Single Nucleotide Polymorphisms of the *IZUMO* Gene in Male Infertile Patients

Shinji IRIE , Junko NAKAMURA , Akira TSUJIMURA , Yasushi MIYAGAWA , and Hiromitsu TANAKA

Life Science Research Laboratory, Toppan Technical Research, Institute,
Corporate Manufacturing, Technology & Research Division
Department of Urology, Graduate School of Medicine, Osaka University
Faculty of Pharmaceutical Sciences, Nagasaki International University
*Corresponding author

Abstract

IZUMO is a surface protein on sperm and essential for egg fusion in mice. To investigate the possible association between variations in the *IZUMO* gene and impaired spermatogenesis in Japanese males, we screened for mutations in the human *IZUMO* gene using DNA from 892 sterile male patients and 172 proven-fertile male volunteers. Single Nucleotide Polymorphisms (SNPs) were identified in the heterozygous state in the infertile patients, and neither variant was identified in fertile subjects using an ABI-PRISM 3730xl Genetic Analyzer and SeqScape® software (Applied Biosystems). Four single nucleotide polymorphisms (SNPs), 417T>G (Cys139Trp), 589A>T (Ser197Cys), 939T>A (Phe313Leu), and 944G>A (Arg315Gln), were found significantly more often in infertile subjects than in fertile controls in the first screening. Otherwise, the four SNPs were not detected by direct sequencing using the other primers. These results show that four SNPs exist as an analytical target of SNPs that associated with the male infertility in the *IZUMO* gene.

Key words

sperm, male infertility, genome, SNPs, spermatogenesis, fusion

Introduction

Infertility affects approximately 15% of couples, and in about half of those cases, the problem resides with the male . The development of novel fertilization treatments, including *in vitro* fertilization and intracytoplasmic injection, has made pregnancy possible regardless of the level of spermatozoa activity, but the etiology of male-factor infertility remains unclear.

Fertilization includes a series of cellular interactions, and infertility might be caused by dysfunctions of the molecules that are engaged in the fusion between the reacted spermatozoa and the mature oocyte. Two ADAM proteins present on sperm were considered as candidates for sperm-egg fusion but deleting the gene in mice showed that fusion still occurred and that these genes were dispensable . In contrast, IZUMO is the only molecule indispensable for sperm-egg fusion because mouse sperm lacking *Izumo* are unable to fuse . IZUMO was identified through the generation of a monoclonal antibody that inhibits sperm-egg fusion . The expression of *Izumo* is specific to testis and the protein is localized in an acrosomal region exposed on the surface only after the acrosome reaction occurs. IZUMO protein is a typical type-1 membrane glycoprotein with one

Shinji IRIE, Junko NAKAMURA, Akira TSUJIMURA, Yasushi MIYAGAWA, and Hiromitsu TANAKA

immunoglobulin-like domain and one N-glycoside link motif . *Izumo*^{-/-} male mice are healthy and produce normal-like sperm that bind to and penetrate the zona pellucida but are incapable of fusing with eggs . From these results, IZUMO appears to be involved in sperm-egg fusion; thus, IZUMO dysfunction may be associated with human male infertility.

We examined single nucleotide polymorphisms (SNPs) as a cause of male infertility in an analysis of genes expressed specifically in spermatogenesis . In the present study, we assessed the prevalence of SNPs in the *IZUMO* coding region by directly sequencing PCR-amplified DNA from male patients who developed azoospermia or severe oligospermia. In total, 1064 DNA samples were analyzed: 892 from infertile patients and 172 from proven-fertile volunteers. Twenty-five variation-induced amino acid substitutions were found in the coding region of *IZUMO*, and the other one was silent. One SNP in the fifth exon, one SNP in the seventh exon, and two SNPs in the tenth exon accumulated significantly in infertile patients. Although the necessity for solving the problem of the primer design has been left, the SNPs associated with the male infertility might exit in the *IZUMO* gene.

Materials and Methods

2.1 Participants

Japanese subjects with non-obstructive infertility ($n = 892$) were assigned to five subgroups: azoospermia (28.4%), oligozoospermia (count < 20 million cells/mL; 26.6%), asthenospermia (count $\geq$ 20 million cells/mL and motility < 50%; 26.6%), teratozoospermia (abnormality > 85%; 0.4%), and normal (18.0%), according to World Health Organization criteria . All patients displayed idiopathic infertility and had no history of prior medical conditions, including but not limited to cryptorchidism, recurrent infections, trauma, orchitis, or varicocele. All subjects were identified with primary idiopathic infertility based on a cytogenetic analysis. The control group consisted of fertile males who had fathered children born at a maternity clinic ($n = 172$). All of the donors were informed of the purpose of

Table   The region amplified by PCR and sequeincing primers for the *IZUMO* gene.

