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ORIGINAL ARTICLE

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Polymorphic alleles of the human *MEI1* gene are associated with human azoospermia by meiotic arrest

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Abstract Genetic mechanisms are implicated as a cause of some male infertility, yet are poorly understood. Mouse meiotic mutant mei1 (meiosis defective 1) was isolated by a screening of infertile mice. Male mei1 mice have azoospermia due to meiotic arrest, and the mouse *Mei1* gene is responsible for the mei1 phenotype. To investigate whether human *MEI1* gene defects are associated with azoospermia by meiotic arrest, we isolated the human *MEI1* cDNA based on the mouse Mei1 amino acid sequence. *MEI1* is expressed specifically in the testis. Mutational analysis by direct sequencing of all *MEI1* coding regions was performed in 27 men (13 European Americans, 13 Israeli and 1 Japanese) having azoospermia due to complete early meiotic arrest. This identified four novel, coding single-nucleotide-polymorphisms (cSNPs), i.e., SNP1 (T909G), SNP2 (A1582G), SNP3 (C1791A) and SNP4 (C2397T) in exons 4, 8, 9 and 14, respectively. Using these cSNPs, an association study was carried out between 26 non-Japanese patients with azoospermia and two sets of normal control men (61 normal European Americans and 60 Israelis). Consequently, SNP3 and SNP4 were shown to be associated with azoospermia among European Americans ($P = 0.0289$ and $P = 0.0299$ for genotype and allele frequencies at both the polymorphic sites, respectively), although no such association was observed among Israelis ($P > 0.05$). Haplotype estimation revealed that the frequencies of SNP3–SNP4 (C–T), SNP3–SNP4 (A–C) and SNP3–SNP4 (A–T) were higher in the European American patients, and the frequency of SNP3–SNP4 (A–T) was also higher than in both control groups. These results suggest that *MEI1* may play a role in meiosis during spermatogenesis, especially in European Americans.

Keywords Azoospermia · Infertility · MEI1 · Meiosis · Polymorphisms

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Introduction

Genetic causes of severe male infertility include chromosomal abnormalities such as Y-chromosome microdeletions and specific gene mutations in *AZF, DAZ, RBMY, USP9Y* and *SYCP3* (Reijo et al. 1995; Elliott et al. 1997; Sun et al. 1999; Matzuk and Lamb 2002;

Miyamoto et al. 2003). Since the Y-chromosome deletions explain only up to 21% of men with infertility (Nakamura et al. 2001), azoospermia in many infertile men may be caused by autosomal gene mutations. Genetic polymorphisms may also be factors susceptible to some forms of male infertility, albeit somewhat controversially, e.g., whether the CAG repeat of the human androgen-receptor gene is linked to male infertility (Dowsing et al. 1999).

Defective meiosis during spermatogenesis is one of the critical causes of azoospermia, although the details remain unknown. In sexually reproducing species, meiosis is a fundamental process that allows a genetic exchange between maternal and paternal genomes (Nasmyth 2002). The genetic regulation of meiosis in mammals is poorly understood compared to that in lower eukaryotes such as yeast. Several key genes expressed in mouse meiosis, including *Dmc1*, *Fkbp6*, *Scp3* (*Sycp3*), *Spo11*, *Msh4* and *Msh5*, have been identified by disruption experiments in embryonic stem (ES) cells (Yoshida et al. 1998; Pittman et al. 1998; Edelmann et al. 1999; Baudat et al. 2000; Kneitz et al. 2000; Romanienko and Camerini-Otero 2000; Yuan et al. 2000; Crackower et al. 2003). In addition, the mouse meiotic mutant mei1 (meiosis defective 1) was isolated by a screening of infertile mice generated by chemical mutagenesis in ES cells (Munroe et al. 2000). The male mice mutant for the *Mei1* gene has small testes and lacks the epididymal sperm and postmeiotic cells. The seminiferous tubules of such mice contain spermatocytes arrested at the zygotene/pachytene stage of meiosis (Libby et al. 2003). In contrast, few genes essential to human meiosis are known.

In the present study, we isolated the human *MEI1* cDNA using the deduced amino acid sequence of the mouse *Mei1* cDNA, and analyzed a possible association of *MEI1* mutations with azoospermia by meiotic arrest in man.

