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ORIGINAL ARTICLE

Male Infertility

The association of stromal antigen 3 (*STAG3*) sequence variations with spermatogenic impairment in the male Korean population

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The stromal antigen 3 *(STAG3)* gene, encoding a meiosis-specific cohesin component, is a strong candidate for causing male infertility, but little is known about this gene so far. We identified *STAG*3 in patients with nonobstructive azoospermia (NOA) and normozoospermia in the Korean population. The coding regions and their intron boundaries of *STAG3* were identified in 120 Korean men with spermatogenic impairments and 245 normal controls by using direct sequencing and haplotype analysis. A total of 30 sequence variations were identified in this study. Of the total, seven were exonic variants, 18 were intronic variants, one was in the 5'-UTR, and four were in the 3'-UTR. Pathogenic variations that directly caused NOA were not identified. However, two variants, c.3669+35C>G (rs1727130) and +198A>T (rs1052482), showed significant differences in the frequency between the patient and control groups (*P* = 0.021, odds ratio [OR]: 1.79, 95% confidence interval [CI]: 1.098–2.918) and were tightly linked in the linkage disequilibrium (LD) block. When pmir-rs1052482A was cotransfected with *miR-3162-5p,* there was a substantial decrease in luciferase activity, compared with pmir-rs1052482T. This result suggests that rs1052482 was located within a binding site of *miR-3162-5p* in the *STAG3* 3'-UTR, and the minor allele, the rs1052482T polymorphism, might offset inhibition by *miR-3162-5p*. We are the first to identify a total of 30 single-nucleotide variations (SNVs) of *STAG3* gene in the Korean population. We found that two SNVs (rs1727130 and rs1052482) located in the 3'-UTR region may be associated with the NOA phenotype. Our findings contribute to understanding male infertility with spermatogenic impairment.

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INTRODUCTION

Azoospermia affects approximately 1% of the male population, accounts for over 15% of all male infertility,[1–3] and includes genital tract obstruction (obstructive azoospermia) and spermatogenic impairment (nonobstructive azoospermia, NOA).[4,5] NOA is mainly caused by severely impaired spermatogenesis and is reported to account for more than 70% of azoospermia in Korean patients.[6] Chromosomal abnormalities, such as Klinefelter syndrome, balanced chromosomal rearrangements, and Yq microdeletions, are well known genetic causes of NOA.[7] In many cases, the genetic etiology remains unknown. Matzuk and Lamb[8] reviewed many genes involved in spermatogenesis and mutations in some of those genes were identified in patients with NOA.[9–11]

The stromal antigen 3 (*STAG3*) gene was mapped to chromosome 7 and consists of 34 exons encoding a protein involved in the meiotic cohesion complex.[12] Human *STAG3* is highly expressed in the testis and several other organs including the ovary.[13,14] During meiosis, STAG3 forms a cohesion core with three other proteins, structural maintenance of chromosome 3 (SMC3), structural maintenance of chromosomes 1β (SMC1β), and Rad21 cohesin complex component like 1 (Rad21L1).[15,16] In mice, defective Stag3 protein causes aberrant meiotic chromosomal features and infertility.[17,18] In humans, a homozygous 1-bp deletion in *STAG3* has been found in a consanguineous family with premature ovarian failure (POF),[18] and a homozygous donor splice-site mutation has been found in two sisters with premature ovarian insufficiency (POI).[19] Therefore, *STAG3* has been suggested as a strong candidate gene target for causing male infertility.[14,20,21] To date, no homozygous or compound heterozygotic mutations of *STAG3* have been identified in patients with spermatogenic impairment, and studies of *STAG3* mutations have not been examined in infertile male populations.

In this study, we investigated whether *STAG3* variations may be a genetic cause of spermatogenic impairment in Korean men.

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PATIENTS AND METHODS

Subjects

A total of 120 Korean men with spermatogenic impairment (43 oligozoospermic and 77 azoospermic) and 245 normal controls were obtained from the Cha Gangnam Medical Center at Cha University, Seoul, Korea, between January 2010 and December 2012. General and clinical characteristics of patients and controls are presented in **Table 1**. Patients with tubule obstruction, chromosomal abnormalities, or microdeletion of the Y chromosome AZF region were excluded. Normal controls had a normal sperm concentration and no history of infertility. Testicular size was measured by a Prader orchidometer (Pro-Health Product Ltd., Guangzhou, China). Serum testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were measured on a Cobas e601 analyzer (Roche Dignostics, Penzberg, Germany) using electrochemiluminescence immunoassay (ECLIA) method. Semen analysis was performed according to the World Health Organization criteria (WHO, 2010).[22] The study was approved by the Institutional Review Board of Cha Gangnam Medical Center, Seoul, Korea, and written informed consent was obtained from all participants.

DNA extraction

Genomic DNA was isolated from peripheral blood of the patient and control samples with the QuickGene DNA blood kit (KURABO industries, Neyagawa, Japan), according to the manufacturer's instructions. DNA yield was quantified with the NanoDrop™ spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The extracted DNA was stored at −80°C until further analysis.

Polymerase chain reaction

Coding regions of *STAG3* (NM_001282716.1) were amplified for genetic screening by polymerase chain reaction (PCR), using primers for the 34 exons and their intron boundaries that were designed by Primer 3 (http://primer3.ut.ee). As this gene has multiple pseudogenes, we performed long-range PCR that produced eleven fragments and designed eleven primer pairs to cover the 34 exons and avoid pseudogene sequences. The locations and sequences of primer sets are presented in **Supplementary Table 1**.

Sequencing analysis

All eleven PCR products were purified with ExoSAP-IT (USB Corporation, Cleveland, OH, USA). To sequence the eleven fragments, we designed 30 sequencing primers to cover 34 exons (**Supplementary Table 1**). All the samples were amplified by PCR and sequenced bidirectionally using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Austin, TX, USA) and BigDye® X-Terminator™ solutions (Applied Biosystems, Bedford, MA, USA) with standard conditions. The sample supernatant was loaded on the ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and processed with a BigDye® X-Terminator run module. All assays were repeated once for confirmation and the results matched over 99.0%.

