ORIGINAL PAPER

Novel SNP of the *VCY2* Gene in Infertile Japanese Patients with Sertoli Cell-only Phenotype

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Abstract. Variable charged Y chromosome (*VCY2*) has been suggested to be involved in susceptibility to spermatogenic failure. Blood samples were collected from 126 proven fertile male volunteers and 271 infertile patients with non-obstructive azoospermia. We discovered three kinds of variant site in the 5' promoter region of the *VCY2* gene located 417 upstream from exon 1. All Y chromosomes are in hemizygous condition. Therefore, the whole Y chromosome behaves as a haplotype. A homozygous A pattern was only observed at a rate of 5.1% (3/59) in Sertoli cell-only (SCO) phenotype, but no variant was detected in idiopathic infertile or proven fertile subjects. Our results suggested that the alteration of total three copies of *VCY2* within the AZFc region on the Y chromosome may markedly affect sperm production.

Key words: SNP, *VCY2, BPY2*, AZF, Male infertility

Introduction

Y chromosomal microdeletion is involved in spermatogenesis impairment. The region required for spermatogenesis is defined as the azoospermia factor (AZF) [1]. Genes in the AZF regions are potential candidates for genes related in spermatogenesis. Microdeletions in these genes are divided into three main regions, AZFa, b, and c, according to the histological phenotype and position [2]. Each AZF region contains some genes involved in spermatogenesis. Among cases with male infertility, microdeletions involving the AZFc gene family represent the most frequent finding [3-7]. The AZFc region is believed to play important roles in normal spermatogenesis. Variable charged Y chromosome (*VCY2*, alias *BPY2*, GenBank accession no. AC006366) is located within the AZFc region in fertile men [8-12]. *VCY2* is composed of nine exons spanning 21 kilobases within the *DAZ* gene cluster. *VCY2* encodes a product of 106 amino acids expressed specifically in the testis, and is located on chromosome Yq in humans (OMIM 400013). Kuroda and Page's group reported the entire, intact sequence of the AZFc region [13,14]. The AZFc region contains a complex of three palindromes (P1, P2, P3), and a conserved highly repetitive sequence. Total AZFc contains four copies of *DAZ* and two copies of both *BPY2* and *CDY1*. According to GenBank, at least 44 SNPs (single nucleotide polymorphisms) were found in the *DAZ* clusters, but there have been no previous reports of SNPs in the *VCY2* gene [15-18].

The present study was performed to investigate genotype frequencies of *VCY2* polymorphisms in 126 healthy and 271 infertile male subjects.

Subjects and methods

1) Subjects

Blood samples were collected from Japanese subjects: 126 proven fertile male volunteers and 271 infertile patients with non-obstructive azoospermia. All patients underwent comprehensive examinations, including detailed history taking, physical examination, at least two semen analyses, endocrinology profile testing, karyotyping, and a molecular test for Y-chromosome microdeletions. Severe oligozoospermia was defined as a sperm count of less than 1×10^6/ml. Non-obstructive azoospermia was defined as germ cell defects on testicular biopsy, elevated serum FSH level, or a

Accepted: August 11, 2004
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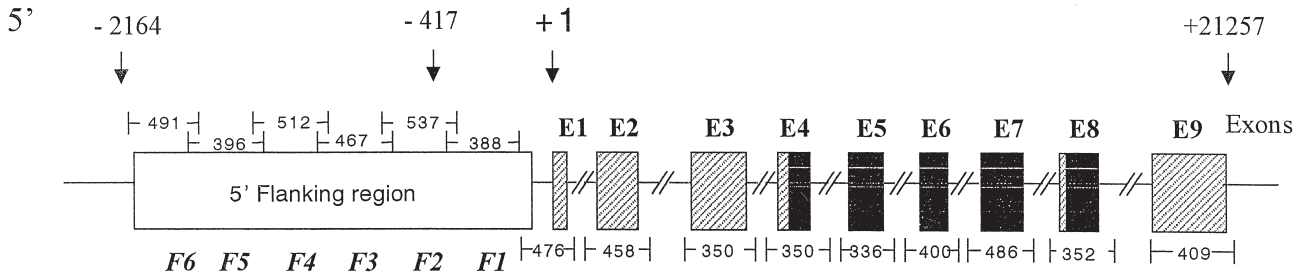

Fig. 1. Targeting strategy
Protein-coding sequences, non-coding sequences, and 5' flanking regions are indicated as solid, striped, and open boxes, respectively.

Table 1. PCR primer,product length, polymorphic sites, and type of polymorphism
The *VCY2* sequence from the completed human genome database (accession no. AC006366) was used as a reference for documenting the locations of the PCR primers, with the first nucleotide of Exon 1 (position 122,361 in AC006366) designated as +1.

