

IS A GENETIC DEFECT IN *Fkbp6* A COMMON CAUSE OF AZOOSPERMIA IN HUMANS?

TOSHINOBU MIYAMATO^{1,*#} HISASHI SATO^{1#}, LEAH YOGEV², SANDRA KLEIMAN², MIKIO NAMIKI³, EITETSU KOH³, NAOKO SAKUGAWA¹, HIROAKI HAYASHI¹, MUTSUO ISHIKAWA¹, DOLORES J. LAMB⁴ and KAZUO SENGOKU¹

¹Department of Obstetrics and Gynecology, Asahikawa Medical College, Midorigaokahigashi 2-1-1-1, Asahikawa 078-8510, Japan, ²Institute for the Study of Fertility, Lis Maternity Hospital, Tel Aviv Sourasky Medical Center, Tel Aviv, 64239, Israel, ³Department of Integrated Cancer Therapy and Urology, Kanazawa University Graduate School of Medical Science, Kanazawa, 920-8641, Japan, ⁴Scott Department of Urology, and Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas, 77030, USA

Abstract: FK506-binding protein 6 (*Fkbp6*) is a member of a gene family containing a prolyl isomerase/FK506-binding domain and tetratricopeptide protein-protein interaction domains. Recently, the targeted inactivation of *Fkbp6* in mice has been observed to result in aspermic males and the absence of normal pachytene spermatocytes. The loss of *Fkbp6* results in abnormal pairing and a misalignment of the homologous chromosomes, and in non-homologous partner switches and autosynapsis of the X chromosome cores in meiotic spermatocytes. In this study, we analyzed whether human *FKBP6* gene defects might be associated with human azoospermia. We performed a mutation analysis in all the coding regions of the human *FKBP6* gene in 19 patients with azoospermia resulting from meiotic arrest. The expression of the human *FKBP6*

* Author for correspondence: e-mail: toshim@asahikawa-med.ac.jp, phone: +81-166-68-2562, fax: +81-166-68-2569

These authors equally contributed to this paper

Abbreviations used: AZF – azoospermia factor; DAZ – deleted in azoospermia; *Fkbp6* – FK506-binding protein 6; RBMY – RNA-binding motif; Y chromosome; RT-PCR – reverse transcription-polymerase chain reaction; SC – synaptonemal complex; USP9Y – ubiquitin-specific protease 9 gene on the Y

gene was specific to the testis, and a novel polymorphism site, 245C→G (Y60X) could be found in exon 3. Our findings suggest that the human *FKBP6* gene might be imprinted in the testis based on an analysis using two polymorphism sites.

Key words: Azoospermia, *FKBP6*, Genomic imprinting, Meiosis, Polymorphism

INTRODUCTION

FK506-binding protein 6 (Fkbp6) belongs to a gene family that contains a prolyl isomerase/FK506-binding domain and tetratricopeptide protein-protein interaction domains [1]. Recently, mouse *Fkbp6* cDNA was isolated by RT-PCR using RNA from the mouse testis [2]. Its expression is restricted to the testis in mice. *Fkbp6* mRNA and protein were detected in the cytoplasm and nucleus of spermatocytes, but not in the cells that had exited prophase I or in spermatids obtained by *in situ* hybridization.

FK506-binding protein localizes to meiotic chromosome cores and regions of homologous chromosome synapsis. The targeted inactivation of *Fkbp6* left both male and female mutant mice healthy and with normal life-spans [2]. However, male mice deficient in *Fkbp6* were completely sterile. The testes of *Fkbp6*^{-/-} males were also reduced in size. A histological analysis revealed a lack of spermatids and mature spermatozoa in the seminiferous tubules of *Fkbp6*^{-/-} males. A loss of *Fkbp6* expression resulted in abnormal pachytene spermatocytes which failed to proceed beyond the pachytene stage. On the other hand, *Fkbp6*^{-/-} females were able to breed up to one year after birth. The overall histological phenotypes of natural mutant aspermic as/as rats are very similar to those observed in *Fkbp6*^{-/-} mice, including the presence of the inclusion bodies in spermatocytes [3-5]. The deletion of *Fkbp6* exon8 was identified as the causative mutation in as/as mutant rats [2]. Loss of the mouse *Fkbp6* results in abnormal pairing and misalignments of homologous chromosomes, and in non-homologous partner switches and autosynapsis of X chromosome cores in meiotic spermatocytes. Mouse *Fkbp6* is not involved in the initiation of synapsis, but it does play a role in monitoring progression and/or maintaining synapsis between homologous pairs. Therefore, Fkbp6 is considered to be a component of the synaptonemal complex essential for sex-specific fertility and the fidelity of homologous chromosome pairing in meiosis.