Statistical analyses and database search

For each sequence variation, the data were statistically analyzed with Statistical Package for the Social Sciences software (SPSS version 22, IBM Software Group, Chicago, IL, USA). To evaluate the association between patient and control groups, odds ratios (OR), 95% confidence intervals (95% CI), and the applied P values were calculated from both the Chi-squared test and Fisher's exact test (two-tailed). Applied $P < 0.05$ were considered statistically significant. Three databases, Polyphen-2, SIFT, and Mutation Tester, were used to predict potentially damaging effects due to amino acid changes.

Multiple hypothesis testing was performed with the Benjamini–Hochberg method[23] to control false discovery rate (FDR) in the logistic regression analysis. Calculating the FDR is a way to address problems associated with multiple comparisons, and FDR provides a measure of the expected proportion of false-positives in the data.

Haplotype block structure was established by HaploView 4.1 software (https://www.broadinstitute.org) using the method of block definition of Gabriel *et al.*[24] Haplotype association tests were also conducted with this software.

Molecular cloning

The entire 3'-untranslated region (3'-UTR) of *STAG3* was amplified from genomic DNA, which contained the rs1052482 A or T alleles, using primers that included *Sac*I and *Xba*I restriction sites. Primer sequences of the *STAG3* 3'-UTR were: forward: 5'-GAGCTCccgttgctgtgtcctgtgta, reverse: 5'-TCTAGAgaccaagaacctgacctcca (for a predicted 476 bp product). PCR products were cloned into the pmirGLO vector (Promega, Madison, WI, USA) via the *Sac*I and *Xba*I sites and all constructs were verified by DNA sequencing.

Dual luciferase assay

HEK293T cells were seeded in 48-well plates (3×10^4 per well). After 24 h, 200 ng pmir-rs1052482A, pmir-rs1052482T, or pmir-empty vector was transiently transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The Renilla vector was used as an internal

Table 1: Participants' clinical characteristics

Characteristics	*Patients with spermatogenic impairment (43 oligozoospermia + 77 azoospermia)*	*Controls*	*P*
Patients (*n*)	120	245	
Age (year), mean±s.d.	33.9±3.7	33.5±2.6	0.783
Semen volume[a] (ml), mean±s.d.	2.9±1.2	3.3±1.5	0.591
Sperm concentration[a] (10^6 ml^{-1}), mean±s.d.	10.0±5.4	74.9±28.0	<0.01
Sperm motility[a] (%), mean±s.d.	26.8±12.6	45.7±11.1	0.013
Sperm morphology[a] (% normal forms), mean±s.d.	2.0±1.3	6.4±2.1	<0.01
Rt. testis volume (ml), mean±s.d.	16.0±6.2	23.0±2.7	0.020
Lt. testis volume (ml), mean±s.d.	17.0±6.2	23.0±2.7	0.025
Serum FSH (mIU ml^{-1}), mean±s.d.	17.6±10.7	4.4±1.6	<0.01
Serum testosterone (ng ml^{-1}), mean±s.d.	4.0±1.1	3.6±1.5	0.486
Serum LH (mIU ml^{-1}), mean±s.d.	5.5±2.4	3.9±2.0	0.106

[a]Data of the azoospermic patients were excluded. s.d.: standard deviation; Rt: right; Lt: left; FSH: follicle-stimulating hormone; LH: luteinizing hormone

control for transfection efficiency. After 48 h, the transfected cells were harvested and lysed with a dual-luciferase reporter assay system (Promega), and the activities of Firefly and Renilla were measured in a Luminometer, Centro XS LB960 (Berthold Technologies, Bad Wildbad, Germany), and MikroWin2000 software (https://mikrowin-20001.software.informer.com). Transfection experiments were performed in triplicate, and activity measurements were done for three times. Relative luciferase activity was determined by normalizing firefly luciferase activity against Renilla luciferase activity. An average value of firefly/Renilla was calculated and then normalized to the average value of the empty vector to yield the vector-normalized ratio.

MicroRNAs (miRNAs)

Computational prediction of putative targets for *STAG3* mRNA was performed by searching mirmap.ezlab.org, www.targetscan.org, www.microrna.org, and www.mirdb.org for the target prediction algorithms. From the potential miRNAs interacting with *STAG3* mRNA 3'-UTR, we selected seven candidate miRNAs (*miR-148a, miR-2909, miR-3162-5p, miR-33a-5p, miR-33b-5p, miR-4739*, and *miR-6508-3p*) that allowed rs1052482A or T alleles to be included in the seed sequence. Seven miRNAs and their inhibitors were constructed by Bioneer (Daejeon, Korea). The prediction score of the seven miRNAs and the 3'-UTR of *STAG3* and details for their *in silico* interactions are presented in **Supplementary Figure 1** and **Supplementary Table 2**. Mimics (5–10 pmol) were transiently cotransfected with pmir-vectors with Lipofectamine 2000 according to the manufacturer's instructions.

RESULTS

Thirty single-nucleotide variations (SNVs) in *STAG3* were identified in this study. The locations, types, frequencies, and *P* values of the variations are presented in **Supplementary Table 3**. The distributions of genotypes of all the SNVs followed the Hardy–Weinberg equilibrium in patients and controls. Seven were exonic, 18 were intronic, one was in the 5'-UTR, and four were in the 3'-UTR of the 30 variations. Three exonic variants were nonsynonymous and the other four variants were synonymous. Three variants were found in a patient but not in controls (c.1269C>T p.Asp423, +112G>A, +315C>T); two variants, c.3669+35C>G and +198A>T, showed significant differences in the frequency between patient and control groups ($P = 0.021$, OR: 1.79, 95% CI: 1.098–2.918). Haplotype analysis by HaploView 4.1 showed that nineteen variants were separated into five linkage disequilibrium (LD) blocks (**Figure 1**). As shown in **Table 2**, in particular, the frequencies of G-C-A (case: control = 0.504: 0.606, $P = 0.009$), G-G-T (case: control = 0.162: 0.075, $P < 0.001$), and C-C-A (case: control = 0.075: 0.018, $P < 0.001$) haplotypes in block 5 were significantly different between patients and controls.

Table 3 shows a genetic model of the 3 SNVs (rs2246713, rs1727130, and rs1052482) between the cases and the controls. We found that the individuals with the CG genotype of rs1727130 and AT genotype of rs1052482 had an increased risk susceptibility to NOA in the codominant model, and those with the minor allele G of rs1727130 and T of rs1052482 had an increased risk susceptibility to NOA in the dominant model ($P = 0.039$, OR: 1.64, 95% CI: 1.030–2.608).