Name	Primer Sequence	Location	Length (bp)	Start	End	Polymorphic site	Sequence variation
F1F	TAAGGCAGGTGCCATGGCAG	5' Flanking region **1**	491	-332	+159		
F1R	GTGCTCAGAGATATGGCCGA						
F2F	GGGCACTGCACATAGGATAG	5' Flanking region **2**	396	-708	-313	-417	G/A
F2R	CTGCCATGGCACCTGCCTTA						
F3F	GACGGATGAAGGCAACTCTT	5' Flanking region **3**	512	-1056	-545		
F3R	AGGTGGGCAGATCCCAAGCA						
F4F	CTGTGTGGACACTCTCCAGC	5' Flanking region **4**	467	-1053	-1037		
F4R	AAGAGTTGCCTTCATCCGTC						
F5F	CTCCTGTACCTAGAGCCAGA	5' Flanking region **5**	537	-1908	-1372		
F5R	CATCACCTAAACCATGAGCC						
F6F	CCAGGCGAATTGCCTAGGAG	5' Flanking region **6**	388	-2164	-1777		
F6R	CTGGGCTATAAATAGGCAGG						
E1F	ATAAGGCAGGTGCCATGGC	Exon **1**	476	-333	+143		
E1R	CCGAATGTCACCAGTAGGC						
E2F	GTCTGGTTGCTGAACAACC	Exon **2**	458	+258	+715		
E2R	CTGTTGGCTGAATCCCGAT						
E3F	GGGTGTGGCTCACCAAAGAAG	Exon **3**	350	+3322	+3671		
E3R	TTGGCCTGTGAGCAGGGACAA						
E4F	CAGAGTCCTAGCCACAAGGA	Exon **4**	350	+7941	+8290		
E4R	CCGAGAACCTGTTAGGACAC						
E5F	TTGTGCCAGGACCGCAGAAG	Exon **5**	336	+10071	+10406		
E5R	CCTAAACCTGGTGGTGAGAGG						
E6F	GTGACTCTCACCTCCTGCCTG	Exon **6**	400	+13001	+13402		
E6R	ACAGGCTGGGCCCAGGTATGA						
E7F	ACCTGTGGTGCCGTGACTCC	Exon **7**	486	+13739	+14224		
E7R	TGCCCTGTAAGAGCATGGCC						
E8F	GATCCTGCCAAGTAGGGA	Exon **8**	352	+20517	+20868		
E8R	CAGAGCAGGAGAGTCTCATC						
E9F	GATGAGACTCTCCTGCTCTG	Exon **9**	409	+20849	+21257		
E9R	CACCTGTGAGTCAGATCCAC						

total testicular volume of less than 30 ml. Semen analysis was performed according to the standard methods outlined by the World Health Organization [19].

A total of 271 infertile men presented with severe oligozoospermia or non-obstructive azoospermia. Of these 271 subjects, 140 showed at least one spermatozoon in the ejaculate. Testicular biopsy was performed in the remaining 108 patients. Twenty-three subjects were not performed testicular biopsy. Histological examination revealed the absence of spermatogenesis (Sertoli cell-only) in 59 of these 108 subjects. The remaining 49 patients had various tissue types, such as hypo-spermatogenesis or maturation arrest. This study was approved by the Ethical Committee of Kanazawa University Hospital, and informed consent was

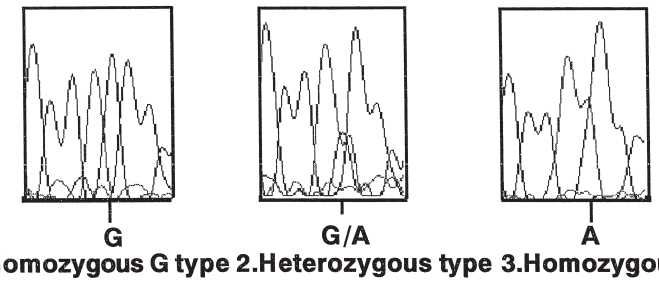

Fig. 2. Sequencing at variant site showed 1) homozygous (G/G), 2) heterozygous (G/A) and 3) homozygous (A/A) patterns

obtained from all volunteers.

