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Genetic Variation in the Testis-Specific *HASPIN* Gene Encoding a Serine/Threonine Protein Kinase in Infertile Japanese Males

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Abstract

HASPIN is a serine/threonine protein kinase predominantly expressed during spermatogenesis and localized in the nucleus. The *HASPIN* gene is conserved from yeast to mammals and plants. To investigate any possible associations between *HASPIN* polymorphisms and impaired spermatogenesis in Japanese males, we screened for mutations in the *HASPIN* coding sequence (CDS) using DNA from 282 sterile male patients and 262 fertile male volunteers. Polymorphisms were found at 10 positions within the *HASPIN* CDS. Among these 10 polymorphisms, 5 were found only in the infertile group, 3 of which were nonsynonymous. These polymorphisms found only in the infertile patients may be a cause of male infertility and thus valuable candidates for further study of this condition.

Keywords

Single-Nucleotide Polymorphism, Spermatogenesis, Meiosis, Sperm, Nucleus, *HASPIN* Gene

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1. Introduction

During spermatogenesis, a range of processes occurs in a timely manner and precise order in the supporting cells of the testis, including proliferation and differentiation of spermatogonia, meiosis of spermatocytes, and drastic morphological changes in haploid cells [1]. It has been reported that many functional genes are predominantly expressed in the testis, and defects in their expression cause male infertility [2]. Mouse *Haspin* (also known as GSG2), encoding a germ cell-specific protein kinase, was cloned from a subtracted cDNA library constructed from mouse testis [3]. Genomic analyses revealed that mouse *Haspin* is an intron-less gene located within the 26th intron of the integrin alpha E (*Itgae*) gene on chromosome 6, which is conserved in rats and humans [4] [5]. However, in contrast to humans, rats, and mice, an intron is present in the *Haspin* genes of other organisms [5]. Analysis of the *Haspin* promoter element showed that a 193-nucleotide (nt) genomic fragment of the *Haspin* promoter specifically, bidirectionally, and synchronously induced transcription of both *Haspin* and *Aed* (an alternative transcript of *Itgae* [6]) in haploid germ cells of the testis [7] [8].

It was shown that human HASPIN phosphorylates histone H3 at threonine 3 and is required for this phosphorylation event in mitotic cells [9] [10]. HASPIN is found at the centrosomes and spindles during mitosis, where it integrates the regulation of chromosome and spindle function during mitosis and meiosis [9] [10]. Recently, in a kinase inhibitor screen using a histone H3 assay with a high-throughput radiometric enzymatic HASPIN kinase, the CHR-6494 compound was identified as a HASPIN kinase inhibitor. CHR-6494 was shown to exhibit antitumor activity [11]. In addition, disordered HASPIN expression was found to arrest cell division [12] [13]. These results indicate that HASPIN kinetics in cells is important for the regulation of cell cycle progression.

In Japan, more than 20% of married couples undergo infertility treatment (http://www.ipss.go.jp/ps-doukou/j/doukou14/doukou14.pdf: 14th birth trend survey, National Institute of Population of Social Security Research, Japan). Infertility may be due to problems with the female, male, or both [14]. More than half of all cases of infertility involve a problem with the male, and in about half of all cases of male infertility the cause is idiopathic [14]. To understand infertility, research has focused on identifying genetic mutations involved in human male infertility. We assessed the prevalence of single nucleotide polymorphisms (SNPs) in germ cell-specific genes by the direct sequencing of PCR amplified DNA from male patients undergoing fertility evaluation [15]. Current data indicate that some SNPs related with male infertility exist in germ cell-specific genes [16] [17]; however, many of the SNPs in these genes are not associated with male infertility [18]-[23].

Building on this earlier work, the present study assessed whether *HASPIN* is a cause of infertility in Japanese males. Specifically, the presence of polymorphisms in the coding sequence (CDS) of *HASPIN* was assessed by direct sequencing of polymerase chain reaction (PCR)-amplified DNA from male patients. This approach led to the identification of potentially important variations in the *HASPIN* CDS associated with male infertility.

2. Material and Methods

2.1. Participants

Japanese subjects with nonobstructive infertility (n = 282) were divided into subgroups according to their degree of defective spermatogenesis: 192 (68%) of these patients had nonobstructive azoospermia, while 90 (32%) had severe oligospermia (<5 × 10^6 cells/ml). All patients displayed idiopathic infertility and had no history of prior medical conditions, including, but not limited to, cryptorchidism, recurrent infections, trauma, orchitis, and varicocele. All subjects were diagnosed with primary idiopathic infertility based on cytogenetic analyses. The control group consisted of fertile males who had fathered children born at a maternity clinic (n = 262). All donors were informed of the purpose of the study and gave permission for their blood samples to be used for genomic DNA analysis. This study was conducted with approval from the institutional review board and an independent ethics committee at Osaka University.