To determine whether variations in the 3'-UTR region affected miRNA-mediated gene expression regulation, miRNAs predicted to interact with *STAG3* were examined by *in silico* analysis, and seven miRNAs, *miR-148a, miR-2909, miR-3162-5p, miR-33a-5p, miR-33b-5p, miR-4739,* and *miR-6508-3p*, were found potentially to interact with the 3'-UTR of *STAG3* mRNA. The effect of these seven miRNAs on +198A>T variation (rs1052482) was examined by a luciferase assay.

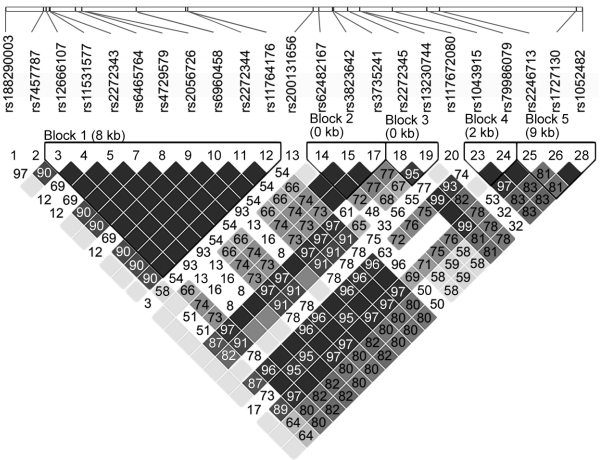

Figure 1: LD pattern in the locus of *STAG3* gene. Nineteen variants were separated into five LD blocks. Numbers in the squares indicate D' index (level of LD) between the corresponding SNPs. *STAG3*: stromal antigen 3; SNPs: single-nucleotide polymorphism; LD: linkage disequilibrium.

There was no significant difference in relative luciferase activities between rs1052482A and rs1052482T (**Figure 2a**), but a substantial decrease in luciferase activity was observed in cells cotransfected with pmir-rs1052482A and *miR-3162-5p* (mean ± standard deviation [s.d.]: 51.6% ± 2.5%, $P = 0.002$), compared with control vector pmir-GLO or pmir-rs1052482T (mean ± s.d.: 85.9% ± 3.6%, $P = 0.061$) (**Figure 2b**). A sequential decrease was observed in cells cotransfected with pmir-rs1052482A and *miR-3162-5p* in proportion to the amount of the mimic (rs1052482A [mean ± s.d.], 63.7% ± 1.7%: 45.0% ± 2.7%; rs1052482T [mean ± s.d.], 89.2 ± 5.0%: 78.3% ± 1.4%, respectively), and this reduction was inhibited when the *mir-3162-5p* inhibitor was cotransfected with pmir-rs1052482A and *miR-3162-5p* (**Figure 2c**). The data indicate that *miR-3162-5p* targets the rs1052482A sequence more efficiently than that of rs1052482T.

DISCUSSION

Many autosomal genes such as ring finger protein 212 (*Rnf212*), testis expressed 15 (*Tex15*), syntaxin 2 (*Stx2*), and siah E3 ubiquitin protein ligase 1A (*Siah1a*) are reported to be crucial factors for the meiotic process and spermatogenesis in mouse studies.[25–28] Human homologs of these genes also play a role in meiosis, and variations of these genes are thought to induce spermatogenic impairment.[8,29–31] In recent studies, the homologous deletion of *Stag*3 has been shown to induce sterility associated with the premature arrest of meiotic prophase I in both male and female mice.[18,32] Therefore, we have investigated the association between *STAG3* gene variations and male infertility.

We identified 30 variations in the coding regions and intron boundaries of *STAG3* in patients with NOA and in control samples. Of the 30 variations, seven were exonic and three were found only in different infertile patients. For these variations, we evaluated the potential pathogenic effects by three prediction methods, Polyphen-2, SIFT, and Mutation Taster (**Table 4**). Most variations (six of nine) were considered benign and three variations did not show consistent results in the three predictive programs. Minor allele frequencies (MAFs) of these variations were not significantly different between patients and controls, and these MAFs were similar to those of other Asian populations on the NCBI SNP database from the 1000 Genomes Project. Considering these data, the above-mentioned variations are unlikely to be related to the spermatogenic impairment in our

Table 2: Haplotype analysis between the SNPs of *STAG3* and nonobstructive azoospermia

db SNP ID	*Haplotype*	*Frequency*		*OR (95% CI)*	*P (Fisher)*	*FDR-P*
		Case	*Control*			
rs12666107 rs11531577 rs2272343 rs6465764 rs4729579 rs2056726 rs6960458 rs2272344 rs11764176	Block 1					
	CGAAGGTTT	0.608	0.622	0.136	0.712	0.812
	GGAGCGGCG	0.329	0.320	0.056	0.812	0.812
	GTCGCAGCG	0.062	0.057	0.083	0.773	0.812
rs62482167 rs3823642 rs3735241	Block 2					
	CCC	0.650	0.645	0.018	0.892	0.949
	CTA	0.287	0.290	0.004	0.949	0.949
	GTA	0.062	0.061	0.005	0.946	0.949
rs2272345 rs13230744	Block 3					
	GG	0.595	0.604	0.047	0.829	0.900
	CA	0.333	0.328	0.016	0.900	0.900
	CG	0.063	0.058	0.086	0.769	0.900
	GA	0.009	0.011	0.050	0.823	0.900
rs1043915 rs79986079	Block 4					
	AC	0.621	0.618	0.004	0.949	0.991
	TC	0.321	0.320	0.000	0.991	0.991
	TT	0.058	0.061	0.264	0.878	0.991
rs2246713 rs1727130 rs1052482	Block 5					
	GCA	0.504	0.606	6.817	0.009	0.012
	CGT	0.259	0.300	1.356	0.244	0.244
	GGT	0.162	0.075	13.028	0.0003	0.001
	CCA	0.075	0.018	14.460	0.0001	0.0004

STAG3: stromal antigen 3; SNPs: single-nucleotide polymorphism; NOA: nonobstructive azoospermia; OR: odds ratio; CI: confidence interval; FDR-*P*: false discovery rate-adjusted *P* value

Table 3: The genotype distributions of *STAG3* rs2246713, rs1727130, and rs1052482 in the cases and the controls

