

SPACA3 gene variants in a New Zealand cohort of infertile and fertile couples

Author

Prendergast, Deborah, Woad, Kathryn J, Chamley, Lawrence W, Holland, Olivia J, Shelling, Andrew N

Published

2014

Journal Title

Human Fertility

Version

Accepted Manuscript (AM)

DOI

[10.3109/14647273.2014.907506](https://doi.org/10.3109/14647273.2014.907506)

Rights statement

© 2014 Taylor & Francis. This is an Accepted Manuscript of an article published by Taylor & Francis in Comparative Strategy on 28 May 2014, available online: <https://doi.org/10.3109/14647273.2014.907506>

Downloaded from

<http://hdl.handle.net/10072/171593>

Griffith Research Online

<https://research-repository.griffith.edu.au>

1 ORIGINAL ARTICLE

2
3
4 **SPACA3 gene variants in a New Zealand cohort of infertile and**
5 **fertile couples**
6

7
8
9 [AQ1] DEBORAH PRENDERGAST¹, KATIE J. WOAD³, LAWRENCE W. CHAMLEY²,
10 OLIVIA J. HOLLAND² & ANDREW N. SHELLING²
11

12 ¹Faculty of Medical and Health Sciences, The University of Auckland, Auckland, New Zealand, ²Department of Obstetrics and
13 Gynaecology, Faculty of Medical and Health Sciences, The University of Auckland, Auckland, New Zealand, and ³School of
14 Veterinary Medicine and Science, Sutton Bonington Campus, University of Nottingham, Loughborough, UK
15
16

17 **Abstract**

18 SPRASA (also referred to as SLLP1) is a protein identified in the acrosome of human sperm and encoded by the gene *SPACA3*.
19 SPRASA is associated with sperm-oocyte recognition and binding, and may play a role in fertility. In order to determine whether
20 variants in the *SPACA3* gene are associated with human infertility, we undertook a genetic analysis of 102 infertile and 104 fertile
21 couples. Three gene variants were identified using PCR-based DNA sequencing; 1) an insertion of TGC within a quadruple tri-
22 nucleotide (TGC) repeat region in the 5' untranslated region (UTR) (g.-22TGC(4_5), 2) a guanine to adenosine transition at
23 position 239 (c.239G>A) resulting in a non-synonymous amino acid substitution from cysteine to tyrosine (p.C80Y) at position
24 80 in the putative transmembrane region, and 3) a novel nucleotide variant (c.691G>C) located in the 3'UTR. A functional effect
25 of the g.-22TGC (4_5) was confirmed by a luciferase expression assay, while the effects of the variants c.239G>A and c.691G>C
26 were predicted using *in silico* analysis. Although the frequencies of these variants were not significantly different between the infertile
27 and fertile populations, we present evidence that the variants could affect the expression levels or function of SPRASA, thereby
28 affecting a couple's fertility. Larger populations, especially individuals/couples with unexplained infertility, need to be screened for
29 these variants to validate a relationship with fertility.
30

31 **Keywords:** *Reproduction, gene mutation, unexplained infertility*
32

33 **Introduction**

34 The prevalence of infertility in Western countries such
35 as New Zealand is estimated to be one in six couples,
36 while one in eight couples require some form of medi-
37 cal assistance to achieve a pregnancy (HFEA, 2006).
38 Of these infertile cases, the precise cause of infertility
39 for approximately 5–15% of couples is unknown, and is
40 referred to as unexplained infertility (Farquhar, 1998;
41 Liu et al., 2007). Standard investigations, including
42 testing for ovulation, tubal patency and semen analysis
43 are routine in these couples (Kamath & Bhattacharya,
44 2012). The causes of unexplained infertility and infertil-
45 ity, in general, are likely to be heterogeneous, and may
46 include endocrine, immunological, and genetic factors
47 (Kamath & Bhattacharya, 2012; Pellicer et al., 1998).
48 It is also common to find multiple potential causes of
49 infertility in an infertile couple (Collins & Crosignani,
50 1992; Marrero & Ory, 1991; Quaas & Dokras, 2008).
51

52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84 SPRASA (also referred to as SLLP1) is a protein
85 identified in the acrosome of human sperm (Chi et al.,
86 2004). SPRASA is a member of the c-type lysozyme/
87 alpha-lactalbumin family (Chiu et al., 2004; Mandal
88 et al., 2003), and human SPRASA is encoded by a gene
89 (*SPACA3*) at chromosomal locus 17q11.2 (Mandal
90 et al., 2003). SPRASA is known to be the target for anti-
91 sperm antibodies in some infertile men, and this protein
92 has been shown to have roles in sperm-oocyte binding
93 and early embryo development in murine, hamster, or
94 bovine models (Chiu et al., 2004; Mandal et al., 2003;
95 Zhang et al., 2005; A. Wagner, unpublished). SPRASA
96 expression was thought to be limited to the sperm/
97 testis and some tumour cells, but we have recently dem-
98 onstrated it is also expressed by oocytes (A. Wagner,
99 unpublished). This pattern of gonad/gamete-specific
100 expression also supports the concept that SPRASA
101 has an important role in fertility (Herrero et al., 2005;
102

Correspondence: Olivia Holland, Department of Obstetrics and Gynaecology, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag 92019 Auckland, New Zealand. Tel: + 64-9-9232314. E-mail: olivia.holland@auckland.ac.nz

(Received 4 September 2013; accepted 3 December 2013)

Mandal et al., 2003; Wagner, 2009). While we discovered SPRASA as the antigen for antisperm antibodies in some infertile men, the association of SPRASA with human infertility has not been investigated further. Because of SPRASA's putative importance in fertility, naturally occurring genetic variation that disrupts the function of SPRASA may have a role in human infertility. This study was undertaken to investigate whether genetic variants in the SPRASA-encoding *SPACA3* gene are associated with infertility in humans.

Methods and materials

Ethics statement and study population

This study was approved by the Northern Regional Ethics Committee (Auckland, New Zealand). Blood samples obtained by venipuncture were collected following written informed consent from 102 infertile couples, (recruited through Fertility Associates, Auckland and Fertility Plus, Auckland; Supplementary Table I available online at: <http://informahealthcare.com/doi/abs/10.3109/14647273.2014.907506>) and 104 fertile couples (recruited from the general Auckland population through media advertisement; Supplementary Table II available online at: <http://informahealthcare.com/doi/abs/10.3109/14647273.2014.907506>). Couples were defined as infertile if they were unable to conceive after one year of regular intercourse without the use of contraception. The clinically diagnosed disorders affecting the infertile cohort recruited for this study are summarised in Supplementary Table I available online at: <http://informahealthcare.com/doi/abs/10.3109/14647273.2014.907506>. Couples were defined as fertile if they had given birth in the previous 2 years.