2.2. Identification of Genetic Variations in *HASPIN* by Direct Sequencing of PCR-Amplified DNA

Genomic DNA was isolated from blood samples by protease treatment and phenol extraction. Two PCR primer sets, *HASPINFr1-HASPINRv1* and *HASPINFr2-HASPINRv2*, were designed to amplify the *HASPIN* gene [National

Center for Biotechnology Information (NCBI) reference sequence NP_114171.2] (**Figure 1**). The *HASPINFr1* primer (5′-TGCGTTTGAACCTCTTGGCGGG-3′) targets nt −30 to nt −9 upstream of the first methionine site, and *HASPINRv*1 (5′-GGCCGGTTTGAACATTCTGATAGGAG-3′) targets nt 1246 to nt 1271 downstream of the first methionine site. The *HASPINFr2* primer (5′-TGGACCAAAACCAGGGCTTCCTTC-3′) targets nt 1141 to nt 1164, and *HASPINRv*2 (5′-ACCAGAGGCTTCAAGACCAGTCTC-3′) targets nt 2433 to nt 2456 downstream of the first methionine start site. PCR reactions were performed using the manufacturer's recommended reaction buffer (50 µl) containing 0.1 µg human genomic DNA; 0.2 µM each primer; 2.5 µM each of dGTP, dATP, dCTP, and dTTP; and Ex Taq polymerase (Takara, Shiga, Japan). The following PCR conditions were used: 40 cycles of denaturation at 96˚C for 45 s, annealing at 65˚C for 45 s, and extension at 72˚C for 90 s for *HASPINFr1-HASPINRv1*; and 40 cycles of denaturation at 96˚C for 45 s, annealing at 65˚C for 45 s, and extension at 72˚C for 75 s for *HASPINFr2-HASPINRv2*. The PCR-amplified fragments were purified using the SUPREC PCR spin column (Takara). The fragments amplified by the *HASPINFr1–HASPINRv1* and *HASPINFr2–HASPINRv*2 primer sets were sequenced independently from both ends using the same PCR primers with thermal-cycle sequencing kits purchased from Applied Biosystems (Foster City, CA, USA). An additional primer, *HASPINRv3* (5′-CCTCCTGTCGCTTCCTCCTG-3′), targeting nt 661 to nt 679 downstream of the translation start site, was used to verify the DNA sequence within the fragment amplified by the *HASPINFr*1–*HASPINRv1* primers. The reaction products were analyzed using the PRISM 310 Genetic Analyzer (Applied Biosystems).

2.3. Statistical Analyses

χ^2 tests were used to compare the genotype distribution between infertile subjects and fertile controls. *P*-values < 0.05 were considered statistically significant.

3. Results

We investigated whether genetic variation in *HASPIN* is associated with male infertility. DNA samples from 282 infertile male patients and 192 fertile male volunteers were screened for *HASPIN* genetic variations (**Table 1**, **Figure 1**). Among the polymorphisms identified in the *HASPIN* CDS, there were six nt changes causing an amino acid substitution, one insertion (TCCCGACGA) leading to the addition of three amino acids (aspartic acid- aspartic acid-proline: DDP) (**Figure 2(a)**), and three silent mutations. There were no correlations among the polymer-

