related variants, and their role yet remain to be investigated. Our findings may improve our understanding of the biologic pathways affected and identify new drug targets

Keywords

Extracellular Matrix; Genetics; PCOS; Whole Exome Sequencing

Introduction

Polycystic ovary syndrome (PCOS) is a prevalent and heterogenous endocrine disease affecting 7–10% women of reproductive age with multi-factorial etiology (1). Based on twin studies, it is highly heritable (over 70%) (2) and first-degree female relatives of PCOS patients show increased prevalence (3–14). In addition to oligogenic/polygenic models and environmental effects on the pathogenesis of PCOS, autosomal dominant mode of inheritance has been recognized with familial clustering of cases (5–8, 10, 11, 13, 15, 16).

The criteria for PCOS diagnosis have been revised several times and there is no universally accepted version. The most widely used is the Rotterdam criteria, and PCOS is diagnosed by two or more of its reproductive features of ovulatory dysfunction (oligomenorrhea-amenorrhea), hyperandrogenism, and polycystic morphology of the ovaries on ultrasound exam, along with exclusion of other etiologies (17–19). Approximately two-third of affected individuals experience subfertility, obesity, and metabolic disorders in the PCOS background (20–29). As future consequences of the syndrome such as diabetes and cardiovascular disease risk are well-known, and also extend to first-degree relatives (30–33), further investigation is needed for possible clinical outcomes of the PCOS (34, 35).

Previous genetic approaches to PCOS, consisting mainly of candidate gene, and to a lesser extent genome-wide association studies, have identified variants that account for only a small percent of inherited PCOS risk and remaining are yet to be identified (36).

Whole-exome sequencing (WES) has been successfully identifying rare mutations that have a greater impact on human diseases since most disease-causing mutations are located within protein coding regions (37). Rare variants with large effects in a specific gene may be found in extreme phenotypes, which can provide insights into the underlying pathophysiology of the common disorder, and eventually lead to the development of risk prediction models and therapeutic strategies for patient care (38). Here, we aimed to identify pathogenic rare coding Mendelian/high-effect size variant(s) using WES in familial PCOS patients to elucidate PCOS related pathways.

Methods

Study co-investigators evaluated the patients at their participating institutions and obtained written consent under Gazi University, Medical Faculty, Ethics Committee (Decision#223).

Study Cohort

20 women and their affected available relatives from unrelated families with two or more individuals diagnosed with PCOS were recruited (Supplementary Table 1). PCOS was diagnosed according to the criteria from The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group (2004) (20).

All probands met the Rotterdam criteria for diagnosis of PCOS, i.e. they had two of the three following features: 1) oligomenorrhea (defined as menstrual cycles >35 days), 2) clinical (hirsutism defined as a modified Ferriman-Gallwey score (FGS) >6 or acne) and/or biochemical evidence of androgen excess (defined as serum levels of dehydroepiandrosterone sulfate (DHEAS), total (TT) or free testosterone (fT) above ±2SD of controls, the upper ±2SD of androgen levels among controls were 404 μg/dL for DHEAS, 0.61 ng/mL for TT and 2.12 pg/mL for fT, and 3) polycystic ovaries (defined as ovarian volume above 10 ml or ovaries having ≥12 follicles measuring 2–9 mm in at least one ovary).

As the Rotterdam ESHRE/ASRM–Sponsored PCOS consensus workshop group (19) suggests using more narrow diagnostic criteria in familial studies to identify affected individuals, such as the presence of PCO alone, or hyperandrogenemia per se, in this study, sisters were affected; 1. if they had PCOS according to Rotterdam criteria, 2. if they had only PCOM or 3. if they had hyperandrogenemia (clinical or biochemical). Mothers were considered affected if they had one of the features of PCOS or if they had a known history of PCOS or history of oligomenorrhea and/or hirsutism in their reproductive years.

