A rare synaptonemal complex protein 3 gene variant in unexplained female infertility

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ABSTRACT: Synaptonemal complex protein 3 (SYCP3) plays a critical role in homologous chromosome pairing and recombination in meiosis, and mice deficient in this gene show infertility in males and subfertility in females. The aim of our current study was to determine whether genetic alterations in the SYCP3 gene are associated with female infertility in humans. We examined sequence variations of the SYCP3 gene in genomic DNA from 88 Japanese women with unexplained infertility and 165 samples obtained from a fertile control group. Case–control study using seven tagging single nucleotide polymorphisms revealed no significant association between common SYCP3 variants and unexplained infertility. However, only infertile women were homozygous for the minor allele of a novel rare variant in the coding region, c.666A>G (222Q>G). The minor allele frequency was significantly higher in the infertile cohort (P<0.05). This variant is predicted to create a cryptic splice site, although the expression of a mini-gene harboring the variant in HeLa cells or mouse testis did not demonstrate any effects on gene splicing. Our current findings therefore suggest that the c.666A>G variant in the SYCP3 gene might possibly contribute to female infertility in humans, although larger studies are needed to assess the possible effects of SYCP3 gene variation on human female infertility.

Key words: infertility / meiosis / rare variant / SYCP

Introduction

It is estimated that ~15% of couples in the world suffer from infertility (Randolph, 2000). The overall incidence of infertility is relatively stable in developed countries, but is increasing in developing countries (Ombel et al., 2008; Quaas and Dokras, 2008; Bhattacharya et al., 2009). Although treatment options, including assisted reproductive technology, have been evolving and treatment outcomes have improved (Quaas and Dokras, 2008), the etiology of many individual cases of infertility have yet to be fully understood. Since a number of factors contribute to reproductive success, it is obvious that infertility causes are heterogeneous. Despite recent advances in sophisticated diagnostic modalities, up to 30% of couples are still diagnosed with unexplained infertility (The Practice Committee of the American Society for Reproductive Medicine, 2006; Quaas and Dokras, 2008).

Accumulating evidence suggests that genetic factors contribute to the etiology of female infertility in humans (The ESHRE Capri Workshop Group, 2008; Matzuk and Lamb, 2008). Genes involved in meiosis are also good candidates for genes contributing to female infertility (Sanderson et al., 2008). This possibility is supported by reports that mice with deficient expression of these genes exclusively manifest reproduction failure without the appearance of any extragonadal symptoms. A defect in meiosis generally results in a chromosome segregation error, leading to oocyte aneuploidy or cell death (Handel and Schimenti, 2010). Indeed, there is some evidence that infertile women have a higher risk of aneuploid oocytes compared with fertile women, suggesting a presence of meiotic defect (Martin, 2008). Furthermore, missense mutations that are potentially responsible for female infertility have been reported in two meiotic genes, Dmc1 and Msh5 (Mandon-Pepin et al., 2008).

Synaptonemal complex protein 3 (SYCP3) is the axial/lateral element of the synaptonemal complex that forms between homologous chromosomes during the prophase stage of meiosis I. Sycp3 gene knock-out mice are characterized by male infertility and female subfertility with reduced numbers of oocytes (Yuan et al., 2000; Yuan et al., 2002). In humans, a frameshift mutation that results in the production of C-terminal truncated SYCP3 protein was identified...
in two azoospermia patients with meiotic arrest (Miyamoto et al., 2003). These observations implicate SYCP3 as a significant candidate gene for a role in female infertility in humans.

In the present study, we employed a candidate gene approach to identify SYCP3 sequence variations that conferred a high risk for unexplained female infertility. We first performed a case–control study using common single nucleotide polymorphisms (SNPs) encompassing the SYCP3 gene on the basis of the ‘common disease—common variant—‘ hypothesis (Altshuler et al., 2008). However, it is also theoretically possible that a risk allele for infertility might be naturally eliminated under strong negative selection and may not have been expanded to constitute a common gene variant. Thus, we also conducted the complete sequence analysis of SYCP3 coding regions in order to identify rare variants or de novo mutations, which contributed to the etiology of female infertility.

