LMTK2 and *PARP-2* gene polymorphism and azoospermia secondary to meiotic arrest

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Received: 18 October 2008 / Accepted: 22 September 2009 / Published online: 6 October 2009
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Abstract

Purpose To investigate whether the human *LMTK2* and *PARP-2* gene defects are associated with azoospermia by meiotic arrest, mutational analysis was performed on Japanese men with azoospermia.

Methods Via direct sequencing, mutational screening was carried out on the exon region of the genes, using genomic DNAs from 18 Japanese men. Statistical analysis was done on the detected single nucleotide polymorphisms (SNPs) in the patients and normal controls.

Results Nine SNPs were detected in *LMTK2* and five SNPs were detected in *PARP-2*. There were no significant differences in the genotype distribution and allele frequencies between the two groups in *LMTK2*. However, the genotype frequency of heterozygotes in SNP1 of *PARP-2* was higher in the patient group. The haplotype analysis revealed that SNP1-SNP4 (T-A) of *PARP-2* was significantly more frequent in the patient group.

Capsule The *PARP-2* gene might play a critical role in human spermatogenesis.

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Conclusion The *PARP-2* gene might be associated with azoospermia by meiotic arrest in humans.

Keywords Azoospermia · *LMTK2* · *PARP-2* · Polymorphism

Introduction

Genetic causes of severe male infertility include chromosomal abnormalities, such as Y-chromosome microdeletions, and several specific gene mutations, such as those in *AZF*, *DAZ*, *RBMY*, *USP9Y*, and *SYCP3* [1–5]. Genetic polymorphism may also be a factor in some forms of male infertility.

In spermatogenesis, there are two different events: meiosis (the major event) and postmeiotic differentiation. Aberrance of either event results in male infertility. To date, a number of genes responsible for azoospermia have been identified in the mouse; however, only a few have been identified in humans. The most common example of a meiotic defect is the aberrance of prophase I (comprising some complicated steps, such as pairing, synapsis, and recombination). Proper synapsis requires a synaptonemal complex (SC) specific to meiosis. Abnormal SC component proteins and incomplete SC formation cause azoospermia in the mouse [6, 7]. Impairment of double-strand break (DSB) initiating recombination and DSB repair also cause meiotic arrest in spermatocytes [8–10].

Several genes, which perform critical roles in postmeiotic differentiation of mouse spermatogenesis, have been reported [11, 12]. One of them is the mouse *Brek/Lmtk2* (brain-enriched kinase/lemur tyrosine kinase 2) gene [13]. The *Lmtk2* gene is expressed in other organs besides the testis, and is particularly high in the brain; *Lmtk2* gene-

deficient mice exhibit normal health except for azoospermia. *Lmtk2* gene deficient female mice possess normal fertility; however, the males are infertile secondary to a defect of spermatid elongation and the germ cells differentiate normally until the round-spermatid stage. The *Lmtk2* gene is essential for late stage-spermatogenesis in the mouse. The mouse poly(ADP-ribose) polymerase-2 (*Parp-2*) gene is known to perform an important role in DNA repair [14]. In addition, it has been demonstrated that *Parp-2* gene deficient mice exhibit severely impaired spermatogenesis [15]. This gene performs essential functions during meiosis and haploid gamete differentiation in mouse spermatogenesis.

In this study, we performed mutational analysis of the human *LMTK2* and *PARP-2* genes on Japanese men with azoospermia secondary to meiotic arrest.