Exons/Introns	Location		Primers		
			Name	Sequence 5'-3	Position of 5'
EXON2	-72	235	1	GGTTTGCGGACCCCATGGATAGGC	-144
EXON3	572	646	2r	GGGTCCACAGCCTAGTATCTGACC	740
EXON4	1321	1407	3	GCATTGCCTAGCCCAGCAGCTGGC	1169
EXON5	1665	1685	4r	AGTGGGGAGGTTGACTATTCAGAG	1746
EXON6	2335	2415	5.1	GTTGGAGCCCACTTACTACTTACC	2176
EXON7	3551	3651	6.1r	GAGACCACACCTAATAGACCACCC	3772
EXON8	3918	4071	7	ACACTCACCTCCCACACTGAGGCC	3817
EXON9	4382	4559	9.5r	CCGCCCCTTACTCCAAGCCAAGCC	4607
EXON10	4832	4972	9.8	ATAGGAGGACTTCGAAGGGCGAGG	4698
			8r	GGGACAGATGGGAAGCAGGTGTGC	5770
EXON5	1665	1685	S417F*	AGAAATCTGACTCTGTCATCTCTTTCTTGCTCTC	1610
			S417R*	GAGGTTGACTATTCAGAGGGCAGGATG	1740
EXON7	3551	3651	S589F*	CCTGGACTGTGAGTTAAACTGGCATCA	3588
			S589R*	AGTTCAAGGGTTACAGAAGTTGAGGCC	3683
EXON10	4832	4972	S939F*	CCAAACCGGTTCCATGATCTGCA	4791
			S939R*	CCAAGGCCAAACAGTGAGGATTTGA	4888

The position for each primer is that of the first nucleotide in relation to the first nucleotide of the ATG start codon.
*Primers for double-check of sequences

Table   Conditions of PCR for SNPs analyses

Region	Primers	Denatureing temp. and time	Annealing temp. and time	Extension time and cycle number	Product size (bp)
EXON 2, 3	1 2r	98℃, 10sec	67℃, 5sec	72℃, 150sec[*] 40cycles	884
EXON 4, 5	3 4r	96℃, 30sec	62℃, 30sec	72℃, 150sec[**] 40cycles	578
EXON 6, 7	5.1 6.1r	96℃, 30sec	65℃, 30sec	72℃, 150sec[**] 40cycles	1597
EXON 8, 9	7 9.5r	96℃, 30sec	67℃, 30sec	72℃, 150sec[**] 40cycles	791
EXON 10	9.8 8r	96℃, 30sec	64℃, 30sec	72℃, 150sec[**] 40cycles	1073
417T>G	S417F S417R	95℃, 30sec	68℃, 30sec	72℃, 150sec[***] 35cycles	131
589A>T	S589F S589R	95℃, 30sec	68℃, 30sec	72℃, 150sec[***] 35cycles	103
939T>A 944G>A	S939F S939R	95℃, 5sec	68℃, 40sec	72℃, 150sec[**] 35cycles	99

[*]PrimeSTAR
[**]Ex TaqHS
[***]Go TaqHS

the study and gave permission for their blood and semen to be used for genomic DNA analysis. This study was carried out with the approval of the institutional review board, independent ethics committee in Osaka University.

2.2 Identification of SNPs in IZUMO by the Direct Sequencing of PCR-Amplified DNA

DNA samples of proven-fertile ($n = 172$) and some of the infertile patients ($n = 247$) were extracted from blood leukocytes. DNA samples from the remainder of the infertile patients ($n = 645$) were extracted from cells in semen. Genomic DNA was isolated from the blood samples using standard protease treatment and phenol extraction procedures. Genomic DNA from sperm was isolated as follows. Freeze-stocked semen (1 mL) was dissolved and added to 1 mL phosphate-buffered saline (PBS). The samples were centrifuged at 10,000 rpm for 10 min at 4℃. The precipitate was used for the extraction of genomic DNA using QuickGene-800 (Fujifilm, To-

kyo, Japan) according to the manufacturer's instructions. The sequences containing the encoded region in the 10 exons of the *IZUMO* gene were amplified by PCR using specific primers (Table 1) under the conditions described in table 2.

The PCR-amplified fragments were purified using AMPure® (Agencourt Bioscience Corporation, Beverly, MA) and then sequenced using the same PCR primers with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The reaction products were purified by the BigDye® XTerminator and analyzed using an ABI-PRISM 3730xl Genetic Analyzer (Applied Biosystems). Any subjects with sequence ambiguities were excluded. The screening of variations in the sequences was performed with SeqScape® software (Applied Biosystems).

