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ORIGINAL ARTICLE

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Screening for deletions in interval D16–22 of the Y chromosome in azoospermic and oligozoospermic Japanese men

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Abstract The genetic basis of infertility remains unclear in a majority of infertile men. In this study, the Y chromosome long arm involving the *DAZ* (deleted in azoospermia) gene was screened in order to evaluate the occurrence of microdeletion in Japanese infertile men. One hundred and fifty-seven infertile Japanese men with azoospermia and oligozoospermia were analyzed for microdeletions in interval D16–22 of the Y chromosome, using polymerase chain reaction with sequence-tagged site markers. Sixteen sets of oligonucleotide primers were synthesized for the polymerase chain reaction, and Southern blot analysis was also performed. The men were divided into five categories on the basis of sperm concentration: functional azoospermia (A; $n = 24$), azoospermia caused by obstruction (AO; $n = 20$), oligozoospermia I (OI, sperm concentration less than 1×10^5/ml; $n = 33$), oligozoospermia II (OII, sperm concentration less than 1×10^6/ml; $n = 30$), and oligozoospermia III (OIII, sperm concentration less than 1×10^7/ml; $n = 50$). Thirty fertile men with a sperm concentration of more than 2×10^7/ml were also analyzed as controls. Microdeletions were identified, in 12 (7.6%) of the 157 infertile men, as follows: 1 man in category A, 1 in category AO, 5 in category OI, 4 in category OII, and 1 in category OIII. No deletion was identified in the fertile men. One common region around sY240 was identified in 11 of the infertile men with microdeletions. This locus may contain specific genes for spermatogenesis. The sperm concentration in the ten oligozoospermic men with microdeletions was below 1×10^6/ml. There were no correlations between the severity of spermatogenic defects and the extent of the microdeletions. These results suggested that genes in the interval D16–22 of the Y chromosome might have important roles in spermatogenesis.

Key words Microdeletion · Y chromosome · Azoospermia · Oligozoospermia · Infertility

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Introduction

Human sperm are produced via a complex developmental process, and spermatogenesis must require many gene products. However, to date, no human genes specifically involving spermatogenesis have been identified at the molecular level (Ma et al. 1993; Nakahori et al. 1994; Reijo et al. 1995; Shinka and Nakahori 1996; Qureshi et al. 1996; Vogt 1998; McElreavey and Krausz 1999). In 1976, Tiepolo and Zuffardi first reported, from microscopic analysis of the Y chromosome in infertile men, that the human Y chromosome played a crucial role in spermatogenesis (Tiepolo and Zuffardi 1976). Since their identification of deletions of the long arm of the Y chromosome in infertile men, the existence of a spermatogenesis gene or azoospermic factor (AZF) on the Y chromosome has been proposed (Vogt et al. 1992), and many investigators have focused on where genes related to spermatogenesis exist in the long arm of Y chromosome (Andersson et al. 1988; Johnson et al. 1989; Vogt et al. 1992).

In order to identify genes involving spermatogenesis, several researchers have analyzed the Y chromosome by various methods, such as Southern blot analysis and polymerase chain reaction (PCR) (Johnson et al. 1989; Vogt et al. 1992). In 1993, Ma et al. reported two cDNAs of a Y-specific gene family named the RNA binding motif (*RBM*) from a region on the long arm of the Y chromosome that was deleted in some infertile patients (Ma et al. 1993). Because *RBM* had a motif characteristic of an RNA-binding protein that may play an important role in gene regulation and because it showed male-specific conser-

vation in several mammalian species, it was considered as a candidate for AZF. In 1995, a gene (*DAZ*; deleted in azoospermia) was identified from analysis of interval D in the long arm of the Y chromosome in azoospermic men (Reijo et al. 1995). *RBM* and *DAZ* had some common characteristics: (1) they had an RNA recognition motif; (2) their expression was testis-specific; and (3) they had a series of tandem repeats. Both *RBM* and *DAZ* are located in the interval D13–22 of Yq, which is in rich Y-specific repetitive sequences, and several investigators have reported microdeletions including RBM and DAZ in azoospermic or oligozoospermic men (Nakahori et al. 1994; Qureshi et al. 1996; Simoni et al. 1997).

These results suggested that about 10% of infertile men with azoospermia of unknown origin had microdeletions in the interval D13–22 of the Y chromosome. In addition, it was also reported that oligozoospermic men had microdeletions in this region (Nakahori et al. 1994; Qureshi et al. 1996; Simoni et al. 1997). However, few reports have demonstrated correlations between the extent of microdeletions and sperm concentration (Vogt et al. 1996; Najmabadi et al. 1996; Kremer et al. 1997; Simoni et al. 1997). Accordingly, in this study, in order to elucidate the relation between Y chromosome deletion and defects of spermatogenesis, including severe and mild oligozoospermia, we analyzed DNAs from azoospermic and oligozoospermic Japanese men, using polymerase chain reaction (PCR) with sequence-tagged site (STS) primers (Vollrath et al. 1992).