Materials and methods

Patients and controls

Idiopathic azoospermia was confirmed by two consecutive semen analyses obtained after 5 to 7 days of sexual abstinence and examination of a centrifuged semen pellet. Patients with defective spermatogenesis following infection, an obstruction of the seminal tract, pituitary failure, or other types of testicular damage were excluded from the study. All patients in this study underwent testicular sperm extraction (TESE) and sperm was absent in all specimens. Final diagnoses were carried out by histological examinations. Histological examinations were meticulously performed by several pathologists and urologists. Chromosome analysis of peripheral lymphocytes revealed a karyotype of 46, XY in all patients; thus, 18 Japanese men with azoospermia secondary to meiotic failure were included in the study. We have accumulated DNA samples from more than 5,000 patients with azoospermia; however, azoospermia caused by meiotic arrest is very rare and our diagnostic criteria were very strict. In general, pathological diagnosis is final diagnosis. However, there are sometimes some discrepancies between clinical diagnosis and pathological one when TESE was performed. As the important tissues judged by seeing with the naked eye are used for looking for sperm and the other parts are used for pathological diagnosis, it may be happened that there are sperm in testis but pathological diagnosis are meiotic arrest or sertoli cell only syndrome (SCOS). All of the analyzed patients in this study have no sperm even in their testis. Because of our strict criteria, the number of analyzed patients was only 18.

A *LMTK2* gene analysis was conducted on 50 healthy, pregnancy-proven fertile controls; a *PARP-2* gene analysis

was conducted on 318 healthy, pregnancy-proven fertile controls. In addition, 86 patients with SCOS were also analyzed on the *PARP-2* gene. All subjects were Japanese and gave written informed consent for molecular analysis of their blood. This study was approved by a local ethics committee.

Mutational screening

A total of 18 Japanese men diagnosed with azoospermia secondary to meiotic arrest were screened for mutations in the *LMTK2* and *PARP-2* genes. These full length cDNA sequences were compared to each human genomic sequences (NT_007933.14 and NT_026437.11), and all exon-intron borders were determined. Nested or semi-nested PCRs were performed using primers with the intronic regions adjacent to each exon. Because of its large size, PCR was performed exclusively on exon 11 of the *LMTK2* gene, using five separated primers sets. The primers listed below were used for mutational analysis. The sequences of oligonucleotide primers are presented in Tables 1 and 2.

Primers of the LMTK gene

Exon 1(A): E1F1, E1R1 and E1R2;
 Exon 1 (B): E1F11, E1F1, E1R11 and E1R1
 Exon 2: E2F1, E2F2, E2R1 and E2R2
 Exon 3: E3F1, E3F2, E3R1 and E3R2;
 Exon 4: E4F1, E4F2, E4R1 and E4R2;
 Exon 5: E5F1, E5F2, E5R1 and E5R2;
 Exon 6: E6F1, E6F2, E6R1 and E6R2;
 Exon 7: E7F1, E7F2, E7R1 and E7R2;
 Exon 8: E8F1, E8F2, E8R1 and E8R2;
 Exon 9: E9F1, E9F2, E9R1 and E9R2;
 Exon 10: E10F3, E10F1, E10R3 and E10R1;
 Exon 11 (A): E11F11, E11F13, E11SR1 and E11SR2;
 Exon 11 (B): E11SF3, E11SF4, E11SR3 and E11SR4;
 Exon 11 (C): E11SF5, E11SF6, E11SR5 and E11SR6;
 Exon 11 (D): E11SF7, E11SF8, E11SR7 and E11SR8;
 Exon 11 (E): E11SF9, E11SF10, E11SR9 and E11SR10;
 Exon 12: E12F1, E12F2, E12R1 and E12R2;
 Exon 13: E13F1, E13F2, E13R1 and E13R2;
 Exon 14 (A): E14F1, E14F2, E14R1 and E14R2;
 Exon 14 (B): E14F3, E14F4, E14R1 and E14R3

Primers of the PARP-2 gene

Exon 1: pE1F1, pE1F2, pE1R1 and pE1R2;
 Exon 2, Exon 3: pE2F1, pE2F2, pE2R1 and pE2R2;
 Exon 4: pE4F1, pE4F2, pE4R1 and pE4R2;
 Exon 5: pE5F1, pE5F2, pE5R1 and pE5R2;
 Exon 6: pE6F1, pE6F2, pE6R1 and pE6R2;

Table 1 Sequences of oligonucleotide primers used for mutational screening of *LMTK2*