2.3 Statistical Analysis

The X -test was used to compare the genotype distribution between infertile subjects and proven-

Shinji IRIE, Junko NAKAMURA, Akira TSUJIMURA, Yasushi MIYAGAWA, and Hiromitsu TANAKA

Table   The identified variants. positions and genotypes in infertile male and controls

Region	Position		Genotype	Number (%) of SNP				P value	Reference
	Nucleotide	Amino acid		Infertile		Proven fertile			NCBI dpSNP #
Exon 2	32	Ala11	C/C	771	(99.9)	172	(100)		
		Ala11[Ala, Glu]	C/A	1	(0.1)	0	(0)	0.637	
	38	Ala13	C/C	768	(99.5)	172	(100)		
		Ala13[Ala, Asp]	C/A	4	(0.5)	0	(0)	0.344	
	41	Gly14	G/G	772	(99.9)	172	(100)		
		Gly14[Gly, Asp]	G/A	1	(0.1)	0	(0)	0.637	
	115	Asp39	G/G	773	(99.9)	172	(100)		
		Asp39[Asp, Asn]	G/A	1	(0.1)	0	(0)	0.637	
	157	Met53	A/A	773	(99.9)	172	(100)		
		Met53Val	G/G	1	(0.1)	0	(0)	0.637	
	169	Val57	G/G	773	(99.9)	172	(100)		
		Val57[Val, Ile]	G/A	1	(0.1)	0	(0)	0.637	
	214	Asp72	G/G	774	(99.9)	172	(100)		
		Asp72[Asp, Asn]	G/A	1	(0.1)	0	(0)	0.637	
Exon 3	285	Lys95	A/A	732	(99.9)	172	(100)		
		Lys95[Lys, Asn]	A/T	1	(0.1)	0	(0)	0.628	
Exon 4	321	Phe107	C/C	17	(2.4)	2	(1.2)	0.314	rs8108468
		-	C/T	140	(19.8)	31	(18.3)	0.616	
		-	T/T	550	(77.8)	136	(80.5)		
			C/C+C/T	157	(22.2)	33	(19.5)	0.448	
Exon 5	407	Pro136	C/C	410	(99.5)	169	(100)		
		Pro136[Pro, Leu]	C/T	2	(0.5)	0	(0)	0.364	
	409	Asn137	A/A	383	(99.2)	169	(100)		
		Asn137[Asn, His]	A/C	3	(0.8)	0	(0)	0.250	
	417	Cys139	T/T	347	(92.8)	169	(100)		
		Cys139[Cys, Trp]	T/G	27	(7.2)	0	(0)	<0.001	
Exon 6	442	Trp148	T/T	351	(997)	170	(100)		
		Trp149[Trp, Arg]	T/A	1	(0.3)	0	(0)	0.487	
	445	Cys149	T/T	351	(99.7)	170	(100)		
		Cys149[Cys, Gly]	T/G	1	(0.3)	0	(0)	0.487	
	497	Gly166	G/G	350	(99.7)	170	(100)		
		Gly166[Gly, Glu]	G/A	1	(0.3)	0	(0)	0.486	
Exon 7	577	Leu193	C/C	352	(99.7)	167	(100)		
		Leu193[Leu, Phe]	C/T	1	(0.3)	0	(0)	0.491	
	589	Ser197	A/A	302	(92.1)	167	(100)		
		Ser197[Ser, Cys]	A/T	26	(7.9)	0	(0)	<0.001	
Exon 8	719	Ser240	C/C	120	(99.2)	158	(100)		
		Ser240[Ser, Phe]	C/T	1	(0.8)	0	(0)	0.252	
Exon 10	937	Phe313	T/T	315	(99.7)	168	(100)		
		Phe313[Phe, Ile]	T/A	1	(0.3)	0	(0)	0.489	
	939	Phe313	T/T	297	(93.7)	168	(100)		
		Phe313[Phe, Leu]	T/A	20	(6.3)	0	(0)	<0.001	
	944	Arg315	G/G	296	(92.5)	168	(100)		
		Arg315[Arg, Gln]	G/A	24	(7.5)	0	(0)	<0.001	
	947	Arg316	G/G	320	(99.4)	162	(96.4)		
		Arg316[Arg, Lys]	G/A	2	(0.6)	6	(3.6)	0.014	
	953	Val318	T/T	326	(99.7)	168	(100)		
		Val318[Val, Glu]	T/A	1	(0.3)	0	(0)	0.473	
	998	Val333	T/T	263	(79.7)	138	(82.1)		rs2307019
		Val333[Val, Ala]	T/C	55	(16.7)	28	(16.7)	0.906	
		Val333Ala	C/C	12	(3.6)	2	(1.2)	0.117	
			C/C+T/C	67	(20.3)	30	(17.9)	0.515	
	999	Val333[Val, Ala]	T/T	258	(78.2)	138	(82.1)		rs2307018
		Val333[Val, Ala]	G/T	58	(17.6)	28	(16.7)	0.685	
		Val333[Val, Ala]	G/G	14	(4.2)	2	(1.2)	0.064	
			G/G+G/T	72	(21.8)	30	(17.9)	0.300	
	1040	Ser347	C/C	332	(99.7)	168	(100)		
		Ser347[Ser, Ter]	C/A	1	(0.3)	0	(0)	0.477	

