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Nucleotide Polymorphism Analysis of Testis-specific *CETN1* in Human Male Infertility

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Abstract: Centrin1 (CETN1) is a member of the EF-hand superfamily of calcium-binding proteins that localizes to centrosomes and regulates cytokinesis. Four *centrin* genes have been reported in mice, of which one, *CETN1*, is specifically expressed in the mouse testis. Computer-assisted analysis showed that *CETN1* was predominantly expressed in the testis. In this study, human *CETN1*, *CETN2*, and *CETN3* mRNAs from various organs were subjected to reverse transcription polymerase chain reaction (RT-PCR) analysis. *CETN1* was specifically detected only in the testis. Next, we identified polymorphisms of human *CETN1* (*centrin1*) as a candidate gene in human male infertility. To investigate the possible association between *CETN1* and male infertility, DNA from 282 infertile male patients and 96 male volunteers proven to be fertile was screened for mutations in *CETN1*. Ten polymorphisms were found in the open reading frame and the 3' untranslated region of *CETN1*. These data provide a basis for further large-scale genetic analysis of the association between genetic background and male infertility.

Keywords: Sperm, centrin, testis, retrogene.

INTRODUCTION

Centrins are a family of calcium binding proteins that contain four EF-hand motifs, and are conserved from yeast to humans [1]. Centrins are present in centrosomes, where they mediate the duplication and segregation of centrosome/basal bodies. Centrioles are required for the formation of axonal microtubule polymerization, cilia and flagellum [2]. Three human centrin genes have been characterized, while four have been characterized in mice.

Many functional retrogenes that have arisen from retroposition are expressed in the testis [3, 4]. Mouse *CENT1* has been reported as one such retrogene [1], and is specifically expressed in the testis, where it localizes to centrosomes of the sperm [1]. It is therefore possible that human *CETN1* is also specifically expressed in testis. In this study, the expression of human centrin genes and single nucleotide polymorphisms of *CETN1* were investigated to identify the role of *CETN1* in human sperm formation. Ten nucleotide polymorphisms were found in 282 Japanese males; seven of these were firsthand. Six nucleotide polymorphisms were found only in males displaying infertility.

MATERIALS AND METHODS

Participants

Japanese infertile subjects (N=282) were divided into subgroups according to the degree of defective spermatogenesis: 192 (68%) patients had non-obstructive azoospermia, and 90 (32%) had severe oligospermia ($<5\times10^6$ cells/ml). All patients displayed idiopathic infertility based on a cytogenetic analysis and possessed no history of prior medical conditions, including but not limited to cryptorchidism, recurrent infections, trauma, orchitis, or varicocele [5]. The control group consisted of fertile males who had fathered children born at a maternity clinic (N = 96). All donors were informed of the purpose of the study and gave permission for their blood to be subjected to genomic DNA analysis. This study was carried out with the approval of the institutional review board and independent ethics committee of Osaka University.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

To examine the tissue-specific expression pattern of *CETN1*, we performed RT-PCR analysis using part of a Rapid-Scan™ gene expression panel containing cDNAs from various human tissues (OriGene Technologies, Rockville, MD, USA). The specific primers used were CETN1F (5'- cgctgcctccaccggccaaaag -3'), CETN1R (5'- catcacggccaggaagtcattg -3'), CETN2F (5'- catggcatcaagttctcagcga -3'), CETN2R (5'- catcacagttaaaaagtcacca -3'), CETN3F (5'- tttgatacagacaaagatgaag -3') and CETN3R (5'- gggatctctttccaatatccag -3'). These primers were designed to amplify fragments with deduced sizes of 253, 252, and 158 bp, representing *CETN1, CETN2* and *CETN3*, respectively. PCR was performed using Prime-STAR or EX Taq hot start (Takara, Shiga, Japan). Cycling conditions were:

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96ºC for 2 min, followed by 35 cycles of denaturation at 98ºC for 10 s, annealing at 60ºC for 30 s, and extension at 72ºC for 60 s. As a control, actin was amplified using the ACTBF (5'- accgaggccccctgaaccc -3') and ACTBR (5'- tccatcatgaagtgtgacgt -3') primers according to the manufacturer's protocol.