SNPs	*Model*	*Genotype*	*Case, n (%)*	*Control, n (%)*	*P (Fisher)*	*OR*	*95% CI*	*FDR-P*
rs2246713	Codominant	GG	52 (43.3)	114 (46.5)				
		GC	55 (45.9)	106 (43.3)	0.638	1.138	0.717–1.806	0.638
		CC	13 (10.8)	25 (10.2)	0.847	1.140	0.541–2.404	0.847
	Dominant	GG	52 (43.3)	114 (46.5)				
		GC + CC	68 (56.7)	131 (53.5)	0.578	0.879	0.566–1.364	0.578
	Recessive	CC	13 (10.8)	25 (10.2)				
		GC + GG	107 (89.2)	220 (89.8)	0.857	1.069	0.526–2.172	0.857
rs1727130	Codominant	CC	36 (30.0)	101 (41.2)				
		CG	67 (55.8)	105 (42.9)	0.021	1.790	1.098–2.918	0.032
		GG	17 (14.2)	39 (15.9)	0.596	1.223	0.617–2.426	0.847
	Dominant	CC	36 (30.0)	101 (41.2)				
		CG + GG	84 (70.0)	144 (58.8)	0.039	1.637	1.030–2.608	0.059
	Recessive	GG	17 (14.2)	39 (15.9)				
		CG + CC	103 (85.8)	206 (84.1)	0.758	0.872	0.471–1.615	0.857
rs1052482	Codominant	AA	36 (30.0)	101 (41.2)				
		AT	67 (55.8)	105 (42.9)	0.021	1.79	1.098–2.918	0.032
		TT	17 (14.2)	39 (15.9)	0.596	1.223	0.617–2.426	0.847
	Dominant	AA	36 (30.0)	101 (41.2)				
		AT + TT	84 (70.0)	144 (58.8)	0.039	1.637	1.030–2.608	0.059
	Recessive	TT	17 (14.2)	39 (15.9)				
		AT + AA	103 (85.8)	206 (84.1)	0.758	0.872	0.471–1.615	0.857

STAG3: stromal antigen 3; SNPs: single-nucleotide polymorphism; OR: odds ratio; CI: confidence interval; FDR-*P*: false discovery rate-adjusted *P* value

Korean male population. Interestingly, two variations, c.3669+35C>G and +198A>T (rs1727130 and rs1052482) located in 3'-UTR, had a significantly different frequency between the patient and control groups. However, there is a discrepancy in this result. According to Yu *et al.*,[33] there is no significant difference in the frequencies of allele and genotype at SNP rs1052482 between patients with NOA and controls and they suggested that this SNP is not associated with azoospermia. Two variants of rs1727130 and rs1052482 are close together (372 bp apart) and tightly linked, as shown in the LD block analysis. The allele distribution of 2 SNVs between the patient and control groups is more evident in the LD block analysis. On the basis of the data, we propose that multiple SNVs linked to a block can interact with each other to regulate gene function, rather than allowing each SNV to function independently.

Table 4: *In silico* analysis for exonic variations and three variations found only in patients

n	*db SNP ID*	*Gene location*	*AA change*	*In silico variant analysis*		
				PolyPhen-2[a]	*SIFT*[b]	*Mutation Taster*[c]
1	rs11531577	c.48G>T	p.Leu16Phe	Benign (1.00/0.00)	Not tolerated	Polymorphism
2	rs2272343	c.106A>C	p.Thr36Pro	Benign (1.00/0.00)	Not tolerated	Polymorphism
3	rs761620488	c.198A>C	p.Lys66Asn	Benign (0.98/0.44)	Tolerated	Polymorphism
4	rs200131656	c.1035A>G	p.Leu345	–	Tolerated	Disease causing
5	rs755877186	c.1269C>T	p.Asp423	–	Tolerated	Polymorphism
6	rs3735241	c.1293A>C	p.Pro431	–	Tolerated	Polymorphism
7	rs1043915	c.2445T>A	p.Ile815	–	Tolerated	Polymorphism
8	rs188384958	+112G>A	3'-UTR	–	–	Polymorphism
9	rs1727131	+315C>T	3'-UTR	–	–	Polymorphism

[a]http://genetics.bwh.harvard.edu/pph 2/; [b]SIFT, http://siftdna.org/; [c]www.mutationtaster.org. AA: amino acid; –: no result; SIFT: sorting intolerant from tolerant

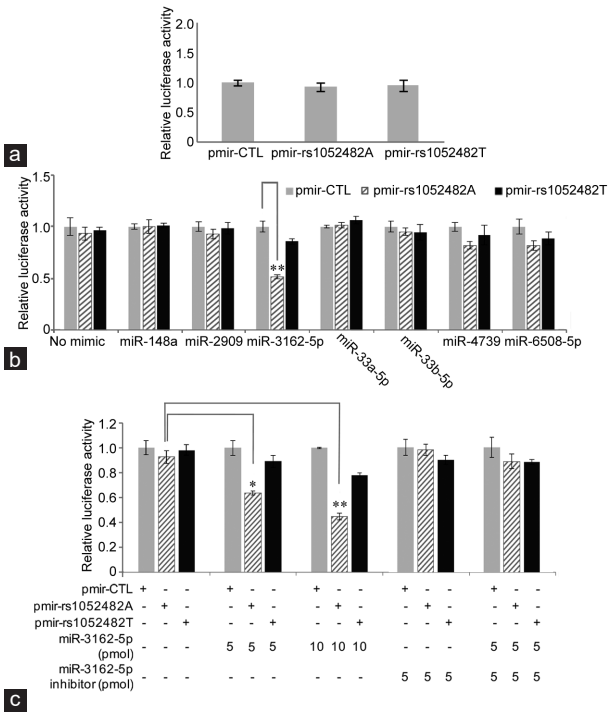

Figure 2: Relative luciferase activities of pmir-rs1052482A and pmir-rs1052482T in HEK293T cells. **a**–**c** represent the mean RLU values ± s.d. of triplicates. (**a**) No significant differences in relative luciferase activity among three vectors. (**b**) Luciferase activity was substantially decreased in cells cotransfected with pmir-rs1052482A and *miR-3162-5p* compared to the cells cotransfected with pmir-control and *miR-3162-5p* ($P < 0.01$). (**c**) A sequential decrease was observed in cells cotransfected with pmir-rs1052482A and *miR-3162-5p* in proportion to the amount of the mimic and this reduction was rescued when the *mir-3162-5p* inhibitor was cotransfected with pmir-rs1052482A and *miR-3162-5p*. -: absence of the indicated one; +: presence of the indicated one; $^{*}P < 0.05$ (pmir-rs1052482A + mir3162-5p [5 pmol] compared to pmir-rs1052482A); $^{**}P < 0.01$ (pmir-rs1052482A + mir3162-5p [10 pmol] compared to pmir-rs1052482A). CTL: control; RLU: relative light unit; s.d.: standard deviation.