Once homologues are paired, the chromosomes are connected by a specific structure known as the synaptonemal complex (SC) [6]. Although SCs were first discovered more than 45 years ago, only three meiosis-specific structural components of the SC have been identified in mammals, namely SCP1 (SYCP1), SCP2 (SYCP2) and SCP3 (SYCP3) [6]. The genetic inactivation of the mouse *Sycp3* gene results in male infertility due to a failure of chromosome synapsis during the meiotic phase [7]. The mouse *Sycp3*^{-/-} results in spermatogenetic arrest at the zygotene stage, suggesting that *Fkbp6* and *Sycp3* have different functions in chromosome synapsis in males. Female *Sycp3*^{-/-} mice

have reduced fertility, and embryos from *Sycp3*^{-/-} mothers have increased incidence of aneuploidy [8].

We recently reported that a 1-bp deletion in human *SYCP3* causes azoospermia resulting from meiotic arrest in two of nineteen patients [9]. In humans, the deletion of the AZF (azoospermia factor) region on the Y-chromosome has been shown to disrupt spermatogenesis, causing infertility in otherwise healthy men. It was reported that disruptions of the following three genes result in azoospermia: *DAZ*, *RBM1* and *USP9Y* [10-12]. All of these are located in the AZF region. In addition, the human *DAZ* and *RBM1* encode RNA-binding proteins. The human *SYCP3* gene encodes DNA-binding protein; however, it is located on chromosome 12. *SYCP3* is therefore the first gene found outside of the AZF region whose mutation may cause azoospermia.

This data was obtained from mouse mutants and spontaneous mutant rats; it would be interesting to investigate whether mutations in the human *FKBP6* gene might account for human azoospermia. In this study, we performed a mutation analysis of the *FKBP6* gene in patients with azoospermia resulting from meiotic arrest. Our findings also suggest that the human *FKBP6* gene might be imprinted in the human testis.

MATERIALS AND METHODS

Expression analysis of the human *FKBP6* using PCR of adult cDNA

For an expression analysis of the human *FKBP6*, PCR of cDNA from various tissue types was done using FKBP6RTF1 and FKBP6RTR1 as primers. cDNA from the following adult human tissue types was examined: spleen, thymus, prostate, testis, ovary, small intestine, colon, leukocyte, brain, heart, kidney, liver, lung, pancreas and placenta (Clontech). The following oligonucleotides were used: FKBP6RTF1 = 5'-GATCAGCACCCCCTGAAGAG-3' and FKBP6RTR1 = 5'-TCTCCTGCTGTAGAACCATC-3'.

All the PCR reactions were carried out as follows: 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 the Advantage 2 PCR Kit (Clontech).

Patients and controls

Idiopathic azoospermia was confirmed by two consecutive semen analyses obtained after five to seven days of sexual abstinence and an examination of a centrifuged semen pellet. Patients with defective spermatogenesis following infection, or due to an obstruction of the seminal tract, pituitary failure, or other causes of possible testicular damage revealed at clinical examinations were excluded from the study. Final diagnoses were carried out based on histological examinations.

A chromosome analysis of peripheral lymphocytes showed a karyotype of 46, XY in all the test subjects. Those found to have microdeletions of the Y chromosome after the examination of 33 loci, including *DAZ*, *USP9Y* and

RBMY, were also excluded from the study. We thus had 19 test subjects with azoospermia caused by meiotic failure. They were of Caucasian, Hispanic, Arab and Asian ethnicity.

Forty healthy and pregnancy-proven fertile control individuals were also analyzed. All the subjects gave their informed consent to undergo a molecular analysis of their blood and testicular tissue, and the study was approved by a local ethics committee.

Testicular histology

For all the subjects with idiopathic azoospermia, bilateral testicular biopsy samples were obtained under local anesthesia using small curved scissors through a small scrotal window. The samples were immediately placed into freshly prepared Bouin's solution. More than 30 different cross-sections cut at right angles to the longitudinal axis of the seminiferous tubules were analyzed.

Mutation screening

Genomic DNA was obtained from peripheral lymphocytes using a QIAGEN Blood and Cell Culture DNA Midi Kit (QIAGEN, Hilden, Germany). The full-length cDNA sequences were compared to human genomic sequences (NT_007758.10), and all the exon-intron borders were determined. Nested PCR was performed using primers homologous with the intronic regions adjacent to each exon, from exon 1 to exon 8, and using human DNA samples.

