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1 **An association study of four candidate loci for human male fertility traits with**
2 **male infertility**

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16 **Running title:** Candidate polymorphisms associate with male infertility

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1 **Abstract**

2 **STUDY QUESTION:** Are the four candidate loci (rs7867029, rs7174015, rs12870438,
3 and rs724078) for human male fertility traits, identified in a genome-wide association
4 study (GWAS) of a Hutterite population in the USA, associated with male infertility in
5 a Japanese population?

6 **SUMMARY ANSWER:** rs7867029, rs7174015, and rs12870438 are significantly
7 associated with the risk of male infertility in a Japanese population.

8 **WHAT IS KNOWN ALREADY:** Recently, a GWAS of a Hutterite population in the
9 USA revealed that 41 single nucleotide polymorphisms (SNPs) were significantly
10 correlated with family size or birth rate. Of these, four SNPs (rs7867029, rs7174015,
11 rs12870438, and rs724078) were found to be associated with semen parameters in
12 ethnically diverse men from Chicago.

13 **STUDY DESIGN, SIZE, DURATION:** This is a case-control association study in a
14 total of 917 Japanese subjects, including 791 fertile men, 76 patients with azoospermia,
15 and 50 patients with oligozoospermia.

16 **PARTICIPANTS/MATERIALS, SETTING, METHODS:** Azoospermia was
17 diagnosed on the basis of semen analysis (absence of sperm in ejaculate), serum
18 hormone levels, and physical examinations. Oligozoospermia was defined as a sperm
19 concentration of less than $20 \times 10^6/\text{mL}$. We excluded patients with any known cause of
20 infertility (i.e., obstructive azoospermia, varicocele, cryptorchidism, hypogonadotropic
21 hypogonadism, karyotype abnormalities, or complete deletion of *AZF* a, b, or c). The
22 SNPs rs7867029, rs7174015, rs12870438, and rs724078 were genotyped using DNA
23 from peripheral blood samples and either restriction fragment length polymorphism
24 PCR or TaqMan probes. Genetic associations between the four SNPs and male

1 infertility were assessed using a logistic regression analysis under three different
2 comparative models (additive, recessive, or dominant)

3 **MAIN RESULTS AND THE ROLE OF CHANCE:** The genotypes of all
4 four SNPs were in HWE in the fertile controls. The SNPs rs7867029 and rs7174015 are
5 associated with oligozoospermia (rs7867029: odds ratio [OR] = 1.70, 95% confidence
6 interval [CI] = 1.07–2.68, $P = 0.024$ [log-additive]; rs7174015: OR = 6.52, 95% CI =
7 1.57–27.10, $P = 0.0099$ [dominant]), and rs12870438 is associated with azoospermia
8 (OR = 10.90, 95% CI = 2.67–44.60, $P = 0.00087$ [recessive]) and oligozoospermia (OR
9 = 8.54, 95% CI = 1.52–47.90, $P = 0.015$ [recessive]). The association between
10 rs7174015 and oligozoospermia under a dominant model and between rs12870438 and
11 azoospermia under additive and recessive models remained after correction for multiple
12 testing. There were no associations between rs724078 and azoospermia or
13 oligozoospermia.

14
15 **LIMITATIONS, REASONS FOR CAUTION:** Even though the sample size of case
16 subjects was not very large, we found that three SNPs were associated with the risk of
17 male infertility in a Japanese population.

18 **WIDER IMPLICATIONS OF THE FINDINGS:** The three infertility-associated
19 SNPs may be contributing to a quantitative reduction in spermatogenesis.

20 **STUDY FUNDING/COMPETING INTEREST(S):** This study was supported in part
21 by the Ministry of Health and Welfare of Japan (1013201) (to T. I.), Grant-in-Aids for
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24 Foundation (to A.T.). None of the authors has any competing interests to declare.