The translation start site was +1on *IZIMO* cDNA.
The whole population could not be fully determined due to the condition of some samples.

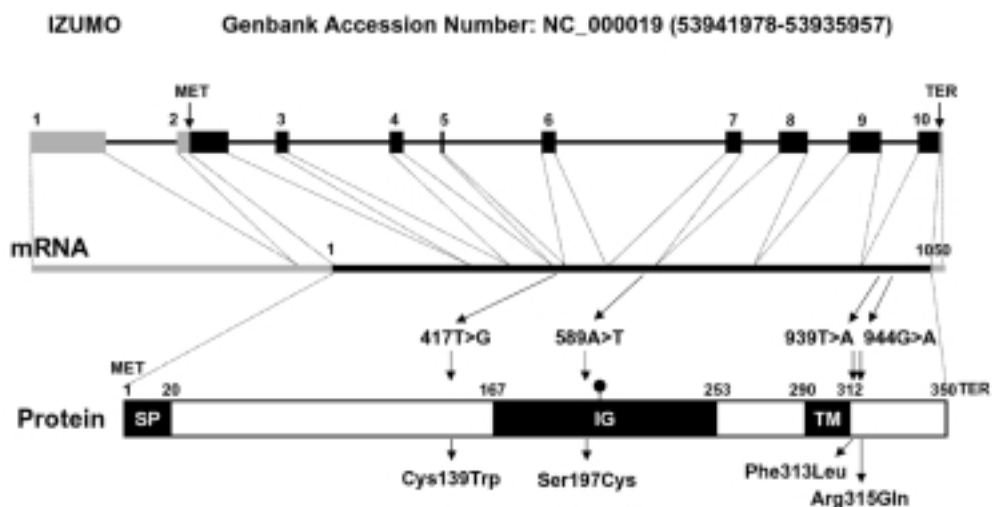IZUMO   Genbank Accession Number: NC_000019 (53941978-53935957)

Figure   Schematic view of the 6-kb *IZUMO* gene *IZUMO* mRNA (1,621 nucleotides), and the IZUMO protein (350 amino acids). Exons 1-10 are depicted as thick boxes, and the introns are shown as lines. Numbers (upper) indicate the positions of the exons. Four SNPs that accumulated significantly in infertile males were designated. Each SNP is named based on its position in relation to the first nucleotide of the start codon. Numbers (lower) indicate the positions of the amino acids in relation to the first methionine (MET). SP, signal peptide; IG, immunoglobulin-like domain; TM, transmembrane region.

fertile controls. A P-value of < 0.05 was considered to be statistically significant.

Results

The entire coding sequence of *IZUMO* (EMBL /DDBJ/GenBank Accession Number NC_000019, 53935957-53941978; IZUMO) and the intronic regions adjacent to each exon were analyzed for sequence variations by direct sequencing as the first screening, and 26 were found: seven variants in exon 2, one variant in exon 3, 4, or 8, three variants in exon 5 or 6, two variants in exon 7, and eight variants in exon 10 (Table 3). The 21 variation-induced amino acid substitutions at 32C>A (Ala11 Glu), 38C>A (Ala13Asp), 41G>A (Gly14Asp), 115 G>A (Asp39Asn), 169G>A (Val57Ile), 214G>A (Asp72Asn), 285A>T (Lys95Asn), 407C>T (Pro 136Leu), 409A>C (Asn137His), 417T>G (Cys139 Trp), 442T>A (Trp148Arg), 445T>G (Cys149Gly), 497G>A (Gly166Glu), 577C>T (Leu193Phe), 589A >T, (Ser197Cys), 719C>T (Ser240Phe), 937T>A