The following primer sets were used for PCR:

Exon 1: FKBPE1F1, FKBPE1F2, FKBPE1R1 and FKBPE1R2;

Exon 2: FKBPE1F1, FKBPE1F2, FKBPE2R1 and FKBPE2R2;

Exon 3: FKBPE3F1, FKBPE3F2, FKBPE3R1 and FKBPE3R2;

Exon 4: FKBPE4F1, FKBPE4F2, FKBPE4R1 and FKBPE4R2;

Exon 5: FKBPE5F1, FKBPE5F2, FKBPE5R1 and FKBPE5R2;

Exon 6: FKBPE6F1, FKBPE6F2, FKBPE6R1 and FKBPE6R2;

Exon 7: FKBPE7F1, FKBPE7F2, FKBPE7R1 and FKBPE7R2;

Exon 8: FKBPE8F1, FKBPE8F2, FKBPE8R1 and FKBPE8R2.

All the oligonucleotide primers are listed in Tab. 1. All the PCR reactions were carried out as follows: initial denaturation at 95°C for 150 s, followed by 32 cycles of denaturation at 95°C for 30 s, annealing at (primers $T_m - 5^\circ\text{C}$) for 90 s and extension at 72°C for 90 s. An aliquot of each PCR product was subsequently subjected to 20 more cycles under the same conditions. The resultant PCR products were subjected to under a direct sequence analysis in both orientations.

Genotyping of the identified polymorphism

A 245C→G polymorphism was found in exon 3 by a mutation analysis of genomic DNA from 19 patients. This polymorphism was analyzed by PCR and direct sequencing using the following primers: FKBPE3F1, FKBPE3F2, FKBPE3R1 and FKBPE3R2. The PCR products of 30 healthy controls were also used for direct sequencing.

Tab. 1. Oligonucleotide primers used in the mutation analysis, and the sizes of the PCR product.

Exon	Primer sequence	Size (bp)
1	FKBPE1F1=5'-CAGAATGGAATGCCGCCGTC-3' FKBPE1F2=5'-CAGAGTCACAGCCAGGAAGG-3' FKBPE1R1=5'-TCTGACTTAACCGCTCGTAC-3' FKBPE1R2=5'-GATGGAGAATGAAAGCCAGG-3'	214
2	FKBPE2R1=5'-ACTGAGCGAGACCCCGTTTC-3' FKBPE2R2=5'-AAATGAGGCCTCTCTCTTCC-3'	405
3	FKBPE3F1=5'-TATTCCTGTACCTCGTGGTG-3' FKBPE3F2=5'-GCTTAAGCTCATCTGATCGC-3' FKBPE3R1=5'-AACCTGCGCTCTGAGCAGTC-3' FKBPE3R2=5'-CTGAGGCATGGTGCCTCATC-3'	266
4	FKBPE4F1=5'-TGTGTGAGGGAGACATGGAG-3' FKBPE4F2=5'-CCAGAGTACTGCTGACTTAG-3' FKBPE4R1=5'-CTGTTGCCCATGCTGGAGTG-3' FKBPE4R2=5'-CAGGGCAGGCCATCTAGAAG-3'	382
5	FKBPE5F1=5'-GTTCTCACTATGTTGCCCAG-3' FKBPE5F2=5'-ACTCCTGGGCTCACGTGTAC-3' FKBPE5R1=5'-GAGCTGAGATTGCGCCACTG-3' FKBPE5R2=5'-CAGAGCGACACTCTTGTCTC-3'	331
6	FKBPE6F1=5'-TCTTGCAACCTGTAACAGCC-3' FKBPE6F2=5'-TGCGTGTGGATTCTTTAGGG-3' FKBPE6R1=5'-ACTCCATCCTGGGCAACAAG-3' FKBPE6R2=5'-AGTTGAGCACTGCTACTACC-3'	331
7	FKBPE7F1=5'-ATCCCTGACCTCAGTTGATC-3' FKBPE7F2=5'-CAAAGTGCTGGGATTACAGG-3' FKBPE7R1=5'-CTGCTTGCTGGGAACTGCTG-3' FKBPE7R2=5'-CACCGCCAGACTGTCCCTTG-3'	295
8	FKBPE8F1=5'-ACTTCCATCCCTCCCAACTC-3' FKBPE8F2=5'-GTTTCTGGATGTGAGTGGAG-3' FKBPE8R1=5'-AGCAAGGAACTTGTCTACCC-3' FKBPE8R2=5'-AGACAGCCACCATTCACTTG-3'	270

Genomic imprinting of the human *FKBP6* gene

Eleven biopsies, one from the testes of one patient with azoospermia and ten from those of healthy controls were studied. The samples were washed with saline solution, rapidly frozen in liquid nitrogen, and then ground to powder.