1

2 **Keywords:** case-control association study/ male infertility/ azoospermia/

3 oligozoospermia/ Japanese population

4

1 **Introduction**

2 Infertility is a major problem worldwide that affects approximately 10% of
3 couples, and 40–50% of these problems are due to male-factor etiology (Skakkebaek *et*
4 *al.*, 1994; McLachlan and Kretser, 2001; Maduro and Lamb, 2002). The main cause of
5 male infertility is spermatogenic failure such as azoospermia and oligozoospermia. In
6 terms of the genetic background underlying male infertility, deletion of the three
7 azoospermia factor (*AZF*) regions (termed *AZF*a, b, and c) of the long arm of the Y
8 chromosome (Yq) has been detected in 10–15% of men with nonobstructive azoospermia
9 or severe oligozoospermia (Vogt *et al.*, 1996; Vogt, 1998; Krausz and McElreavey, 1999;
10 Maurer and Simoni, 2000; McElreavey *et al.*, 2000). Aside from the genes in the Y
11 chromosome, polymorphisms in certain genes, such as those encoding glutathione S-
12 transferases (Pajarinen *et al.*, 1996; Chen *et al.*, 2002; Finotti *et al.*, 2009; Polonikov *et*
13 *al.*, 2010), 5-methylenetetrahydrofolate reductase (Bezold *et al.*, 2001; Park *et al.*, 2005;
14 Singh *et al.*, 2005), and ADP-ribosyltransferase 3 (Okada *et al.*, 2008; Norambuena *et al.*,
15 2012), have been reported to be associated with male infertility.

16 To date, there have been four genome-wide association studies (GWASs)
17 regarding male fertility and infertility (Aston *et al.*, 2009; Hu *et al.*, 2012; Zhao *et al.*,
18 2012; Kosova *et al.*, 2012). Of these GWASs, a GWAS in a Hutterite population in the
19 USA revealed that 41 single nucleotide polymorphisms (SNPs) are significantly
20 correlated with family size or birth rate ($P < 1 \times 10^{-4}$). Hutterites comprise a founder
21 population of European descent that traditionally proscribes contraception and uniformly
22 desires large families. Of 41 SNPs, the following were found to be associated with sperm
23 concentration or total sperm count in ethnically diverse men from Chicago, USA:
24 rs7867029, which is downstream of *PSATI*, the gene that encodes phosphoserine

1 aminotransferase 1; rs7174015, which is in *USP8*, the gene that encodes ubiquitin specific
2 peptidase 8; rs12870438, which is in *EPSTH1*, the gene that encodes the epithelial stromal
3 interaction protein 1; and rs724078, which is upstream of *MAS1L*, the gene that encodes
4 the MAS1 oncogene-like protein, and downstream of *UBD*, the gene that encodes
5 ubiquitin D (Kosova *et al.*, 2012).

6 Associated conditions, azoospermia and oligozoospermia, were defined as the
7 absence of sperm in ejaculate and a sperm concentration of less than $20 \times 10^6/\text{mL}$,
8 respectively. We hypothesized that these four aforementioned SNPs might also be
9 associated with the risk of male infertility in a Japanese population. Hence, in this study,
10 we conducted a case-control association study to assess whether the SNPs rs7867029,
11 rs7174015, rs12870438, and rs724078 were associated with infertility in Japanese males.

1 **Materials and Methods**

2 **Subjects**

3 This study was approved by the ethics committees of the University of
4 Tokushima and St. Marianna Medical University. All participants provided written
5 informed consent.

6 The 791 fertile Japanese men (31.2 ± 4.8 years; mean \pm SD) were used as the
7 control sample. The fertile subjects in this study have been described in previous reports
8 (Iwamoto *et al.*, 2013). Briefly, fertile men were recruited from the partners of pregnant
9 women who attended obstetric clinics in four cities in Japan (Sapporo, Kanazawa,
10 Osaka, and Fukuoka). The eligibility criteria for the male participants were as follows:
11 the participants had to have been aged 20–45 years at the time of invitation by the
12 hospital at which they were recruited, and both the man and his mother had to have been
13 born in and living in Japan. In addition, the pregnancy of the female partner had to have
14 been the result of conception by sexual intercourse and not by fertility treatment.

