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Supplemental Data

Homozygous mutations in *C14orf39*/*SIX6OS1* cause non-obstructive azoospermia and premature ovarian insufficiency in humans

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Supplemental Note: Case Reports

The two affected males (IV-2 and IV-3) in the family PK-INF-543 were 40 and 37 years old (Figure 1A), respectively. They had normal height and body weight and had no other serious diseases except suffered from primary infertility. At least two semen analyses indicated that they had normal semen volume but no sperm, and reproductive hormone levels within the normal range (Table 1). After obtaining informed consent, we obtained a testicular biopsy from individual IV-3 and performed H&E staining on the testicular sections. For the control, many spermatogenic cells including spermatogonia, spermatocytes, and spermatozoa were observed in the seminiferous tubules, but for individual IV-3, spermatogonia and spermatocytes were found, but post-meiotic cells were absent (Figure 1B). These results suggested that the affected males (IV-2 and IV-3) in this family were individuals with NOA. The father (III-1) and mother (III-2) in this family were first cousins. Their siblings married outside of the family, and each has multiple children with no history of infertility. The unaffected married brothers (IV-1 and IV-4) of the proband (IV-2) were fertile and fathered five and one offspring, respectively. The other two brothers (IV-6 and IV-7, 28 and 24 years old, respectively) and one sister (IV-5, 30 years old) were unmarried with unknown fertility status. Cytogenetic studies indicated that all the affected individuals, their siblings, and mother had normal karyotypes (Table 1).

For the unmarried sister (IV-5) in this Pakistani family, we noticed that the levels of her reproductive hormones were abnormal (Table 1). Specifically, she had elevated

levels of follicle-stimulating hormone (FSH, 49.06 mIU/ml) and luteinizing hormone (LH, 47.69 mIU/ml), as well as dramatically reduced levels of anti-Müllerian hormone (AMH, 0.021 ng/ml). This sister had menarche at the age of 17, followed by a history of irregular menstrual cycles, and reached menopause at the age of 24. Furthermore, her uterus and ovaries were small, with the left ovary being smooth and without follicular activity; in the expected location of the right ovary, there was only a small cyst (18 mm). These features indicated that the sister (IV-5) suffered from POI.[1]

The two affected Chinese men (P3907 and P6032) were 37 and 30 years old, respectively (Figure 1A). Individual P3907 was born into a consanguineous marriage. However, his parents were unwilling to provide blood samples. These two individuals also had normal karyotypes (46, XY) and no Y-chromosome microdeletions, but no sperm in their semen. Through histological examination of their testicular sections, we found no spermatozoa in the seminiferous tubules (Figure 1B).