(Phe313Ile), 939T>A (Phe313Leu), 944G>A (Arg 315Gln), 953T>A (Val318Glu), and 1040C>A (Ser 347Ter) were either in the major homozygous or heterozygous states; no minor homozygous SNPs were identified. The one variation-induced amino acid substitution at 157A>G (Met53Val) was either the major or minor homozygous state; no heterozygous SNPs were observed. These 22 variations were not detected in any of the fertile subjects. The four different sequence changes in these variations, 417T >G, 589A>T, 939T>A, and 944G>A, were significantly (P<0.001) more frequent in the heterozygous state in the infertile subjects (Fig. 1). Because 939T >A and 944G>A almost always occurred in the same subjects, these two SNPs seemed to be linked. The three known SNPs were observed in exon 4 (rs 8108468) and exon 10 (rs2307019 and rs2307018). Because subjects showing the 998T>C homozygous state were almost always the same as the subjects showing the 999T>G homozygous state, these two SNPs also seemed to be linked. The amino acids Ala

```
HUMAN       MGPH-FTLLCAALAGCLLPAEGCVICDPSVVLALKSLEKDYLPGHLDAKH   49
CHIMPANZEE  MGPH-FTLLCAALAGCLLPAEGCVICDPSVVLALKSLEKDYLPGHLDAKH   49
CATTLE      MGPRGLPLLVATLAGCLFPARGCVICDPKVREALNSLEMDYLPGHLEANH   50
MOUSE       MGPH-FTLLLAALANCLCPGRPCIKCDQFVTDALKTFENTYLNDHLPHDI   49
RAT         MGLH-FTLLLAALANCLCPARLCIICDPFVVAAIKTLEQNYLPTHLAPEH   49
            **----**-*-**-**-*---*--**--*--*---*--**--**----

HUMAN       HKAMMERVENAVKDFQELSINEDAYMGVVDEATLQKGSWSLLKDLKRITD   99
CHIMPANZEE  HKAMMERVENAVKDFQELSINEDAYMGVVDEATLQKGSWSLLKDLKRITD   99
CATTLE      HKKVMEKIKQALEDFKDLPIDEDSYMGVVDEATLEKSSWSLLKDMKRITD  100
MOUSE       HKNVMRMVNHEVSSFGVVTSAEDSYLGAVDENTLEQATWSFLKDLKRITD   99
RAT         HEDVMKRVEQEVRNFADLPLNQNTFLGVVDEDTLEQASWSFLKDLKRITD   99
            *---*-------------*------*-*-***-**---**-***-*****

HUMAN       SDVKGDLFVKELFWMLHLQKETFATYVARPQKEAYCPNKCGVMLQTLIWC  149
CHIMPANZEE  SDVKGDLFVKELFWMLHLQKETFATYVARPQKEAYCPNKCGVMLQTLIWC  149
CATTLE      SDAKGELFVKEMLWMLHLAKNTFASYAAQPQKEAFCPNKCGLMLQPLIWC  150
MOUSE       SDLKGELFIKELLWMLRHQKDIFNNLARQPQKEVLCPNKCGVMSQTLIWC  149
RAT         SDVKGELFVKELFWMLRLQKDIFATLVARPQKEVYCPNQCGTMSQTLIWC  149
            **-**-**-**--***---*--*--**--***-**-*-*-*WC

HUMAN       KNCKKEVHACRKSYDCGERNVEVPQMEDMILDCELNWHQASEGLTDYSFY  199
CHIMPANZEE  KNCKKEVHACRKSYDCGERNVEVPQMEDMILDCELNWHQASEGLTDYSFY  199
CATTLE      STCQKQVHACRKSKNCGEREVKVHHMEDMILDCELNWHKISQGLTDYSPY  200
MOUSE       LKCEKQLHICRKSLDCGERHIEVHRSEDLVLDCLLSWHRASKGLTDYSFY  199
RAT         NKCEKQMHFCRKSMDCGERQIEVHRLEDMVLDCQLSWHHASEGLTDYSFY  199
            --*-*--*-****--****---*---**-***-*-**----*******

HUMAN       RVWGNNTETLVSKGKEATLTKPMVGPEDAGSYRCELGSVNSSPATIINFH  249
CHIMPANZEE  RVWGNNTETLVSKGKEATLTKPMVGPEDAGSYRCELGSVNSSPATIINFH  249
CATTLE      RVWKNNSETLVSKGKEPILTKTMVRPKDAGTYRCELGSIQSSPATIIYFH  250
MOUSE       RVWENSSETLIAKGKEPYLTKSMVGPEDAGNYRCVLDTINQGHATVIRYD  249
RAT         RVWGNSSETLMSKGKEPYLTKTMVGPEDAGNYRCELDTVNAGPATIIYYH  249
            ***-*--***--****--***-***-*-***-***-*------**-*---

HUMAN       VTVLPKMIKEEKPSPNIVTPGEAT-------------------------  273
CHIMPANZEE  VTVLPRMIKEEKPSPNIVTPGEAT-------------------------  273
CATTLE      VTVLPKRIQEEIPSPNTETQDETALGEVALDRP----------------  283
MOUSE       VTVLPPKHSEENQPPNIITQEEHETPVHVTPQTPPGQEPESELYPELHPE  299
RAT         VTVLPPRSVEEKPPPNIVTQEEEETPVQVIVPT------------LEPE  286
            *****----**---**-----*------------------

HUMAN       -TESSISLQPLQPEKMLASRLLGLLICGSLALITGLTPAIFRRRKVIDFI  322
CHIMPANZEE  -TESSITLQPLKPEKMLASRLLGLLICGSLALITGLTPAIFRRRKVIDFI  322
CATTLE      HTTTDFEPQTPKPEQLLRRRLLGVLIWGLVVLTVGVLTA            322
MOUSE       LYPELIPTVAQNPEKKMKTRLLILLTLGFVVLVASIIISVLHFRKVSAKL  349
RAT         PEPEPIPTVTHRPEKKLKSRLLILLILGFVVLVASVIASVLHFRKTRVKS  336
            ------------*------**--**-*---------

HUMAN       KSS----------------LFGLGSGVVEQTQVPKEKATDSRQQ       350
CHIMPANZEE  KSS----------------LFGLGSGAAEQTQVPKEKATDSRQQ       350
CATTLE      
MOUSE       KNASDEVKPTASGSKSDQSLSQQMGLKKASQADFNSDYSGDKSEATEN   397
RAT         KNSNVENKTSAAEFKSEAESPQKMGSRKLSQAEFHTDSS-DKVEEADN   383
            *----------------------*----------*------*-------
```