15 Some of the subjects in this study have been described in previous reports (Sato
16 *et al.*, 2013). Briefly, 126 patients who consecutively presented as infertile at the
17 Department of Urology, St. Mariana University Hospital, Kanagawa Prefecture, Japan,
18 were enrolled from 2000 to 2011; of these patients, 76 (aged 33.2 ± 5.6 years; mean \pm
19 SD) were diagnosed as having azoospermia and 50 (aged 35.1 ± 6.1 years; mean \pm SD)
20 were diagnosed as having oligozoospermia. Semen analysis was performed in
21 accordance with the 4th edition WHO Laboratory Manual for the Examination of
22 Human Semen (World Health Organization, 1999). According to the 4th edition WHO
23 guidelines (1999) criteria, azoospermia patients were diagnosed on the basis of semen
24 analysis (absence of sperm in ejaculate), serum hormone levels, and the results of

1 physical examinations. Oligozoospermia was defined as a sperm concentration of less
2 than $20 \times 10^6/\text{mL}$. We excluded patients with any known cause of infertility (i.e.,
3 obstructive azoospermia, varicocele, cryptorchidism, hypogonadotropic hypogonadism,
4 karyotype abnormalities, or complete deletion of *AZF* a, b, or c). Deletions in *AZF* a, b,
5 and c were analyzed according to European Academy of Andrology and the European
6 Molecular Genetics Quality Network best practice guidelines (Simoni *et al.*, 2004).

7

8 **Genotyping**

9 Genomic DNA was extracted from the peripheral blood samples of subjects
10 using a QIAamp DNA blood kit (Qiagen; Tokyo, Japan). From SNPs previously
11 reported to show associations with sperm concentration, semen volume, total sperm
12 count, total motile sperm count, or sperm motility (Kosova *et al.*, 2012), 4 SNPs
13 (rs7867029, rs12870438, rs7174015, and rs724078) with minor allele frequencies $>$
14 0.05 in the HapMap-JPT population were selected for genotyping. The rs12870438 SNP
15 was detected by restriction fragment length polymorphism -PCR using the following
16 primer sets: 5' - GCAAACAGGAGAAGGGTGTT -3' (forward) and 5' -
17 GCTTTGGAGCATGTTTTCCC -3' (reverse). DNA from each subject was amplified
18 using Taq DNA polymerase (Promega; Tokyo, Japan) under the appropriate
19 amplification conditions. The resulting PCR products were then digested using the *HhaI*
20 restriction enzyme (New England Biolabs Japan Inc.; Tokyo, Japan). The digested
21 products were separated by electrophoresis on a 2.5% agarose gel. The following
22 fragment sizes were used for allele identification on gels: 488 bp (A-allele) and 278 +
23 210 bp (G-allele). The rs7867029, rs7174015, and rs724078 SNPs were genotyped
24 using TaqMan probes rs7867029 (C_31364474_20), rs7174015 (C_32072246_10), and

1 rs724078 (C_2500858_10; Applied Biosystems; Tokyo, Japan) with the ABI 7900HT
2 real-time PCR system (Applied Biosystems).

3

4 **Statistical analysis**

5 Hardy–Weinberg equilibrium (HWE) was assessed in control samples by using
6 an internet-based HWE calculator (<http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>). Odds ratios
7 (ORs) and their 95% confidence intervals (CIs) were calculated using logistic regression
8 analysis. All statistical analyses were performed using R version 3.0.2 (The R Project for
9 Statistical Computing [<http://www.r-project.org>]), and statistical significance was
10 considered at P -value < 0.05 . Correction for multiple testing was performed with a factor
11 of eight (four SNPs and two phenotypes).

12

1 **Results**

2 The genotype and allele frequencies of the four SNPs among 791 fertile
3 controls, and 76 azoospermia and 50 oligozoospermia patients are shown in Table I. The
4 genotyping of the SNPs was complete except for rs12870438 (the missing genotyping
5 rate was 0.3%), and the genotypes of all four SNPs were in HWE in the fertile controls.
6 Next, we assessed genetic associations between the four SNPs and male infertility in a
7 case-control study design using a logistic regression analysis under three different
8 comparative models (additive, recessive, or dominant) to verify whether the genetic
9 model effects were consistent with the male fertility trait associations reported
10 previously. The results of the logistic regression analysis from different comparative
11 genetic models are summarized in Table II. There was a statistically significant
12 association between rs7867029 and oligozoospermia in two models: log-additive (OR =
13 1.70, 95% CI = 1.07–2.68, $P = 0.024$) and recessive (OR = 3.14, 95% CI = 1.16–8.55, P
14 = 0.025). However, there was no association between rs7867029 and azoospermia.
15 Similarly, rs7174015 showed a significant association with oligozoospermia in two
16 models: log-additive (OR = 1.56, 95% CI = 1.02–2.39, $P = 0.042$) and dominant (OR =
17 6.52, 95% CI = 1.57–27.10, $P = 0.0099$), but not with azoospermia. SNP rs12870438
18 showed significant associations with azoospermia in three models: log-additive (OR =
19 1.92, 95% CI = 1.21–3.05, $P = 0.0059$), recessive (OR = 10.90, 95% CI = 2.67–44.60, P
20 = 0.00087), and dominant (OR = 1.71, 95% CI = 1.01–2.89, $P = 0.046$). In addition,
21 rs12870438 showed a significant association with oligozoospermia in the recessive
22 model (OR = 8.54, 95% CI = 1.52–47.90, $P = 0.015$). Among these, the association
23 between rs7174015 and oligozoospermia under a dominant model and between
24 rs12870438 and azoospermia under additive and recessive models remained after