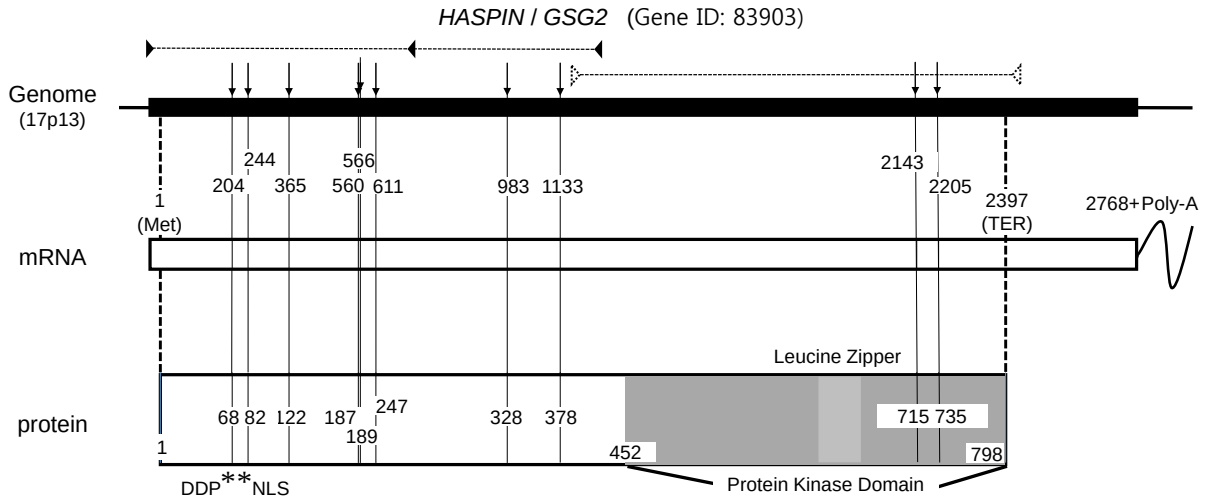

Figure 1. Schematic view of the *HASPIN* gene, mRNA, and protein. The heavy line represents intronless *HASPIN* transcription from the genome. The numbers on the gene and mRNA indicate the nucleotide positions relative to the first nucleotide of the start codon. The numbers at the bottom indicate the amino acid positions relative to the first methionine. Triangles indicate the primers used for PCR and sequencing. Dotted lines between the triangles indicate the DNA fragments amplified by PCR. Shaded areas within the protein indicate the protein kinase and leucine zipper domains. Stars indicate the aspartic acid-aspartic acid-proline (DDP)-repeat region and the nuclear localization signal.

Table 1. Prevalence of single nucleotide polymorphisms (SNPs) in *HASPIN* in infertile or proven fertile populations.

	Position			Genotype	Number (%) of SNP				Reference
	Nucleotide	Amino acid			Infertile		Proven fertile		(NCBI dbSNP rs#)
HASPIN	204	68	-	-	281	(99.6)	262	(100)	
			-/DDP	-/tcccgacga	1	(0.4)	0	(0)	
	244	82	R	c/c	97	(34.4)	89	(34.0)	rs9907144
			R/C	c/t	125	(44.3)	121	(46.2)	
			C	t/t	60	(21.3)	52	(19.8)	
	365	122	P	c/c	281	(99.6)	262	(100)	
			P/H	c/a	1	(0.4)	0	(0)	
			H	a/a	0	(0)	0	(0)	
	560	187	H	t/t	280	(99.3)	262	(100)	
				t/c	2	(0.7)	0	(0)	
				c/c	0	(0)	0	(0)	
	566	189	H	t/t	276	(97.9)	259	(98.9)	rs201447225
				t/c	6	(2.1)	3	(1.1)	
				c/c	0	(0)	0	(0)	
	611	247	G	g/g	121	(42.9)	118	(45.0)	rs220462
			G/D	g/a	120	(42.6)	104	(39.7)	
			D	a/a	41	(14.5)	40	(15.3)	
	983	328	I	t/t	121	(42.9)	104	(39.7)	rs220461
			I/T	t/c	120	(42.6)	118	(45.0)	
			T	c/c	41	(14.5)	40	(15.3)	
	1133	378	V	t/t	60	(21.3)	89	(34.0)	rs3809806
			V/A	t/c	96	(34.0)	117	(44.6)	
			A	c/c	126	(44.7)	56	(21.4)	
	2143	715	G	g/g	281	(99.6)	262	(100)	rs376754182
			G/S	g/a	0	(0)	0	(0)	
			S	a/a	1	(0.4)	0	(0)	
	2205	735	E	a/a	281	(99.6)	262	(100)	
				a/g	1	(0.4)	0	(0)	
				g/g	0	(0)	0	(0)	
Total					282		262		

phisms in terms of their co-occurrence. Three single nucleotide polymorphisms (SNPs) (c365C > A, c560T > C, and c2205A > G) and the insertion resulting in three additional amino acids (c204-/TCCCGACGA) were identified in Japanese males for the first time. Unexpectedly, c2143G > A (rs376754182) was present only in homozygous form in the infertile group (**Figure 2(b)**). Other SNPs reported in the NCBI dbSNP were not identified in the Japanese males of this study.