Figure   Alignment of the putative amino acid sequences of humans, chimpanzees, cows, mice, and rats. Amino-acid identity is indicated by an asterisk. Amino acids indicated by arrows correspond to the SNP substitutions that accumulated significantly in infertile subjects. Amino acids designated by an underline correspond to the other SNPs specific to infertile men. The immunoglobulin-like domain is boxed. The putative transmembrane region is boxed in gray. The cysteine residues that might form a disulfide bridge are indicated by open arrowheads. The asparagine residue, which is an N-glycosylation site, is indicated by a closed arrowhead.
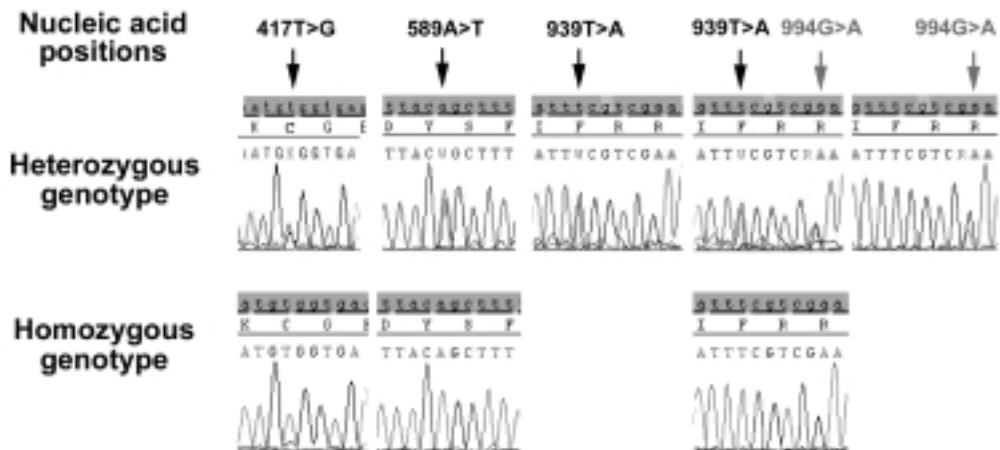
Figure   Detection of SNPs in *IZUMO* gene. The four nucleic acid positions were distinctly identified as heterozygous genotypes (arrows) by the first screenng. The translation start site was +1 on *IZUMO* cDNA.

13, Lys95, Pro136, Asn137, Cys139, Trp148, Cys 149, Gly166, Leu193, and Ser197 were conserved in the IZUMO sequence (Fig. 2). Because a conserved amino acid sequence within different species means that the region has a common and important function, these substitutions may influence activity of IZUMO. However, the heterozygous variations showing low significance might only be rare mutations unrelated to IZUMO function. Otherwise, significant variations, such as Cys139Trp and Ser197 Cys, might function as dominant-negative. IZUMO may be involved in sperm-egg fusion; thus, it is possibly associated with human male infertility. Therefore, variations that affect functioning of the *IZUMO* gene would appear in the genome sequence in infertile human males. The four variations inducing the substitution of amino acids Cys139Trp, Ser197Cys,

Phe313Leu, and Arg315Gln were strongly associated with male infertility. No patient had these SNPs more than once. The sperm of almost all patients having these SNPs showed normal sperm count and morphology (Table 4).