Supplemental Figures

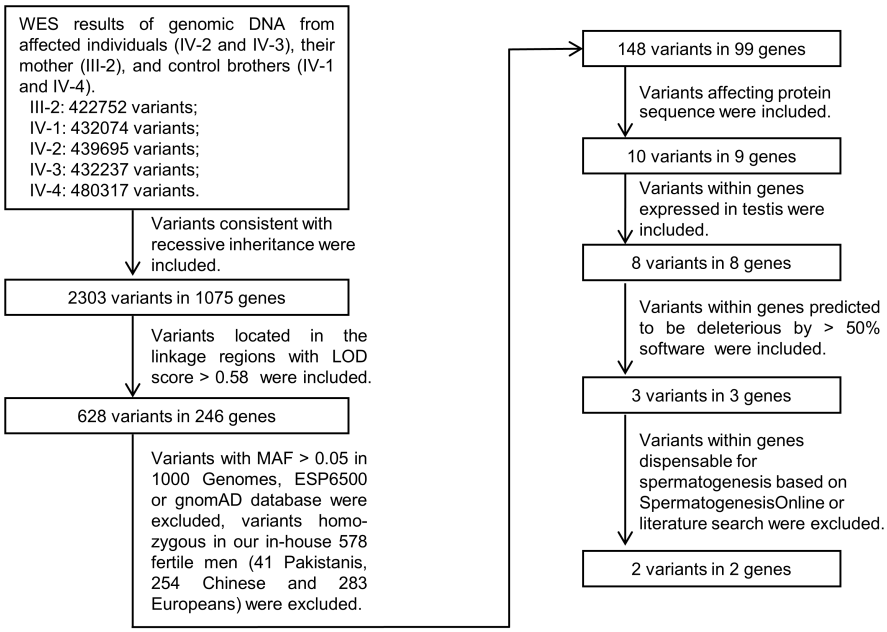

Figure S1. The workflow for whole-exome sequencing data analysis

The flowchart shows the strategy to filter the candidate variants for the Pakistani family. LOD, the logarithm of the odds; WES, whole-exome sequencing; MAF, minor allele frequency.

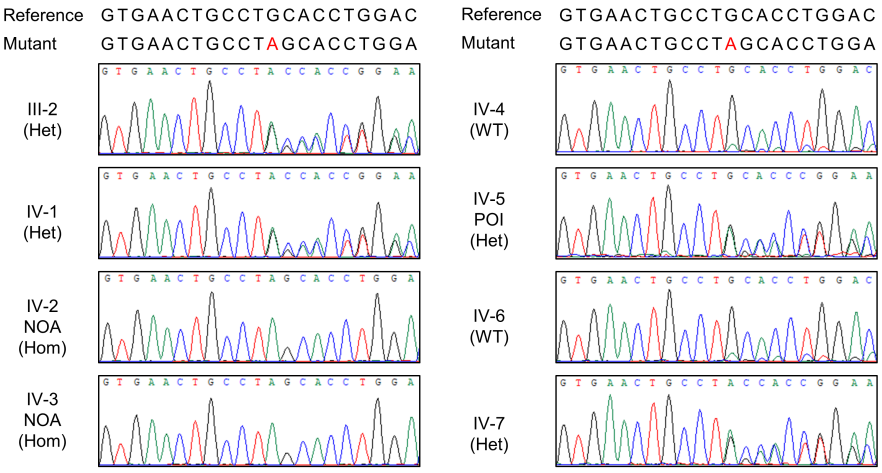

Figure S2. Detection of *DHRS4L2* variant by Sanger sequencing in all available family members

Genomic DNA extracted from peripheral blood was used as the PCR template to detect the variant (1-bp insertion) in *DHRS4L2*. The two brothers with NOA are homozygous for *DHRS4L2* mutation, but the sister (IV-5) who was diagnosed with POI carries a heterozygous *DHRS4L2* mutation, as is identified in her mother (III-2). Other unaffected family members are either heterozygous or wild type (WT). Hom, homozygous; Het, heterozygous; NOA, non-obstructive azoospermia; POI, premature ovarian insufficiency.