	Forward primer	Reverse primer
Exon1	E1F1 = 5'-gatggtgtttctgcgactgg-3'	E1R1 = 5'-cttaaggcaacagggtccagg-3'
	E1F11 = 5'-ttgctgctgttgagaggcgg-3'	E1R2 = 5'-ctccctaatactcgcctcc-3'
Exon2	E2F1 = 5'-ctgggtgaaggctgtatag-3'	E1R11 = 5'-acatctggaaagggaagctg-3'
	E2F2 = 5'-atcctggatggatcatcagc-3'	E2R1 = 5'-ctggcagagttgagctgctg-3'
Exon3	E3F1 = 5'-ggcaacagagcaaaccttg-3'	E2R2 = 5'-gagactgtatggcccaaat-3'
	E3F2 = 5'-ctgctgttgagtcagaga-3'	E3R1 = 5'-acaatggcctgaccactag-3'
Exon4	E4F1 = 5'-ttggcctggtgattggtgtg-3'	E3R2 = 5'-cactgggagcagaaggaaga-3'
	E4F2 = 5'-catgagagtagctggatcgg-3'	E4R1 = 5'-gccgtaagacatctttctg-3'
Exon5	E5F1 = 5'-gcttttgtaggtggtgaag-3'	E4R2 = 5'-acagcaaggctgaagctcac-3'
	E5F2 = 5'-gaagtcacctgcttattgc-3'	E5R1 = 5'-ctgagttctcctggttctg-3'
Exon6	E6F1 = 5'-gtgagctatgattgcaccac-3'	E5R2 = 5'-gatttcagatgacgcctgg-3'
	E6F2 = 5'-ggtgacagaacgagaccctg-3'	E6R1 = 5'-ggactgcagtgtaacaacc-3'
Exon7	E7F1 = 5'-agtgtgagtgaccatttc-3'	E6R2 = 5'-ctatcaccatcaccaagg-3'
	E7F2 = 5'-cgtgtcatctcctaggtaac-3'	E7R1 = 5'-caatgaagcccaactcaacc-3'
Exon8	E8F1 = 5'-ccttgacacatgtcgtcag-3'	E7R2 = 5'-catgagctgggttcattctg-3'
	E8F2 = 5'-gaacaagccatacatccgg-3'	E8R1 = 5'-gcaaacctcggatgcaag-3'
Exon9	E9F1 = 5'-ggatgacttagcaagcgttc-3'	E8R2 = 5'-cgcagacttacacaagccac-3'
	E9F2 = 5'-gcagatgaggtcatgaagcc-3'	E9R1 = 5'-ctattacatgtcaggccctg-3'
Exon10	E10F1 = 5'-ggcaggagaatcactgaac-3'	E9R2 = 5'-gcaggggcaggtattcaaac-3'
	E10F3 = 5'-tgagtcaggactttgagag-3'	E10R1 = 5'-gaggatcacttgagccagga-3'
Exon11	E11F1 = 5'-gatgtcaccatgagctgaac-3'	E10R3 = 5'-attgtcctgcccgaatcag-3'
	E11F13 = 5'-tcatgccactgcactccaga-3'	E11SR1 = 5'-cccaggtatctgaagcgaac-3'
	E11SF3 = 5'-cacaggctgctgacttacct-3'	E11SR2 = 5'-gcctccagacatactcga-3'
	E11SF4 = 5'-acagcagagactcctccaac-3'	E11SR3 = 5'-tcgtcaccctttgtgttac-3'
	E11SF5 = 5'-agtgcctctctgctatc-3'	E11SR4 = 5'-cctgcaacaagacaagcct-3'
	E11SF6 = 5'-ggctcattgtccagcaaga-3'	E11SR5 = 5'-tgttcgtagccactgtctg-3'
	E11SF7 = 5'-ggaagaccatcagtcac-3'	E11SR6 = 5'-agagtcagtagcgttcgt-3'
	E11SF8 = 5'-caaccagtcaggacgcctc-3'	E11SR7 = 5'-tctgctgcctcaaatggca-3'
	E11SF9 = 5'-cttcgagacacaggacgac-3'	E11SR8 = 5'-gtcctcctctgtctgcatcca-3'
	E11SF10 = 5'-ctccttgctacaccaattc-3'	E11SR9 = 5'-accaaaaccttagccagcca-3'
Exon12	E12F1 = 5'-cccttctcttctgagc-3'	E11SR10 = 5'-agcattcgggtctcttctc-3'
	E12F2 = 5'-attgtggaggagaagccgac-3'	E12R1 = 5'-ccccacccttttatcactg-3'
Exon13	E13F1 = 5'-tgttcttaatgaccccc-3'	E12R2 = 5'-ggggcgtcattaaagcaaca-3'
	E13F2 = 5'-ctgacatgggcacagaactg-3'	E13R1 = 5'-gtaacgagcctgagatgac-3'
Exon14	E14F1 = 5'-cgaggtatctgaacatagg-3'	E13R2 = 5'-ctcctgagtagctgggatta-3'
	E14F2 = 5'-ctgctatctgccagtctgtc-3'	E14R1 = 5'-agaatgacaaggagaggctg-3'
	E14F3 = 5'-ccccgtcactagattgaaag-3'	E14R2 = 5'-caagacctctagctcctca-3'
	E14F4 = 5'-gcatttgagcctagcagatg-3'	E14R3 = 5'-gacaaggagaggctgcaaga-3'