Mutation nomenclature

In accordance with current mutation nomenclature (den Dunnen & Antonarakis, 2001), the adenosine of the ATG (initiator methionine codon) of *SPACA3* is denoted nucleotide +1. Nucleotide numbers refer to GenBank accession number NM_173847.

Polymerase chain reaction

Genomic DNA was extracted from 5 to 10 mL of blood using the salting out protocol (Miller et al., 1988). Eight sets of Polymerase chain reaction (PCR) primers were designed to flank exons and the putative promoter regions of *SPACA3* (Supplementary Table III available online at: <http://informahealthcare.com/doi/abs/10.3109/14647273.2014.907506>). Genomic DNA (100ng) was amplified using standard PCR conditions (Supplementary Tables III and IV available online at: <http://informahealthcare.com/doi/abs/10.3109/14647273.2014.907506>). All PCR amplifications were performed in an iCycler (BioRad, Hempstead, UK). The PCR products were analysed using agarose gel electrophoresis, stained

with ethidium bromide (10mg/ml; Sigma, Australia) and visualised under UV light.

DNA sequencing

The PCR products were purified with the Roche High Pure PCR Product Purification Kit (Roche, Basel, Switzerland) following the manufacturer's instructions. Sequencing reactions were performed using the ABI prism Big Dye Terminator Sequencing Kit v3.1 (ABI, Foster City, USA) following the manufacturer's instructions in a 20 µL volume with approximately 5 ng per 100 bp of PCR product. All primers used in the sequencing reactions allowed for 100% coverage of the coding region for each sample. Sequencing reactions for exon four were supplemented with betaine at a final concentration of 1.4M to assist sequencing through secondary structures (Haqqi et al., 2002). The variants were confirmed by sequence replication. Sequencing of the promoter variants was carried out in both directions using internal primers (Supplementary Table III available online at: <http://informahealthcare.com/doi/abs/10.3109/14647273.2014.907506>). Sequencing was amplified using standard conditions (Supplementary Table VI available online at: <http://informahealthcare.com/doi/abs/10.3109/14647273.2014.907506>). Sequencing extension products were precipitated by MgSO₄. Capillary electrophoresis was analysed on a 3100 Genetic Analyser (Applied Biosystems, Life Technologies New Zealand Limited, Auckland) at the Genome Research Facility at the School of Biological Sciences, The University of Auckland.

Luciferase assay for promoter function: variant g.-22TGC(4_5)

The three putative promoters isolated in this study, Promoter A (located five-prime of exon one; major allele; 983bp), Promoter A g.-22TGC(4_5) variant (located five-prime of exon one; 986bp) and Promoter B (located in intron one; invariant; Figure 1), were investigated for their capacity to support transcriptional activity using luciferase assay.

Promoter A, Promoter A g.-22TGC(4_5) variant and Promoter B were cloned upstream of the firefly luciferase gene of the pGL3-enhancer reporter plasmid (Promega, Wisconsin, USA). The cell lines HEK-293T (non-*SPACA3* expressing negative control; human embryonic kidney; American Type Culture Collection, Manassas, USA), NCCIT (*SPACA3* expressing teratocarcinoma; human pluripotent embryonal carcinoma; American Type Culture Collection, Manassas, USA) and KGN (*SPACA3* expressing granulosa; human granulosa cell tumour originated from a stage III granulosa cell tumour of the ovary; provided by Dr Ashwini Chand, Prince Henry's Institute of Medical Research, Victoria, Australia) were co-transfected with the RL-Tk vector (Promega, In vitro Technologies, Auckland) and either the (1) pGL3-Promoter A construct, (2) pGL3-Promoter A g.-22TGC(4_5) variant construct,

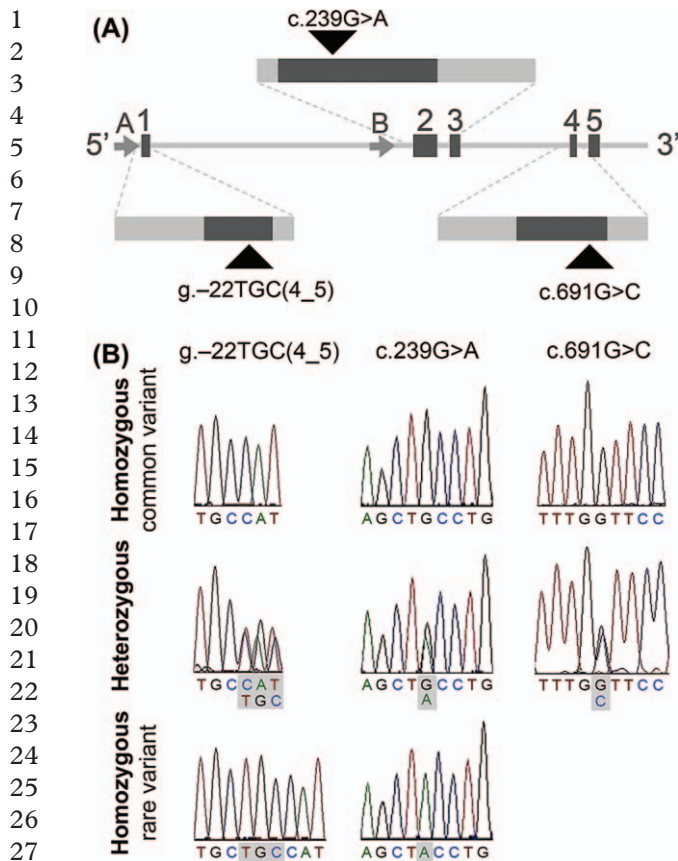


Figure 1. The genomic organisation of human *SPACA3* and the location of variants identified in this study. (A) Schematic diagram of *SPACA3*; exons are shown in dark grey, introns are shown in light grey and the putative promoters (A and B) are indicated by grey arrows. The location of the variants g.-22TGC(4_5), c.239G>A and c.691G>C are indicated by black triangles on magnified sections. (B) Representative electropherograms of the variants g.-22TGC(4_5), c.239G>A and c.691G>C.

(3) Promoter B construct or (4) pGL3 empty vector. Cells were transfected using Lipofectamine 2000 (Invitrogen, Auckland) according to the manufacturer's instructions. Cells were cultured for 24 h in RPMI 1640 medium (Invitrogen, Auckland) supplemented with 2mM L-glutamine (Invitrogen, Auckland) and 10% fetal calf serum (Invitrogen, Auckland). All cell lines were maintained at 37°C in 5% CO₂.

Luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega, In vitro Technologies, Auckland) following the manufacturer's instructions. The promoter assays were normalised to Renilla luciferase activity from the co-transfected pRL-Tk vector. The means and standard error (SE) were calculated from identical transfections in triplicate from three independent experiments, and the two *SPACA3* promoter variants were compared to the empty control plasmid transfected into the appropriate cell lines.

In silico analysis for function: variants g.-22TGC(4_5), c.239G>A and c.691G>C

The putative effects of the sequence variations on genetic association were predicted using the SHEsis

programme (Shi & He, 2005). The TESS (transcription element search software) prediction tool was used to determine whether the g.-22TGC(4_5) variant affected transcription factor binding (Schug & Overton, 1997). The possible impact of the amino acid substitution p.C80Y (genetic variant c.239G>A) on the structure, function and expression of the SPRASA protein was tested using the three prediction tools; SIFT (sorts intolerant from tolerant) scores were classified as intolerant (0.00–0.05), potentially intolerant (0.051–0.10), borderline (0.101–0.20) or tolerant (0.201–1.00) (Ng & Henikoff, 2003; Xi et al., 2004); PolyPhen II (polymorphism phenotyping), scores were designated probably damaging (≥ 2.00), possibly damaging (1.50–1.99), potentially damaging (1.25–1.49), borderline (1.00–1.24) or benign (0.00–0.99) (Xi et al., 2004); Grantham matrix scores categorise codon replacement into classes of increasing chemical dissimilarity and are designated conservative (0–50), moderately conservative (51–100), moderately radical (101–150) or radical (≥ 151) (Grantham, 1974; Li et al., 1984; Rudd et al., 2005). The potential effect on microRNA (miRNA) binding to the variant c.691G>C was analysed using the TargetScanHuman version 6.2 programme. TargetScan predicts biological targets of miRNAs by searching for the presence of conserved 8mer and 7mer sites that match the seed region of each miRNA (Lewis et al., 2005), also identified are sites with mismatches in the seed region that are compensated by conserved 3' pairing (Friedman et al., 2009). In mammals, predictions are ranked based on the predicted efficacy of targeting as calculated using the context scores of the sites (Grimson et al., 2007).

Sequence alignments

In order to obtain a measure of the level of evolutionary conservation of the variants, multiple sequence alignments of *SPACA3* were performed as relevant for each variant using known orthologs with complete sequences in the programme ClustalW2. Thirteen higher primates possess known orthologs to human exon one containing the g.-22TGC(4_5) variant, 42 mammalian species possess known orthologs to human exon two containing the c.239G>A variant, and 23 mammalian species possess known orthologs to human exon five containing the c.691G>C variant.

Statistical analysis

The chi-squared statistic was used to detect the Hardy-Weinberg equilibrium (HWE) for the three identified sequence variations. The odds ratios and 95% confidence interval were used to measure the association in the genotype and allele distribution of the three sequence variations between the infertile and fertile groups. The p values were estimated using chi-square test. Statistical analysis from the results of the luciferase assay comparing the promoter variants was determined using unpaired Student's T-test in the Graph Pad Prism

1 5.02 programme. All p values were two-tailed and con- 59
 2 sidered significant if $p < 0.05$. 60

3 Results 61

4
 5
 6
 7 In order to investigate the population level variation in 62
 8 *SPACA3* and to determine whether this variation is as- 63
 9 sociated with infertility, 102 infertile couples ($n = 204$ 64
 10 individuals) and 104 fertile couples ($n = 208$ individu- 65
 11 als) were analysed. Three sequence variants were identi- 66
 12 fied in this population; g.-22TGC(4_5), c.239G>A 67
 13 and c.691G>C (Figure 1; Table I). 68

14 *SPACA3* is well conserved in the cohort investigat- 69
 15 ed; the minor allele frequencies for the variants were 70
 16 g.-22TGC(4_5) 0.12, c.239G>A 0.02 and c.691G>C 71
 17 0.001. The variant c.691G>C was found at a popula- 72
 18 tion frequency of less than 0.01, and is thus defined as 73
 19 a mutation (den Dunnen & Antonarakis, 2001). There 74
 20 were a number of couples from the infertile and fertile 75
 21 cohorts who had a combination of multiple variants 76
 22 (Supplementary Table VII available online at: [http://](http://informahealthcare.com/doi/abs/10.3109/14647273.2014.907506) 77
 23 [informahealthcare.com/doi/abs/10.3109/14647273.](http://informahealthcare.com/doi/abs/10.3109/14647273.2014.907506) 78
 24 [2014.907506](http://informahealthcare.com/doi/abs/10.3109/14647273.2014.907506)). The variants identified consisted of one 79
 25 in-frame insertion and two single nucleotide polymor- 80
 26 phisms (details below). 81

27 *SPACA3*/SPRASA is highly evolutionarily conserved. 82
 28 A multiple sequence alignment of *SPACA3* orthologs 83
 29 showed 100% conservation of the major variant in exons 84
 30 one and five (Figure 2). Analysis of *SPACA3* orthologs 85
 31 in mammals showed that the guanosine/cytosine is con- 86
 32 served (100% conservation in 42 species; Figure 2). 87

33 The first variant in the *SPACA3* gene consists of the 88
 34 insertion of an additional TGC within a quadruple tri- 89
 35 nucleotide TGC repeat region, g.-22TGC(4_5), and is 90
 36 situated in the 5' UTR of exon one (Figure 1). Allele 91
 37 frequencies for infertile and fertile individuals are shown 92
 38 in Table II. Diagnoses of infertile individuals with this 93
 39 variant are shown in Supplementary Table VIII available 94
 40 online at: [http://informahealthcare.com/doi/abs/10.3109/](http://informahealthcare.com/doi/abs/10.3109/14647273.2014.907506) 95
 41 [14647273.2014.907506](http://informahealthcare.com/doi/abs/10.3109/14647273.2014.907506). The frequencies of this variant 96
 42 in the infertile and fertile individuals were calculated to 97
 43 be in HWE and were not significantly different between 98
 44 the infertile and fertile individuals ($p = 0.24$). 99