1 correction for multiple testing (P -value < 0.0063). There were no associations between
2 rs724078 and azoospermia or oligozoospermia.
3

1 Discussion

2 A recent GWAS found that 41 SNPs were significantly correlated with family
3 size or birth rate ($P < 1 \times 10^{-4}$) in 269 Hutterite men in the USA. Of these SNPs, rs7867029,
4 rs7174015, rs12870438, and rs724078 were found to be associated with semen
5 parameters (including sperm concentration, semen volume, total sperm count, total motile
6 sperm count, or sperm motility) in 123 ethnically diverse men from Chicago, USA
7 (Kosova *et al.*, 2012). Recently, we performed replication analyses of these four SNPs to
8 assess their association with five semen parameters; however, none of the four SNPs
9 displayed a significant association with any semen parameters in a total of 2015 Japanese
10 men (Sato *et al.*, submitted). In contrast, we found that the polymorphisms rs7867029,
11 rs7174015, and rs12870438 were significantly associated with more severe disease
12 phenotype(s) in male infertility in this case-control study. SNPs rs7867029, rs7174015,
13 and rs12870438 were associated with the risk for developing oligozoospermia, and
14 rs12870438 was also associated with azoospermia. Meanwhile, there were no
15 associations between rs724078 and either azoospermia or oligozoospermia. In the
16 previous GWAS in 269 Hutterite men (Kosova *et al.*, 2012), rs7867029, rs7174015, and
17 rs12870438 were significantly associated with family size, and rs724078 was
18 significantly associated with birth rate. There have been no previous studies that
19 examined family size and oligozoospermia. This study therefore provides the first
20 evidence that the family size-associated SNPs (rs7867029, rs7174015, and rs12870438),
21 but not the birth rate-associated SNP (rs724078), are associated with the risk of
22 oligozoospermia in a Japanese population.

23 Two (rs7174015 and rs12870438) of the three associated SNPs are located in the
24 introns of *USP8* and *EPSTII*, respectively. *Usp8* is highly expressed in male germ cells

1 and contributes to the formation of the mouse acrosome, which is indispensable for
2 fertilization (Berruti *et al.*, 2010), while *EPSTII* is highly expressed in the testes (Nielsen
3 *et al.*, 2002). Although the relationship between these SNPs and the function of these
4 genes is unknown, they may be biologically compelling candidates for further exploration
5 into the genetics of human male infertility.

6 The present findings imply that three infertility-associated SNPs may be
7 contributing to a quantitative reduction in spermatogenesis rather than to spermatogenesis
8 failure. Although there has been no report available to indicate relationships between
9 these three SNPs and sperm parameters in Hutterite men, men with these risk alleles might
10 have associated reproductive outcomes, leading to a decrease in family size in the
11 Japanese population.

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16

17 **Authors' roles**

18 Y.S. and A.T.: study design and data analysis; Y.S. and K.T.: genotyping; S.N., M.Y., E.K.,
19 J.K., M.N., K.M., A.T., K.K., N.I., J.E., and T.I.: cohort collection and characterization;
20 Y.S., A.T., K.T., S.N., M.Y., E.K., J.K., M.N., K.M., A.T., K.K., N.I., J.E., I.I., A.Y., and
21 T.I.: preparation and approval of the final version of the manuscript.