Exon 7: pE7F1, pE7F2, pE7R1 and pE7R2;
 Exon 8: pE8F1, pE8F2, pE8R1 and pE8R2;
 Exon 9: pE9F1, pE9F2, pE9R1 and pE9R2;
 Exon 10, Exon 11: pE11F1, pE11F2, pE11R1 and pE11R2;
 Exon 12, Exon 13: pE12F1, pE12F2, pE12R1 and pE12R2;
 Exon 14, Exon 15, Exon 16: pE14F1, pE14F2, pE14R1 and pE14R2;

Nested or semi-nested PCRs were performed using primers for each intronic region and 10-fold diluted first PCR products were used as templates. PCR was performed on a final volume of 25 μ l, consisting of genomic DNA (10 ng), dNTP (0.32 mM each; TaKaRa, Shiga, Japan), each primer (0.2 μ M), Taq polymerase (0.625 U; Roche, Tokyo, Japan), and a reaction buffer containing MgCl₂ (Roche). Nested and semi-nested PCRs were carried out for 20 cycles under the same conditions above but with 2 μ l of

Table 2 Sequences of oligonucleotide primers used for mutational screening of *PARP-2*

	Forward primer	Reverse primer
Exon1	pE1F1=5'-cgtgagctctgttccaagctc-3' pE1F2=5'-ccagaacacatagcgacatg-3'	pE1R1=5'-gctccgcaaggctttatgca-3' pE1R2=5'-aggttatagggagctggaag-3'
Exon2 and 3	pE2F1=5'-cagatcacttgagggtcacga-3' pE2F2=5'-cacgtattcgggtgtgtact-3'	pE2R1=5'-ctaccactctctatcttgc-3' pE2R2=5'-ctaacagcatccagacagg-3'
Exon4	pE4F1=5'-aggttacaccaggttactg-3' pE4F2=5'-cctgggagatgaatgttga-3'	pE4R1=5'-tcctgagagatgaagtgaga-3' pE4R2=5'-gatctgaacactgtgatgct-3'
Exon5	pE5F1=5'-accagctgctatgccttacc-3' pE5F2=5'-caagtcaggtcaggttaggt-3'	pE5R1=5'-cctgccattcccaatttca-3' pE5R2=5'-aaggctctccagacttcag-3'
Exon6	pE6F1=5'-gagcagccattgagctga-3' pE6F2=5'-cttctgcctgtttgtcag-3'	pE6R1=5'-cttagagaaggcagggcacg-3' pE6R2=5'-caacatggcgaaaccccatc-3'
Exon7	pE7F1=5'-agctaggagtttgagaccag-3' pE7F2=5'-gggtttgggaaggacaagc-3'	pE7R1=5'-ttgagccagggaattcgaga-3' pE7R2=5'-aggtgggagatcatcttgag-3'
Exon8	pE8F1=5'-ccagatgctgaaactgtcc-3' pE8F2=5'-gctgtctttacactgacg-3'	pE8R1=5'-ccacttcagcttcgaagta-3' pE8R2=5'-ccatacaactgtcaccca-3'
Exon9	pE9F1=5'-gccccacttgtaggacttc-3' pE9F2=5'-tggggtgacaggtgtatgg-3'	pE9R1=5'-gtcacggggctctataaac-3' pE9R2=5'-tacttgggtggttgatagc-3'
Exon10 and 11	pE11F1=5'-ctttatagaccccgtagcc-3' pE11F2=5'-gtcatgcgccttttagccac-3'	pE11R1=5'-tggcagacctttctgtccc-3' pE11R2=5'-ctagaaaagcttggccttc-3'
Exon12 and 13	pE12F1=5'-tgtttgggagcagaaaggtc-3' pE12F2=5'-cagtcagctcagctcttg-3'	pE12R1=5'-gcagtaattggcactcttgg-3' pE12R2=5'-tgagctggcttaactcttgg-3'
Exon14,15 and 16	pE14F1=5'-gtgaggggaaatggagaagg-3' pE14F2=5'-gtcagtggtatgcacctct-3'	pE14R1=5'-aggatgttgagtgtggag-3' pE14R2=5'-ctttgtgcctctacccttc-3'