Materials and Methods

Subjects

A total of 253 Japanese women who lived in the Nagoya area of Japan were included in the present study. The infertility cohort comprised 88 women (median age: 33.3 years, range: 24–41 years) with unexplained infertility (duration of infertility: 39.6 months, range: 12–144 months) who were recruited from private infertility clinics in the Nagoya area of Japan. The diagnosis of unexplained infertility was made by exclusion of known causes of infertility using standard clinical investigations (tubal patency, analysis of semen and ovulation). We also analyzed a total of 165 fertile control subjects (86 pregnant women at second or third trimester and 79 women who had one or more healthy children), for whom the inclusion criteria were no history of infertility and no discernible medical complications by standard examination. The objective of the study was clearly explained to each of these individuals and written informed consent was obtained from each subject. The study protocol was approved by the Ethical Review Boards for Human Genome Studies at Fujita Health University (accession numbers 39 and 82, approved on 4 October 2004 and 22 May 2009, respectively).

SNP selection and genotyping

We consulted the HapMap database (release#23.a.phase2, Mar 2008, www.hapmap.org, population: Japanese Tokyo) to survey common SNPs with minor allele frequencies (MAFs) of >0.05. The surveyed SYCP3 5′-flanking regions ranged from ~4.63 kb upstream of the initial exon to about 14.1 kb downstream of the last exon (HapMap database contig number chr 12: 25619952-25590536). Upstream and downstream boundaries were determined by those of linkage disequilibrium blocks including the promoter region or the last exon. From the 28 SNPs identified in this region, 7 tagging SNPs (rs10778147, rs3751248, rs10778146, rs10860779, rs11110986, rs4764650, and rs3205421) were selected for subsequent association analyses (Fig. 1) based on an \( r^2 \) threshold >0.8 in a pair-wise tagging-only mode using the HAPLOVIEW Tagger.
program (http://www.broadinstitute.org/mpg/haploview; Barrett et al., 2005). TaqMan assays (Applied Biosystems, Foster City, CA, USA) were used to determine specific genotypes. All TaqMan assays were tested for quality control using positive and negative control samples.

**Statistical analysis**

Genotype deviations from a Hardy–Weinberg equilibrium were evaluated using the chi-square test (SAS/Genetics, Release 8.2, SAS Japan, Inc., Tokyo, Japan). Marker-trait association analysis was used to evaluate allele- and genotype-wise associations with the chi-square test (SAS/Genetics). We conducted a seven-marker haplotype analysis in sliding-window fashion and log-likelihood ratio tests were performed for establishing a global P-value with the COCPHASE2.403 program (Dudbridge, 2003).

In these haplotype-wise analyses, rare haplotype (<0.05) cases and controls were excluded. A SYCP3 gene mutation screening detected three variants, which were excluded in the haplotype-wise analyses. The significance level for all statistical tests was set at 0.05. A Bonferroni’s correction was used to control for inflation of the Type I error rate. Since we employed 31 multiple tests (7 tests for tagging SNPs, 21 for haplotype and 3 for rare variants) for each sample, the corrected significance level was set at 0.0016.