22

23

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5

6 **Conflicts of interest**

7 None declared.

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Table I. Allele and genotype frequencies of the subjects in a study of candidate loci for human male fertility traits

SNP	Chr	Position (NCBI Build 36.3)	Closest Genes ^a	Location	Allele ^b	Control		Case			
						Genotypes ^b	AF ^b	Azoospermia		Oligozoospermia	
								Genotypes ^b	AF ^b	Genotypes ^b	AF ^b
rs7867029	9	80,210,238	<i>PSAT1</i>	dwnst.	G	27/256/508	0.20	4/27/45	0.23	5/19/26	0.29
rs7174015	15	48,504,360	<i>USP8</i>	intron	T	226/396/169	0.54	22/38/16	0.54	16/32/2	0.64
rs12870438	13	42,378,205	<i>EPSTII</i>	intron	A	4/148/638	0.098	4/18/54	0.17	2/6/40	0.10
rs724078	6	29,597,027	<i>MASIL</i> , <i>UBD</i>	upst., dwnst.	T	61/334/396	0.29	7/27/42	0.27	2/26/22	0.30

SNP: single nucleotide polymorphism, Chr, chromosome; dwnst., downstream; upst., upstream.

^aGene names: *PSAT1*, phosphoserine aminotransferase 1; *EPSTII*, epithelial stromal interaction 1; *USP8*, ubiquitin specific peptidase 8; *MASIL*, MAS1 oncogene-like; *UBD*, ubiquitin D.

^b“Allele” indicates the Hutterite minor allele reported in previous genome wide association studies (Kosova et al., 2012). “Genotypes” and “AF” indicate genotype counts (2/1/0) and the frequencies of the Hutterite minor alleles, respectively.

Table II. The associations from different comparative genetic models between four SNPs and azoospermia or oligozoospermia

Model	Case	OR (95% CI)	<i>P</i>
rs7867029			
Log-additive ^a	Azoospermia	1.23 (0.83–1.85)	0.31
(<i>Risk allele, G</i>)	Oligozoospermia	<u>1.70 (1.07–2.68)</u>	<u>0.024</u>
Recessive	Azoospermia	1.57 (0.54–4.62)	0.41
(<i>GG vs. GC+CC</i>)	Oligozoospermia	<u>3.14 (1.16–8.55)</u>	<u>0.025</u>
Dominant	Azoospermia	1.24 (0.77–2.00)	0.39
(<i>GG+GC vs. CC</i>)	Oligozoospermia	1.66 (0.93–2.94)	0.084

rs7174015			
Log-additive	Azoospermia	1.01 (0.73–1.42)	0.94
(<i>Risk allele, T</i>)	Oligozoospermia	<u>1.56 (1.02–2.39)</u>	<u>0.042</u>
Recessive	Azoospermia	1.02 (0.61–1.71)	0.95
(<i>TT vs. TC+CC</i>)	Oligozoospermia	1.18 (0.64–2.17)	0.60
Dominant	Azoospermia	1.02 (0.57–1.81)	0.95
(<i>TT+TC vs. CC</i>)	Oligozoospermia	6.52 (1.57–27.10)	0.0099

rs12870438			
Log-additive	Azoospermia	1.92 (1.21–3.05)	0.0059
(<i>Risk allele, A</i>)	Oligozoospermia	1.06 (0.54–2.11)	0.86
Recessive	Azoospermia	10.90 (2.67–44.60)	0.00087
(<i>AA vs. AG+GG</i>)	Oligozoospermia	<u>8.54 (1.52–47.90)</u>	<u>0.015</u>
Dominant	Azoospermia	<u>1.71 (1.01–2.89)</u>	<u>0.046</u>
(<i>AA+AG vs. GG</i>)	Oligozoospermia	0.84 (0.39–1.83)	0.66

rs724078			
Log-additive	Azoospermia	0.91 (0.62–1.33)	0.63
(<i>Risk allele, T</i>)	Oligozoospermia	1.06 (0.68–1.67)	0.80
Recessive	Azoospermia	1.21 (0.54–2.76)	0.64
(<i>TT vs. TC+CC</i>)	Oligozoospermia	0.50 (0.12–2.10)	0.34
Dominant	Azoospermia	0.81 (0.51–1.30)	0.39
(<i>TT+TC vs. CC</i>)	Oligozoospermia	1.28 (0.72–2.27)	0.41

Underlines indicate *P*-value < 0.05 and bold numbers indicate *P*-value < 0.0063 (0.05/8 test: four SNPs and two phenotypes) to account for multiple testing.

^aLog-additive, additive model in log-odds scale.