For all participants medical history, signs of hirsutism and menstrual irregularities were recorded. Weight and height were determined, and body mass index (BMI) was calculated as weight (kg)/height(m^2). The presence of hirsutism was defined as a Ferriman-Gallwey score of >6 and acne were noted as present or absent. Blood sampling was performed during the follicular phase from all the subjects for the measurement of serum follicle stimulating hormone (FSH), luteinizing hormone (LH), androstenedione (A), 17-hydroxy progesterone (17-OHP), sex hormone binding globuline (SHBG), total testosterone (TT), dehydroepiandrostenodione sulfate (DHEAS) and anti-mullerian hormone (AMH). Hormone assays of LH, FSH, E2, DHEAS, TT was measured using electro-chemiluminescence immune assay with the Roche e Cobas 601 immunoassay analyzer, using the Roche kit. Androstenedione, SHBG and 17-OHP were measured using the Dia.Metra kit by “Enzyme-linked imunosorbent assay” (ELISA) method manually with μ-Quant Bio-Tek analyzer (μ-Quant Bio-Tek Instruments Inc. USA). Serum AMH was assayed by ELISA using Beckman Coulter AMH Gen 2 kits. Serum levels of total cholesterol, HDL-C, LDL-C, and TG were determined with the use of an AU680 Chemistry System (Beckman– Coulter). On the same day ovarian morphology was evaluated with Siemens Acuson Antares (Mountain View,

CA, USA) ultrasound machine equipped with a CH6-2 MHz abdominal or an EC9-4 MHz transvaginal probe.

Whole-Exome Sequencing (WES) and Analysis

After DNA extraction from whole blood using Gentra Puregene Blood Kit (QIAGEN, Hilden, Germany), whole-exome sequencing was performed with targeted enrichment of coding genome with NimbleGen 2.1M human exome array (Roche Nimblegen, Inc.) according to the manufacturer's protocol with modifications, described previously (37). Sequencing of the prepared libraries was performed on Illumina's HiSeq2000 using 75bp reads and paired-end chemistry. Base calling was performed with Illumina Casava pipeline version 1.8, and sequencing data were analyzed using BWA for alignment (39), GATK for variant calling and local pipelines for annotation (40). Main steps of bioinformatics pipeline are given in Supplementary Figure 1.

In-slico Analysis of Candidate Variants

Online database PCOSKB (http://www.pcoskb.bicnirrh.res.in), a curated set of genes and phenotype associations, along with biochemical pathways were (41) interrogated for phenotype/genotype correlations and sex specific validations. Chemogenomics analysis was performed with QuartataWeb server (42) and DrugBank (43). We used VarCards for functional predictions (44). Candidate variants were annotated for impact on 3D structures in the Protein Data Bank (PDB) through VarSite (45).

Results

Study Population

Central tendency measures as compared to standard distributions are outlined in Table 1. Mean age of the patients was 32.04 (±11.95) years and mean modified Ferriman Galways Scores were 10.13 (±7.83). High BMI, and increased hip and waist circumference values were observed. Insulin levels and OGTTs were normal. TT, SHBG, DHEASO4, 17OH progesterone, triglycerides, cholesterol, HDL and LDL measurements were all in normal ranges with the exception of high androstenedione levels.

Whole Exome Sequencing

31 germ line DNA were processed for WES from twenty unrelated families (2 patients from 11/20 families) (Supplementary Table 1). While 4 out of 20 families had a single affected individual, the remaining 16 (80%) had two or more affected individuals. Across the cohort, an average of 68,176,055 reads were obtained with 46.55% targeting coding (RefSeq) and flanking sequences. Mean target coverage was 65.17 and an average coverage of 10X or greater was achieved for 93.14% of the targeted bases, generating sufficient support to detect dominant and recessive single nucleotide and indel variants (Supplementary Table 2).