**DNA sequencing**

Genomic DNA was extracted from blood samples, and then all 8 SYCP3 gene exons and exon–intron boundaries were amplified by PCR using 6 SYCP3-specific primer pairs. These SYCP3-specific primers were designed using the human SYCP3 genomic sequence (Genbank accession number NC_000012.11). The PCR products were directly sequenced using an ABI Prism Sequencer 310XL (Applied Biosystems). The frequency of SNPs was performed as described for the tagging SNPs. The effects of genetic alterations upon gene splicing function were analyzed using Human Splicing Finder (http://www.umd.be/HSF/) and ACESCAN2 (http://genes.mit.edu/acescan2/index.html). The frequencies of codon usage among synonymous codons were analyzed using codon usage and gene exons and exon–intron boundaries were amplified by PCR using 6 SYCP3-specific primer pairs. These SYCP3-specific primers were designed using the human SYCP3 genomic sequence (Genbank accession number NC_000012.11). The PCR products were directly sequenced using an ABI Prism Sequencer 310XL (Applied Biosystems). The frequency of SNPs was performed as described for the tagging SNPs. The effects of genetic alterations upon gene splicing function were analyzed using Human Splicing Finder (http://www.umd.be/HSF/) and ACESCAN2 (http://genes.mit.edu/acescan2/index.html) software. The frequencies of codon usage among synonymous codons were analyzed using codon usage and gene identification table (http://genome.crg.es/courses/SeqAnalysis/GenIDentification/).

**RNA analysis**

The SYCP3 genomic region encompassing exon 8, intron 8 and exon 9 was amplified for both wild-type and mutant alleles. The resulting PCR products were then cloned into the intron region of a mammalian expression vector (Exontron, Mobitec). These plasmids were then transfected to HeLa cells using Lipofectamine 2000 (Invitrogen). Cells were harvested at 48 h after transfection, and total RNA extracts were isolated using RNAeasy (Qiagen). After treatment with DNase I, first-strand cDNAs were synthesized using an oligo-(dT) primer and Superscript (Invitrogen). RT–PCR was then performed using primers designed with vector sequences and SYCP3 sequences.

The resulting PCR products were first analyzed by direct sequencing. We also established a high-sensitivity detection system using TaqMan real-time quantitative PCR amplification specific for wild-type or mutant transscripts. The PCR primers and probes were as follows. PYCP3-ex9-Rv: 5′-TCTACTTACTGGTGCACTAAGTGAATTT-3′, PYCP3-ex9-Fw: 5′-GTGATGAGATATGTGAATTGAAAG-3′, wild type: VIC-5′-TCTTCTGCCTGTAGTCT-3′, c.666A>G (9-bp deletion): FAM-5′-CTTCTGTACCTTCAGTTC-3′.

For expression in meiotic cells, the vector promoter was replaced with the mouse Sycp1 promoter (−722/+102) (Vidal et al., 1998). The resultant plasmids were then injected into the rete tests of a 4-week-old DDY mouse. The plasmids were introduced into the meiotic cells by in vivo electroporation using a CUY21 electroporator (BEX) (Shojo et al., 2005). The electroporation settings involved an eight pulse of 30 V with a pulse length of 50 ms and an interval of 950 ms. Testicular tissue was resected at 24 h after transfection and RT–PCR was performed essentially as described above. The protocols adopted for the use of laboratory mice were approved by the Animal Care and Use Committee at the Fujita Health University.

**Results**

To identify new candidate SYCP3 gene variants associated with female unexplained infertility, we first genotyped 88 females with unexplained infertility and 165 control fertile females using 7 common SNPs that were selected as tagging SNPs. In both groups, genotype frequencies were in Hardy–Weinberg equilibrium for all 7 SNPs. SYCP3 genomic region linkage disequilibrium block structures were developed based on the information from the HapMap database (Fig. 1). Evaluation of the seven selected tagging SNPs revealed no association with female infertility by either allele/genotype-wise analysis or haplotype-wise analysis (Tables I and II).

Next, we sequenced the entire coding regions as well as the exons–intron boundaries of the SYCP3 gene for the 88 females with unexplained infertility. Sequence analyses identified three sequence variants, including two intronic variants (c.353+194T>C, c.454-10delTTTTA) and one synonymous variant (c.666A>G, Q222Q) (Fig. 2A). Among these variants, c.454-10delTTTTA was a common variant in linkage disequilibrium with the tagging SNP (SNP2 in Table I). c.353+194T>C was a novel rare variant, which was present in the infertile and control groups at a similar frequency (Table III).