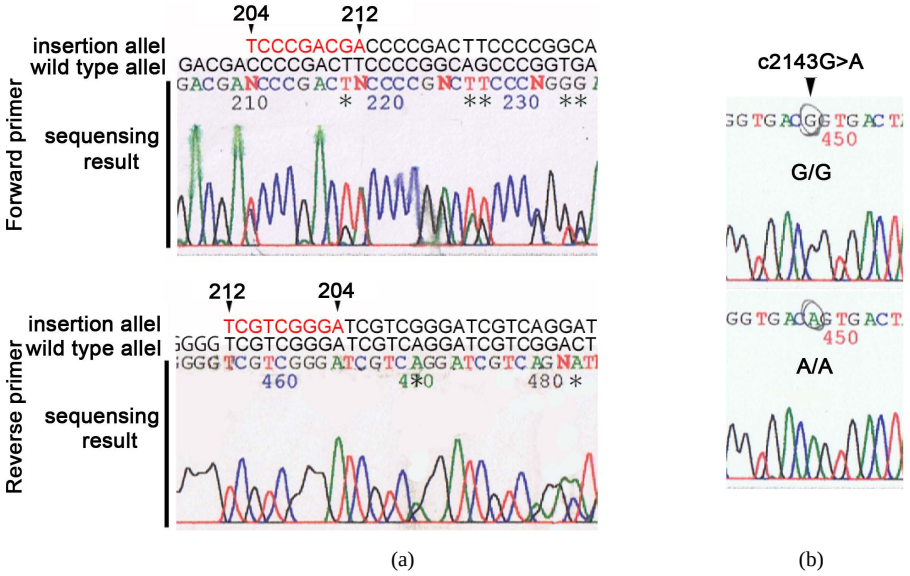

Figure 2. Detection of a TCCCGACGA DNA insertion at c204 and the minor homozygous allele (c2143G > A). To determine the DNA sequence in the region of the c204-/TCCCGACGA insertion, amplified DNA fragments were sequenced using the *HASPINFr1* (forward) or *HASPINRv3* (reverse) primer (a). Stars represent the positions of the errors introduced by the PRISM 310 Genetic Analyzer (Applied Biosystems). Homozygous G/G (upper) and A/A (lower) signals at c2143G > A (rs376754182) were clearly distinguished by the PRISM 310 Genetic Analyzer (b).

The silent SNP c566T > C (rs201447225) was found in heterozygous form in both the fertile and infertile groups, but minor homozygous SNPs were not found. Although c244C > T (rs9907144), c611G > A (rs220462), c983T > C (rs220461), and c1133T > C (rs3809806) cause an amino acid substitution, both forms of these four variants were found to be homozygous in the fertile and infertile groups, suggesting that these SNPs are not associated with male fertility. However, five other polymorphisms were found only in the infertile group. Among these, the c365C > A and c2143G > A SNPs affect the amino acid sequences around the basic region and protein kinase domain of HASPIN, respectively.

4. Discussion

Recent studies have shown that HASPIN is an important molecule involved in cell division [9] [10] [12] [13] and is conserved from yeast to animals and plants [24] [25]. The entire CDS of HASPIN in humans (NCBI reference sequence NP_114171.2), rats, and mice (**Figure 1**) is intron-less, although it contains an intron in other organisms. Defects in HASPIN associated with spermatogenesis are easily identified, as HASPIN is predominantly expressed in the testis [3] [5].

As the expression profile of HASPIN, it is possible that disorder of *HASPIN* activity due to genetic polymorphisms in *HASPIN* may cause some impairment of spermiogenesis and/or sperm function and result in male infertility. In this study, the 5 genetic polymorphisms observed only in infertile patients. Insertion of the TCCCGACGA sequence results in the introduction of three amino acids (DDP) within the DDP-repeat region of *HASPIN*. These three different polymorphisms influence the function of HASPIN. In addition, two nonsynonymous SNPs (c560T > C and c2205A > G) were identified. Nonetheless, the fact that all five of these polymorphisms were found only in the infertile group suggests their potential association with male infertility, by influencing transcription and/or translation. Four genetic polymorphisms were not found in NCBI dbSNP database although many SNPs in the NCBI dbSNP database were registered. These results may be due to race differences.

5. Conclusion

In this study, significant differences in the frequencies of the genotypes associated with *HASPIN* genetic polymorphisms in the infertile subjects were not identified ($P > 0.05$). Nonetheless, as this is the first analysis of *HASPIN* genetic polymorphisms in males with nonobstructive azoospermia, these results will contribute significantly to

future large-scale studies on the genetic background of infertility in Japanese males and on functional analyses of the role of HASPIN in cell cycle progression.

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