45 In order to investigate the potential consequences 100
 46 of this *SPACA3* variant on promoter function, we con- 101
 47 ducted luciferase reporter experiments using three cell 102
 48 lines. *In vitro* luciferase reporter assays comparing Pro- 103
 49 moter A (located five-prime of exon one; major allele) 104
 50 and Promoter A g.-22TGC(4_5) variant (located five- 105
 51 prime of exon one) found expression from Promoter 106
 52 A in the *SPACA3*-expressing granulosa cell line KGN, 107
 53 with an approximately 1.4-fold increase in expression 108
 54 compared to the control. There was no expression 109
 55 above the normalisation-control from Promoter A in 110
 56 the *SPACA3*-expressing seminoma cell line NCCIT or 111
 57 the non-*SPACA3*-expressing embryonic kidney cell line 112
 58 HEK-293T. Promoter A g.-22TGC(4_5) variant did 113
 59 not induce expression above the normalisation-control 114
 60 in any cell line; there was a significant difference be- 115
 61 tween Promoter A and Promoter A g.-22TGC(4_5) 116
 62 variant expression in the reproductively relevant KGN 117
 63 cell line ($p = 0.01$; Figure 3). The function of Promoter 118
 64 B (located in intron one; invariant) was also assessed. 119
 65 Expression from Promoter B was found in the NNCIT 120
 66 (approximately 2.7-fold increase compared to the con- 121
 67 trol) and KGN (approximately 5.6-fold increase com- 122
 68 pared to the control) cell lines. There was no expression 123
 69 above the normalisation-control from Promoter B in the 124
 70 HEK-293T cell line (Figure 3). 125

71 The second variant, a guanosine to adenosine tran- 126
 72 sition at position 239, c.239G>A, was identified in 127
 73 exon two, which encodes the putative transmembrane 128
 74 region of the SPRASA protein (Figure 1). This tran- 129
 75 sition occurred at the second residue of the codon 130
 76 and resulted in a non-synonymous amino acid sub- 131
 77 stitution at codon position 80 from a cysteine to a 132
 78 tyrosine (p.C80Y). Allele frequencies for infertile and 133
 79 fertile individuals are shown in Table II. Diagnoses of 134
 80 infertile individuals with this variant are shown in 135
 81 Supplementary Table VIII available online at: [http://](http://informahealthcare.com/doi/abs/10.3109/14647273.2014.907506) 136
 82 [informahealthcare.com/doi/abs/10.3109/14647273.](http://informahealthcare.com/doi/abs/10.3109/14647273.2014.907506) 137
 83 [2014.907506](http://informahealthcare.com/doi/abs/10.3109/14647273.2014.907506). The frequencies of this variant in the 138
 84 infertile and fertile individuals were calculated to be in 139
 85 HWE and were not significantly different between the 140
 86 infertile and fertile individuals ($p = 0.36$). 141

87 To determine possible effects that the c.239G>A 142
 88 variant may have on the function of the transmembrane 143
 89 region, online prediction tools were used to examine the 144
 90 effect of the amino acid substitution p.C80Y. Bioinforma- 145
 91 tic analysis by SIFT (Ng & Henikoff, 2001; Xi et al., 146
 92 2004) and PolyPhen II (Ramensky et al., 2002; Xi et al., 147
 93 2004) programmes suggested that the substitution is not 148
 94 tolerated, and is predicted to be deleterious to protein 149
 95 function. 150

49 Table I. Variations in *SPACA3* identified in all subjects. 107

50 Nucleotide change	51 Amino acid change	52 Domain	53 Previous report	54 dbSNP ID	55 PolyPhen II ^a prediction	56 SIFT ^b prediction	57 Grantham ^b score	58 Sequence conservation ^c
52 g.-22TGC(4_5)	53 -	54 5'UTR	55 Yes	56 rs3052914	57 -	58 -	59 -	60 -
52 c.239G>A	53 p.C80Y	54 Transmembrane	55 Yes	56 rs16967845	57 0.96	58 0.02	59 194	60 High (100% in 42 species)
52 c.691G>C	53 -	54 3'UTR	55 No	56 -	57 -	58 -	59 -	60 -

56 UTR, untranslated region 114

57 ^aPolyPhen II score (0 least; 1 most) and SIFT score (0–1; ≤ 0.05 damaging, > 0.05 tolerated) predicted the damage of the variant. 115

57 ^bGrantham score classifies the chemical dissimilarity of the variant (0–50 conservative, 51–100 moderately conservative, 101–150 moderately 116
 58 radical, ≥ 151 radical). 117

58 ^cProtein sequence alignment of all known orthologs ($n = 42$). 118

mono for print colour online

1	Exon two		Exon one		59
2	c.239G>A variant		g.-22TGC(4_5) variant		60
3	Human	AGCTGCCTG	Human	CTGC---CATT	61
4	Human c.239G>A variantA....	Human g.-22TGC(4_5) variantTGC....	62
5	Chimpanzee	Chimpanzee	63
6	Pygmy chimpanzee	Pygmy chimpanzee	64
7	Orangutan	Orangutan	65
8	Squirrel monkey	Gorilla	66
9	Capuchin monkey	Gorilla (western lowland)	67
10	Gorilla (western lowland)	Gibbon	68
11	Gorilla	Baboon	69
12	Gibbon	Rhesus macaque	70
13	Baboon	Pigtailed macaque	71
14	Babbon (olive)	Capuchin monkey	72
15	Pigtailed macaque	Squirrel monkey	73
16	Rhesus macaque	Marmoset	74
17	Spider monkey			75
18	Marmoset	Exon five		76
19	Marmoset (white-tufted-ear)	c.691G>C variant		77
20	Galago (primate)	Human	TTTGGITCC	78
21	Madagascar hedgehog	Human c.691G>C variantC....	79
22	Mole (star-nosed)	Chimpanzee	80
23	PikaAG	Pygmy chimpanzee	81
24	Rat (rodent)	Orangutan	82
25	Mouse (rodent)	Gorilla	83
26	Jerboa (rodent)	Gorilla (western lowland)	84
27	Degu (rodent)T..	Gibbon	85
28	Chinchilla (rodent)T..	Baboon	86
29	Hamster (rodent)	Baboon (olive)	87
30	Guinea pig (rodent)T..T	Pigtailed macaque	88
31	Prairie vole (rodent)	Rhesus macaque	89
32	Armadillo (nine-banded)	Squirrel monkey	90
33	Rhinoceros	Capuchin monkey	91
34	Horse	Marmoset	92
35	PigT..	Mouse (rodent)	AC.T....	93
36	Sheep	...A....	Rat (rodent)	GC.T....	94
37	Bovine	...A....	Hamster (rodent)	...T....	95
38	Orca	Rhinoceros	96
39	Dolphin (bottle-nosed)	Bovine	CC.....	97
40	Walrus (Pacific)	G.....	Dolphin (bottle-nosed)	98
41	ManateeT.A	FerretT.	99
42	Cat	Walrus (Pacific)T.	100
43	Ferret	G.....	DogT.	101
44	Dog	G.....			102
45	Platypus	G.....			103

Figure 2. Multiple sequence alignments of SPACA3 orthologs. Sequence alignments of all known SPACA3 orthologs were performed in the programme ClustalW2 on exons one containing the g.-22TGC(4_5) variant, exon two containing the c.239G>A variant, and exon five containing the c.691G>C variant. Four nucleotides either side of each variant are shown. The variant region is boxed. Dots indicate agreement with the header sequence (human major allele).

structure or function. A similar prediction was observed using the Grantham matrix score (Abkevich et al., 2004; Grantham, 1974; Lee et al., 2008).