The presence of SNVs in the 3'-UTR of genes may interfere with mRNA stability and translation through effects on polyadenylation and regulatory protein–mRNA and miRNA–mRNA interactions, or may locally alter secondary structures of mRNAs, affecting the accessibility of binding sites for interacting transelements.[34–36]

We investigated whether rs1052482, the SNV in the 3'-UTR of *STAG3,* could affect interaction with miRNAs and thus affect posttranscriptional repression of *STAG3*. When pmir-rs1052482A was cotransfected with *miR-3162-5p,* a substantial decrease was observed in luciferase activity compared with pmir-rs1052482T. This result suggests that rs1052482 is located within a binding site for *miR-3162-5p* in the *STAG3* 3'-UTR, and the minor rs1052482T allele may offset the inhibition by *miR-3162-5p*.

According to Fukuda *et al.*,[17,37] STAG3 interacts with the three different α-kleisin subunits present in mammalian meiotic cells, depending on the temporal and spatial distribution. STAG3 combined with meiotic recombination protein (REC8), one of the α-kleisin subunits, and promoted synapsis between homologous chromosomes, while the same complexes inhibited synaptonemal complex assembly between sister chromatids. Therefore, we hypothesize that *STAG3* with the rs1052482T variation reduces the normal inhibitory function of *mir-3162-5p,* thereby increasing the amount of STAG3 protein and ultimately disturbing synapses between homologous chromosomes or sister chromatids. However, it is unclear how elevated STAG3 may affect meiotic chromosome dynamics. In previous studies, *mir-3162-5p* was identified as a regulator of prostate or cervical antigen in cell carcinomas.[38–40] However, little is known about *mir-3162-5p*'s regulation of human meiosis, because of the difficulties in uncovering the spatiotemporal and sequential expression of miRNAs in human germ cells, and identifying which miRNAs are the actual operators for the onset of meiosis or spermatogenesis.

CONCLUSION

We have identified 30 SNVs of *STAG3* in the Korean population. Pathogenic variations that directly cause NOA were not identified. However, we found that two SNVs, rs1727130 and rs1052482, located in the 3'-UTR region may be associated with the NOA phenotype through the regulation of miRNA. Further studies are needed to determine whether variations in the 3'-UTR region of *STAG3* actually affect gene expression through miRNAs, including *mir-3162-5p* in germ cells. While there is still much to learn about the exact mechanisms regulating human meiosis or spermatogenesis, our findings contribute to the understanding of spermatogenic impairment, as well as the identification of predictive susceptibility biomarkers.

AUTHOR CONTRIBUTIONS

SH Shim conceived and designed the article; SH Shim, YN, and KMK drafted the manuscript; YN, KMK, and SRS designed and performed the experiments; SRS, JEP, and YJS analyzed data and interpreted findings. SH Song collected the samples and performed andrology workup. JTS and TKY prepared the publication. All authors read, edited, and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

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Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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Supplementary Table 1: The list of polymerase chain reaction primer and sequencing primer sequences