Part of the powdered tissue was used to obtain RNA. RNA was extracted with guanidinium thiocyanate, followed by centrifugation in a caesium chloride gradient. The total RNA was treated with *DNase* I (Roche, Tokyo, Japan) at 37°C for 30 min to prevent contamination. The cDNA was synthesized from total RNA, using a cDNA synthesis kit (Roche). RT-PCR was carried out with total RNA, under the same conditions as for PCR using genomic DNA. Negative controls without oligo (dT) primer or reverse transcriptase were used.

To examine whether the human *FKBP6* gene is imprinted or not, PCR and direct sequence analysis were performed using genomic DNA and testicular cDNA from ten healthy controls. First, PCR was carried out with the primers FKBPRTF1 and FKBPRTR1. Nested PCR was done with the primers FKBPRTF3 and FKBPRTR3. The following oligonucleotides were used:

FKBPRTF1 = 5'-GCCACAGGGAGGGCAGCTAGGACATGGG-3';

FKBPRTF3 = 5'-CCTGTACGAGCGGTTAAGTCAGAGGATG-3';

FKBPRTR1 = 5'-CAGCTCAATCTCAAACAGGACAGTGGTG-3'; and

FKBPRTR3 = 5'-TCAGAAGGCCAGCTCCATGCCCCACAG-3'.

First PCR reactions were carried out under the following conditions: 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 the Advantage 2 PCR Kit (Clontech). Nested PCR was performed under the same conditions, but for 20 cycles. Following this, PCR and direct sequence analysis were carried out with genomic DNA and testicular cDNA from one patient with azoospermia caused by meiotic arrest.

When a mutation analysis was performed, one more SNP (single-nucleotide-polymorphism) (656G→A) was found in exon 6. To confirm whether or not the human *FKBP6* gene was indeed imprinted, PCR and a direct sequence analysis were carried out using genomic DNA from the twenty controls. The primers used were FKBPE6F1, FKBPE6F2, FKBPE6R1 and FKBPE6R2. Next, RT-PCR and a direct sequence analysis was carried out with testicular RNA from each, including a heterozygous sequence. First PCR was done with the primers FKRTF1 and FKRTF1. Nested PCR was performed with the primers FKRTF3 and FKRTF3. The following oligonucleotides were used:

FKRTF1 = 5'-ACTGTGCTGAGTCAGACAAG-3';

FKRTF3 = 5'-GTCCTGAAAGTGGCAGCTAC-3';

FKRTR1 = 5'-TGTAGAACCATCGCCACAGG-3'; and

FKRTR3 = 5'-TCCTTCTGGGCTCGAACTAG-3'.

First PCR and nested PCR were carried out under the same conditions. The conditions were as follows: 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 the Advantage 2 PCR Kit.

RT-PCR and direct sequence analyses with testicular RNA from a subject with azoospermia due to meiotic arrest

Two biopsies from the testes of a subject with azoospermia and one from a healthy control were studied. The samples were washed with saline solution,

rapidly frozen in liquid nitrogen, and then ground to powder. Part of the powdered tissue was used to obtain RNA, which was extracted with guanidinium thiocyanate followed by centrifugation in a caesium chloride gradient. Total RNA was treated with *DNase* I at 37°C for 30 min to prevent contamination. The cDNA was synthesized from total RNA using a cDNA synthesis kit. RT-PCR was carried out with total RNA using FKFULLF1 and FKFULLR1 as primers. Nested PCR was performed with FKFULLF2 and FKFULLR2 as primers. The following oligonucleotides were used:

FKFULLF1 = 5'-ACCAGGCCGAAGGCTCACGCCAC-3';

FKFULLF2 = 5'-GCCACAGGGAGGGCAGCTAGGAC-3';

FKFULLR1 = 5'-CCACTCCCCAATGATGGAGAACCAC-3'; and

FKFULLR2 = 5'-CTCCCCTCGTTGGTAGGTGAAGAACC-3'.

First PCR reactions were carried out as follows: 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. An aliquot of each PCR product was subsequently subjected to 20 more cycles under the same conditions. The resultant PCR products were subjected to a direct sequence analysis in both orientations. Negative controls without oligo (dT) primer or reverse transcriptase were used.