A third variant was identified in exon five and resulted in a guanosine to cytosine transversion at nucleotide position 691, c.691G>C (Figure 1; Table I). This novel SNP occurred in the 3'UTR in the heterozygous form in one individual, an infertile female (Table II) diagnosed as having unexplained infertility (Supplementary Table VIII available online at: <http://informahealthcare.com/doi/abs/10.3109/14647273.2014.907506>). The observed frequencies for this variant were not significantly different between the infertile and fertile individuals ($p=0.31$). The TargetScanHuman programme version 6.2 (Lewis

et al., 2005) identified that the novel variant c.691G>C is located in the seed region of the human miRNA 873 (hs-miR873).

Discussion

Currently there is only limited knowledge about the specific proteins that are involved in human fertility, and how genetic variation in these proteins may affect fertility. In this study we investigated variations in the gene *SPACA3* which encodes SPRASA, a little studied protein that has a role in sperm-oocyte binding (Chiu & Chamley, 2002; Chiu et al., 2004; Herrero et al., 2005; Mandal et al., 2003) and early embryonic development

Table II. Allele frequencies and levels of homozygosity/heterozygosity in *SPACA3* variants identified in all subjects.

Nucleotide change	Minor allele frequency		Number in infertile patients (n = 204)		Frequency in infertile patients		Number in fertile controls (n = 208)		Frequency in fertile controls	
	Infertile patients	Fertile controls	HO	HE	HO	HE	HO	HE	HO	HE
g.-22TGC(4_5)	0.137	0.098	5	46	0.025	0.226	3	35	0.014	0.168
c.239G>A	0.032	0.017	1	11	0.005	0.054	0	7	0	0.034
c.691G>C	0.002	0	0	1	0	0.005	0	0	0	0

HO, homozygote; HE, heterozygote

(A. Wagner, unpublished). Levels of population genetic variation in *SPACA3* were determined in a cohort of New Zealand males and females ($n = 412$). Limited variation was found in *SPACA3*, suggesting that this gene may be well conserved, possibly because its potential role in fertilisation (Herrero et al., 2005; Mandal et al., 2003) means that variation would be detrimental to fertility.

To explore whether variants in *SPACA3* are associated with infertility in the New Zealand population, variation was investigated in infertile and fertile couples. Twenty-nine variants in *SPACA3* have previously been recorded (NCBI SNP database <http://www.ncbi.nlm.nih.gov/>). In the New Zealand population, three variants were found. All variants were seen at higher frequencies in individuals belonging to infertile, compared to fertile, couples. However, none of these differences were statistically significant. When investigating infertility, couples should be considered together (Campana et al., 1995). Thus, analysis of couples found three variant combinations in the infertile couples only, suggesting that these variant combinations are possibly detrimental to fertility, although the variant combinations were not statistically associated with infertility. Given that infertility has many

causes and is often multifactorial, it may be difficult to show an association with infertility in such a broad collection of individuals. The variants identified may therefore be important in the pathogenesis of infertility in the affected individuals. Indeed, we determined that the variant encoding a cysteine to a tyrosine (p.C80Y) transition in the putative transmembrane region may affect the function of SPRASA. Further, the other two variants may affect the expression levels of SPRASA.

The functional activity of Promoter A (major allele) in the KGN cell line was abolished when Promoter A g.-22TGC(4_5) variant was transfected instead suggesting that the g.-22TGC(4_5) variant may reduce the expression of *SPACA3*, and could lead to an effect on fertility. Further, the major allele at this locus is highly conserved, suggesting that any variation in this region may be deleterious. However, the g.-22TGC(4_5) variant was found in the homozygous state in three fertile couples, indicating that it does not lead to frank infertility in all individuals, and may have a subtle effect on fertility. A second promoter, Promoter B, is located in intron one. This promoter may allow the continuing expression of an isoform of SPRASA and, therefore, the continuation of some functions of SPRASA in fertility.

The c.239G>A variant results in a non-conservative change of a cysteine to tyrosine and is located in the putative transmembrane region of SPRASA. *In silico* analyses suggest that this substitution is deleterious to the function of SPRASA, and analysis of *SPACA3* orthologs confirms that the cysteine is highly conserved, further indicating that it may be crucial for protein function. The c.239G>A variant was only seen in the homozygous state in a single infertile female, which may indicate an effect on fertility of non-functional SPRASA in this individual. The allele frequency of the c.239G>A variant in the New Zealand population (0.02) is similar to the European and Asian frequencies (0.03 and 0.01, respectively; refer to cluster reports ss227519113, ss23645285, and ss237222685 on dpSNP <http://www.ncbi.nlm.nih.gov/snp>), indicating the potential of this variant to affect fertility in multiple populations.

The heterozygous variant c.691G>C was identified in a single female with unexplained infertility. The major variant at this locus is conserved across all known *SPACA3* orthologs, indicating its importance to the functioning of SPRASA. This variant is located within the seed region for hs-miR873, a miRNA

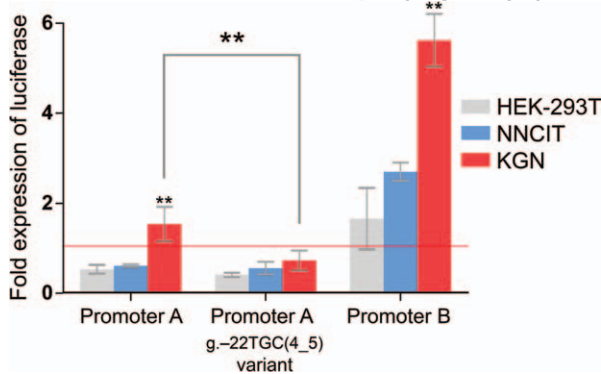


Figure 3. Functional analysis of the human *SPACA3* gene promoter variants isolated in this study. Luciferase reporter assay of Promoter A (located five-prime of exon one; major allele; 983bp), Promoter A g.-22TGC(4_5) variant (located five-prime of exon one; 986bp) and Promoter B (located in intron one; invariant) transfected into HEK-293T (negative control; grey), NNCIT (*SPACA3*-expressing teratocarcinoma; blue) and KGN (*SPACA3*-expressing granulosa; red) cell lines. Data are presented as fold luciferase activity calibrated to the appropriate cell line transfected with the empty pGL3-enhancer. The red line shows the level of the normalisation control. Results shown as mean \pm SE of three independent experiments performed in triplicate. ** $p < 0.01$.