Materials and methods

Isolation of the human *MEI1* cDNA and analysis of its expression in various tissues

The mouse *Mei1* cDNA was isolated previously (Libby et al. 2003). Using the mouse Mei1 amino acid sequence (GenBank accession no. AY270177), we identified its homologous region in the human genome (GenBank BX391221). We designed a pair of primers (MEI1F2 and MEI1R2) in the human homologous region encompassing the putative introns, and carried out PCR on a human testis cDNA library (Clontech, Tokyo, Japan). The sequences (5′–3′) of the oligonucleotides used were as follows: MEI1F2, GCTGGAAGAAGCCATGCAGG; and MEI1R2, AGTCCGGTCCCTGGTCATTG. Semi-nested PCR was performed with MEI1F2/MEI1R3 and a 10-fold dilution of the first PCR product as a template. The other oligonucleotide (5′–3′) used was MEI1R3, TGCAGAACCTCCTGGTGCAG. The product from the semi-nested PCR was subcloned into a T-Easy vector (Promega, Madison, WI), and several representative clones were sequenced in both directions. The 5′- and 3′-RACE were performed with primers 5RAFUL1, 5RAFUL8, 3RA1, 3RA5, AP1 (Clontech) and AP2 (Clontech). Their sequences (5′–3′) were as follows: 5RAFUL1, GTACTGGCGATCAGACAGGAAGGCAAGG; 5RAFUL8, AAGGATGAGGAAGCTTCAGAGCCGTGGG; 3RA1, TGGATGCTGGAGAGAATTCCTTCCTCAG and 3RA5, TTTGGCTGACCTGTCTACCCTCTCGAAC. Both RACE products were also subcloned, and several representative clones were sequenced in both directions. The isolated full-length cDNA sequences were compared with human genomic sequences. All PCRs were carried out using an Advantage 2 PCR Kit (Clontech) under the following conditions and according to the manufacturer's instructions: initial denaturation at 95°C for 150 s, followed by 32 cycles of denaturation at 95°C for 15 s, annealing and extension at 68°C for 90 s.

For expression analysis of *MEI1*, PCR of cDNA from various tissue types (spleen, thymus, prostate, testis, ovary, small intestine, colon, leukocyte, brain, heart, kidney, liver, lung, pancreas and placenta) purchased from Clontech was performed with EXP2F1 and EXP2R6 as primers. The sequences (5′–3′) of the primers were: EXP2F1, CTGGGAAGAGAGCAGCTATG; and EXP2R6, CTGCTGGGTGTGGTCTGATG. PCR conditions were initial denaturation at 95°C for 150 s, followed by 32 cycles of denaturation at 95°C for 15 s, annealing and extension at 68°C for 90 s using an Advantage 2 PCR Kit.

Patients and control individuals

All patients and donors participating in this study gave informed consent for molecular analysis of their blood samples, and the study protocol was approved by the Committee for the Ethical Issues on Human Genome and Gene Analysis, Tel Aviv Sourasky Medical Center and Kanazawa University. To test the hypothesis that human *MEI1* gene mutations are associated with human azoospermia, we screened 27 patients diagnosed as having azoospermia due to complete meiotic arrest. Genomic DNA from 13 of these patients was obtained from the NIH funded tissue bank at Baylor College of Medicine with the patients' written informed consent and the full oversight of the Institutional Review Boards for the Protection of Human Subjects at Baylor College of Medicine. These men underwent clinical evaluations for diagnosis and treatment of their azoospermia by an expert diagnostician, Dr. Larry Lipshultz. They provided an extensive history and underwent a physical examination, as well as state-of-the-art andrology testing (showing a normal karyotype by high resolution banding chromosome analysis and by testing for Y-chromosome microdeletions). Consequently, azoospermia was

diagnosed to be idiopathic in all cases. Of the 27 patients, 13 were European Americans, 13 were from Israel and the remaining 1 was from Japan, and all had normal karyotypes and no Y-chromosome microdeletions. Pathological examination of bilateral testicular biopsy specimens from 26 (13 European Americans and 13 Israelis) of the 27 men showed an early maturation arrest testicular phenotype (the most mature spermatogenic cell type present was the spermatocyte), which was consistent with that seen in mei1 mutant mice. Sixty-one and 60 healthy and pregnancy-proven fertile men (European American and Israeli, respectively) were used as normal control individuals.

MEI1 mutation screening

To test the hypothesis that *MEI1* gene mutations are associated with human non-obstructive azoospermia, we screened 27 patients for mutations in *MEI1*. Genomic DNA was obtained from peripheral blood lymphocytes using a Qiagen Blood and Cell Culture DNA Midi Kit (Qiagen, Hilden, Germany). After all exon–intron borders were identified by comparison between the full-length cDNA and genomic sequences (NT_007758.10), all *MEI1* coding regions and intronic sequences adjacent to exons of the patients were analyzed by direct sequencing. Nested or semi-nested PCRs were performed using primers for each intronic region (Table 1) and 10-fold diluted first PCR products as templates. PCR was performed in a final volume of 25 μl, consisting of genomic DNA (10 ng), dNTP (0.32 mM each) (TaKaRa, Shiga, Japan), each primer (0.2 mM), *Taq* polymerase (0.625 U) (Roche, Tokyo, Japan) and reaction buffer containing $MgCl_2$ (Roche). Nested and semi-nested PCRs were carried out for 20 cycles under the same conditions above but with 2 μl of 10-fold diluted first PCR products as templates, using a programmable PC 960G gradient thermal cycler (Cosmo Bio, Tokyo, Japan) using the following PCR conditions: initial denaturation at 95°C for 150 s, followed by 32 cycles of denaturation at 95°C for 30 s, annealing at (primer T_m-5°C) for 90 s and extension at 72°C for 90 s. PCR products were purified using a QIAquick PCR Purification kit (Qiagen), and direct sequencing of each product was carried out in both directions.