2) Polymerase chain reaction (PCR)

Amplification genotyping was performed using allele-specific probes. Oligonucleotide primer and probe sets were designed based on gene sequences from the Gene Bank, July 2003. *VCY2* genomic structure and positions of primers are shown in Fig. 1. Primers for each locus are listed in Table 1. In each reaction, 19.2 μl of PCR Master Mix (Toyobo, Osaka, Japan) containing KOD DNA Polymerase, dNTPs, and $MgCl_2$ were mixed with 10 nmol of each forward and reverse primer. DNA was allowed to stand at 94℃ for 1 min, followed by 30 cycles of amplification at 94℃ for 30 s, 60℃ for 30 s, and 72℃ for 30 s, with a final extension at 72℃ for 10 min and stored at 4℃. PCR was carried out with a thermocycler (BIOMETRA, Goettingen, Germany).

3) Restriction cleavage site

To detect a G > A variant at -417, we amplified 396-bp fragments using oligonucleotide primers F2F/R. The products were digested with StyI, electrophoresed on 1.5% agarose gels, and visualized under UV by staining with ethidium bromide. The mismatch-containing primer introduced a restriction site for StyI only in homogygous A, which was cleaved into two fragments of 290 and 106 bp (Fig. 3a).

4) Nucleotide sequence analysis

PCR products were purified for sequencing using a Microcon PCR purification kit (Millipore, Bedford, MA, USA). DNA sequencing was carried out on an ABI (Applied Biosystems, Foster City, CA) 377 DNA Sequencer using a BigDye Terminator v3.1 Cycle Sequencing Kit (ABI).

5) Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences statistical software (version 11.0). The Fisher's exact test was used to determine whether there was any significant difference in genotype frequencies between normal control and infertile male group. The value of $P<0.05$ was taken to be statiscally significant.

Results and Discussion

We analyzed 271 infertile male patients with non-obstructive azoospermia and 126 proven-fertile male volunteers. No novel mutations of the *VCY2* gene were identified in any of the exons. However, we discovered three kinds of variant site in the 5' flanking region of the *VCY2* gene located 417 upstream from exon 1 (Fig. 1). The sequences of these three variants showed homozygous (G/G and A/A) and heterozygous (G/A) patterns (Fig. 2). All Y chromosomes are in hemizygous condition, and therefore the whole Y chromosome behaves as a haplotype. These variants were mono-allelic on the Y chromosome.

The human *DAZ* gene cluster, which included the AZFc region, is located on the distal part of the long arm of the Y chromosome and forms a huge repetitive region and palindrome complex [13, 14]. The genes in this region, such as *DAZ*, *VCY2*, and *CDY*, exist as multiple copies. *VCY2* is present as three copies as shown in Fig. 3. The distal two genes are characterized by a palindrome structure in a head-to-head orientation. The variants with homozygous A or G patterns contain three identical copies in the upstream region, but we cannot determine which copies of *VCY2* are heterozygous.

To detect a G > A variant at -417, we performed PCR using oligonucleotide primers F2F/R. The products after digestion with StyI were electrophoresed. The mismatch-containing primer introduced a restriction site for StyI only in the homozygous (A) product, which was cleaved into

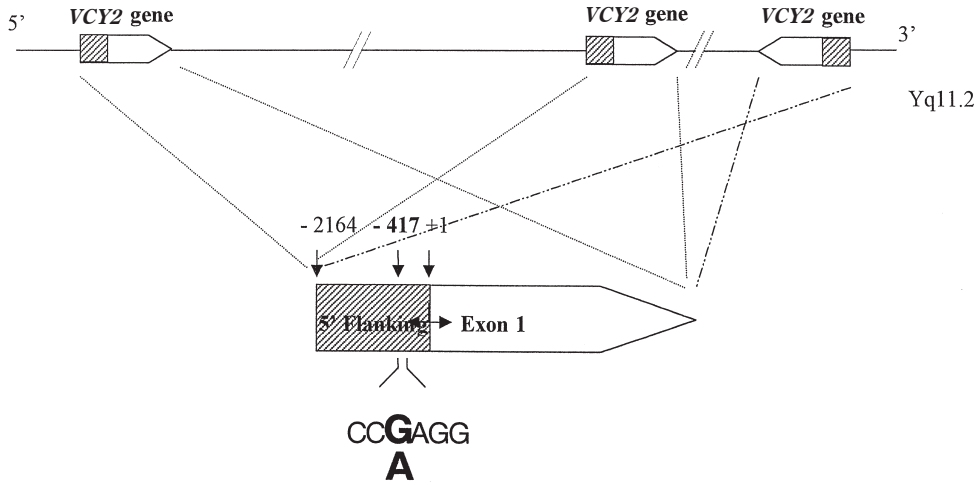

Fig. 3. Positions and orientations of three copies of *VCY2* on the Y chromosome and a novel SNP in the 5' flanking region of *VCY2*