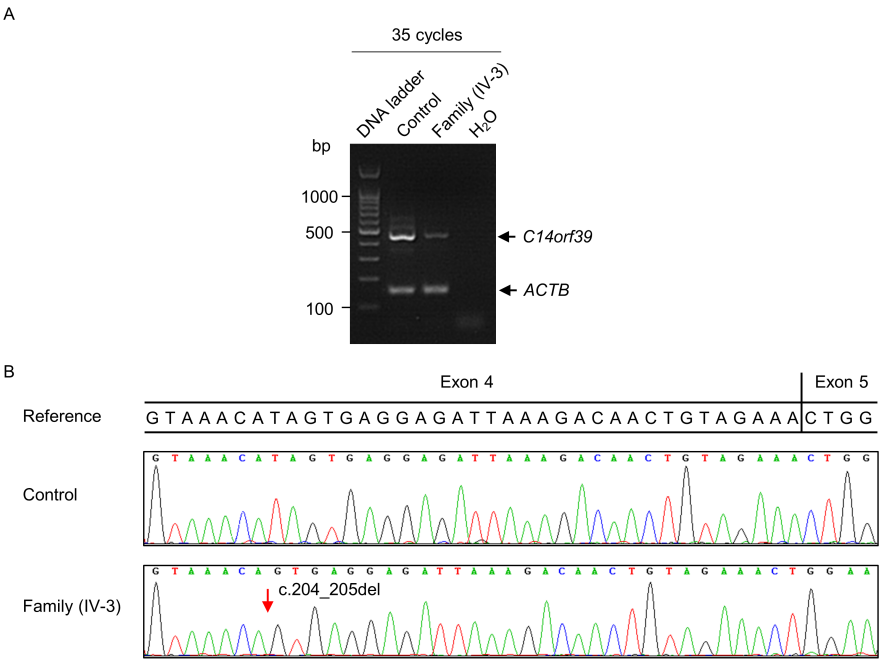

Figure S3. Detection of *C14orf39* mutation at the cDNA level by RT-PCR and Sanger sequencing

(**A**) The total RNAs extracted from the testicular tissues of a man with obstructive azoospermia and individual IV-3 were used to synthesize the cDNAs by RT-PCR. *C14orf39* and *ACTB* primer pairs were added simultaneously in the PCR mixture. The *C14orf39* band can be observed under 35 cycles of amplification in both control and individual IV-3. *ACTB* was used as an internal control. (**B**) Sanger sequencing chromatograms of testicular cDNAs of control and the individual IV-3. The red arrow indicates the 2-bp deletion in *C14orf39*.