Genotyping and statistical analyses

Single-locus analysis

To investigate the role of *MEI1* polymorphisms in azoospermia, 26 patients (13 European Americans and 13 Israelis) were genotyped for polymorphic alleles, and compared to the genotype and allele frequencies in the normal control men. To match ethnic populations, the single Japanese patient was excluded from the study.

Table 1 Sequences of oligonucleotide primers used for mutation analysis

		Forward primer		Reverse primer
Exon2	E2F1	5′-CATAGCCACCTTAAGACCTC-3′	E2R1	5′-ATTCCTCTGGGCAGCCTCAG-3′
	E2F2	5′-ACTAGCCTCTGGCACAAAGC-3′	E2R2	5′-TCGTGGCTGTACTAACTCAC-3′
Exon3	E3F1	5′-TCCCGGTAGGTTGAACCTTG-3′	E3R1	5′-CAGTGTCTACCCACCTGTTC-3′
	E3F2	5′-AAGAGGAGCCTCTGATTTCC-3′	E3R2	5′-AAGATATGAGCCTCTCAGGG-3′
Exon4	E4F1	5′-GAGTATTTGGCCCCTAACTG-3′	E4R1	5′-CTCCACACTCCTAACACCTC-3′
	E4F2	5′-GCACAAGGCACAGAACAATG-3′	E4R2	5′-CTCTCTCTACCACCCGCTTG-3′
Exon5	E5F1	5′-TCTCACTAAAGTGCCTTGGG-3′	E5R1	5′-GAGGTGGGCTGAAGCAGATG-3′
	E5F2	5′-GCCAAGGTGTCCTGGAATAC-3′	E5R2	5′-CTCAGTAAACCGGCTCTCAG-3′
Exon6	E6F1	5′-GGATGGACCTATTCTAAGGG-3′	E6R1	5′-GCAGTGAGAGAAAGGAGGTG-3′
	E6F2	5′-GCACCTAGAAGAGCCTTTGG-3′	E6R2	5′-CTTGATGCCACAATGGCCTG-3′
Exon7	E67F1	5′-GAAGTCACTCAGCCTCTCTG-3′	E67R1	5′-AGAGAAAGCCCTGAGAGGTC-3′
	E67F2	5′-TACCAACCTATGTCTGGCAC-3′	E67R2	5′-GGACAGACTGACCTACTAGC-3′
Exon8	E7F1	5′-CTAGATGGAGGGTGTGAATG-3′	E7R1	5′-GAGCCAGAAAGGACCTTATG-3′
	E7F2	5′-TTAGTTTGTGCTTGCTCCCC-3′	E7R2	5′-ACCAGCCACACACACAACCC-3′
Exon9	E8F1	5′-GAGCTTCTGTTATGAAGCTG-3′	E8R1	5′-CTAATAAGCCCCCAGAGCTG-3′
	E8F3	5′-TCAGGTGCTAAGCACCATAG-3′	E8R3	5′-TGCCACCCCATGCCTCAAAG-3′
Exon10	E9F1	5′-TGATTCTCCGCATGCTCCTG-3′	E9R1	5′-ATTATGGAGTGACCATTGGC-3′
	E9F2	5′-TTGGAGGACTCTGTGTGGCC-3′	E9R2	5′-GCTGGTCTTATCTGAAAACC-3′
Exon11	E10F1	5′-CCACAAGTCTCCAGCAGAAG-3′	E10R1	5′-GTCTTAATCCACCCTGGAAG-3′
	E10F2	5′-CAGATAAGACCAGCACCCTG-3′	E10R2	5′-GAGTATCAGGCTCTCGACAG-3′
Exon12	E11F1	5′-TACAGGCGTGAGCCACTCTG-3′	E11R1	5′-AGGGAATGGAAGGCTGGAGC-3′
	E11F3	5′-TGAGGGTGAGAGATGAAGCC-3′	E11R3	5′-CTCTCTCATGGGAACATCAG-3′
Exon13	E12F2	5′-CCTTGGGTCAGCTTGTTCAG-3′	E12R2	5′-TCTTCAGCACCACCCAGCTC-3′
	E12F3	5′-CCTGTCTCTAACTGGGTAGC-3′	E12R3	5′-GACACTCCTCAGACCACAAG-3′
Exon14	E13F1	5′-ACTCTGAGCTCCTTGAAGGC-3′	E13R1	5′-TTGCTGTGGCCAGAGCTCCC-3′
	E13F2	5′-CAGGTTCTGTGGGCTTCAGG-4′	E13R2	5′-AGAGAAGATGGTGTTGAGGG-3′
Exon15	E14F1	5′-TGCAGCCACTGTAAAGCTAG-3′	E14R1	5′-GAGAGAGGACACTGCTACTC-3′
	E14F2	5′-TTCTCTTGGAGGGTGGGAGC-3′	E14R2	5′-AACTGGCCCAGGAAGAGAAG-3′
Exon16	E15F1	5′-CATCCCACTATTCTCAGGAG-3′	E15R1	5′-CTCCTTGCTGAAACAATCCC-3′
	E15F2	5′-ACTTCTGTTCTTGCCTGACC-3′	E15R2	5′-ATCCAACTGGCTGAGAAACC-3′