Data Analysis

WES data set generated 403,773 variants from 31 patients which were initially filtered based on following criteria: (i) GnomAD v.2.1.1 was used to filter out variants with higher

than 1% minor allele frequency, (ii) only heterozygous variants with no multiple allelic sites were retained, (iii) variants labelled as frameshift variant, in-frame indel, missense variant, deleterious initiator codon variant, splice acceptor/donor variant or stop gain/lost variant were selected. A resulting set of 25,353 variants were further selected by excluding the variants that are not shared between the family members. These 11,823 variants were distributed across 5,514 genes with 1,827 genes harbored variants in more than one family (Figure 1). 43 common genes from OMIM and PCOSK curations were found to overlap with this set, are given in Table 2. When we manually interrogated the variants related to these 43 genes, we identified two heterozygous *FBN3* variants in three patients from two different families. Applying American College of Medical Genetics and Genomics and the Association for Molecular Pathology variant pathogenicity criteria to *FBN3*:c.4823A>G and *FBN3*:c.4498G>A variants, we classified these variants as variants of unknown significance with PM2 and PP3 (46) (Supplementary Table 3). These variants are rare, according to publicly available databases comprising multiethnic individuals. *FBN3*:c.4823A>G is never reported in GnomAD and GME Variome databases and for *FBN3*:c.4498G>A, minor allele frequency is $2{,}4\times10^{-5}$ in GnomAD, and 5×10^{-4} in GME Variome, respectively, and predicted to be pathogenic by in slico prediction tools. We have observed consensus for pathogenicity across functional prediction tools (FATHMM (47), MutationTaster (48), PolyPhen2 (49) and SIFT (50), VEST3 (51)), for conservation (GERP++, phastCons (52) and PhyloP (53)), eight ensemble methods (CADD (54), DANN (55), Eigen (56), FATHMM-MKL (57), REVEL (58), MetaLR (59), and MetaSVM (59). No other candidate variants were detected for phenotype causality (44, 60). Based on identified variants' rarities, in slico patogenicity predictions and previous studies implicating *FBN3* gene's role in pathogenesis of PCOS, *FBN3* variants in these families may be disease causing. In pathway analysis (STRING (61)), *FBN3* first and second neighbors were identified (Figure 1 and Supplementary Table 4) and investigated for variants. However, there was no candidate gene mutation identified among other primary and secondary FBN3 interactors in the study cohort. A heterozygous rare ovarian expressed *FN1* gene variant was identified in a familial PCOS patient. Applying same criteria to *FN1*:c.1802C>T, PM2 and PP3 led again to the classification of variant of unknown significance (Supplementary Table 3) (44, 46). The residue 601 is a proline with a rigid side chain predicted to restrict the conformation of the protein at this point (45). Patient' variant leads to a leucine replacement with an aliphatic and hydrophobic side chain. While the missense mutation is predicted to have a low 'disease propensity' value of 0.95 (45), it is very highly unfavoured in terms of conserved amino acid properties. The *FN1* variant is in collagen binding region of Fibronectin type-I 9 domain and expressed ubiquitously including uterus, fallopian tubes, and ovary. Previously *FN1* heterozygous disease-causing variants were shown to be associated with glomerulopathy with fibronectin deposits (GFND2; MIM#601894) (62), and the corner fracture type of spondylometaphyseal dysplasia (SMDCF; MIM#184255) (63). Interestingly, in SMDCF reported *FN1* mutations affect disulfide bond of Fibrin- and heparin-binding 1 region of FN1 and GFND2 related FN1 variants affect Fibronectin type-III domains 4 and 15.

FBN3 and *FN1* variants detail were given in Supplementary Tables 3 and 5. Although we were not able to check segregation of these variants due to lack of their consents, identified *FBN3* and *FN1* variants were rare, conserved and predicted pathogenic in almost

all prediction tools. We then compared three individuals who have *FBN3* or *FN1* variant(s) according to their clinical and laboratory findings (Supplementary Table 6). Although classic presentation of PCOS was observed in patients with *FBN3* variants, increased level SHBG was detected in the patient with *FN1* variant. (Supplementary Table 6).