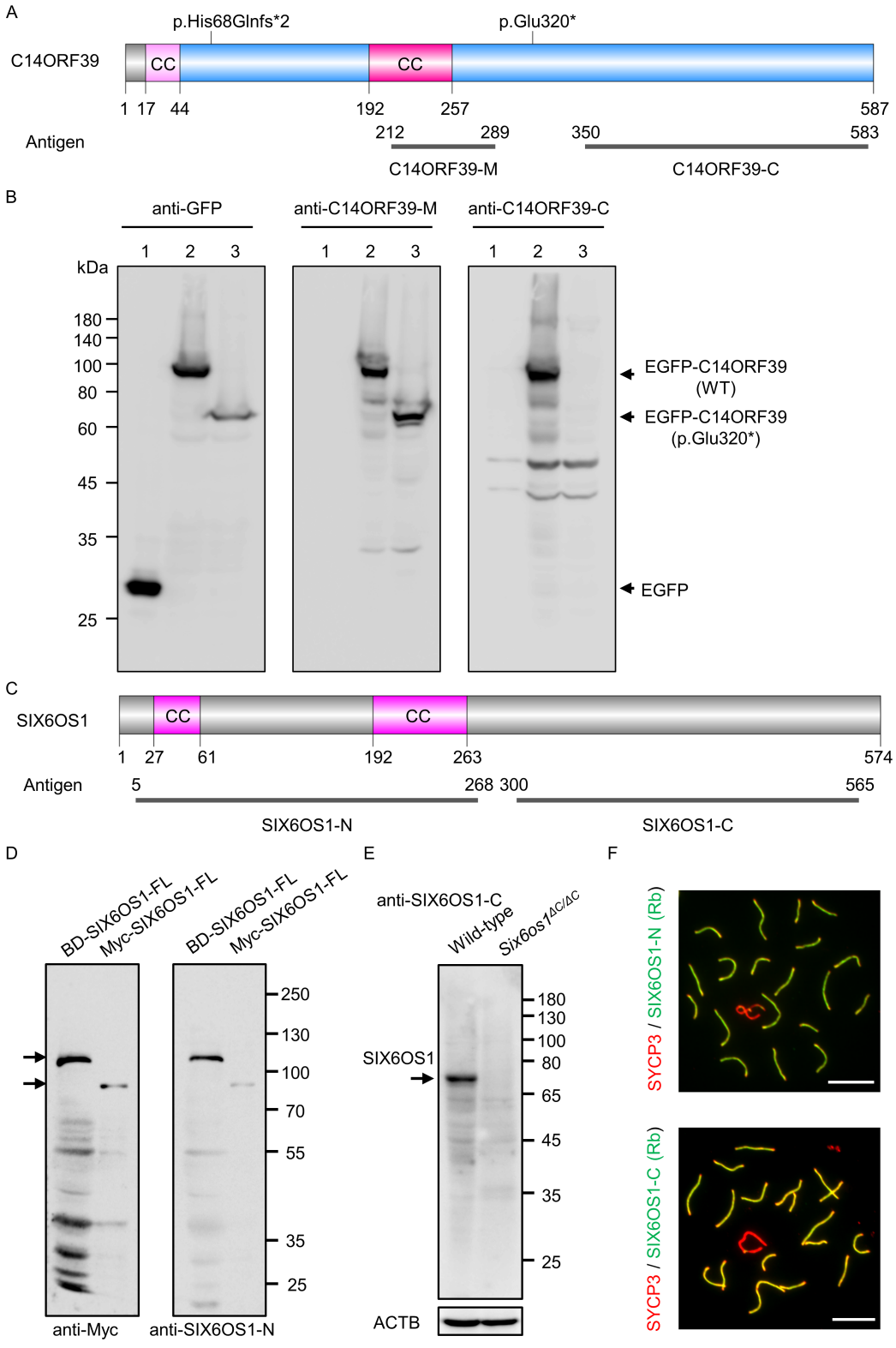

Figure S4. Validation of human C14ORF39 and mouse SIX6OS1 antibodies

(**A**) Schematic representation of the antigen information about human C14ORF39 antibodies. (**B**) Validation of C14ORF39 antibodies by Western blot. The whole-cell

lysates were extracted from transfected HEK293T cells. Lane 1: EGFP; lane 2: EGFP-C14ORF39; lane 3: EGFP-C14ORF39-E320X. Wild-type and mutated C14ORF39 fusion proteins were detected by C14ORF39-M and –C antibodies. The GFP antibody was used as a positive control. The arrows indicate the target proteins. (**C**) Schematic representation of the antigen information about the antibodies against SIX6OS1-N and SIX6OS1-C. Two putative coiled-coil domains (CC, pink) are indicated in the SIX6OS1 protein. (**D**) Validation of C14ORF39-N antibody by Western blot. Gal4-DNA binding domain tagged SIX6OS1 protein (BD-SIX6OS1-FL) was obtained from the transformed yeast cells, and Myc-tagged SIX6OS1 protein was extracted from the transfected HEK293T cells. The Myc antibody was used as a positive control. (**E**) Validation of C14ORF39-C antibody by Western blot. Testicular lysates were obtained from wild-type and *Six6os1*$^{\Delta C/\Delta C}$ mice at 16 dpp. ACTB was used as an internal control. Arrows indicate the target proteins. (**F**) Immunofluorescence staining of surface-spread spermatocytes from 10-week-old wild-type mice using SYCP3 (red) and different SIX6OS1 antibodies (green). Rb, rabbit. Scale bars denote 10 μm.