Fisher's exact test was used to determine the significance of differences. *P* values of 0.05 or less were considered statistically significant. The Hardy–Weinberg equilibrium (HWE) was tested using a commercial program (SNPAlyze ver 5.0 Standard; Dynacom, Chiba, Japan).

Pairwise locus disequilibrium analysis

The measure of linkage disequilibrium (LD) known as D' (Lewontin 1988), which is corrected for allele frequencies of loci, was computed for allele at pairs of SNP loci using the 26 patients. Tests of departures from linkage equilibrium were performed using the composite test for the overall SNPs. *P* values were determined via χ^2 approximation. As described above, significance was determined at the $P = 0.05$ level. These calculations were performed using a commercial program (SNPAlyze ver 5.0 Standard).

Haplotype frequency estimation

Haplotype frequencies were estimated by the method of maximum likelihood from genotype data through the use of the expectation–maximization (E–M) algorithm under the assumption of HWE (Excoffier and Slatkin 1995). Haplotype-based hypothesis tests focused on the case and control groups. Haplotypes of SNP were assessed using the EH package soft (http://linkage.rockefeller.edu/soft/). Chi-square statistics were derived from a series of simple 2×2 tables based on the frequency of each haplotype versus all others combined between the case and control groups. *P* values were determined via χ^2 approximation. Significance was determined at the $P = 0.05$ level.

Results

Based on the amino acid sequence deduced from the mouse *Mei1* cDNA, we have identified the human *MEI1* cDNA. Comparison of the cDNA and its corresponding genomic sequence revealed that *MEI1* is located to chromosome 22q13.2, consists of 16 exons encompassing over 47.1 kb in the genome, with an open reading frame (ORF) from nt 634 to 2,562 (Fig. 1). *MEI1* has at least two alternative transcripts of 2,714 and 2,609 bp (annotated as AY952376 and AY952377 in GenBank, respectively), encoding proteins of 642 and 607 amino acids, respectively. The shorter 2,609-bp cDNA lacks exon 7 as a result of alternative splicing. Furthermore, the 642 amino acid protein shares 77% homology with the mouse Mei1 protein (Fig. 2) and the cDNA in the coding region shares 61% homology with the mouse *Mei1* cDNA. However, no previously known functional domains could be found in human MEI1. Analysis of expression patterns of *MEI1* in various adult tissues revealed that it was predominately expressed in the testis and weakly in the spleen and thymus (Fig. 3).

Mutation analysis of *MEI1* revealed four nucleotide changes among the 27 patients, i.e., T909G in exon 4, A1582G in exon 8, C1791A in exon 9 and C2397T in exon 14. As the four coding single nucleotide polymorphisms (cSNPs) were hitherto undescribed or have not been registered in the NCBI database, they are novel cSNPs (Table 2). Among SNP1 (T909G), SNP2 (A1582G), SNP3 (C1791A) and SNP4 (C2397T), the latter three SNPs were observed in the heterozygous state in all but the Japanese patient and SNP2 is nonsynonymous (Thr–Ala). We did not find any of these SNPs in the Japanese patient.