Later, chemogenomics analyses of drug targets for the possible treatment of *FBN3* and *FN1* linked PCOS resulted with 3 known interactions (drugs) with *FN1* gene including Zn (42) (Supplementary Table 7). Indeed, zinc and its role in female reproductive system has recently been studied (64). A meta-analysis investigating PCOS and Zinc relationship showed lower zinc levels in patients with PCOS than healthy controls and this association should be further investigated (65). Further, PCOS related diseases such as type 2 diabetes (T2DM), and cardiovascular disease (CVD) prevalence increased in patients with zinc deficiency (66). Lastly, we performed enrichment analysis to of 1,827 variant-carrying genes that were identified in multiple families and mostly extracellular matrix related molecules and pathways were emerged. Since extracellular matrix provides infrastructure for specific ovarian cells, our findings highlight detected variants importance (Supplementary Table 8).

Discussion

We identified two heterozygous rare *FBN3* missense variants in three patients, and one FN1 missense variant in one patient from three different PCOS families. *FBN3* encodes an extracellular matrix (ECM) protein (67) and the variants are in TB 6 and EGF-like 25 calcium-binding domains. Previous linkage and immunohistochemical analyses strongly suggest a role for *FBN3* in the pathogenesis of PCOS (68–75). The *FBN3* expression was found in perifollicular stroma of follicles (71, 72, 76, 77), and several changes were reported in the ovarian ECM in PCOS patients including thickening of the tunica albuginea, ovarian stromal hyperplasia, stromal cell luteinization, and large cystic antral follicles (78–80).

Fibronectins (FNs) are multi-domain glycoproteins which allow cells to interact with other ECM proteins (81), and play important roles during follicle development (82). Ambekar et. al. (2015) found downregulated levels of Fibronectin by comparing the follicular fluid protein repertoire of PCOS with healthy women (83). Similarly, Hassani et al. (2019) found that downregulation of FN1 levels in the cumulus cells seemed to be related to PCOS (84).

FN1 heterozygous mutations are responsible for glomerulopathy with fibronectin deposits (GFND2; MIM#601894) (62) and the corner fracture type of spondylometaphyseal dysplasia (SMDCF; MIM#184255) (63). It has been noted that GFND2-associated mutations tend to cluster in more C-terminally located regions, whereas the SMDCF-associated mutations are more N-terminally located. We identified a heterozygous, rare, missense variant predicted to be deleterious and located in collagen binding region of Fibronectin type-I 9 domain. Interestingly, the patient with *FN1* variant has increased level of SHBG (Supplementary Table 5). Of note, since PCOS patients are expected to have low serum SHBG levels, and we thought this finding might be a coincidental finding.

In spite of researchers' best endeavors, the etiology (or etiologies) of PCOS remain unknown (85). Efforts have been made to which genes are involved the PCOS pathogenesis

via candidate gene approaches (86–89), genome-wide association studies (GWAS) from different populations with replication studies (1, 90–100) and more recently next generation sequencing (NGS)(87, 101). These studies are clearly bringing important novel information with limitations due to background genetic heterogeneity (102), phenotype heterogeneity of the PCOS, limited power and replication, along with limited understanding of disease pathophysiology to guide more informed candidate gene/targeted approaches (89, 103–105).

A wide array of genes have previously been associated with PCOS including those genes related to the biosynthesis and the action of androgens, metabolism and inflammatory cytokines (38, 106), however, it is yet to be elucidated how these genes/variants contribute to PCOS phenotype, and further exploration is warranted (38).

The small number of participants is the main limitation of the study. Technical limitations of whole exome sequencing should be also taken into considerations.

We prioritizated and focused on variants in genes implicated in PCOS pathogenesis including androgen, insulin and lipid metabolism, folliculogenesis, oxidative stress and inflammation and hemostasis. We found three novel *FBN3* and *FN1* variants and their role yet remain to be investigated. Further functional studies may identify causality of these newly discovered PCOS related variants and may improve our understanding of the biologic pathways affected and identify new drug targets.