Subjects and methods

Subjects

One hundred and fifty-seven infertile Japanese men with azoospermia and oligozoospermia were analyzed to determine whether they had microdeletions. All patients consulted our infertility clinic. They had normal male karyotype (46,XY). Semen analysis was performed according to the World Health Organization guidelines. These infertile men were divided into five categories: functional azoospermia (A; $n = 24$), azoospermia caused by obstruction (AO; $n = 20$), oligozoospermia I (OI, sperm concentration less than 1×10^5/ml; $n = 33$), oligozoospermia II (OII, sperm concentration less than 1×10^6/ml; $n = 30$), and oligozoospermia III (OIII, sperm concentration less than 1×10^7/ml; $n = 50$). Thirty fertile men who had children and had a normal sperm concentration of more than 2×10^7/ml were also analyzed as controls.

PCR and Southern blot analysis

Genomic DNA was prepared from peripheral blood samples of the infertile and fertile Japanese men, after informed consent had been obtained, in order to screen for Y-chromosome deletions by PCR. Sixteen sets of primers, located in the interval D16–22 of the Y chromosome, were synthesized: sY138, sY233, sY240, sY245, sY277, sY254, sY283, sY255, sY236, sY267, sY272, sY269, sY273, sY158, sY159, and sY160 (Vollrath et al. 1992). The primers E76 and E77, as follows, were used as positive controls for PCR: E76, ACACAGAGCAAGGCCAGAAT; and E77, CTTCATGGGCCTGAACTGAT (Ma et al. 1993). Genomic DNAs were digested with *Eco*RI before PCR to allow for a smooth reaction. Genomic DNA in PCR buffer (50 mmol/l KCl, 15 mmol/l $MgCl_2$, 0.1% gelatin, 10 mmol/l Tris HCl pH 8.3) and dNTP (20 μmol/l dATP, 20 μmol/l dTTP, 20 μmol/l dGTP, 20 μmol/l dCTP) were mixed with 1 μmol/l oligonucleotide as primer, and 1.87 units of *Thermus aquaticus* DNA polymerase (Applied Biosystems, Foster City, CA, USA) was added and mixed. After a preheating step at 95°C for 5 min, the PCR was performed with denaturing at 95°C for 1 min, annealing at 57°C for 2 min, and primer extension at 72°C for 3 min, in 35 cycles, with final incubation at 72°C for 5 min. In order to check the amplified DNAs, gel electrophoresis was performed with 2% NuSieve GTG agarose (FMC BioProducts, Rockland, ME, USA) and 1% Seakem GTG agarose (FMC BioProducts) gel in TAE buffer (40 mmol/l Tris-acetate, 1 mmol/l ethylenediaminetetraacetic acid [EDTA]). After electrophoresis, the DNAs were transblotted onto nylon membranes (Hybond; Amersham Japan, Tokyo, Japan) and Southern blot analysis was performed with various ^{32}P-labeled amplified DNAs as probes. In order to confirm microdeletions, genomic Southern blot analysis was performed. Genomic DNAs were digested with *Eco*RI, and gel electrophoresis was performed with 1% Seakem GTG agarose (FMC BioProducts) gel in TAE buffer (40 mmol/l Tris-acetate, 1 mmol/l EDTA). After the DNAs were transblotted onto the nylon membrane, genomic Southern blot analysis was performed as described above.

Results

The DNAs amplified by PCR were analyzed by gel electrophoresis (Fig. 1). As shown in Fig. 1, analysis showed which gene could be amplified by PCR, and Southern blot analysis was performed in order to confirm the results of PCR (Fig. 2). Microdeletions of the Y chromosome were detected in 12 of the 157 infertile men studied (Table 1). No Y chromosome microdeletions were detected in the normal fertile men. The microdeletions in the 12 infertile men, on the basis of sperm concentration showed 1 man in category A, 1 in category AO, 5 in category OI, 4 in category OII, and 1 in category OIII (Table 2). In the 24 azoospermic men without any seminal tract obstruction (group AO), microdeletions of the regions around sY240 and sY283 were identified in only 1 man (4%). In the 113 oligozoospermic men, the microdeletions were identified in 10 patients (8.8%); 5 in category OI, 4 in category OII, and 1 in category OIII. The sperm concentration of the 1 infertile man with a microdeletion in category OIII was 1×10^6/ml.