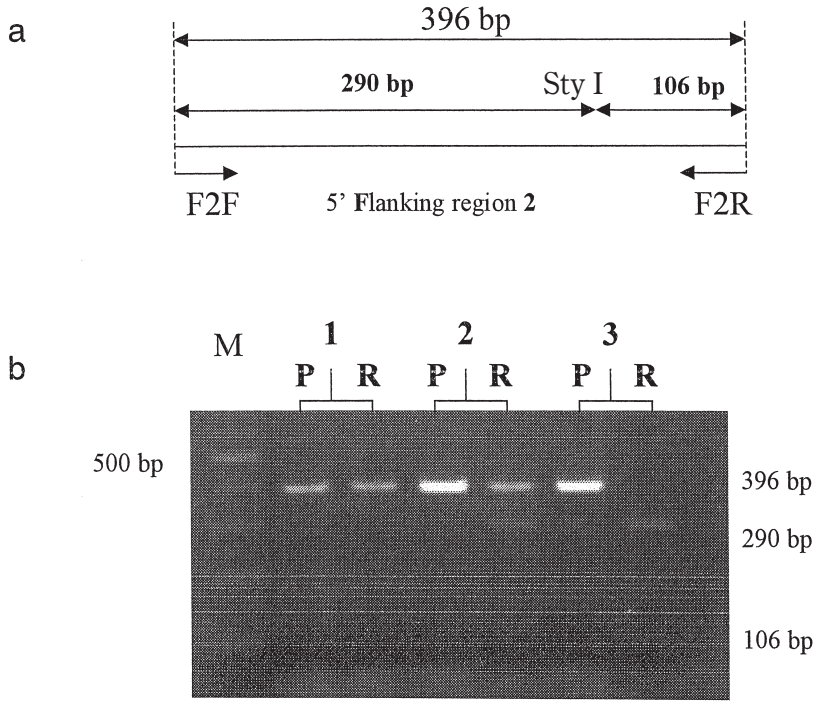

Fig. 4 Restriction fragment length polymorphism
a. The 396-bp PCR product was digested into two fragments.
b. PCR products of the 5' flanking region using primers F2F/R were digested with StyI.
1, Homozygous G type; 2, Heterozygous type; 3. Homozygous A type. P, PCR product; R, Restriction fragments

two fragments of 290 and 106 bp (Fig. 4a). There is no cleavage site for the homozygous (G) product (Fig. 4b).

We examined the relationships between three *VCY2* variants and male infertility. As the Y chromosome is hemizygous and contains repetitive sequences, the 5' upstream sequence in the promoter region exists in all three copies of the gene. A homozygous G/G pattern was observed at a rate of 91.5% (54/59) in the SCO phenotype, 97.2% (206/212) in idiopathic infertile, and 96.0% (121/126) in proven fertile subjects. A heterozygous G/A pattern was observed at a rate of 3.4% in the SCO phenotype, 2.8% in idiopathic infertile, and 5.0% in proven fertile subjects. On

Table 2. Number of SNPS

Genotype	Number (%) of SNP		
	Infertile		Proven fertile
	SCO*	Idiopathic infertile	
G/G	54 (91.5)	206 (97.2)	121 (96.0)
G/A	2 (3.4)	6 (2.8)	5.0 (4.0)
A/A	3 (5.1)	0†	0†
total	59	212	126

SCO*: Sertoli cell-only syndrome
†: P<0.05 versus SCO corresponding genotype A/A

the other hand, a homozygous A/A pattern was observed at a rate of only 5.1% (3/59) in the SCO phenotype, but no variants were detected in idiopathic infertile or proven fertile subjects (Table 2). These polymorphisms were not found in the normal population of unrelated healthy subjects and idiopathic infertile without SCO, suggesting that these variants may be associated with the SCO phenotype. Therefore the alteration of all three copies of *VCY2* (homozygous A/A) within the AZFc region on the Y chromosome may greatly affect sperm production.

The *VCY2* -417G>A polymorphism is in the promoter region, and it is therefore likely that this polymorphism may alter the level of *VCY2* expression. However, this polymorphic site had no consensus sequence for transcription factors by Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter/). Further studies in larger numbers of subjects with idiopathic male infertility may be necessary to detect the influence of the *VCY2* -417G>A polymorphism on the incidence of azoospermia. Further analysis of the gene regulation system is necessary to elucidate the mechanism of spermatogenesis.

Although the precise function of *VCY2* is not yet known, this gene appears to have some links to DNA repair, the ubiquitin system, and regulation of meiotic chromatin structure [20,21].

In summary, the present study provided the first evidence of a relationship between the common *VCY2* -417G>A polymorphism and male infertility. This finding represents an important contribution to the molecular diagnosis of idiopathic azoospermia in Japan and may be useful for genetic counseling.

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