The c.666A>G variant in exon 9 was also a novel rare variant. Women who were homozygous for c.666A>G were present only in the infertile group (2/88) and the minor allele frequency was significantly higher in the infertile group (0.046 versus 0.009, P = 0.00755), although it was not significant after Bonferroni’s correction. The nucleotide substitution did not change the coding amino acid (Gln222Gln) and did not dramatically alter the frequencies of codon usage in humans (CCA 0.27 versus CAG 0.73). However, this variant creates a potential cryptic splice acceptor site at a position nine nucleotides downstream of the original intron 8/exon 9 junction. The score predicting the acceptor site in the Human Splicing Finder was higher than that of the original site (80.39 versus 78.54). This variant could possibly affect the normal splicing of intron 8, resulting in an in-frame, 11 nucleotide deletion within the SYCP3 transcript (Fig. 2B).

When an expression vector harboring the c.666A>G variant was introduced into human HeLa cells, PCR direct sequencing of the RT–PCR product revealed only transcripts that had undergone splicing at the original junction (data not shown). To examine splicing within meiotic cells, we replaced the vector promoter with the mouse Sycp1 promoter and injected the new vector directly into mouse testis. Again, the expected splicing aberration was not detected (data not shown). Finally, we established a highly sensitive quantitative PCR assay for detecting small amounts of the splice aberration. However, even with this sensitive system, we could not detect any aberrant PCR products in the RNA obtained from either HeLa cells or mouse testis (Fig. 2C).
In the current study, we identified a genomic variation in the SYCP3 genomic region that is possibly associated with female infertility. This variant, c.666A>G, is a rare variant but does not appear to be a de novo variation based on the fact that more than two infertile women as well as a small number of healthy individuals harbored the same variation. Thus, this variant was most likely inherited from a fertile parent. In this context, the variant cannot be a sole determinant for infertility in the women with the variation, but might be one of the multiple factors responsible for female infertility. Furthermore, it is formally possible that the ability of this rare variant to increase disease risk is greater than that of more common genomic variations (Manolio et al., 2009). Thus, c.666A>G will likely not increase in prevalence in the general population, since women with the risk allele will probably have a reproductive problem. This conclusion suggests that the variant possibly arose recently during human evolution and will soon disappear under negative selection pressure.

Although in silico analysis predicted that the c.666A>G variation would possibly create a cryptic splice acceptor site, functional analyses did not demonstrate the splicing aberration in the transcript from the variant allele. However, the experimental strategy utilized herein possesses an

### Table I Association of SYCP3 SNPs with infertility.

<table>
<thead>
<tr>
<th>SNP ID*</th>
<th>Phenotypeb</th>
<th>MAF</th>
<th>n</th>
<th>Genotype deviation</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M/M</td>
<td>M/m</td>
<td>m/m</td>
<td>HWE</td>
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<td>SNP1, G&gt;A</td>
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<td>Cases</td>
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<td>88</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Controls</td>
<td>0.136</td>
<td>165</td>
<td>121</td>
</tr>
<tr>
<td>SNP2, G&gt;T</td>
<td>rs3751248</td>
<td>Cases</td>
<td>0.244</td>
<td>88</td>
<td>52</td>
</tr>
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<td></td>
<td></td>
<td>Controls</td>
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<td>83</td>
</tr>
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<td>44</td>
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<tr>
<td></td>
<td></td>
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<td>165</td>
<td>67</td>
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<tr>
<td>SNP4, C&gt;A</td>
<td>rs10860779</td>
<td>Cases</td>
<td>0.415</td>
<td>88</td>
<td>31</td>
</tr>
<tr>
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<td></td>
<td>Controls</td>
<td>0.355</td>
<td>165</td>
<td>70</td>
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<tr>
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<td>0.443</td>
<td>88</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Controls</td>
<td>0.497</td>
<td>165</td>
<td>41</td>
</tr>
<tr>
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<td>88</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Controls</td>
<td>0.206</td>
<td>165</td>
<td>100</td>
</tr>
<tr>
<td>SNP7, T&gt;C</td>
<td>rs3205421</td>
<td>Cases</td>
<td>0.352</td>
<td>88</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Controls</td>
<td>0.312</td>
<td>165</td>
<td>79</td>
</tr>
</tbody>
</table>