Analysis of the Region 5' Upstream of the Genomic Region

The genomic region 5' upstream of the transcription start site of *CETN1* was subjected to computer analysis (TFSEARCH) (http://www.cbrc.jp/research/db/TFSEARCH.html). GENETYX ver. 10 (GENETYX Inc., Tokyo, Japan) was used for a homology search of DNA sequences of the genomic regions 5'upstream of the putative promoter region of the *CETN1* exon and *HASPIN* promoter [6].

Identification of Nucleotide Polymorphisms in *CETN1*

Genomic DNA was isolated from blood samples by protease treatment and phenol extraction [7]. Sequences corresponding to the region encoded by the exon of *CETN1* were amplified by PCR using the following primers: CETNF1 (5'- gcgttggcagtgagcgaggg -3') and CETNR1 (5'- tacatactagccctagataaaccc -3') (Fig. **1**). PCRs were performed using Prime-STAR or EX Taq hot start (Takara). The following PCR conditions were used: 40 cycles of denaturation at 96ºC for 45 s, annealing at 68ºC for 45 s, and extension at 72ºC for 80 s. PCR-amplified fragments were purified using the SUPREC PCR spin column (Takara). DNA fragments were sequenced independently from both ends using the same PCR and internal primers: CETNF2 (5'- aagtgactctctgggttacc -3') and CETNR2 (5'- tacatactagccctagataaaccc -3') with thermal cycle sequencing kits purchased from Applied Biosystems (Foster City, CA, USA) (Fig. **1**). The reaction products were analyzed using an ABI-PRISM 310 Genetic Analyzer (Applied Biosystems).

Statistical Analysis

Differences between the experimental and control conditions were compared using Fisher's exact test ($P<0.05$).

RESULTS

Expression of CETN1, CETN2, & CETN3

CETN1, *CETN2*, and *CETN3* mRNAs from different organs were examined using RT-PCR analysis (Fig. **2**). *CETN2* was detected ubiquitously in all organs without plasma blood leucocytes. *CETN3* was detected in the kidney, liver, muscle, testis, adrenal gland, prostate and plasma blood leucocytes. *CETN1* was specifically detected only in the testis. The translation of many testis-specific genes is regulated via the CREM-t transcription factor [8]. The putative promoter region of *CETN1* was therefore subjected to computer-assisted analysis. The results indicated that the 2 kb sequence of the region 5' upstream of *CETN1* did not contain a consensus CRE DNA sequence, consisted of GC-rich sequences (79%), and contained AP2 and SP1 binding sites (Fig. **3**). These features resembled the promoter region of the testis-specific gene *Haspin* [6].

Nucleotide Polymorphisms of *CETN1*

The entire coding sequence of *CETN1* (NCBI accession number: NC_000018.9 (580369-581524) *CETN1*, Fig. **1**) was intron-less, similar to mouse *Cetn1*. As *CETN1* is specifically expressed in human testis, we investigated whether single-nucleotide polymorphisms (SNPs) and C*ETN1* mutations were associated with male infertility. Nucleic acid base exchanges introducing three amino acid substitutions, one non-sense mutation and one silent mutation were found in the CETN1 open reading frame (Table **1**). Four SNPs were detected in the 3'-untranslated region (3'-UTR) and five continuous nucleic acid sequences collectively differed (tgtt/agaga; Table **1**). Three nucleic acid base changes (c215T>C, c715T>C, and c793-797TGTT797del>AGAGA) were found in the homozygous or heterozygous state; the others were not detected in the homozygous state. Three nucleic acid base changes introduced amino acid substitutions, and three nucleic acid changes in the 3'-UTR were detected only in infertile males. However, no significant differences in genotype frequency were identified in infertile subjects ($P>0.05$). SNPs were found in the dbSNP of The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/SNP/). SNPs of dbSNP