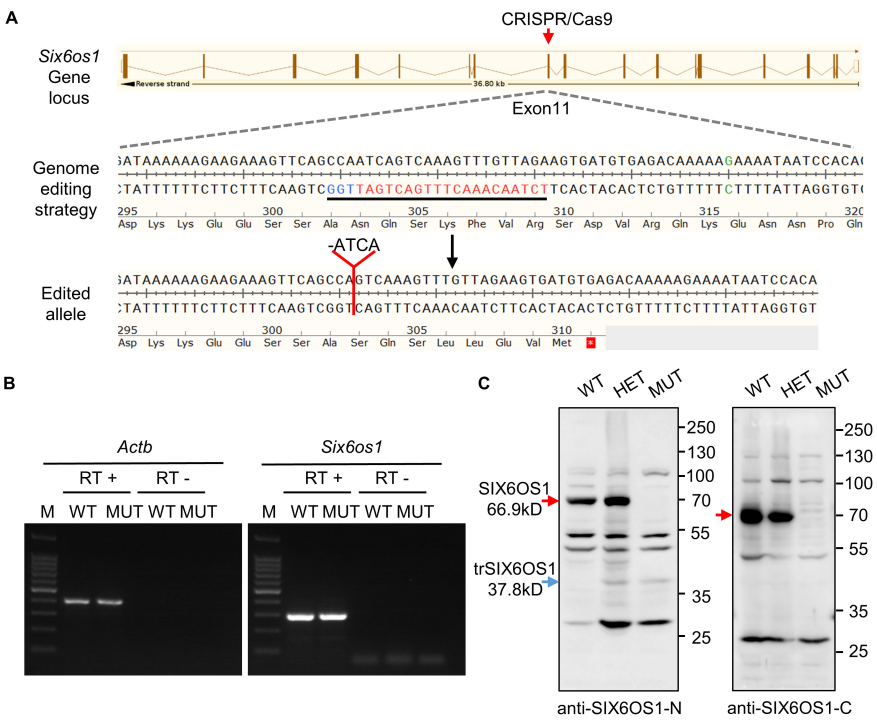

Figure S5. Generation and verification of the *Six6os1*$^{\Delta C/\Delta C}$ mouse model

(**A**) The strategy for the generation of *Six6os1*$^{\Delta C/\Delta C}$ mice by CRISPR/Cas9 technology. The colored nucleotides with a black line indicate the single guide RNA (sgRNA) targeting sequence. Four base pair deletion introduces a premature stop codon (red asterisk). (**B**) The expression of *Six6os1* was evaluated by RT-PCR in testes of 10-week-old WT and *Six6os1*$^{\Delta C/\Delta C}$ mice. *Actb* was served as internal control and underwent 28 cycles of amplification. *Six6os1* was amplified for 35 cycles. RT+, with reverse transcriptase; RT-, without reverse transcriptase. (**C**) Detection of SIX6OS1 protein from the testis lysates of 16 dpp mice by Western blot using antibodies against the N-terminal and C-terminal of SIX6OS1, respectively. Red arrows indicate the full-length SIX6OS1 and blue arrow indicates the truncated SIX6OS1 (trSIX6OS1). WT, wild-type; HET, heterozygous; MUT, homozygous.

Supplemental Tables

Table S1. The antibodies used in this study

Primary antibodies				
Target	**Dilution**	**Host species**	**Supplier**	**Catalog number**
Human SYCP3	1:200	Mouse	Proteintech	66409-1-Ig
Human SYCP1	1:80	Rabbit	Novus Biologicals	NB300-228
Human SYCE1	1:50	Rabbit	Proteintech	11063-1-AP
Human C14ORF39-M	1:50	Rabbit	Novus Biologicals	NBP2-32473
Human C14ORF39-C	1:30	Rabbit	This study	-
Mouse SYCP3	1:200	Mouse	Abcam	ab97672
Mouse SYCP1	1:200	Rabbit	Novus Biologicals	NB300-229
Mouse SYCE1	1:200	Rabbit	Proteintech	11063-1-AP
Mouse SIX6OS1-N	1:500	Rabbit	This study	-
Mouse SIX6OS1-C	1:500	Rabbit	This study	-
γH2AX	1:5000	Rabbit	Novus Biologicals	NB100-384
GFP	1:2000 (for WB)	Mouse	Abmart	M20004
Myc-Tag	1:1000	Mouse	Clontech	631206
Secondary antibodies				
Target	**Dilution**	**Host species**	**Supplier**	**Catalog number**
Mouse (Alexa-488)	1:100	Goat	Molecular Probes	A-21121
Rabbit (Alexa-555)	1:300	Donkey	Molecular Probes	A31572
Rat (Alexa-568)	1:150	Goat	Thermofisher	A11077
Mouse (HRP)	1:10000	Goat	Biolegend	405306
Rabbit (HRP)	1:10000	Donkey	Biolegend	406401
Rat (HRP)	1:10000	Goat	Biolegend	405405