(Pasten-Hidalgo et al., 2008) that can specify post-transcriptional repression by transcript destabilisation/translational repression (Friedman et al., 2009). The identification of hs-miR873 and the conservation of this region suggest that this locus may be important in the regulation of *SPACA3* by affecting the stability and/or translation of the mRNA, thereby affecting levels of the SPRASA protein.

Summary

In this investigation, we have determined that *SPACA3* has low levels of variation in our study population. Three variants were detected; although none were statistically associated with infertility; this is not a surprising finding, given the multifactorial nature of infertility. Furthermore, because infertility has multiple causes, the explanation of an individual's infertility may not be associated with infertility when examined on a population basis. All three of the variants were found at higher frequencies in the infertile cohort and one of the variants was only found in the homozygous state in an infertile individual, suggesting that this could be associated with the pathogenesis of infertility in some individuals/couples. Two of the variants may affect the expression levels of *SPACA3*, and the other variant may affect the function of SPRASA. Results from multiple models indicate that SPRASA is important in fertility (Chiu et al., 2004; Mandal et al., 2003; Zhang et al., 2005; A. Wagner, unpublished), and thus variants that affect SPRASA's function or expression levels may compromise fertility for some couples. Further functional studies are required to determine whether the presence of these variants has a role in infertility.

Acknowledgement

We would like to thank the infertile and fertile couples for their involvement in this study, staff at Fertility Plus and Fertility Associates, as well as Irma Heijboer for recruiting the couples. This work was supported by a grant from the Marsden Fund of the Royal Society of New Zealand.

Declaration of interest: The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

References

Abkevich, V., Zharkikh, A., Deffenbaugh, A.M., Frank, D., Chen, Y., Shattuck, D., et al. (2004). Analysis of missense variation in human BRCA1 in the context of interspecific sequence variation. *Journal of Medical Genetics*, 41, 492-507.

Campana, A., de Agostini, A., Bischof, P., Tawfik, E., & Mastroianni, A. (1995). Evaluation of infertility. *Human Reproduction Update*, 1, 586-606.

Chiu, W.W. & Chamley, L.W. (2002). Use of antisperm antibodies in differential display Western blotting to identify sperm proteins important in fertility. *Human Reproduction*, 17, 984-989.

Chiu, W.W., Erikson, E.K., Sole, C.A., Shelling, A.N., & Chamley, L.W. (2004). SPRASA, a novel sperm protein involved in immune-mediated infertility. *Human Reproduction*, 19, 243-249.

Collins, J.A. & Crosignani, P.G. (1992). Unexplained infertility: a review of diagnosis, prognosis, treatment efficacy and management. *International Journal of Gynaecology and Obstetrics*, 39, 267-275.

den Dunnen, J.T. & Antonarakis, S.E. (2001). Nomenclature for the description of human sequence variations. *Human Genetics*, 109, 121-124.

Dieffenbach, C.W. & Dveksler, G.S. (eds.) (1995). *PCR Primer: A Laboratory Manual*, 1st ed. New York: Cold Spring Harbor Laboratory Press.

Farquhar, C. (1998). Infertility: causes, investigations and treatments. *New Zealand Family Physician*, 25, 11-15.

Friedman, R.C., Farh, K.K., Burge, C.B., & Bartel, D.P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome Research*, 19, 92-105.

Grantham, R. (1974). Amino acid difference formula to help explain protein evolution. *Science*, 185, 862-864.

Grimson, A., Farh, K.K., Johnston, W.K., Garrett-Engle, P., Lim, L.P., & Bartel, D.P. (2007). MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Molecular Cell*, 27, 91-105.

Haqqi, T., Zhao, X., Panciu, A., & Yadav, S.P. (2002). Sequencing in the presence of betaine: Improvement in sequencing of the localized repeat sequence regions. *Journal of Biomolecular Techniques*, 13, 265-271.

Herrero, M.B., Mandal, A., Digilio, L.C., Coonrod, S.A., Maier, B., & Herr, J.C. (2005). Mouse SLLP1, a sperm lysozyme-like protein involved in sperm-egg binding and fertilization. *Developmental Biology*, 284, 126-142.

HFEA. (2006). Facts and Figures: Fertility Problems and Treatment. <http://www.hfea.gov.uk/5777.html>

Kamath, M.S. & Bhattacharya, S. (2012). Demographics of infertility and management of unexplained infertility. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, 26, 729-738.

Lee, E., McKean-Cowdin, R., Ma, H., Chen, Z., Van Den Berg, D., Henderson, B.E., et al. (2008). Evaluation of unclassified variants in the breast cancer susceptibility genes BRCA1 and BRCA2 using five methods: results from a population-based study of young breast cancer patients. *Breast Cancer Research*, 10, R19.

Lewis, B.P., Burge, C.B., & Bartel, D.P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, 120, 15-20.

Li, W.H., Wu, C.I., & Luo, C.C. (1984). Nonrandomness of point mutation as reflected in nucleotide substitutions in pseudogenes and its evolutionary implications. *Journal of Molecular Evolution*, 21, 58-71.

Liu, D.Y., Liu, M.L., Garrett, C., & Baker, H.W. (2007). Comparison of the frequency of defective sperm-zona pellucida (ZP) binding and the ZP-induced acrosome reaction between subfertile men with normal and abnormal semen. *Human Reproduction*, 22, 1878-1884.

Mandal, A., Klotz, K.L., Shetty, J., Jayes, F.L., Wolkowicz, M.J., Bolling, L.C., et al. (2003). SLLP1, a unique, intra-acrosomal, non-bacteriolytic, c lysozyme-like protein of human spermatozoa. *Biology of Reproduction*, 68, 1525-1537.

Marrero, M.A. & Ory, S.J. (1991). Unexplained infertility. *Current Opinion in Obstetrics & Gynecology*, 3, 211-218.

Miller, S.A., Dykes, D.D., & Polesky, H.F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research*, 16, 1215.

Ng, P.C. & Henikoff, S. (2001). Predicting deleterious amino acid substitutions. *Genome Research*, 11, 863-874.

Ng, P.C. & Henikoff, S. (2003). SIFT: Predicting amino acid changes that affect protein function. *Nucleic Acids Research*, 31, 3812-3814.