The list of PCOS-related candidate genes is long and still open to new entries. Remaining cases without previously implicated presumptive candidate genes must also be investigated for genomic structural variations and epigenetic factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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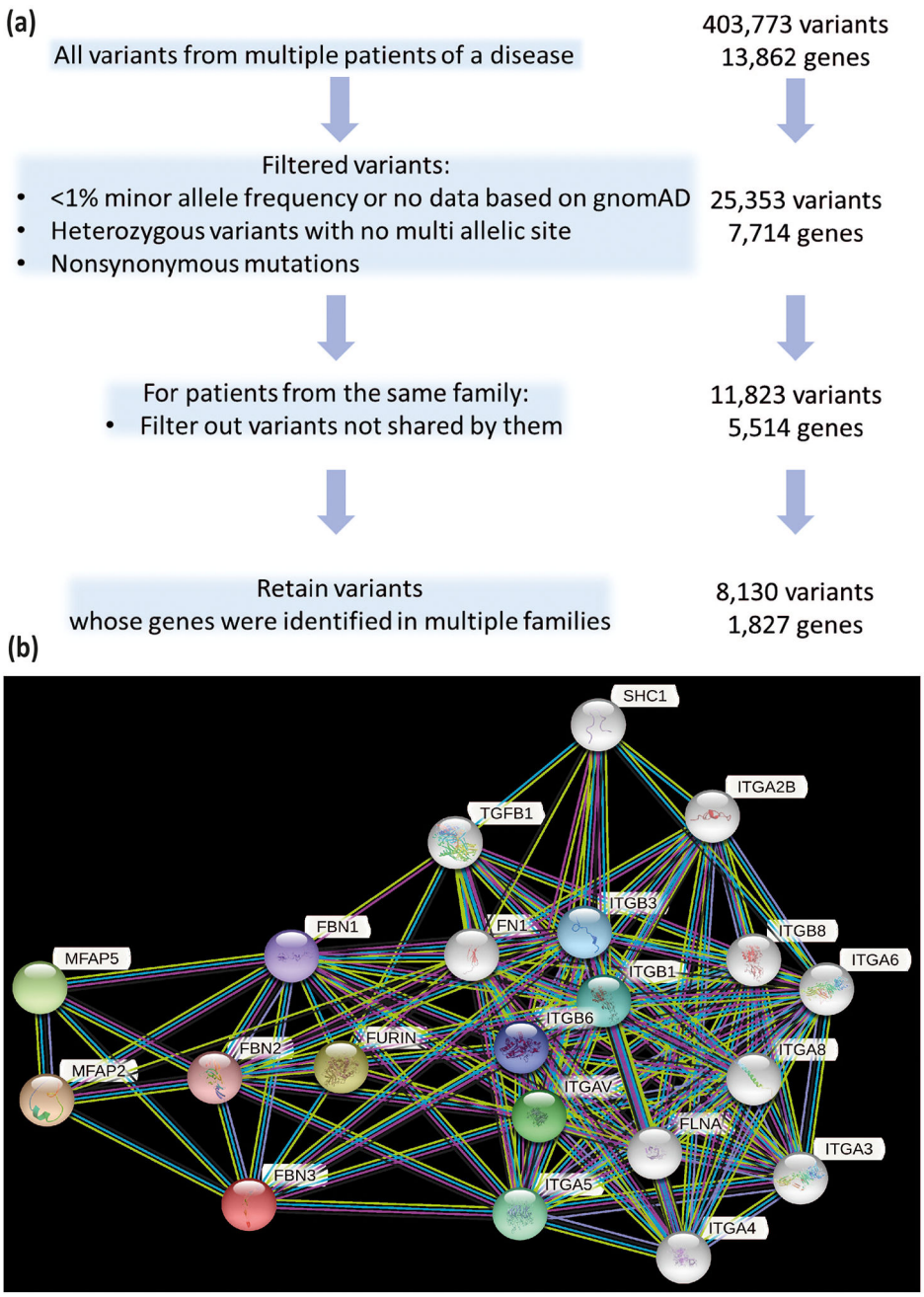

Figure 1.