Genotyping for *MEI1* SNP alleles among the 26 patients (13 European Americans and 13 Israelis) and 61 control individuals (European Americans) revealed that the genotype distribution and the allele frequency of SNP3 and SNP4 were significantly different between the two groups (Table 2). At the 1791A/C site (SNP3), the proportions of AA homozygote/AC heterozygote/CC homozygote in the patient and control groups were 0.00/0.154/0.846 and 0.000/0.000/1.000, respectively ($P = 0.0067$). The allele frequencies for 1791A/C in the two groups were 0.077/0.923 and 0.00/1.000, respectively, and were significantly different ($P = 0.0073$). Likewise, at the 2397T/C site (SNP4), the proportions of

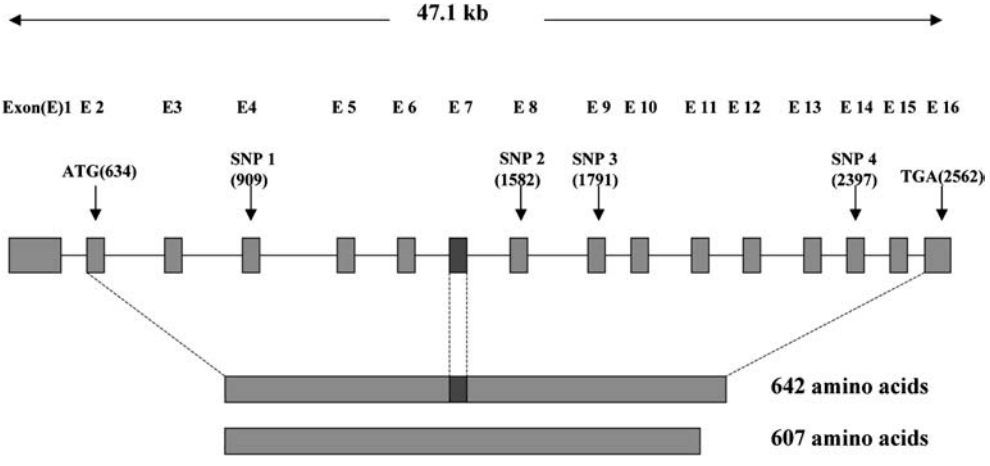

Fig. 1 Gene structure and four novel coding single-nucleotide-polymorphism (cSNP) sites in the human *MEI1* gene. The gene consists of 16 exons, and alternative splicing events generate two mRNA products. The shorter cDNA lacks exon 7. *Arrows* indicate four novel SNPs (SNP1, SNP2, SNP3 and SNP4), the start codon and the stop codon

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human: 1   MCLNLLSAPEKTGPPSKEELSAVSELLQHGLPQISSRSPESLAFLSDRQYMEGAARQRQY 60
           |||||||||||| | | |||||||| |||||| |||| |||||||||||| | | |||||
mouse: 254 MCLNLLSAPEKTEPLSQEELSAVSEFLQHGLPHISSRTPESLAFLSDRQYVEAATRQRQY 313

human: 61  CILLLFYLAYIHEDRFVSEAELFEAVQSFLLSLQDQGERPPLVVFKASIYLLAICQDKDN 120
           ||||||||| || |||| ||||| |||||||||||||| || || |||||||| | |||
mouse: 314 CILLLFYLAHIHDDRFVPEAELFVAVQSFLLSLQDQGECPPPVVCKASMYLLAVCGDKDS 373

human: 121 TLRETMVSAIRKFLEGIPDLQLVYTHHPLLLRFFLLYPELMSRYGHRVLELWFFWEESSY 180
            | |   |||||||||||||  |||||||||||| || |||||||||||||| |||| |
mouse: 374 ALAEAVISAIRKFLEGIPDLRGVYTHHPLLLRFFLAYPGLMSRFGHRVLELWFSWEESGY 433

human: 181 EELDDVTSAGQPALPASLVVLFQLLRSIPSILLILLDLIYSSPVDTAHKVLISLRTFLRR 240
           | |||  | |    || |  ||  | | |||||||||| |||||||| |||| || ||
mouse: 434 ENLDDDSSPGRTVFPANLAALFRVLQSTPSILLILLDLVYSSPVDTARKVLIVLRVFLWE 493

human: 241 NEDIQVGGLIRGHFLLILQRLLVEHGASPSGASGNLPLLLSLLSLMQLRNVSEQELDSVA 300
           |||  |||||||||||||||||| ||  |  ||||||| |||| | || ||||||| |
mouse: 494 NEDVKVGGLIRGHFLLILQRLLVEYGA--STSGGNLPLLLNLLSLVQMRNESEQELDSMA 551

human: 301 MKLLHQVSKLCGKCSPTDVDILQPSFNFLYWSLHQTTPSSQKRAAAVLLSSTGLMELLEK 360
           |||||||| |||||||  |||||||||||||||||||||||||||||||||| | |||||
mouse: 552 MKLLHQVSMLCGKCSPAHVDILQPSFNFLYWSLHQTTPSSQKRAAAVLLSSTALLELLEK 611

human: 361 MLALTLAK-ADSPRTALLCSAWLLTASFSAQQHKGSLQVHQTLSVEMDQVLKALSFPKKK 419
           |||||      |||| || |||||||||||||| | |||| |||||  ||||||||||
mouse: 612 MLALTWTETGSSPRTPLLSSAWLLTASFSAQQHNGNLQVHRTLSVELNQVLKALSFPKKM 671