n	*Range*	*PCR primer Sequence (5′ → 3′)*		*Range*	*Sequencing primer Sequence (5′→3′)*	
P1	Exon1 (404bp)	F	CGCCCAATGGAGTAGGAGAT	Exon1 (404bp)	F	CGCCCAATGGAGTAGGAGAT
		R	ACCTGTCAGAGCCTGGAAGA		R	ACCTGTCAGAGCCTGGAAGA
P2	Exon2-Exon5 (7,413bp)	F	TACCACACCCAGTGTGCAAT	Exon2 (294bp)	F	GCCCTTTCTTCTCTTTCTTCC
		R	GGGGGTACCACAGCTACAGA		R	TCCCACGCATATTATCATCAA
				Exon3 (394bp)	F	AAAAAGACTTTGTCCCAACTTCC
					R	CGGCTCACTGCAAGCTCT
				Exon4 (538bp)	F	GGTTCAGGTGATACGGTTCAT
					R	TGTTCACGTCAAATCAAGTTTGT
				Exon5 (450bp)	F	TTGTTTACCTCCCAGGGTTG
					R	AGTGCCCGGCCTAAATAAGT
P3	Exon6-Exon8 (7,971bp)	F	CTTATTGCCATGGCTTCGTT	Exon6 (297bp)	F	CCCACCTAAGCTCTTTGCAG
		R	CCTGTGGCACATTTTGGTAA		R	TTCCTCCTTCTAAAAGCTACCC
				Exon7 (365bp)	F	GCCCCTATGACTTCATGGAC
					R	AGCCAAGATGCAGGTAGGAA
				Exon8 (395bp)	F	TCATTGCCCTTCTTTCCTTC
					R	ACCCCTTACAGGATGGGTCT
P4	Exon9-Exon13 (3,748bp)	F	TCCGAATAACCACATGCAGA	Exon9 (330bp)	F	CGGGGGTTCACACTATCCTA
		R	GCTCAGCACAACAGGAAACA		R	ATTTTTGCTCCAGCTGCATT
				Exon10 (433bp)	F	CCATGAGAGGGAGTTATCTGG
					R	CTCCCCGTACCTCAGGTTTT
				Exon11 (300bp)	F	AATGAGGGATCGGAGAGG
					R	GCTGGGATAGCCAAGACATC
				Exon12 (399bp)	F	TCTTGGCTATCCCAGCATCT
					R	CCCCCTCAACATACTGCAAC
				Exon13 (425bp)	F	TGCAGTATGTTGAGGGGGTA
					R	GCTGCGAGAAGAAAGGAGAC
P5	Exon14-Exon17 (1,893bp)	F	ATCTGCTGCTGCCCTACCTA	Exon14 (417bp)	F	TCTCCCTGGTGTCTCCTTTC
		R	AAGCAGCTGAGAAGCTGGAG		R	AGGCTGGTCTCAAACTCCTG
				Exon15 (352bp)	F	AATGGAGAAGGATGGGAGTG
					R	CACCTTCCAACTCCAAGCTC
				Exon16 (460bp)	F	TGCTGGAGAAGGACCAGAGT
					R	TGCTGGGATTATAGGCGTAA
				Exon17 (400bp)	F	AAATCTCGTGGGAGCTACTGA
					R	AAGCAGCTGAGAAGCTGGAG
P6	Exon18-Exon21 (1,082bp)	F	GGGGGTGGGAGTAGGAATTA	Exon18 (248bp)	F	GGGGGTGGGAGTAGGAATTA
		R	CTTCCTCGCTTTGTCCACTC		R	GGAACCCAAGTTCTTAGGAAAAA
				Exon19 (387bp)	F	AATGCTTTTAACCCCGTTCC
					R	CAATAGCATTTCCCCCAGAA
				Exon20~21 (526bp)	F	AGCAGGAGCTTGAAGAGCTG
					R	CTTCCTCGCTTTGTCCACTC
P7	Exon22-Exon25 (1,237bp)	F	GAGTGGACAAAGCGAGGAAG	Exon22~23 (553bp)	F	GATGCCTCTGAAGAATGTCCA
		R	TTGGATATCCCCCACCTGTA		R	AAAAGCCTGTAGGGGGAAAA
				Exon24~25 (626bp)	F	GGAGCAACAAGGCGAGTATC
					R	TTGGATATCCCCCACCTGTA
P8	Exon26-Exon29 (1,565bp)	F	TTATTTTGGGCTTTGCACCT	Exon26 (344bp)	F	GGAGTTTGGGAGGGAGACAT
		R	TACCCACACACAGCACCCTA		R	AAGAATGAAGGAACCTATCACG
				Exon27 (371bp)	F	CAAGGCCTTTGGAATTTCTG
					R	AAGGCATACCCACCCCTAAC
				Exon28~29 (602bp)	F	GGGTATGCCTTTGGAGACAA
					R	CCCTGAATGACAGTAGATGCTC
P9	Exon30-Exon32 (2,057bp)	F	AGCCCAGGGGTATGTCTCTT	Exon30 (427bp)	F	TAGGGCTATGCCCATTTGAG
		R	GGAGGATAGGGGGTCATGTT		R	ACAGCAGGGAACCATGAAAC
				Exon31 (387bp)	F	CTCCCACATTGTTGGGTTCT
					R	TGACAGGAAGTGCTCTGTGG
				Exon32 (394bp)	F	CTCACCCATTGCCTCTCTGT
					R	TCTAGATTCATTCAGCTTTTCCA

Contd...

Supplementary Table 1: Contd...

n	*Range*	*PCR primer Sequence (5′ → 3′)*		*Range*	*Sequencing primer Sequence (5′→3′)*	
P10	Exon33 (307bp)	F	TTTGCGAAGTGACAGGAGTG	Exon33 (307bp)	F	TTTGCGAAGTGACAGGAGTG
		R	TACACAGGACACAGCAACGG		R	TACACAGGACACAGCAACGG
P11	Exon34 (490bp)	F	GGGCTTTGAGGGTAACCCAGGG	Exon34 (490bp)	F	GGGCTTTGAGGGTAACCCAGGG
		R	CGATCTCAAGCCACACCTTGG		R	CGATCTCAAGCCACACCTTGG

PCR: polymerase chain reaction

Supplementary Table 2: miRNA target-site prediction software score

Software	*mirmap.org*	*TargetScan*	*microrna.org*	*mirdb.org*
	Relevant score threshold			
	>90	*<–0.4*	*<–0.1*	*>60*
miR-148a	87.6	–0.20	–0.27	
miR-2909	26.3			
miR-3162-5p	93.0	–0.38		
miR-33a-5p	7.8			
miR-33b-5p	8.2			
miR-4739	90.6	–0.40		55
miR-6508-3p	46.7	–0.19		

miRmap: miRmap score; TargetScan: context ++ score; microrna.org: mirSVR score; mirdb.org: Target Score

Supplementary Table 3: Genotypes and allele distributions of STAG3 gene variations

n	*db SNP ID*	*Gene Location*	*SNP function*	*Genotype*	*Case (n=120) (%)*	*Control (n=245) (%)*	*OR*	*95% CI*	*P (Fisher)*	**FDR-p*
1	rs188290003	c.-125C>A	5'-UTR	CC	117 (97.5)	239 (97.6)	1.000			
				CA	3 (2.5)	6 (2.4)	1.021	0.251–4.156	1.000	1.000
				AA	0 (0)	0 (0)	–	–	–	–
				A allele	3	6	1.021	0.253–4.119	1.000	1.000
2	rs7457787	c.-64-247A>C	Intron	AA	101 (84.2)	198 (80.8)	1.000			
				AC	19 (15.8)	42 (17.1)	0.887	0.490–1.604	0.767	0.767
				CC	0 (0.0)	5 (2.0)	–	–	0.174	0.432
				C allele	19	52	0.724	0.418–1.255	0.288	0.432
3	rs12666107	c.-64-97G>C	Intron	GG	16 (13.3)	37 (15.1)	1.000			
				GC	62 (51.7)	111 (45.3)	1.292	0.665–2.508	0.511	1.000
				CC	42 (35.0)	97 (39.6)	1.001	0.503–1.995	1.000	1.000
				C allele	146	305	0.942	0.686–1.294	0.746	1.000
4	rs11531577	c.48G>T	p.Leu16Phe	GG	105 (87.5)	218 (89.0)	1.000			
				GT	15 (12.5)	26 (10.6)	1.198	0.609–2.357	0.725	0.867
				TT	0 (0.0)	1 (0.4)	–	–	–	
				T allele	15	28	1.1	0.576–2.101	0.867	0.867
5	rs2272343	c.106A>C	p.Thr36Pro	AA	105 (87.5)	218 (89.0)	1.000			
				AC	15 (12.5)	26 (10.6)	1.1978	0.609–2.357	0.725	0.867
				CC	0 (0.0)	1 (0.4)	–	–	–	
				C allele	15	28	1.1	0.576–2.101	0.867	0.867
6	rs6465764	c.219+71G>A	Intron	GG	16 (13.3)	37 (15.1)	1.000			
				GA	62 (51.7)	111 (45.3)	1.2917	0.665–2.508	0.511	1.000
				AA	42 (35.0)	97 (39.6)	1.0013	0.503–1.995	1.000	1.000
				A allele	146	305	0.9421	0.686–1.294	0.746	1.000
7	rs761620488	c.198A>C	p.Lys66Asn	AA	120 (100.0)	244 (99.6)	1.000			
				AC	0 (0.0)	1 (0.4)	–	–	–	
				CC	0 (0.0)	0 (0)	–	–	–	
				C allele	0	1	–	–	1.000	1.000
8	rs4729579	c.220-64C>G	Intron	CC	16 (13.3)	37 (15.1)	1.000			
				CG	62 (51.7)	111 (45.3)	1.292	0.665–2.508	0.511	1.000
				GG	42 (35.0)	97 (39.6)	1.001	0.503–1.995	1.000	1.000
				G allele	146	305	0.942	0.686–11.294	0.746	1.000