MAF, minor allele frequency; n, number; M, major allele; m, minor allele; HWE, Hardy–Weinberg equilibrium.

*Tag SNPs, major allele > minor allele.

bCases = infertility.

### Table II Sliding-window analysis of tagging SYCP3 SNPs.

<table>
<thead>
<tr>
<th></th>
<th>Global P-value</th>
<th>2 Window</th>
<th>3 Window</th>
<th>4 Window</th>
<th>5 Window</th>
<th>6 Window</th>
<th>7 Window</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs10778147</td>
<td>0.572</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>rs3751248</td>
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<td>0.442</td>
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<td></td>
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<tr>
<td>rs10778146</td>
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<td>0.340</td>
<td>0.509</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs10860779</td>
<td>0.401</td>
<td>0.541</td>
<td>0.512</td>
<td>0.678</td>
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<td></td>
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<tr>
<td>rs11110986</td>
<td>0.419</td>
<td>0.525</td>
<td>0.531</td>
<td>0.686</td>
<td>0.678</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs4764650</td>
<td>0.479</td>
<td>0.628</td>
<td>0.618</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>rs3205421</td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

Discussion

In the current study, we identified a genomic variation in the SYCP3 genomic region that is possibly associated with female infertility. This variant, c.666A>G, is a rare variant but does not appear to be a de novo variation based on the fact that more than two infertile women as well as a small number of healthy individuals harbored the same variation. Thus, this variant was most likely inherited from a fertile parent. In this context, the variant cannot be a sole determinant for infertility in the women with the variation, but might be one of the multiple factors responsible for female infertility. Furthermore, it is formally possible that the ability of this rare variant to increase disease risk is greater than that of more common genomic variations (Manolio et al., 2009). Thus, c.666A>G will likely not increase in prevalence in the general population, since women with the risk allele will probably have a reproductive problem. This conclusion suggests that the variant possibly arose recently during human evolution and will soon disappear under negative selection pressure.

Although in silico analysis predicted that the c.666A>G variation would possibly create a cryptic splice acceptor site, functional analyses did not demonstrate the splicing aberration in the transcript from the variant allele. However, the experimental strategy utilized herein possesses an
inherent limitation due to the use of heterologous expression systems in human cell lines or mouse testis. Unfortunately, it is still possible that these experiments did not reveal what actually happened in the oocytes of women harboring the rare variant. Further studies incorporating micro-injection into mouse fetal ovary are needed to elucidate the impact of the variant on female meiosis, although it is still unclear whether the human transcript will be properly processed in mouse ovary.

There are some noteworthy limitations to our present study that need to be considered. First, we could not exclude the possibility of Type I error in our data. Second, the data did not explain the etiology of infertility in the majority of the patients since the minor allele frequency of c.666A>G variant was low. Further, the small sample size that we used may have impacted upon our findings. Provided that human infertility is a polygenic disease, a larger sample size would enable significant

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**Figure 2** Genetic variations in the coding region of the SYCP3 gene. (A) Location of three variants. Variations are depicted by arrowheads, while exons are indicated by boxes. (B) Junction sequence between intron 8 and exon 9. The original splice acceptor site is indicated by the solid box, while the putative cryptic splice acceptor is indicated by the dotted box. Normal and putative variant transcripts are depicted by thick arrows below the panels. (C) High-sensitivity quantitative PCR for normal and aberrant transcript production. Four serially diluted PCR products were used as templates for positive controls. Left panels indicate PCR results for normal transcripts, while right panels indicate the results for aberrant transcripts with a 9-bp deletion. Mini-genes harboring either allele-type produced similar levels of normal transcripts, but no aberrant transcripts.