RESULTS

To determine the expression patterns of human *FKBP6* in normal tissue, PCR was performed with cDNAs of various tissues as templates. PCR was done with the primers FKBP6RTF1 and FKBP6RTR1. A 367-bp sized band was clearly detected specifically in the testis on the *FKBP6* (Fig. 1). No bands could be detected in the other 14 tissue specimens.

Human *FKBP6* cDNA was first isolated in 1998 [1]. The nucleotide sequence is available from GenBank under the accession number NM_003602. This 1546-bp cDNA spans an open reading frame (ORF) from nucleotide 63 to 1046, encoding a putative 327-amino acid protein. It is located on human chromosome 7q11.23. The coding region is from exon 1 to exon 8 (Fig. 1). The mutation screen of *FKBP6* in 19 patients with azoospermia due to meiotic arrest was done via a direct sequence analysis in all the coding regions. *as/as* rats harbor a genomic deletion of a 9357-bp region including exon 8 of the rat *Fkbp6* gene [2]. However, no such gross mutations were detected in any of the patients (data not shown). When a mutation analysis was performed, one novel polymorphism site, 245C→G (Y60X), could be found in exon 3 in four of the patients (Fig. 2A).

To confirm the role of this polymorphism in azoospermia, 30 healthy and pregnancy-proven fertile control individuals were also analyzed by the direct sequencing of exon 3. 10 people in the control group were also heterozygous for the sequences analyzed (data not shown). At the 245G/C site, the proportions of GG homozygote/CG heterozygote/CC homozygote were 0.00/0.211/0.789 in the patient group and 0.000/0.333/0.667 in the control group ($p > 0.05$). The allele

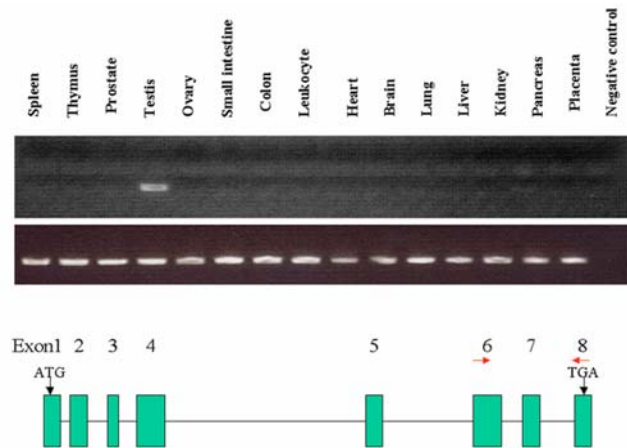


Fig. 1. PCR analysis of human *FKBP6* cDNA using the primers FKBP6RTF1 and FKBP6RTR1 (upper panel). The distribution patterns of *FKBP6* in 15 adult human tissue cDNAs were examined by PCR. β -Actin was used as a positive control (middle panel). The lower panel indicates the structure of the human *FKBP6* gene. The black arrows indicate the start and stop codons. The red arrows indicate the positions of the primers used.

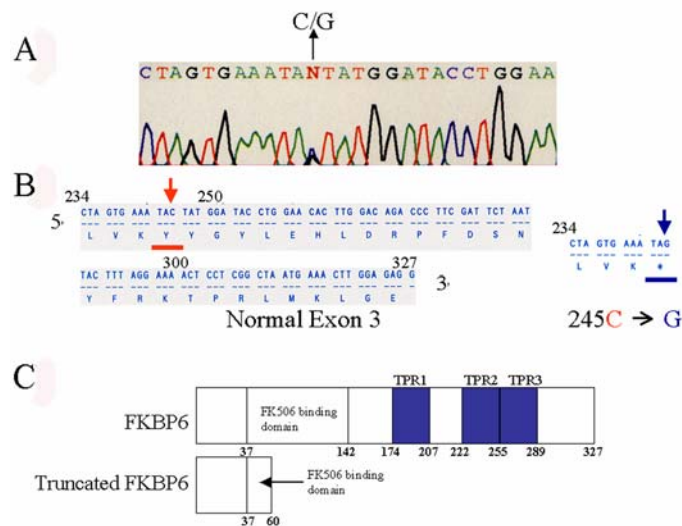


Fig. 2. Genotyping of the identified polymorphism. A – Partial sequences of exon 3 of human *FKBP6* by direct sequence analysis using genomic DNA from a subject with azoospermia. The arrow indicates heterozygotes (cytosine and guanine) (Y60X). B – The deduced amino acid sequences of exon3. The left panel shows normal amino acid sequences, while the right panel shows a premature stop codon. Arrows indicate the position of Y60X. The area underlined in red shows the normal amino acid sequence, while the area underlined in blue shows the stop codon. C – The structure of the FKBP6 protein. The upper panel shows the normal structure and the lower panel shows the truncated version. The TPRs indicate the positions of the tetratricopeptide repeat domains.