Contd...

Supplementary Table 3: Contd...

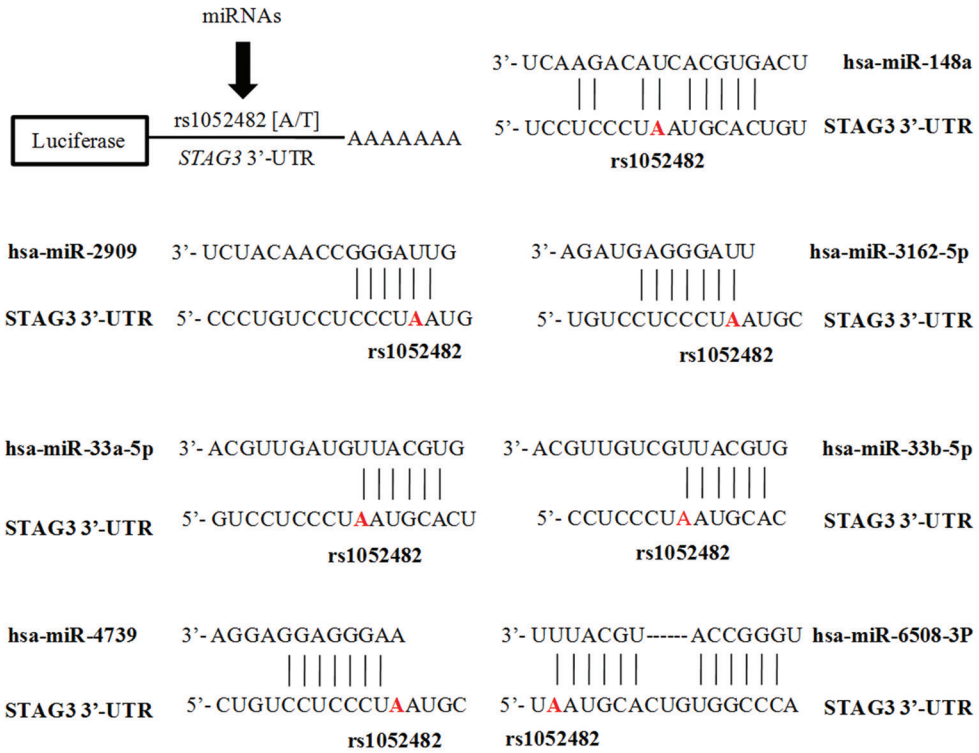
n	*db SNP ID*	*Gene Location*	*SNP function*	*Genotype*	*Case (n=120) (%)*	*Control (n=245) (%)*	*OR*	*95% CI*	*P (Fisher)*	**FDR-p*
9	rs2056726	c.220-63G>A	Intron	GG	105 (87.5)	218 (89.0)	1.000			
				GA	15 (12.5)	26 (10.6)	1.198	0.609–2.357	0.725	0.867
				AA	0 (0.0)	1 (0.4)	–	–	–	
				A allele	15	28	1.100	0.576–2.101	0.867	0.867
10	rs6960458	c.337-109G>T	Intron	GG	16 (13.3)	37 (15.1)	1.000			
				GT	62 (51.7)	111 (45.3)	1.2917	0.6651–2.5084	0.511	1.000
				TT	42 (35)	97 (39.6)	1.001	0.503–1.995	1.000	1.000
				T allele	146	305	0.942	0.686–1.294	0.746	1.000
11	rs2272344	c.715+180C>T	Intron	CC	16 (13.3)	37 (15.1)	1.000			
				CT	62 (51.7)	111 (45.3)	1.292	0.665–2.508	0.511	1.000
				TT	42 (35.0)	97 (39.6)	1.001	0.503–1.995	1.000	1.000
				T allele	146	305	0.942	0.686–1.294	0.746	1.000
12	rs11764176	c.716-104G>T	Intron	GG	16 (13.3)	37 (15.1)	1.000			
				GT	62 (51.7)	111 (45.3)	1.292	0.665–2.508	0.511	1.000
				TT	42 (35.0)	97 (39.6)	1.001	0.503–1.995	1.000	1.000
				T allele	146	305	0.942	0.686–1.294	0.746	1.000
13	rs200131656	c.1035A>G	p.Leu345	AA	111 (92.5)	225 (91.8)	1.000			
				AG	9 (7.5)	20 (8.2)	0.912	0.402–2.069	1.000	1.000
				GG	0 (0)	0 (0)	–	–	–	
				G allele	9	20	0.916	0.410–2.042	1.000	1.000
14	rs62482167	c.1066-186C>G	Intron	CC	105 (87.5)	215 (87.8)	1.000			
				CG	15 (12.5)	30 (12.2)	1.024	0.528–1.985	1.000	1.000
				GG	0 (0)	0 (0)	–	–	–	
				G allele	15	30	1.022	0.539–1.939	1.000	1.000
15	rs3823642	c.1245-26T>C	Intron	TT	17 (14.2)	39 (15.9)	1.000			
				TC	50 (41.7)	96 (39.2)	1.195	0.615–2.322	0.621	0.934
				CC	53 (44.2)	110 (44.9)	1.105	0.573–2.133	0.868	0.934
				C allele	156	316	1.023	0.740–1.413	0.934	0.934
16	rs755877186	c.1269C>T	p. Asp423	CC	119 (99.2)	245 (100.0)	1.000			
				CT	1 (0.8)	0 (0.0)	–	–	–	
				TT	0 (0)	0 (0)	–	–	–	
				T allele	1	0	–	–	0.329	0.329
17	rs3735241	c.1293A>C	p.Pro431	AA	17 (14.2)	38 (15.5)	1.000			
				AC	50 (41.7)	96 (39.2)	1.195	0.615–2.322	0.621	0.934
				CC	53 (44.2)	111 (45.3)	1.105	0.573–2.133	0.868	0.934
				C allele	156	318	1.023	0.740–1.413	0.934	0.934
18	rs2272345	c.1573+41C>G	Intron	CC	18 (15.0)	41 (16.7)	1.000			
				CG	59 (49.2)	107 (43.7)	1.256	0.663–2.379	0.526	1.000
				GG	43 (35.8)	97 (39.6)	1.010	0.522–1.954	1.000	1.000
				G allele	145	301	0.958	0.699–1.315	0.809	1.000
19	rs13230744	c.1678-67A>G	Intron	AA	12 (10.0)	35 (14.3)	1.000			
				AG	58 (48.3)	96 (39.2)	1.762	0.847–3.665	0.162	0.485
				GG	50 (41.7)	114 (46.5)	1.279	0.613–2.668	0.588	0.882
				G allele	158	324	0.987	0.713–1.367	1.000	1.000
20	rs117672080	c.1678-58A>G	Intron	AA	107 (89.2)	221 (90.2)	1.000			
				AG	13 (10.8)	23 (9.4)	1.167	0.569–2.394	0.710	0.860
				GG	0 (0)	1 (0.4)	–	–	–	
				G allele	13	25	1.065	0.535–2.121	0.860	0.860
21	rs200967267	c.2133-36C>A	Intron	CC	117 (97.5)	242 (98.8)	1.000			
				CA	3 (2.5)	3 (1.2)	2.068	0.411–10.405	0.399	0.401
				AA	0 (0)	0 (0)	–	–	–	
				A allele	3	3	2.055	0.412–10.258	0.401	0.401
22	rs1043915	c.2445T>A	p. Ile815	TT	14 (11.7)	39 (15.9)	1.000			
				TA	63 (52.5)	109 (44.5)	1.61	0.812–3.194	0.189	0.556
				AA	43 (35.8)	97 (39.6)	1.235	0.608–2.508	0.600	0.900
				A allele	149	303	1.011	0.735–1.389	1.000	1.000