human: 420 AALLSAAILCFLRTALRQSFSSALVALVPSGAQPLPATKDTVLAPLRMSQVRSLVIGLQN 479
            |||||||| |||||| |||||||| ||||| |||    | |||||  |||  | |||||
mouse: 672 SALLSAAILRFLRTALQQSFSSALVVLVPSGDQPLSTPEDAVLAPLGKSQVLALLIGLQN 731

human: 480 LLVQKDPLLSQACVGCLEALLDYLDARSPDIALHVASQPWNRFLLFTLLDAGENSFLRPE 539
           |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
mouse: 732 LLVQKDPLLSQACIGCLEALLDYLHARSPDIALHVASQPWNRFLLFTLLDAGENSFLRPE 791

human: 540 ILRLMTLFMRYRSSSVLSHEEVGDVLQGVALADLSTLSNTTLQALHGFFQQLQSMGHLAD 599
           ||||||||  |||| ||| |||| ||| || ||| ||| ||||||||  | |||| | |
mouse: 792 ILRLMTLFVQYRSSCVLSREEVGLILQGAALVDLSALSNDTLQALHGFLLQVQSMGLLND 851

human: 600 HSMAQTLQASLEGLPPSTSSGQPPLQDMLCLGGVAVSLSHIRN* 642
             | |||| |||||    |   ||  ||||||||| ||  |||
mouse: 852 QHMTQTLQSSLEGLCSRTFPAQPLFQDMLCLGGVSVSQAHIRG* 894
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Fig. 2 Comparison of amino acid sequences between human (upper sequence) and mouse MEI1 (lower sequence). There is 77% amino acid identity between the two sequences. *Vertical lines* Identical amino acids, *asterisks* stop codons

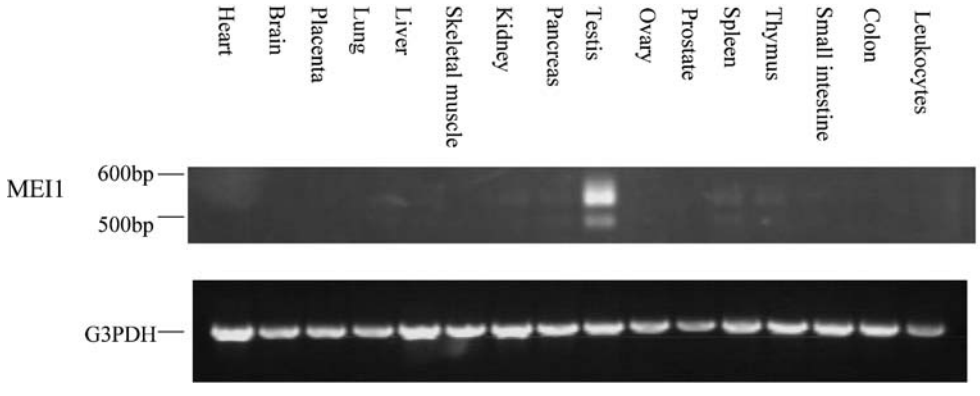

Fig. 3 RT–PCR analysis of human *MEI1* cDNA using primers EXP2F1 and EXP2R6. Expression patterns of *MEI1* in 15 human adult tissue cDNA samples were examined by PCR (*upper panel*). Two clear bands were detected in the testis and very weak bands were present in spleen and thymus. The upper and lower bands in the testis lane indicate the longer and shorter cDNAs, respectively. G3PDH was used as a positive control (*lower panel*)

Table 2 Genotype and allele frequencies of four coding single-nucleotide-polymorphism (cSNPs) in the human *MEI1* gene in 26 azoospermic patients (13 European Americans and 13 Israelis) and European American control individuals. *HWE* Hardy–Weinberg equilibrium

SNPs	Alterations		Genotype frequency					Allele frequency			
	Nucleotide	Amino acid	No of samples with genotype/total no of samples (%)			*P*-value[a]	HWE test[b]	No of chromosome with minor allele/total no of the chromosomes (%)			*P*-value[a]
			Genotype	Patients	Controls			Allele	Patients	Controls	
SNP1	909T/G	Synonymous	TG	15/26 (57.7)	16/53 (30.2)	0.0271*		G	17/52 (32.6)	40/106 (37.7)	0.4799
			GG	1/26 (3.8)	4/53 (7.5)	0.6622	0.37602				
SNP2	1582A/G	Thr/Ala	AG	1/26 (3.8)	0/56 (0)	0.3171	< 0.05	G	1/52 (1.9)	0/112 (0)	0.3171
SNP3	1791C/A	Synonymous	AC	4/26 (15.4)	0/60 (0)	0.0067*	0.33904	A	4/52 (7.7)	0/122 (0)	0.0073*
SNP4	2397C/T	Synonymous	TC	3/26 (11.5)	0/61 (0)	0.0245*	0.13582	T	3/52 (5.7)	0/122 (0)	0.0256*