Additionally, we analyzed the 4 SNPs significantly more frequent identified in the infertile subjects by using the additional primer sets (Table 1). These SNPs in figure 3 were not identified in each two samples of 4 SNPs by additional primers.

Discussion

IZUMO is the sperm protein confirmed to be essential for egg fusion in mice, and male infertile patients may have mutations in the *IZUMO* gene. In this study, we found several differences between infertile subjects and fertile controls in genotypes that

Table   The case of total infertile patients and the number (%) of the infertile patients having each SNP in the case

Case of infertility	Number of a patient	Nucleic acid position of SNPs			
		T>G	A>T	T>A	G>A
Azoospermia					
Oligozoospermia					
Asthenospermia					
Teratozoospermia					
Normal sperm					
Total					

caused amino acid substitutions in the first screening. Heterozygous Cys139Trp, Ser197Cys, Phe313 Ile, and Arg315Gln accumulated significantly in infertile patients and were strongly associated with normal sperm count and morphology. This association is consistent with the function of the IZUMO protein . We analyzed these four SNPs by the additional primer sets. However, the SNPs were not able to be detected. It is not clear why the sequences including these SNPs were different between the screening and the confirmation of SNPs. But the specific allele in homologous chromosomes might be amplified by effects of the setting position of the primers because of the allele-specific modification of DNA. The results indicated that the design of the primer is important for analyzing SNPs. As for this cause, a further analysis is necessary.

Recently, the four SNPs in exon 4, intron 4, and exon 10 were identified, but they showed no statistical differences in infertile populations . We also identified the same polymorphisms in exon 4 (rs8108468) and exon 10 (rs2307018, rs2307019), but 321C>T (rs8108468) did not result in modification of the protein sequence and showed no significant different in genotype frequency in the infertile subjects in their study. The rs2307019 was linked to rs2307018, and the minor homozygote C998T (Val 333Ala) was also not significantly different.

Until now, no study had reported on the relationships between IZUMO and male infertility. In 12 male infertile patients, a change in IZUMO expression on sperm was not detected by immunocytochemistry . Because we screened for variations in the sequences coding the IZUMO protein, we may not have found any variations that affected IZUMO expression.

This is the first analysis of *IZUMO* mutations in men with non-obstructive azoospermia. The significant differences in genotype frequency were observed to be specific to the infertile subjects in the

first screening in this study. Although the deleterious effects of these heterozygous mutations on fertility are unclear and may be influenced by genetic background, these SNPs were found only in the infertile patient group.

Previously, it was reported that a dominant, recombination-defective allele of *DMC1* (*DMC1^mei1*) causes male-specific infertility . DMC1 is a meiosis-specific homolog of bacterial RecA and eukaryotic that can catalyze homologous DNA strand invasion . The *DMC1^mei1* mutation causes an alanine to proline change at amino acid 272 within the counterpart of the structurally disordered "Loop 2" of RecA for DNA binding . The DMC1 null mutation introduced infertility in both sexes by arresting in meiotic prophase I . When one allele of *DMC1* was expressed, no dysfunction in meiosis was detected . When *DMC1^mei1* was expressed from a separate mutant allele, abnormalities were specifically observed in male . These dominant negative autosomal mutations that affects spermiogenesis might be transferred to the next generation via females. Heterozygous SNPs identified in male infertile patients may cause male infertility by altering *IZUMO* function.

The Cys139 was located in the region between the signal peptide and the immunoglobulin-like domain , the Ser197 was located in the immunoglobulin-like domain, and the remaining two significant variations were located in the C-terminal cytoplasmic region. The substitution of Ser 197 to Cys might influence the formation of the disulfide bridge (Cys182-Cys233) and result in improper protein folding. In mammals, the C-terminal sequences showed the lowest homology compared to the N-terminus region, indicating that intracellular signaling via the IZUMO cytoplasmic region is not a common mechanism among these species. The Phe313Leu and Arg315Gln variations were located in this region, but the effect of these substitutions

could not be deduced.

Our findings indicated that *IZUMO* might be associated with male infertility, although not only further investigation and functional studies of these variants are needed to determine how the IZUMO mutation causes infertility in human males but also the appearance of these variations should be confirmed to be valid.