Contd...

Supplementary Table 3: Contd...

n	*db SNP ID*	*Gene Location*	*SNP function*	*Genotype*	*Case (n=120) (%)*	*Control (n=245) (%)*	*OR*	*95% CI*	*P (Fisher)*	**FDR-p*
23	rs150085849	c.2395-20C>T	Intron	CC	118 (98.3)	241 (98.4)	1.000			
				CT	2 (1.7)	4 (1.6)	1.021	0.184–5.655	1.000	1.000
				TT	0 (0)	0 (0)	–	–	–	
				T allele	2	4	1.021	0.186–5.614	1.000	1.000
24	rs79986079	c.2803-206C>T	Intron	CC	106 (88.3)	216 (88.2)	1.000			
				CT	14 (11.7)	28 (11.4)	1.019	0.515–2.016	1.000	1.000
				TT	0 (0.0)	1 (0.4)	–	–	–	
				T allele	14	30	0.95	0.494–1.827	1.000	1.000
25	rs2246713	c.3081-38G>C	Intron	GG	52 (43.3)	114 (46.5)	1.000			
				GC	55 (45.8)	106 (43.3)	1.138	0.717–1.806	0.638	0.847
				CC	13 (10.8)	25 (10.2)	1.140	0.541–2.404	0.847	0.847
				C allele	81	156	1.091	0.786–1.514	0.614	0.847
26	rs1727130	c.3669+35C>G	Intron	CC	36 (30.0)	101 (41.2)	1.000			
				CG	67 (55.8)	105 (42.9)	1.79	1.098–2.918	0.021	0.063
				GG	17 (14.2)	39 (15.9)	1.223	0.617–2.426	0.596	0.596
				G allele	101	183	1.219	0.890–1.670	0.226	0.339
27	rs188384958	+112G>A	3'-UTR	GG	119 (99.2)	245 (100.0)	1.000			
				GA	1 (0.8)	0 (0.0)	–	–	0.329	0.329
				AA	0 (0)	0 (0)	–	–	–	
				A allele	1	0	–	–	0.329	0.329
28	rs1052482	+198A>T	3'-UTR	AA	36 (30.0)	101 (41.2)	1.000			
				AT	67 (55.8)	105 (42.9)	1.79	1.098–2.918	0.021	0.063
				TT	17 (14.2)	39 (15.9)	1.223	0.617–2.426	0.596	0.596
				T allele	101	183	1.219	0.890–1.670	0.226	0.339
29	rs1727131	+315C>T	3'-UTR	CC	119 (99.2)	245 (100.0)	1.000			
				CT	1 (0.8)	0 (0.0)	–	–	0.329	0.329
				TT	0 (0)	0 (0)	–	–	–	
				T allele	1	0	–	–	0.329	0.329
30	rs12056000	+370G>A	3'-UTR	GG	105 (87.5)	216 (88.2)	1.000			
				GA	13 (10.8)	18 (7.3)	1.486	0.701–3.147	0.322	0.483
				AA	2 (1.7)	11 (4.5)	0.374	0.081–1.718	0.238	0.483
				A allele	17	40	0.858	0.476–1.547	0.662	0.662

STAG3: stromal antigen 3; SNPs: single-nucleotide polymorphism; NOA: nonobstructive azoospermia; OR: odds ratio; CI: confidence interval; FDR-*P*: false discovery rate-adjusted *P* value; –: 0, NaN (not a number) or infinity



Supplementary Figure 1: Schematic diagram of luciferase reporter construct and *in silico* interaction of the potential microRNAs with rs1052482 of *STAG3* Seven candidate miRNAs (miR-148a, miR-2909, miR-3162-5p, miR-33a-5p, miR-33b-5p, miR-4739, and miR-6508-3p) that allow rs1052482A or T alleles to be included in the seed sequence. *STAG3*: stromal antigen 3.