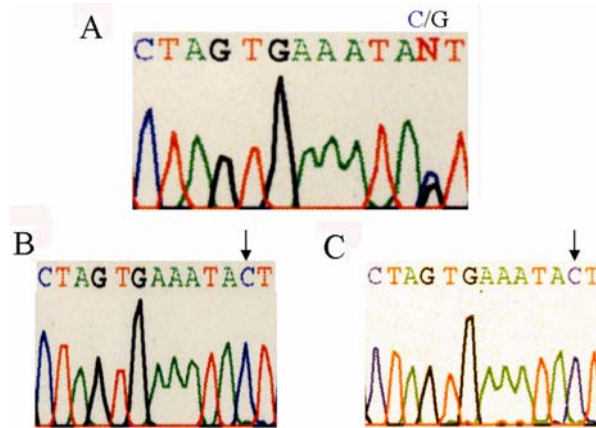


Fig. 3. Genomic imprinting of the *FKBP6* gene. A – A direct sequence analysis of genomic DNA shows heterozygous (cytosine and guanine) (Y60X) sequences. B – A direct sequence analysis of testicular cDNA from a normal control subject demonstrates only cytosine expression (arrow). C – A direct sequence analysis of testicular cDNA from the patient with azoospermia due to meiotic arrest demonstrates only cytosine expression (arrow).

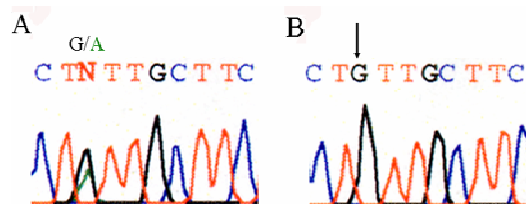


Fig. 4. Genomic imprinting of *FKBP6*. A – A direct sequence analysis of genomic DNA shows the heterozygous (guanine and adenine) sequences. B – A direct sequence analysis of testicular cDNA from one normal control subject demonstrates only guanine expression (arrow).

frequencies for 245G/C in the two groups were 0.105/0.895 and 0.167/0.833, respectively, and no significant difference was observed ($p > 0.05$). The human *FKBP6* gene encodes 327 amino acids. However, if a change in this sequence (245C→G) (Y60X) exists, a premature stop codon appears (Fig. 2B). FK506-binding protein 6 (Fkbp6) contains a prolyl isomerase/FK506-binding domain and tetratricopeptide protein-protein interaction domains (Fig. 2C). If the premature stop codon appears, only 60 amino acids are transcribed, and the encoded FK-506-binding domain is incomplete (Fig. 2C). Heterozygous genomic DNA (C/G) was observed at the 245 nt position in the people analyzed in this study (Fig. 3A).

An incomplete amino acid is translated when guanine exists at the 245 nt position. We therefore suspect that the human *FKBP6* gene is imprinted in a manner by which guanine is not translated into RNA. A direct sequence analysis was performed using cDNA from the testes of ten healthy control subjects that were heterozygous at the genomic level. Only cDNA containing cytosine was expressed in the testicular tissue from the ten controls (Fig. 3B). Therefore, it was suggested that the human *FKBP6* gene might be imprinted. We also hypothesized that guanine might be expressed, thus resulting in a premature stop codon and azoospermia by meiotic arrest in patients with azoospermia. However, a direct sequence analysis using the cDNA from one subject with azoospermia demonstrated the expression of cytosine in this patient (Fig. 3C). When a mutation analysis was performed, one more novel polymorphism site, 656G→A (silent) could be found in exon 6 in the two patients (data not shown). To confirm whether or not the human *FKBP6* gene is imprinted, twenty normal controls were analyzed. Only one person in the control group was also heterozygous for the sequences analyzed (Fig. 4A). A direct sequence analysis was carried out using cDNA from the testis of a person heterozygous for the gene under study. Only cDNA containing guanine was expressed (Fig. 4B). This result also suggested the possibility that the *FKBP6* gene is imprinted in humans. To assess whether the patient with polymorphism (Y60X) has a deletion at the RNA level, RT-PCR was done with the testicular RNA of one patient and one healthy control. Next, a direct sequence analysis was performed using both PCR products. However, no deletion at the RNA level was detected in the analyzed patient (data not shown).