Acknowledgement

References

Skakkebaek, N. E., Jørgensen, N., Main, K. M., Rajpert-De Meyts, E., Leffers, H., Andersson, A. M., Juul, A., Carlsen, E., Mortensen, G. K., Jensen, T. K. & Toppari, J. (2006) Is human fecundity declining? *International Journal of Andrology* 29, 2-11.

Rubinstein, E., Ziyyat, A., Wolf, J. P., Le Naour, F. & Boucheix, C. (2006) The molecular players of sperm-egg fusion in mammals. *Seminars in cell & developmental biology* 17, 254-263.

Okabe, M., Adachi, T., Takada, K., Oda, H., Yagasaki, M., Kohama, Y. & Mimura, T. (1987) Capacitation-related changes in antigen distribution on mouse sperm heads and its relation to fertilization rate *in vitro*. *Journal of Reproductive Immunology* 11, 91-100.

Inoue, N., Ikawa, M. & Okabe, M. (2008) Putative sperm fusion protein IZUMO and the role of N-glycosylation. *Biochemical and biophysical research communications* 377, 910-914.

Inoue, N., Ikawa, M., Isotani, A. & Okabe, M. (2005) The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. *Nature* 434, 234-238.

Nishimune, Y. & Tanaka, H. (2006) Infertility caused by polymorphisms or mutations in spermatogenesis-specific genes. *Journal of Andrology* 27, 326-334.

Tanaka, H., Hirose, M., Tokuhiro, K., Matsuoka, Y., Miyagawa, Y., Tsujimura, A., Okuyama, A. & Nishimune, Y. (2007) Single nucleotide polymorphisms: discovery of the genetic causes of male infertility. *Society of Reproduction and Fertility supplement* 65, 531-534.

Tokuhiro, K., Hirose, M., Miyagawa, Y., Tsujimura, A., Irie, S., Isotani, A., Okabe, M., Toyama, Y., Ito, C., Toshimori, K., Takeda, K., Oshio, S., Tainaka, H., Tsuchida, J., Okuyama, A., Nishimune, Y. & Tanaka, H. (2008) Meichroacidin containing the membrane occupation and recognition nexus motif is essential for spermatozoa morphogenesis. *Journal of Biological Chemistry* 283, 19039-19048.

Irie, S., Tsujimura, A., Miyagawa, Y., Ueda, T., Matsuoka, Y., Matsui, Y., Okuyama, A., Nishimune, Y. & Tanaka, H. (2009) Single nucleotide polymorphisms of the PRDM9 (MEISETZ) gene in patients with non-obstructive azoospermia. *Journal of Andrology*, In press.

World Health Organization. (1999) Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction, 4th edn. Cambridge University Press, Cambridge.

Granados-Gonzalez, V., Aknin-Seifer, I., Touraine, R. L., Chouteau, J., Wolf, J. P. & Levy, R. (2008) Preliminary study on the role of the human IZUMO gene in oocyte-spermatozoa fusion failure. *Fertility & Sterility* 90, 1246-1248.

Hayasaka, S., Terada, Y., Inoue, N., Okabe, M., Yaegashi, N. & Okamura, K. (2007) Positive expression of the immunoglobulin superfamily protein IZUMO on human sperm of severely infertile male patients. *Fertility & Sterility* 88, 214-216.

Bannister, L. A., Pezza, R. J., Donaldson, J. R., de Rooij, D. G., Schimenti, K. J., Camerini-Otero, R. D. & Schimenti, J. C. (2007) A dominant, recombination-defective allele of Dmc1 causing male-specific sterility. *PLoS Biology* 5, e105.

Habu, T., Taki, T., West, A., Nishimune, Y. & Morita T. (1996) The mouse and human homologs of DMC1, the yeast meiosis-specific homologous recombination gene, have a common unique form of exon-skipped transcript in meiosis. *Nucleic Acids Research* 24: 470-477.

Yoshida, K., Kondoh, G., Matsuda, Y., Habu, T., Nishimune, Y. & Morita, T. (1998) The mouse RecA-like gene Dmc1 is required for homologous chromosome synapsis during meiosis. *Molecular Cell* 1: 707-718.

Shinji IRIE, Junko NAKAMURA, Akira TSUJIMURA, Yasushi MIYAGAWA, and Hiromitsu TANAKA

IZUMO

$$\left(\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \right)$$

IZUMO

IZUMO

DNA   IZUMO

ABI-PRISM 3730xl Genetic Analyzer and SeqScape®software   Applied

Biosystems

T>G Cys   Trp   A>T Ser   Cys   T>A Phe   Leu   G>A Arg   Gln

SNPs