A. Prioritization strategy of identified variants. **B.** *FBN3* gene' first and second neighbors were shown by STRING database.

Table 1.

Measures of central tendency *[median (min-max values)]*

Variable (metric; normal values)	All patients (n=27)
Age (years)	32.04 (±11.95)[a]
AMH (ng/mL; 0,07–7,35)	2.4 (0.1 – 8.5)[b]
FGS (Ferriman Gallwey score)	10.13 (±7.83)[a]
BMI (kg/m^2 ; 18,5–24,9)	28.44 (±7.38)[a]
Waist / hip circumference (cm; <80)	86.5 (61 – 144)[b]
Hip perimeter (cm; <105)	107.6 (±15.6)[a]
Insulin (IU/mL; 1,9–23)	8.49 (1.77– 43.3)[b]
Oral glucose tolerance test 75gr (mmol/L; <100 fasting blood sugar)	97 (60 – 168)[b]
Oral glucose tolerance test 75gr_2 hours ((mmol/L; 2 hour<153)	84.22 (±14.52)[a]
Dehydroepiandrosterone sulfate (µg/dL; 23–266)	173.96 (±8.83)[a]
Sex hormone-binding globulin (nmol/L; 18–144)	37,31 (6.96 – 153)[b]
Total Testesterone (ng/L; 0.15–0.7)	0.395 (0.071 – 1.09)[b]
17-hydroxyprogesterone (ng/L; <8)	0.96 (0.42 – 2.815)[b]
Androstenedione (ng/dL; 0,3–3,3)	4.26 (0.66 – 11.41)[b]

[a]"Mean" and "standard deviation" values in parentheses were specified for the data that fit the normal distribution.

[b]"Median" and "lowest - highest values" in parentheses were specified for data that do not fit the normal distribution.

Table 2.

PCOS-related genes in the study cohort obtained from OMIM and/or PCOSKB databases

Gene Symbol	Gene Name
ABCA1	ATP binding cassette subfamily A member 1
ACE	Angiotensin I converting enzyme
ADIPOQ	Adiponectin, C1Q and collagen domain containing
ALDH1A3	Aldehyde dehydrogenase 1 family member A3
ANGPTL1	Angiopoietin like 1
APC	APC regulator of WNT signaling pathway
APOB	Apolipoprotein B
AR	Androgen receptor
ATF4	Activating transcription factor 4
CAPN10	Calpain 10
CD14	CD14 molecule
CPZ	Carboxypeptidase Z
CR1	Complement C3b/C4b receptor 1 (Knops blood group)
CYP11B2	Cytochrome P450 family 11 subfamily B member 2
DENND1A	DENN domain containing 1A
ESR1	Estrogen receptor 1
F5	Coagulation factor V
FASN	Fatty acid synthase
FBN3	Fibrillin 3
FGA	Fibrinogen alpha chain
FOS	Fos proto-oncogene, AP-1 transcription factor subunit
HLA-DRB1	Major histocompatibility complex, class II, DR beta 1
HSD3B2	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2
IGF2R	Insulin like growth factor 2 receptor
INSR	Insulin receptor
LIPE	Lipase E, hormone sensitive type
LPA	Lipoprotein(a)
MAP3K4	Mitogen-activated protein kinase kinase kinase 4
NFKB1	Nuclear factor kappa B subunit 1
NID2	Nidogen 2
NPPB	Natriuretic peptide B
PEPD	Peptidase D
PIK3CG	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma
PKD1	Polycystin 1, transient receptor potential channel interacting
PLCB3	Phospholipase C beta 3
PRDX2	Peroxiredoxin 2
RPS6KA1	Ribosomal protein S6 kinase A1

Gene Symbol	Gene Name
SLC2A4	Solute carrier family 2 member 4
SRA1	Steroid receptor RNA activator 1
TH	Tyrosine hydroxylase
THADA	THADA armadillo repeat containing
TLR2	Toll like receptor 2
VEGFB	Vascular endothelial growth factor B