10-fold diluted first PCR products as templates, using a programmable PC 960G gradient thermal cycler using a PCR system (Cosmo Bio, Tokyo, Japan) as follows: initial denaturation at 95°C for 150 s, followed by 32 cycles of denaturation at 95°C for 30 s, annealing (primers $T_m - 5^\circ\text{C}$) for 90 s and extension at 72°C for 90 s. PCR products were purified using a QIAquick PCR Purification kit (Qiagen, Tokyo, Japan), and direct sequencing of each product was carried out. To confirm the role of the detected polymorphism in azoospermia, groups of 50, 150 and 318 healthy and fertile controls were also analyzed by direct sequencing analysis.

Genotyping and statistical analyses (single-locus analysis)

To investigate the role of *LMTK2* and *PARP-2* polymorphisms in azoospermia, 18 patients were compared to the genotype and allele frequencies found in the 50, 150, and 318 normal controls. In addition, 86 patients with SCOS were compared to the genotype and allele frequencies on the *PARP-2*. Fisher's exact test was used to determine significant differences. A $P < 0.05$ was considered to be statistically significant. The Hardy-Weinberg equilibrium (HWE) was tested using SNPlayze software (DYNACOM, Chiba, Japan). Linkage disequilibrium (LD) of all possible two-way combinations of SNP with the absolute value of the correlation coefficient (D') was tested.

P -values were determined via χ^2 approximation. Significance was determined with a $P < 0.05$ as described above. Haplotype frequencies were estimated by the method of maximum likelihood based on the expectation–maximization (E–M) algorithm under the assumption of HWE. Linkage disequilibrium and haplotype frequencies were tested using SNPalyze software (DYNACOM). P values were determined via χ^2 approximation; significance was determined at the $P = 0.05$ level.

Results

Using direct sequence analysis of the *LMTK2* gene of the 18 Japanese men, we identified nine single-nucleotide-polymorphisms (SNPs). The SNPs were: C381G (SNP1) in exon 1; T2371C, T2631A, G2726A, A2780T, C3164T, G3641A and A4145G (SNP2–8) in exon 11; and T5277C (SNP9) in exon 14. By comparing our results to the NCBI dbSNP database, four of nine SNPs (SNP2, SNP5, SNP6, and SNP9) were unique.

Genotyping for *LMTK2* SNP alleles in the 18 patients and 50 control individuals revealed that there were no significant differences in the genotype distribution and the allele frequency in all SNPs (Table 3). Minor allele frequencies ranged from 1.0% to 30.6%. Although there were CC, AA and GG homozygotes at the SNP2, SNP7

Table 3 Genotype and allele frequencies of single-nucleotide-polymorphisms (SNPs) in the human *LMTK2* gene in 18 azoospermic men patients and control individuals