DISCUSSION

Chromosomal defects and specific gene mutations result in male infertility, and many idiopathic cases are also thought to have a genetic basis [13]. Unfortunately, while numerous genes associated with male infertility are known in mice, the translation of this information to humans has been slow. Karyotypic abnormalities have been reported in up to 13% of men with azoospermia, and Yq chromosome deletions are present in up to 21% of men with infertility [14]. Defects of meiosis during spermatogenesis are significant causes of azoospermia, but these causes remain largely unknown.

The loss of mouse *Fkbp6* causes azoospermia with abnormal pachytene spermatocytes. These spermatocytes fail to proceed beyond the pachytene stage due to a failure to maintain homologous synapsis. The natural mutation of rat *Fkbp6* also results in such azoospermia. In addition, in this study, we demonstrated that human *FKBP6* is expressed specifically in the testis. These results suggest that human *FKBP6* plays a critical role in meiosis during spermatogenesis.

One novel polymorphism (245C→G) (Y60X) was found in exon 3 in the patients. The expression of the exon 3 polymorphism (245C→G) results in

a premature stop codon. If guanine is then translated into RNA, an incomplete protein of only 60 amino acids is encoded. We therefore suspected genomic imprinting of human *FKBP6* in the testis. Unfortunately, we were unable to obtain the genomic DNA from the parents of the test subjects. Therefore, we could not analyze whether inheritance was from the mother or father. Genomic imprinting is a form of non-mendelian inheritance in mammals, where imprinted genes are expressed depending on whether they are inherited from the mother or father. Therefore, it is absolutely necessary to demonstrate that the alleles expressed in the subjects analyzed in this study have the same parental origin. In 11 different individuals (healthy controls and patients), only alleles expressing the cytosine at position 245 in exon 3 were detected. The presence of guanine at this position leads to the formation of a premature stop codon. As a result, a failure to detect alleles containing guanine could thus be explained by some other mechanism, such as nonsense mediated decay (NMD). NMD is an mRNA surveillance pathway that detects and eliminates mRNAs harboring premature translation termination codons [15]. We therefore analyzed another polymorphism site in exon 6, 656G→A (silent). Only cDNA containing guanine was expressed. This result suggested the possibility that the *FKBP6* gene is imprinted in humans. However, as we could not analyze the parents, the results in this study do not conclusively show that the human *FKBP6* gene is imprinted. To test the hypothesis that human *FKBP6* gene defects are associated with human azoospermia, we screened 19 patients diagnosed with azoospermia caused by complete meiotic arrest. We also investigated whether the expression of guanine at the 245 nt position (Y60X) might be responsible for azoospermia by producing a premature stop codon and inducing meiotic arrest. However, as in the normal controls, only cytosine was expressed in the subjects with azoospermia. These results did not support our hypothesis; however, it should be noted that the cytosine expression was only examined in one patient with azoospermia in this study. Azoospermia caused by meiotic arrest is a relatively infrequent event and it is difficult to obtain testicular tissue; thus, the number of samples examined in this study was limited. It is known that SNPs in the promoter and intron region were associated with several diseases [16]. However, as the amounts of the genomic DNAs of the patients with azoospermia were limited, we could not analyze such regions. It was recently reported that a mutation screen was performed in 51 men with non-obstructive azoospermia, and no homozygous mutations were identified [17]. However, a histological analysis was not performed on the analyzed patients. Based on these results, we could not conclude that mutations of human *FKBP6* do not cause azoospermia by meiotic failure. To confirm our results, a further analysis of human *FKBP6* expression in a greater number of patients is needed.

In summary, we determined that the human *FKBP6* gene is expressed specifically in the testis and our findings suggested that the gene might be imprinted. This data suggests that human *FKBP6* might play an important role in human spermatogenesis. Further analysis of the human *FKBP6* gene is needed.

Acknowledgments. This study was supported in part by Grants-in-Aid for Scientific Research (Nos. 16390471, 15591725 and 16790934) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, from the Ministry of Health, Labour and Welfare of Japan, and from the Kanzawa Medical and Akiyama foundation.