*Statistically significant
[a]Fisher's exact test between patients and controls
[b]Genotypes significantly different from HWE proportions at $P = 0.05$ level

the respective zygosity in the two groups were 0.000/0.115/0.885 and 0.00/0.00/1.000 ($P = 0.0245$), and the allele frequencies were 0.057/0.943 and 0.000/1.000 ($P = 0.0256$). The most common genotypes for SNP3 and SNP4 in both groups were 1791C/C and 2397C/C. However, AC heterozygotes and TC heterozygotes were strikingly higher at the SNP3 and SNP4 sites in the patient group, respectively. There were no statistical differences for SNP1 and SNP2 in the two groups ($P > 0.05$ for both SNPs). Tests of HWE carried out for all SNPs among patients revealed that SNP2 of patients showed a significant deviation from HWE ($P < 0.05$).

We next analyzed a possible association of the *MEI1* SNPs with azoospermia separately among European Americans and among Israelis (Table 3). At the SNP3 site, the proportions of AA homozygote/AC heterozygote/CC homozygote in the 13 European American patients and their 61 controls were 0.00/0.154/0.846 and 0.000/0.000/1.000, respectively ($P = 0.0289$), and the frequencies for alleles A/C were 0.077/0.923 and 0.00/1.00, respectively, showing a significant difference ($P = 0.0299$). Similarly, at the SNP4 site, the proportions of the respective zygosity in the two groups were 0.000/0.154/0.846 and 0.000/0.000/1.000 ($P = 0.0289$), and the allele frequencies were 0.077/0.923 and 0.00/1.00 ($P = 0.0299$). However, no such association at the two polymorphic sites was observed between the 13 Israelis and their 60 control individuals ($P > 0.05$).

Haplotype analysis revealed that haplotype frequencies estimated for all four polymorphisms in the groups were close to each other, with no significant differences (data not shown). Haplotype estimation and LD analysis revealed different distributions of the haplotypes with SNP3 and SNP4 (Tables 4, 5). The SNP3–SNP4 (C–T), SNP3–SNP4 (A–C) and SNP3–SNP4 (A–T) haplotypes were revealed to be significantly more frequent in the European American patient group than in the European American control group. The SNP3–SNP4 (A–T) haplotype was also revealed to be significantly more frequent in the Israeli patient group than in the Israeli control group.

Discussion

We have isolated the human *MEI1* cDNA, which shares 61 and 77% homology at the nucleotide and amino acid levels, respectively, to the mouse *Mei1* cDNA. The

Table 3 Genotype and allele frequencies of SNPs 3 and 4 between 13 European American azoospermic patients and 61 controls, and between 13 Israeli patients and 60 controls

Populations	Genotype frequency					Allele frequency			
	SNP	Genotype	No of samples with genotype/total no of samples (%)		*P*-value[a]	Allele	No of chromosome with minor allele/total no of the chromosomes (%)		*P*-value[a]
			Patients	Controls			Patients	Controls	
Americans	SNP3	AC	2/13 (15.4)	0/61 (0)	0.0289*	A	2/26 (7.7)	0/122 (0)	0.0299*
	SNP4	TC	2/13 (15.4)	0/61 (0)	0.0289*	T	2/26 (7.7)	0/122 (0)	0.0299*
Israelis	SNP3	AC	2/13 (15.4)	1/60 (1.7)	0.0798	A	1/26 (3.8)	1/120 (8.3)	0.0819
	SNP4	TC	1/13 (7.7)	1/60 (1.7)	0.3265	T	1/26 (3.8)	1/120 (8.3)	0.3255

*Statistically significant
[a]Fisher's exact test between patients and controls

Table 4 Pairwise linkage disequilibrium (D' above diagonal) and statistical significance (*P*-value below diagonal) for the four SNPs in the human *MEI1* gene among 26 azoospermic patients (13 European Americans and 13 Israelis). *P*-values based on χ^2 distribution

	SNP1	SNP2	SNP3	SNP4
SNP1		−1	0.4003	0.1539
SNP2	0.5008		1	1
SNP3	0.3857	< 0.05*		1
SNP4	0.921	< 0.05*	< 0.05*	

*Significant at $P = 0.05$

Table 5 Estimated haplotype frequencies of SNP3 and SNP4 between 13 European American azoospermic patients and 61 controls, and between 13 Israeli patients and 60 controls. *P*-values based on χ^2 distribution

Haplotype	European American		Israeli	
	Patients ($n = 13$; %)	Control ($n = 61$; %)	Patients ($n = 13$; %)	Control ($n = 60$; %)
SNP3–SNP4				
C–C	81.9	100	88.8	98.7
C–T	6.8*	0	3.6	0.8
A–C	10.6*	0	7.4	0.8
A–T	8.9*	0	0.2*	0.1