SNPs	Alterations		Genotype frequency			Allele frequency			P-value
	Nucleotide	Amino acid	No of samples with genotype/total no of samples (%)		P-value	HWE test	No of chromosome with minor allele/total no of chromosomes (%)		
			Patients	Controls			Allele	Patients	
SNP1	381 C/G	Pro/Ala	CG	1/18(5.6)		<0.05	G	1/36(2.8)	
SNP2	2371T/C	Ile/Thr	TC	9/18(50.0)	0.7570	0.8410	C	11/36(30.6)	21/100(21.0)
			CC	1/18(5.6)	0.5910				
SNP3	2631T/A	Leu/Met	TA	5/18(27.8)	0.3789	0.7633	A	5/36(13.9)	9/100(9.0)
SNP4	2726G/A	Synonymous	GA	5/18(27.8)	0.3789	0.7633	A	5/36(13.9)	9/100(9.0)
SNP5	2780A/T	Synonymous	AT	1/18(5.6)	0.5910	<0.05	T	1/36(2.8)	0/100(0)
SNP6	3164C/T	Synonymous	CT	1/18(5.6)	0.9618	<0.05	T	1/36(2.8)	1/100(1.0)
SNP7	3641G/A	Synonymous	GA	7/18(38.9)	0.8274	0.6373	A	9/36(25.0)	18/100(18.0)
			AA	1/18(5.6)	0.5910				
SNP8	4145A/G	Synonymous	AG	9/18(50.0)	0.8710	0.8410	G	11/36(30.6)	22/100(22.0)
			GG	1/18(5.6)	0.5910				
SNP9	5277T/C	3'UTR	TC	2/18(11.1)	0.7442	<0.05	C	2/36(5.6)	6/100(6.0)
									0.7521

Fisher's exact test between patients and controls
HWE Hardy–Weinberg equilibrium

Table 4 Pairwise linkage disequilibrium (D' -value above right triangle) and statistical significance (P -value below left triangle) for the five SNPs in the *LMTK2*

	SNP2	SNP3	SNP4	SNP7	SNP8
Case group					
SNP2		−0.1158	−0.1158	1	1
SNP3	0.5808		1	0.2595	−0.1158
SNP4	0.5808	<0.05 ^a		0.2595	−0.1158
SNP7	<0.05 ^a	0.4039	0.4039		1
SNP8	<0.05 ^a	0.5808	0.5808	<0.05 ^a	
Control group					
SNP2		−1	−1	1	1
SNP3	0.1049		1	−1	−1
SNP4	0.1049	<0.05 ^a		−1	−1
SNP7	<0.05 ^a	0.1406	0.1406		1
SNP8	<0.05 ^a	0.1002	0.1002	<0.05 ^a	

Significant at $P=0.05$ level (χ^2 test)

^a Statistically significant

and SNP8 sites in the patient group, no homozygotes were found in the control group (0.056 vs. 0.000). However, there were no significant differences between the two groups. Hardy-Weinberg equilibrium of the distribution of genotypes for each SNP for the case group was evaluated by the χ^2 test; this test revealed that SNP1, SNP5, SNP6 and SNP9 of patients exhibited a significant deviation ($P<0.05$).

Pair-wise LD between SNPs was tested for all possible two-way comparisons; a strong LD was verified in four pair SNPs with a D' value of 1.00 and a P value of <0.05 by the χ^2 test (both patient and control groups). The result of LD assessment of SNP2, SNP3, SNP4, SNP7 and SNP8 via Hardy-Weinberg equilibrium is summarized in Table 4. SNP2, SNP7 and SNP8 as well as SNP3 and SNP4 sites were in complete LD.