Table S2. The sgRNAs and primers used in this study

Primers used for Sanger sequencing		
Primer name	**Sequence (5'- 3')**	**Product (bp)**
hC14orf39-MT1-Fw	TCTTAGCTTCTGGCTCTAGG	471
hC14orf39-MT1-Rv	ACATAGTGGCATAACAAGGC	
hDHRS4L2-Fw	TTAGCCACAAGACAGTTTCC	476
hDHRS4L2-Rv	CCTCAGTCTCCAGCCTATAC	
hC14orf39-MT2-Fw	CATTGGCTGGTTTCTTTGTC	682
hC14orf39-MT2-Rv	TTCCTTCAGGGATTTCTCAC	
hC14orf39-MT3-Fw	AGAAGTGGAAGATGGAACTGGA	670
hC14orf39-MT3-Rv	GCAGGAAAAATGGGGAAGAGG	
Primers used for RT-PCR		
Primer name	**Sequence (5'- 3')**	**Product (bp)**
hC14orf39-MT1-rtPCR-Fw	GCCTGTTTGTCAGTTTGGACA	455
hC14orf39-MT1-rtPCR-Rv	ACATGCCAACACTCTGCTTTG	
hC14orf39-MT2-rtPCR-Fw	TCTAGGCATAATGAAACTAAGGCTC	430
hC14orf39-MT2-rtPCR-Rv	CCCCTTTTTCCGACCACTGA	
hACTB-rtPCR-Fw	AATGAGCTGCGTGTGGCTC	148
hACTB-rtPCR-Rv	ATAGCACAGCCTGGATAGCAAC	
Primers used for qPCR		
Primer name	**Sequence (5'- 3')**	**Product (bp)**
hC14orf39-qPCR-Fw	GCCTGTTTGTCAGTTTGGACA	178
hC14orf39-qPCR-Rv	TCAATTTCCTCATCTGTTGCATT	
hACTB-qPCR-Fw	AATGAGCTGCGTGTGGCTC	148
hACTB-qPCR-Rv	ATAGCACAGCCTGGATAGCAAC	
Primers used for generation and genotyping of *Six6os1* mutant mice		
Primer name	**Sequence (5'- 3')**	**Product (bp)**
mSix6os1-exon11-sgRNA-Fw	GAAATTAATACGACTCACTATAGGGAGATCTAACAAACTTTGACTGATGTTTTAGAGC	122
mSix6os1-sgRNA-Rv	AAAAAAGCACCGACTCGGTG	
mSix6os1-exon11-Fw	ATGGTTGGAGAAATGAGGGC	476
mSix6os1-exon11-Rv	CCTGTCCTGAAACTCACACT	
mSix6os1-exon11-S-Fw	CAGATATTGTTTGGTTTTAGCTTCAG	134
mSix6os1-exon11-S-Rv	CCTGTGGATTATTTTCTTTTTGTCTC	
Primers used for plasmid construction		
Primer name	**Sequence (5'- 3')**	**Product (bp)**
AD-hC14orf39-Fw	GCCATGGAGGCCAGTGAATTCATGAATGACAGCCTGTTTGTCAG	1764
AD-hC14orf39-Rv	CAGCTCGAGCTCGATGGATCCTCAAAAAAAAGTAAACTGTGTTGTATTTTGTG	