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**Table III** SYCP3 sequencing and association of variants with infertility.

<table>
<thead>
<tr>
<th>Location</th>
<th>Polymorphism</th>
<th>Phenotype</th>
<th>MAFb</th>
<th>n</th>
<th>Genotype</th>
<th>P-value</th>
<th>HWE</th>
<th>Genotype</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intron 5</td>
<td>c.353+194T&gt;C</td>
<td>Cases</td>
<td>0.006</td>
<td>88</td>
<td>M/M</td>
<td>0.957</td>
<td>NA</td>
<td>0.486</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Controls</td>
<td>0.012</td>
<td>165</td>
<td>M/m/m</td>
<td>0.875</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intron 6b</td>
<td>c.454-10delTTTTA</td>
<td>Cases</td>
<td>0.244</td>
<td>88</td>
<td>M/M</td>
<td>0.313</td>
<td>0.369</td>
<td>0.295</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Controls</td>
<td>0.288</td>
<td>165</td>
<td>M/m/m</td>
<td>0.798</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 9</td>
<td>c.666A&gt;G (Q222Q)</td>
<td>Cases</td>
<td>0.046</td>
<td>88</td>
<td>M/M</td>
<td>7.93E−06</td>
<td>0.0658</td>
<td>0.00755</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Controls</td>
<td>0.009</td>
<td>165</td>
<td>M/m/m</td>
<td>0.906</td>
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<td></td>
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</tbody>
</table>

MAF, minor allele frequency; n, number; M, major allele; m, minor allele; HWE, Hardy–Weinberg equilibrium.

"Cases = infertility.

rs33217308.
differences to be detected more readily and also allow us to identify SNPs responsible for a low relative risk of disease. To overcome these limitations, a replication study using a larger cohort may be required for conclusive results.

We recently reported two dominant-negative mutations in the SYCP3 gene in women with recurrent pregnancy loss (Bolor et al., 2009). Indeed, Sycp3-deficient female mice display DNA recombination errors and non-disjunction between homologous chromosomes during meiosis, leading to a reduced oocyte pool and embryonic death due to aneuploidy of the embryos, which die in utero at an early stage of development (Yuan et al., 2002). The phenotype appears analogous to early pregnancy loss rather than infertility in humans. However, humans might manifest a more severe phenotype than mice, since it is generally acknowledged that the typical rate of human aneuploidy is an order of magnitude higher than rates observed in mice due to differences in checkpoint robustness (Hassold et al., 2004). It is possible that a severe recombination defect results in early embryonic loss around the early implantation period, which manifests as infertility. This possibility has clinically important implications, since infertile women with c.666A>G variation can choose an option of assisted reproductive technology combined with pre-implantation screening to exclude aneuploid embryos. Thus, the possible effects of SYCP3 gene variation on human female infertility deserve further investigation.

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Authors’ roles
S.N.: Involvement in conception and design of the project. Important contribution to critical aspects of the research. Drafted and revised the important intellectual content of the submitted article. T.K.: Important contribution to critical aspects of the research. M.S.: Important contribution to critical aspects of the research. H.B.: Important contribution to critical aspects of the research. H.N.: Important contribution to critical aspects of the research. N.I.: Involvement in conception and design of the project. Helped to draft and revise the important intellectual content of the submitted article. Y.U.: Involvement in conception and design of the project. Helped to draft and revise the important intellectual content of the submitted article. H.K.: Involvement in conception and design of the project. Helped to draft and revise the important intellectual content of the submitted article. Approval of the final version of the submitted manuscript.

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