Haplotype frequencies for various combinations were estimated via an expectation maximization algorithm. The results of combinations of two and three loci in HWE and in the strong LD are presented in Table 5. Two haplotypes of those combinations (SNP2-SNP7-SNP8 and SNP3-SNP4

Table 5 Haplotype frequency estimation in patients and controls in the *LMTK2*

SNP2–SNP7–SNP8	Case	Control	P-value
T–G–A	0.6944	0.7857	0.2719
C–A–G	0.2500	0.1735	0.3209
C–G–G	0.0556	0.0306	0.4984
SNP3–SNP4	Case	Control	P-value
T–G	0.8611	0.9100	0.4078
A–A	0.1389	0.0900	0.4078

Table 6 Genotype and allele frequencies of single-nucleotide-polymorphisms (SNPs) in the human *PARP-2* gene in 18 azoospermic patients and controls

SNPs	Alterations	Genotype frequency				Allele frequency				P-value	
		Nucleotide	Amino acid	No of samples with genotype/total no of samples (%)		HWE test	No of chromosome withinor allele/total no of the chromosomes (%)				
				Patients	Controls			Allele	Controls		
SNP1	1159C/T	Synonymous	CT	11/18(61.1)	14/50(28.0)	0.0124 ^a	0.1897	T	11/36(30.5)	24/100(24.0)	0.5828
			TT	0/18(0.0)	5/50(10.0)	0.3857					
SNP2	1359G/A	Arg/Gln	GA	1/18(5.6)	0/318(0.0)	0.0470 ^a	<0.05	A	1/36(2.8)	0/636(0.0)	0.0472 ^a
SNP3	1469A/C	Asn/His	AC	1/18(5.6)	2/50(4.0)	0.6938	<0.05	C	1/36(2.8)	2/100(2.0)	0.6971
SNP4	1789A/C	3'UTR	AC	3/18(16.6)	14/50(28.0)	0.5255	0.2175	C	7/36(19.4)	24/100(24.0)	0.5764
			CC	2/18(11.1)	5/50(10.0)	0.7495					
SNP5	1790T/C	3'UTR	TC	1/18(5.6)	2/146(1.3)	0.7502	<0.05	C	1/36(2.8)	2/292(0.68)	0.7513

Fisher's exact test between patients and controls

HWE Hardy–Weinberg equilibrium

^a Statistically significant**Table 7** Pairwise linkage disequilibrium (D' -value above right triangle) and statistical significance (P -value below left triangle) for the five SNPs in the *PARP-2*

	SNP1	SNP2	SNP3	SNP4	SNP5
Case group					
SNP1		−1	1	0.2750	1
SNP2	0.5011		−1	−1	−1
SNP3	0.1263	0.8639		1	−1
SNP4	0.4312	0.6183	<0.05 ^a		−1
SNP5	0.1263	0.8639	0.8639	0.6183	
Control group					
SNP1		−	1	0.5329	0
SNP2	−		−	−	−
SNP3	<0.05 ^a	−		1	0
SNP4	<0.05 ^a	−	<0.05 ^a		
SNP5	1	−	1	1	

Significant at $P=0.05$ level (χ^2 test)^a Statistically significant

(T-G-A, C-A-G, and T-G, A-A)), comprised more than 95% of the population. No statistical significance of the each haplotype was observed between the patient and control groups.

In these Japanese men, we identified five SNPs in the human *PARP-2* as follows: C1159T (SNP1) in exon 11; G1359A (SNP2) in exon 13; A1469C (SNP3) in exon 14; and A1789C (SNP4) and T1790C (SNP5) in exon 16. By comparing this result to the NCBI dbSNP database, all detected SNPs were unique. Although the major allele of SNP1 is T in the genomic sequence listed in NCBI database, in this study, the major allele of SNP 1 was C.

The genotype frequencies of heterozygotes in SNP1 and SNP2, as well as the minor allele frequency in SNP2, were higher in the patient group with significant differences (Table 6). However, the TT homozygote in SNP 1 was detected only in the control group; therefore, there were no significant differences in the minor allele frequencies of SNP1 between the patient and control group. There were no significant differences between the patient and control groups in regard to genotype and minor allele frequencies

Table 8 Haplotype frequency estimation in patients and controls in the *PARP-2*

SNP1–SNP4	Case	Control	P-value
C–A	0.5965	0.6748	0.3977
T–C	0.0965	0.1548	0.3864
T–A	0.2090	0.0852	0.0483 ^a
C–C	0.0979	0.0852	0.8183

^a Statistically significant

in other SNPs. In the HWE test of the distribution of genotypes for each SNP of the patient group, SNP2, SNP3 and SNP5 showed a significant deviation ($P < 0.05$).