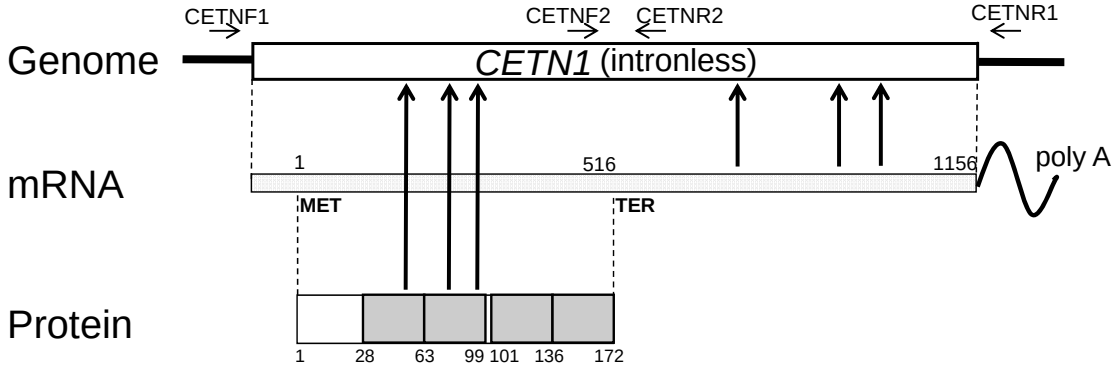

Fig. (1). Schematic view of the *CETN1* genome, mRNA, and protein. The *CETN1* intron-less gene is localized at 18p11.32: NCBI accession number NC_000018.9 (580369-581524). mRNA numbers indicate nucleic acid sequence positions relative to the first nucleotide of the start codon. The numbers (bottom) indicate amino acid positions relative to the first methionine. Horizontal arrows indicate the primers used for PCR and sequencing. Gray boxes indicate EF-hand motifs. Vertical arrows indicate the six SNPs detected in the infertile males.

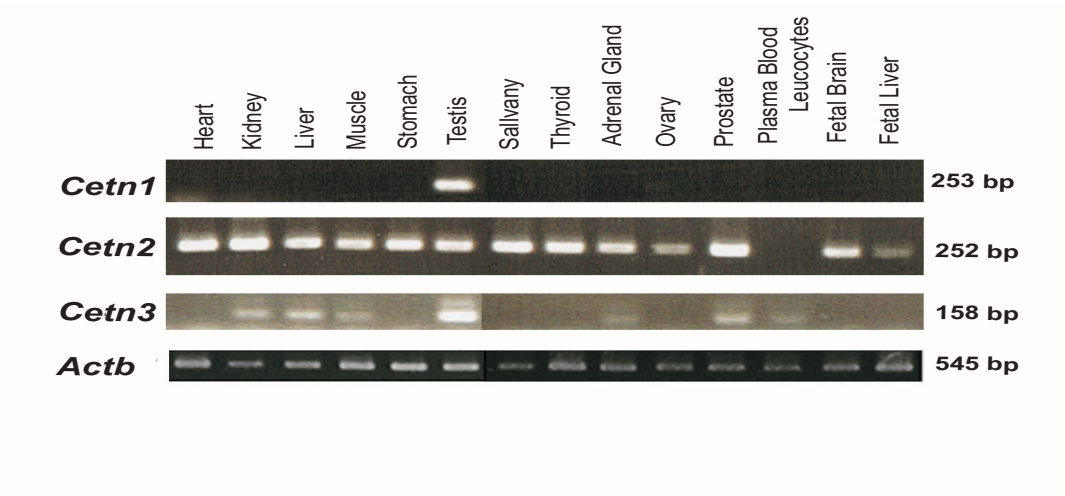

Fig. (2). Expression of *CETN1, CETN2,* and *CETN3* mRNAs in various human organs. Multiple human tissue cDNAs were subjected to PCR analysis. Fragments of *CETN1* were specifically detected in the testes. Numbers in the right margin indicate the lengths of the amplified fragments. The expression of actin mRNA was assessed as an internal control.

Fig. (3). Putative Promoter Regions of *CETN1*. The putative promoter regions of *CETN1* possess a high GC content (79%). The box indicates AP2 and SP1 binding sites. Arrows indicate transcription and translation start sites, respectively.

accession number 202167220, 2201991506, 201239078, 201796390, 201589440, 200221316, 199682997, 199595244, 143489830, 114933134, 11875237, 144780218, 139722524, 199826197, 3216112, 113182453, 67964388, 67007997, 3542329, 183402734, 62088229, 62088230, 114728825, 72863071, 113711943, 111457289, 115842359, 144514549, 116290875, 115993932, 114926253, 114552098, 139897799, 571200, 147834042, and 146097459 were not found in Japanese males.