Pasten-Hidalgo, K., Hernandez-Rivas, R., Roa-Espitia, A.L., Sanchez-Gutierrez, M., Martinez-Perez, F., Monroy, A.O., et al. (2008). Presence, processing, and localization of mouse ADAM15 during sperm maturation and the role of its disintegrin domain during sperm-egg binding. *Reproduction*, 136, 41-51.

Pellicer, A., Albert, C., Mercader, A., Bonilla-Musoles, F., Remohí, J., & Simón, C. (1998). The follicular and endocrine environment in women with endometriosis: local and systemic cytokine production. *Fertility and Sterility*, 70, 425-431.

Quaas, A. & Dokras, A. (2008). Diagnosis and treatment of unexplained infertility. *Reviews in Obstetrics and Gynecology*, 1, 69-76.

1	Ramensky, V., Bork, P., & Sunyaev, S. (2002). Human non-synonymous SNPs: server and survey. <i>Nucleic Acids Research</i> , 30, 3894-3900.	59
2		
3	Rudd, M.F., Williams, R.D., Webb, E.L., Schmidt, S., Sellick, G.S., & Houlston, R.S. (2005). The predicted impact of coding single nucleotide polymorphisms database. <i>Cancer Epidemiology, Biomarkers & Prevention</i> , 14, 2598-2604.	60
4		
5	Schug, J. & Overton, G.C. (1997). TESS: Transcription Element Search Software on the WWW CBIL-TR-1997-1001-v0.0.	61
6		
7	Shi, Y.Y. & He, L. (2005). SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. <i>Cell Research</i> , 15, 97-98.	62
8		
9		
10		
[AQ8]	Supplementary material available online	63
11		
12	Supplementary Tables I-IX.	64
13		
14		
15		
16		
17		
18		
19		
20		
21		
22		
23		
24		
25		
26		
27		
28		
29		
30		
31		
32		
33		
34		
35		
36		
37		
38		
39		
40		
41		
42		
43		
44		
45		
46		
47		
48		
49		
50		
51		
52		
53		
54		
55		
56		
57		
58		

Wagner, A. (2009). The Role of *SPRASA* in Fertility Obstetrics and Gynaecology. 2009. University of Auckland, Auckland, pp. 247.

Xi, T., Jones, I.M., & Mohrenweiser, H.W. (2004). Many amino acid substitution variants identified in DNA repair genes during human population screenings are predicted to impact protein function. *Genomics*, 83, 970-979.

Zhang, K., Gao, R., Zhang, H., Cai, X., Shen, C., Wu, C., et al. (2005). Molecular cloning and characterization of three novel lysozyme-like genes, predominantly expressed in the male reproductive system of humans, belonging to the c-type lysozyme/alpha-lactalbumin family. *Biology of Reproduction*, 73, 1064-1071.

PROOF ONLY

1 *Supplementary material for Prendergast, D., et al. (2014). SPACA3 gene variants in a New Zealand cohort of infertile* 59
 2 *and fertile couples, Human Fertility, doi: 10.3109/14647273.2014.907506.* 60
 3 61

4 Supplementary Table I. Infertility diagnosis, duration of infertility and age of the infertile couples analysed in this study. 62

Classification	Sex/diagnosis	Range (mean)	Number/percentage
Age (years)	Female	24–42 (35.0)	n = 102
	Male	26–52 (36.8)	n = 102
Duration of infertility (years)		1–14 (4)	
Male factor infertility	Oligospermia		n = 38
	Teratospermia		37.3%
	Asthenospermia		
	Testicular cancer		
	Vasectomy reversal		
	Antisperm antibodies		
Female factor infertility	Endometriosis		n = 17
	PCOS		16.7%
	Raised FSH		
Couples with both a female and male factor infertility			n = 18
			17.6%
Couples with unexplained infertility			n = 29
			28.4%

20 The percentages indicate the number of male, females or couples with a diagnosed cause for their infertility. Male n = 102, female n = 102 and 78
 21 couples n = 102. 79

22 Oligospermia, teratospermia and asthenospermia as defined by World Health Organization standards. 80

23 PCOS = polycystic ovary syndrome, FSH = follicle stimulating hormone. 81

24 82

25 83

26 84

27 Supplementary Table II. Age of the fertile couples analysed in this 85

28 study. 86

29

30

31

32

33

34

35

36

37

38

39

40

41

42

43

44

45

46

47

48

49

50

51

52

53

54

55

56

57

58

Supplementary Table III. Summary of PCR primers used for mutation screening and promoter amplification.

Fragment	Size	Primers (5'–3')	Location
Exon 1	253 bp	FOR GTGGCGCTGTTTGTGGAAGATGAG	60349–60372
		REV TGTTAACAGCCCCAGGAAGAAGG	60556–60578
Exon 2	544 bp	FOR GCCTTCTGCCACCCCTTCTCT	63939–36960
		REV GGTTGCCTGCTGCCTGCCTACT	64461–64482
Exon 3	223 bp	FOR CTGGGGTGGCTGTAACCATCTGAC	65360–65383
		REV AGGCCAGCTACCTGAGCAGTA	65561–65582
Exon 4	268 bp	FOR ACAGGATTGGATTTAGGCGAGTGG	65857–65882
		REV ACCGCTGCGGGGCTCCAG	66108–66125
Exon 5	366 bp	FOR GTGGGCAGCAGCAGGGAACAAAC	66141–66163
		REV AACGGAGGTGCTCTGGCTCTGACA	66483–66506
Promoter A	983 or 986	FOR TATATAACGCGTCCAACACTACCATCCCTG	59514–59531
		REV ATATACTCGAGGTGACAATGGCAGCAGCA	60479–60496
ProInt	616	FOR CTATTCTGGGCACCAACCA	59881–59898
Promoter A		REV ATATACTCGAGGTGACAATGGCAGCAGCA	60479–60496
Promoter A	537	FOR TATATAACGCGTCCAACACTACCATCCCTG	59514–59531
ProInt		REV GGGATGTTAACAGGTGTGCAA	60030–60050
Promoter BF	926	FOR GGTATAACGCGTGGTGAGGACAAGAAGCAG	63224–63241
Promoter BR		REV TATATCTCGAGATCCCAGCTGGGCACCA	64133–64149

Forward (FOR) and reverse (REV) PCR primers spanning the five exons of the SPACA3 gene and the promoter region. Size of PCR products generated by each set of primer pairs is indicated. Locations of fragments are indicated with reference to the reverse complements of Genbank accession number AC003687. Bases flanking restriction sites Mlu I and Xho I are highlighted in grey, the restriction sites Mlu I and Xho I are in green and yellow respectively and the SPACA3 gene sequence is in black.

Supplementary Table IV. Summary of PCR conditions.