*Significant at $P = 0.05$

longest ORF of the mouse *Mei1* comprises 2,685 bp and is predicted to encode a protein of 894 amino acids. Two alternative *MEI1* transcripts consisting of 2,714 and 2,609 bp encode proteins of 642 and 607 amino acids, respectively, as in the mouse *Mei1* gene (Libby et al. 2003). The human *MEI1* is not assigned to the Y chromosome but is located to 22q13.2. The predominant expression of *MEI1* in the testis is consistent with its putative role in spermatogenesis, as seen in the mouse *Mei1* gene (Libby et al. 2003). Positional cloning showed that mouse *Mei1* is responsible for the mutant mei1 phenotype. The mei1 mice lack the first 58 bp in exon 12 or entirely skip exon 12 of *Mei1*, resulting in a frameshift leading to a predicted truncated Mei1 protein. Male mice with such a mutated *Mei1* show spermatocytes arrested at meiosis, and the RAD51 protein does not load onto chromosomes bearing mutated *Mei1*, suggesting that there is a defect either in recombinational repair or in the production of double-strand breaks (DSBs) that require such repair (Libby et al. 2003). Recent studies on meiosis in ($Mei1^{-/-}$) and ($Dmc1^{-/-}$) mice of both sexes have demonstrated that their phenotypes are identical to those of $Mei1^{-/-}$ mice (Reinholdt and Schimenti 2005). Therefore, *Mei1* can be positioned upstream of *Dmc1* in the genetic pathway that operates during mouse meiosis. Further analysis is needed to determine the relationship between *MEI1* and *DMC1* in man.

We have identified four novel cSNPs in the *MEI1* gene. The present association study has revealed that the genotype distributions for SNP3 (A/C) and SNP4 (T/C) are significantly different between the European American azoospermic patients and their controls: 0.154/0.846 vs 0.000/1.000 for AC heterozygotes/CC homozygotes at the SNP3 site; and 0.154/0.846 vs 0.000/1.000 for TC heterozygotes/CC homozygotes at the SNP4 site, respectively ($P < 0.05$). Likewise, the frequencies of alleles A/C at SNP3 were 0.077/0.923 and 0.00/1.000; and those of alleles T/C at SNP4 were 0.077/0.923 vs 0.000/1.000 in the patients and controls, respectively ($P < 0.05$). These findings suggest that allele C at nucleotide 1,791 in exon 9 and allele C at nucleotide 2,397 in exon 14, or their flanking regions, may play a role in the disruption of spermatogenesis in the European American patients, although the number of patients analyzed was not large enough to allow a definitive conclusion to be drawn; no such association was found in Israeli patients.

HWE tests performed for the four cSNPs in European American and Israeli patients ruled out the equilibrium of SNP2 by its *P* value. This deviation is most likely due to the small sample size in the present study. We performed haplotype analysis on the synonymous SNPs identified as well as their combinations, and found no significant difference between the haplotype frequencies estimated for all four polymorphisms between the patient and control groups. We then performed LD analysis using pairs of SNPs. Consequently, we detected three pairs of SNPs (SNP2–SNP3, SNP2–SNP4 and SNP3–SNP4) with LD values of 1.00 and with *P* values of < 0.05 by χ^2 tests. We carried out haplotype analysis on the SNP3–SNP4 pair containing statistical differences in genotype and allelic levels. All analyses were performed both in European American and Israeli patients. Haplotype analysis demonstrated that three haplotypes, SNP3–SNP4 (C–T), SNP3–SNP4 (A–C) and SNP3–SNP4 (A–T), were markedly associated with azoospermia among the European American patients. Furthermore, such an association of a SNP3–SNP4 (A–T) haplotype was also found among the Israeli patients.

In vitro fertilization (IVF) has been proven to be an efficient way to resolve infertility due to female factors

(Edwards et al. 1980), but it has not been so effective for problems due to severe oligospermia in the male partner (Devroey and Van Steirteghem 2004). Although testicular sperm extraction (TESE)–intracytoplasmic sperm injection (ICSI) is now available for patients with azoospermia, it cannot help patients lacking spermatozoa in their testes due to a complete failure in spermatogenesis. Therefore, treatment for infertility due to non-obstructive azoospermia is an important immediate goal in assisted reproductive technology (ART).

In conclusion, this is the first report showing that *MEI1* SNPs may predispose men to a defect in spermatogenesis, although the mechanism by which these SNPs result in azoospermia remains uncertain. Our results may also advance a better understanding of the molecular basis of early meiotic arrest as a cause of non-obstructive azoospermia. It remains to be confirmed whether the association is seen in larger sample numbers and in similar patients from other ethnic groups, although men with azoospermia caused by meiotic arrest are very rare.

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