AD-hC14orf39-Fw	GCCATGGAGGCCAGTGAATTCATGAATGACAGCCTGTTTGTCAG	215
hC14orf39 (c.204_205del)-Rv	ATCTCCTCACTGTTTACAGTAATGATCAATTTCCTCATCTG	
hC14orf39 (c.204_205del)-Fw	ACTGTAAACAGTGAGGAGATTAAAGACAACTGTAG	1571
AD-hC14orf39-Rv	CAGCTCGAGCTCGATGGATCCTCAAAAAAAAGTAAACTGTGTTGTATTTTGTG	
AD-hC14orf39-Fw	GCCATGGAGGCCAGTGAATTCATGAATGACAGCCTGTTTGTCAG	978
hC14orf39 (c.958G>T)-Rv	AAATATCTGTGTATCATTTTaTTTTTGTCTAAAGTCAATATTGGCAAGC	
hC14orf39 (c.958G>T)-Fw	TAAAATGATACACAGATATTTAATGACTCTGC	806
AD-hC14orf39-Rv	CAGCTCGAGCTCGATGGATCCTCAAAAAAAAGTAAACTGTGTTGTATTTTGTG	
BD-mSyce1-Fw	ATGGCCATGGAGGCCGAATTCATGGCCACCAGACCGCAGCC	1032
BD-mSyce1-Rv	CCGCTGCAGGTCGACGGATCCTTAGGTCCTGCTTGATGGGCGCTC	
EGFP-hC14orf39-Fw	GCATGGACGAGCTGTACAAGTCCAAGATGAATGACAGCCTGTTTGTC	1810
EGFP-hC14orf39-Rv	GATTATGATCTAGAGTCGCGTCAAAAAAAAGTAAACTGTGTTGTATTTTGTG	
EGFP-C1-hC14orf39-Fw	TCGAGCTCAAGCTTCGAATTCTATGAATGACAGCCTGTTTGTCAG	235
hC14orf39 (c.204_205del)-N-Rv	ATCTCCTCACTGTTTACAGTAATGATCAATTTCCTCATCTG	
hC14orf39 (c.204_205del)-N-Fw	ACTGTAAACAGTGAGGAGATTAAAGACAACTGTAG	1590
EGFP-C1-hC14orf39-Rv	TTATCTAGATCCGGTGGATCCTCAAAAAAAAGTAAACTGTGTTGTATTTTGTG	
EGFP-hC14orf39-Fw	GCATGGACGAGCTGTACAAGTCCAAGATGAATGACAGCCTGTTTGTC	998
hC14orf39 (c.958G>T)-N-Rv	CTGTGTATCATTTTATTTTTGTCTAAAGTCAATATTGGCAAGC	
hC14orf39 (c.958G>T)-C-Fw	GACTTTAGACAAAAATAAAATGATACACAGATATTTAATGACTCTGC	842
EGFP-hC14orf39-Rv	GATTATGATCTAGAGTCGCGTCAAAAAAAAGTAAACTGTGTTGTATTTTGTG	
mCherry-hSyce1-Fw	GCATGGACGAGCTGTACAAGggcggcggcgggtcgATGGCGGGGAGGTCCCTGAC	1111
mCherry-hSyce1-Rv	GATCTAGAGTCGCGGCCGCTTCAAAATAGCTCCTTTATTTCCTGAAAGCC	
BD-mSix6os1-Fw	GCATATGGCCATGGAGGCCGAATTCATGAATGATAATCTGTTTGTCAGTTTG	1769
BD-mSix6os1-Rv	CCGCTGCAGGTCGACGGATCCCAAAAAACATAAATTGTGTTTTATTTTGTGA	
Myc-mSix6os1-Fw	TCGAGCTCAAGCTTCGAATTCATGGAGGAGCAGAAGCTGATC	1880
Myc-mSix6os1-Rv	TTATGATCTAGAGTCGCGGCCGCAGGCCCCAAGGGGTTATGCTA	

Table S3. Overview of *C14orf39* variants identified from the affected individuals

Subjects	Genomic Position on chr14 (bp)	cDNA Change	Protein Change	Genotype	Allele Frequency in Population		
					1KGP	ESP6500	gnomAD
IV-2, IV-3 & IV-5	60483719-60483720	c.204_205del	p.His68Glnfs*2	Homozygous	0	0.0187	4.22×10^{-6}
P3097	60465993	c.958G>T	p.Glu320*	Homozygous	0	0	0
P6032	60457095	c.1180-3C>G	p.?	Homozygous	0	0	0

NCBI accession number of *C14orf39* is NM_174978.3. 1KGP, 1000 Genomes Project; gnomAD, the Genome Aggregation Database.

Supplemental References

1. Qin, Y., Jiao, X., Simpson, J.L., and Chen, Z.J. (2015). Genetics of primary ovarian insufficiency: new developments and opportunities. Hum. Reprod. Update 21, 787-808.