Cycle	Step	Temperature & Duration	Function
One (1x)		94.0°C for 5.0 minutes	Initial denaturation
Two (30x)	One	94.0°C for 1.0 minutes	Denaturation
	Two	64.5°C for 1.0 minutes	Annealing
	Three	72.0°C for 1.0 minutes	Elongation
Three (1x)		72.0°C for 10.0 minutes	Final elongation
Four (1x)		7.0°C for ∞	Hold

Supplementary Table V. Summary of Touchdown PCR conditions.

Cycle	Step	Temperature & Duration	Function
One (1x)		94.0°C for 5 minutes	Initial denaturation
Two (20x)	One	94.0°C for 0.45 minutes	Denaturation
	Two	70.0°C for 0.45 minutes	Annealing
Three (15x)		Decrease temperature after cycle one by 0.5°C every 1 cycle	Annealing
	Three	72.0°C for 1.0 minutes	Elongation
	One	94.0°C for 0.45 minutes	Denaturation
	Two	60.0°C for 0.45 minutes	Annealing
Four (1x)	Three	72.0°C for 1.0 minutes	Elongation
		72.0°C for 10.0 minutes	Final elongation
Five (1x)		7.0°C for ∞	Hold

Due to non-specific primer binding, exon two, four and the promoter were amplified by Touchdown PCR 70°C–60°C.¹ The promoter amplicons followed the same Touchdown PCR protocol as exon two and four with the exception that the time for the first 20 cycles was extended to one minute.

¹Dieffenbach CW, Dveksler GS (eds.): PCR Primer: A Laboratory Manual, First Edition: Cold Spring Harbor Laboratory Press; 1995.

Supplementary Table VI. Summary of sequencing reaction conditions.

Cycle	Step	Temperature & Duration	Function
One (1x)		96.0°C for 1.0 minutes	Initial denaturation
Two (25x)	One	96.0°C for 0.1 minutes	Denaturation
	Two	50.0°C for 0.05 minutes	Annealing
	Three	60.0°C for 1.0 minutes	Elongation
Three (1x)		15.0°C for ∞	Hold

Primers (5µM) used for sequencing the human SPRASA exons were the forward PCR primers for exons one, two, three and five. Due to the presence of repetitive sequences in the 5' end of the amplicon, the reverse primer for exon four was used.

Supplementary Table VII. Variant frequencies of g.-22TGC(4_5) and c.239G>A in fertile and infertile couples.

Variant frequencies in couples				
Variant combination		Number		p-values
Male	Female	Fertile	Infertile	
Wildtype	Wildtype	69	56	0.43
Wildtype	5x	11	17	0.32
Wildtype	A	1	2	0.64
Wildtype	5x/A	1	0	0.50
5x	Wildtype	13	15	0.84
A	Wildtype	1	2	0.62
5x/A	Wildtype	2	0	0.25
5x	5x	4	3	1.00
5x	5x/A	1	0	0.50
5x	A	0	2	0.25
5x/A	5x	0	3	0.25
A	5x	1	1	1.00
5x/A	A	0	1	0.50
5x/A	5x/A	0	0	-
A	A	0	0	-
A	5x/A	0	0	-

Possible combinations and the frequencies of the 5x TGC repeat (g.-22TGC(4_5)) and the A allele (c.239G>A) in the fertile and infertile couples.

Statistics were performed with Fisher's exact t-test (2-tailed).

Supplementary Table VIII. Diagnosis of infertility in infertile patients with identified *SPACA3* variants.

Diagnosis	g.-22TGC(4_5)		c.239G>A		c.691G>C	
	HO	HE	HO	HE	HO	HE
Male factor infertility ^a	0	9	0	5	0	0
Unexplained (male)	4	11	0	2	0	0
Endometriosis	0	4	1	2	0	0
PCOS	1	3	0	0	0	0
Unexplained (female)	0	19	0	2	0	1
TOTAL	5	47 ^b	1	11	0	1

^aTesticular cancer, oligospermia, antisperm antibodies, teratospermia or asthenospermia.

^bForty six individuals were heterozygous for g.-22TGC(4_5), one female was diagnosed with both endometriosis and PCOS.

PCOS = polycystic ovary syndrome; HO = homozygote, HE = heterozygote.

Supplementary Table IX. The genotype analysis of the three variants identified in the infertile and fertile cohorts.

				Genotype analysis				OR	95% CI	P Value
	Cohort		4_4	4_5	5_5	5X allele				
g.-22TGC(4_5)	Male	Fertile Controls	84 (0.808)	18 (0.173)	2 (0.019)	22 (0.106)	0.71	0.40–1.29	0.55	
		Infertile Cases	77 (0.755)	21 (0.206)	4 (0.039)	29 (0.142)				
	Female	Fertile Controls	86 (0.827)	17 (0.163)	1 (0.010)	19 (0.091)	0.66	0.35–1.23	0.35	
		Infertile Cases	76 (0.745)	25 (0.245)	1 (0.010)	27 (0.132)				
	Couples	Fertile Controls	170 (0.817)	35 (0.168)	3 (0.014)	41 (0.099)	0.69	0.45–1.05	0.24	
		Infertile Cases	153 (0.750)	46 (0.225)	5 (0.025)	56 (0.137)				
c.239G>A	Male		GG	GA	AA	A allele	0.55	0.16–1.1	0.34	
		Fertile Controls	100 (0.962)	4 (0.038)	0 (0.000)	4 (0.019)				
	Female	Fertile Controls	101 (0.971)	3 (0.029)	0 (0.000)	3 (0.014)	0.48	0.12–1.96	0.55	
		Infertile Cases	97 (0.951)	4 (0.039)	1 (0.010)	6 (0.029)				
	Couples	Fertile Controls	201 (0.966)	7 (0.034)	0 (0.000)	7 (0.017)	0.52	0.21–1.32	0.36	
		Infertile Cases	192 (0.941)	11 (0.054)	1 (0.005)	13 (0.032)				
c.691G>C	Male		GG	GC	CC	C allele	1.00			
		Fertile Controls	104 (1.000)	0 (0.000)	0 (0.000)	0 (0.000)				
	Female	Fertile Controls	104 (1.000)	1 (0.010)	0 (0.000)	0 (0.000)	0.31			
		Infertile Cases	201 (0.990)	0 (0.000)	0 (0.000)	1 (0.005)				
	Couples	Fertile Controls	208 (1.000)	0 (0.000)	0 (0.000)	0 (0.000)	0.31			
		Infertile Cases	203 (0.995)	1 (0.005)	0 (0.000)	1 (0.002)				

OR = odds ratio; CI = confidence interval