DISCUSSION

Numerous retrogenes are specifically expressed in the testis, where they contribute to spermatogenesis [4]. Single nucleotide polymorphisms (SNPs) in male germ-cell specific genes have been identified as the genetic background of male infertility [9-13]. Recently, it was suggested that SNPs in the testis-specific retrogene PGAM4 are a major cause of male infertility [14]. It is thought that PGMA4 is maintained in females, as defective gene exchange in male germ-cell-specific genes, which causes male infertility, has no effect in females. It has been reported that mouse *CETN1* is retrotranslocated from *CETN2* [1]. The genomic regions of *CETN1* and *CETN2* in mice and humans are conserved. In this study we confirmed by RT-PCR that human *CETN1* is specifically expressed in the testis, in a similar fashion to mouse *Cetn1*. These results suggest that *CETN1* functions during spermatogenesis. To examine the specific functions of *CETN1*, SNP discovery was performed using genomic DNA from infertile male patients and fertile volunteers. We found 10 nucleic acid changes in *CETN1;* six SNPs were specific to infertile patients. These six SNPs were heterozygous and no significant differences between infertile patients and fertile volunteers were observed, because the appearance ratio was low. Those SNPs that induced amino acid substitutions may cause male infertility as dominant-negative SNPs. Six SNPs were found only in the male infertility patients. These results show that *CETN1* in male infertility patients may contain more SNPs than *CETN1* in fertile volunteers. Such an association may be evident through the analyses of a larger number of subjects. Although many SNPs in the NCBI dbSNP database were registered, SNPs of dbSNP (without accession numbers 114739741, 61734344, and 568365) were not found in Japanese males. These results may be due to race differences. This is the first analysis of *CETN1* mutations in males with non-obstructive azoospermia. Further investigations and functional studies of these variants are necessary to confirm their contributions to male infertility.

Table 1. Prevalence of Single Nucleotide Polymorphisms (SNPs) in *CETN1* in Infertile or Proven Fertile Populations

	Position			Genotype	Number (%) of SNP				Reference
	Nucleotide	Amino acid			Infertile		Proven fertile		(NCBI dbSNP rs#)
CETN1	120	40	F	c/c	271	(96.1)	93	(96.9)	114739741
				c/t	11	(3.9)	3	(3.1)	
				t/t	0	(0)	0	(0)	
	164	55	V	t/t	281	(99.6)	96	(100)	
			V/A	t/c	1	(0.4)	0	(0)	
			A	c/c	0	(0)	0	(0)	
	215	72	M	t/t	208	(73.8)	66	(68.8)	61734344
			M/T	t/c	65	(23.0)	27	(28.1)	
			T	c/c	9	(3.2)	3	(3.1)	
	235	79	E	g/g	281	(99.6)	96	(100)	
			E/K	g/a	1	(0.4)	0	(0)	
			K	a/a	0	(0)	0	(0)	
	283	95	Q	c/c	281	(99.6)	96	(100)	
			Q/Ter	c/t	1	(0.4)	0	(0)	
			Ter	t/t	0	(0)	0	(0)	
	715			t/t	235	(83.3)	82	(85.4)	568365
				t/c	45	(16.0)	12	(12.5)	
				c/c	2	(0.7)	2	(2.1)	
	765			c/c	280	(99.3)	96	(100)	
				c/t	2	(0.7)	0	(0)	
				t/t	0	(0)	0	(0)	
	793-X-797			tgtt	118	(41.8)	35	(36.5)	
				tgtt/agaga	132	(46.8)	48	(50.0)	
				agaga	32	(11.3)	13	(13.5)	
	935			t/t	281	(99.6)	96	(100)	
				t/c	1	(0.4)	0	(0)	
				c/c	0	(0)	0	(0)	
	1001			g/g	281	(99.6)	96	(100)	
				g/a	1	(0.4)	0	(0)	
				a/a	0	(0)	0	(0)	
Total					282		96		

Each SNP is named based on its position relative to the first nucleotide of the start codon.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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