REFERENCES

1. Meng, X., Lu, X., Morris, C.A. and Keating, M.T. A novel human FKBP6 is deleted in Williams syndrome. **Genomics** 52 (1998) 130-137.
2. Crackower, M.A., Kolas, N.K., Noguchi, J., Sarao, R., Kikuchi, K., Kaneko, H., Kobayashi, E., Kawai, Y., Kozieradzki, I., Landers, R., Mo, R., Hui, C.C., Nieves, E., Cohen, P.E., Osborne, L.R., Wada, T., Kunieda, T., Moens, P.B. and Penninger, J.M. Essential role of Fkbp6 in male fertility and homologous chromosome pairing in meiosis. **Science** 300 (2003) 1291-1295.
3. Ikadai, H., Noguchi, J., Yoshida, M. and Imamichi, T. An aspermia rat mutant (as/as) with spermatogenic failure at meiosis. **J. Vet. Med. Sci.** 54 (1992) 745-749.
4. Atagi, Y., Ikadai, H., Kurohmaru, M. and Hayashi, Y. Testicular disruption in the As (aspermia) mutant rat, with special reference to the aggregate of ribosomes. **J. Vet. Med. Sci.** 55 (1993) 301-306.
5. Noguchi, J., Kobayashi, E., Shimada, A., Kikuchi, K., Kaneko, H., Takahashi, H., Ikadai, H. and Kunieda, T. A locus responsible for arrest of spermatogenesis is located on rat chromosome 12. **Mamm. Genome** 10 (1999) 189-190.
6. Zickler, D. and Kleckner, N. Meiotic chromosomes: integrating structure and function. **Annu. Rev. Genet.** 33 (1999) 603-754.
7. Yuan, L., Liu, J.G., Zhao, J., Brundell, E., Daneholt, B. and Hoog, C. The murine SCP3 gene is required for synaptonemal complex assembly, chromosome synapsis, and male fertility. **Mol. Cell** 5 (2000) 73-83.
8. Yuan, L., Liu, J.G., Hoja, M.R., Wilbertz, J., Nordqvist, K. and Hoog, C. Female germ cell aneuploidy and embryo death in mice lacking the meiosis-specific protein SCP3. **Science** 296 (2002) 1115-1118.
9. Miyamoto, T., Hasuike, S., Yogev, L., Maduro, M.R., Ishikawa, M., Westphal, H. and Lamb, D.J. Azoospermia in patients heterozygous for a mutation in SYCP3. **Lancet** 362 (2003) 1714-1719.
10. Reijo, R., Lee, T.Y., Salo, P., Alagappan, R., Brown, L.G., Rosenberg, M., Rozen, S., Jaffe, T., Straus, D., Hovatta, O., de la Chapelle, A., Silber, S. and Page, D.C. Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. **Nat. Genet.** 10 (1995) 383-393.
11. Elliott, D.J., Millar, M.R., Oghene, K., Ross, A., Kiesewetter, F., Pryor, J., McIntyre, M., Hargreave, T.B., Saunders, P.T., Vogt, P.H., Chandley, A.C.

- and Cooke, H. Expression of RBM in the nuclei of human germ cells is dependent on a critical region of the Y chromosome long arm. **Proc. Natl. Acad. Sci. USA** 94 (1997) 3848-3853.
12. Sun, C., Skaletsky, H., Birren, B., Devon, K., Tang, Z., Silber, S., Oates, R. and Page, D.C. An azoospermic man with a de novo point mutation in the Y-chromosomal gene USP9Y. **Nat. Genet.** 23 (1999) 429-432.
 13. Matzuk, M.M. and Lamb, D.J. Genetic Dissection of Mammalian Fertility Pathways. **Nat. Med.** 8(S1) (2002) S41-S49.
 14. Nakamura, Y., Kitamura, M., Nishimura, K., Koga, M., Kondoh, N., Takeyama, M., Matsumiya, K. and Okuyama, A. Chromosomal variants among 1790 infertile men. **Int. J. Urol.** 8 (2001) 49-52.
 15. Lejeune, F. and Maquat, L.E. Mechanistic links between nonsense-mediated mRNA decay and pre-mRNA splicing in mammalian cells. **Curr. Opin. Cell Biol.** 17 (2005) 309-315.
 16. Ozaki, K., Ohnishi, Y., Iida, A., Sekine, A., Yamada, R., Tsunoda, T., Sato, H., Sato, H., Hori, M., Nakamura, Y. and Tanaka, T. Functional SNPs in the lymphotoxin- α gene that are associated with susceptibility to myocardial infarction. **Nat. Genet.** 32 (2002) 650-654.
 17. Westerveld, G.H., Repping, S., Lombardi, M.P. and van der Veen, F. Mutations in the chromosome pairing gene FKBP6 are not a common cause of non-obstructive azoospermia. **Mol. Hum. Reprod.** 11 (2005) 673-675.