Pair-wise LD between SNPs was tested for all possible two-way comparisons and revealed that LD was limited in SNP1 and SNP4 in HWE (D' value: 0.5329; and $P < 0.05$ by the χ^2 test) in the control groups (Table 7). In analysis of haplotype frequencies, SNP1-SNP4 (T-A) haplotype was found to be significantly more frequent in the patient group than in the control group (Table 8). The 86 patients with SCOS were also analyzed on SNP1 and SNP4 in the *PARP-2*. However, there are no statistically significant differences on the frequencies of genotypes, alleles and haplotypes between the patients with meiotic arrest and SCOS ($p > 0.05$).

Discussion

Via mutational analysis of the *LMTK2* gene, nine genetic variations could be found including the four unique SNPs. Exon 11 is the longest exon and almost all of the nine SNPs were identified in this region. Genotype and minor allele frequencies were found to have no significant differences between the patient and control groups. The reason that three were ruled out by HWE was probably due to the fact that the population was insufficient. The five SNPs (SNP2, SNP3, SNP4, SNP7, and SNP8) were in HWE. There is an amino acid substitution in SNP2 and SNP3; however, the others are synonymous. By LD analysis, SNP2 is in complete linkage disequilibrium with SNP7 and SNP8. In addition, SNP3 also has complete linkage disequilibrium with SNP4. Amino acid substitution is induced in both of these haplotypes; however, no statistically significant difference could be detected between the patient and control groups. The *Lmtk2*^{-/-} male mice are infertile due to azoospermia; however, differentiation normally takes place until the round-spermatid stage [13]. In the present study, we analyzed the patients with azoospermia secondary to early meiotic arrest; therefore, no differences might be found between the patient and control groups.

By mutational analysis of the *PARP-2* gene, SNP2 and SNP3 of the five detected SNPs resulted in amino acid substitutions. In addition, both genotype and minor allele frequencies were significantly different between the patient and control groups only in SNP2. However, three SNPs (SNP2, SNP 3 and SNP5) were ruled out by HWE because of their P values. Therefore, we have to analyze more samples especially on the SNP2. Conversely, SNP1 and SNP4 are in HWE, and the SNP1-SNP4 (T-A) haplotype was higher with statistical significance in the patient group. SNP4 (A) was a major allele and SNP1 had no amino acid substitution.

Poly(ADP-ribosylation) is a post-translational modification of nuclear proteins in response to DNA damage [14]. PARPs contribute to the DNA repair and apoptosis by detection and signaling of the strand-break [15]. In addition, it was demonstrated that the *Parp-2* gene play essential roles in both meiotic and postmeiotic processes in the mouse [16]. *Parp-2* deficient mice exhibit severely impaired spermatogenesis, with a defect in prophase of meiosis I (characterized by massive apoptosis at pachytene and metaphase I stages). In this study, we demonstrated that the genotype frequency of heterozygote of SNP1 and the haplotype frequency of SNP1-SNP4 were higher in the patient than the control groups. In addition, SNP2 of *PARP-2* was found only in patient groups with significant differences. We believe that the patient group of 18 men is far too small for an association study. However, azoospermia by meiotic arrest is very rare and our histological diagnostic criteria are very strict. We have the DNA samples from more than 5,000 patients with azoospermia; however, only 18 patients had azoospermia secondary to meiotic arrest. In addition, we could not find a significant difference between the patients with meiotic arrest and SCOS.

Therefore, these results suggest that the human *PARP-2* gene might also be associated with azoospermia caused by meiotic arrest.

In conclusion, this is the first report demonstrating that *PARP-2* SNPs may predispose men to a defect in spermatogenesis; however, the mechanism whereby these SNPs result in azoospermia remains uncertain. Our results may also advance a better understanding of the molecular basis of meiotic arrest as a cause of non-obstructive azoospermia. The study of a larger sample and/or other ethnic groups is clearly indicated.

Acknowledgements This study was supported by Grants-in-Aid for Scientific Research (No. 19591887 and 20591902) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Ministry of Health, Labour and Welfare of Japan.

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