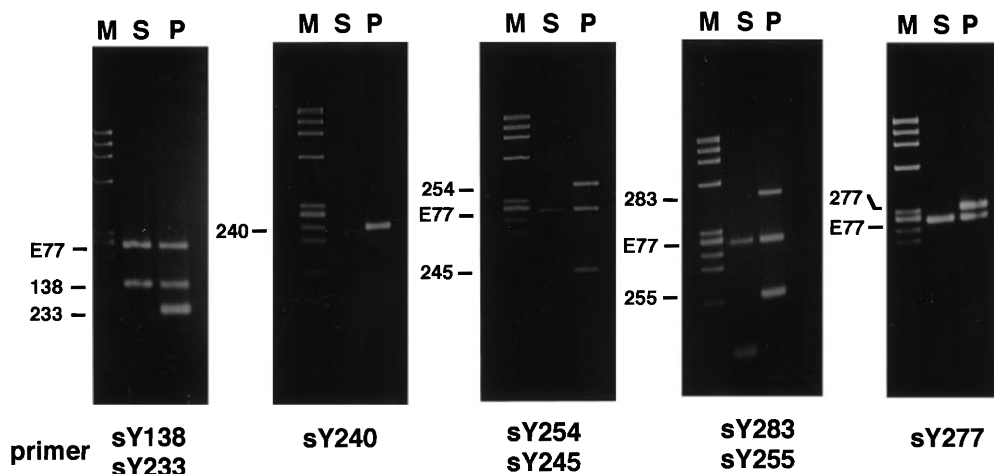

Fig. 1. Polymerase chain reaction (PCR) analysis of infertile Japanese men with azoospermia and oligozoospermia. *Numbers to the left of each photograph* show the positions of the DNA amplified with each primer. Names of primers used are indicated *under each photograph*. E77 was used with E76 as an internal control for PCR, as described in "subjects and methods." *M*, Size marker ($\phi \times 174$/*Hae*III digest); *S*, subject (infertile man), *P*, normal fertile man

Therefore, no infertile men with sperm concentrations above 1×10^6/ml had any microdeletions.

In all 12 of the infertile men with microdeletions, one common region around the sY240 primer was deleted. A microdeletion around *DAZ* (sY283) was also detected in 6 (50%) of these 12 infertile men with microdeletions. In this study, 10 (83%) of the 12 infertile men with microdeletions were oligozoospermic. Endocrinological data for these 10 men, as shown in Table 2, showed that their serum luteinzing hormone (LH) levels (1.5–9.2 IU/l) and serum follicle-stimulating hormone (FSH) levels (4.0–27.6 IU/l) were lower than those (LH, 11.2 IU/l and FSH, 30.1 IU/l) of the azoospermic man without obstruction with microdeletion. Serum testosterone levels in the 10 oligozoospermic men and the azoospermic man with obstruction who had microdeletions were within the normal range.

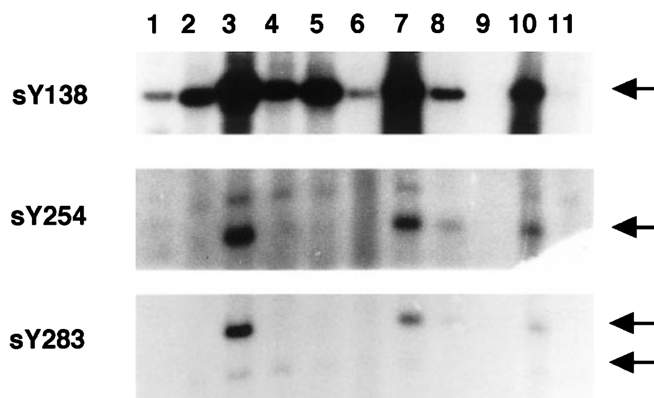

Fig. 2. Genomic Southern blot analysis for infertile males with microdeletions. Genomic DNAs were digested with *Eco*RI, and Southern blot hybridization was performed. PCR products (sY138, sY254, and sY283) were used as probes. *Arrows* indicate positive bands. *Lane 1*, Patient 191; *lane 2*, patient 207; *lane 3*, patient 342; *lane 4*, patient 390; *lane 5*, patient 430; *lane 6*, patient 132; *lane 7*, patient 157; *lane 8*, patient 389; *lane 9*, marker DNA (λ *Hind*III); *lane 10*, normal male; *lane 11*, normal female. All patients are numbered when we collect blood samples in our clinic. The numbers given to the 157 patients chosen for this study thus have no particular meaning

Discussion

Since Tiepolo and Zuffardi first reported deletion of the Y chromosome in azoospermic men, many reports have confirmed the presence of different genes critical for the regulation of male infertility on the long arm of the Y chromosome, and the concept of the azoospermia factor (AZF) was defined (Tiepolo and Zuffardi 1976; Andersson et al. 1988). To date, three regions on the Y chromosome have been identified as possibly important for spermatogenesis; they were named AZFa (interval D3–D6), AZFb (interval D13–D16), and AZFc (interval D20–D22) (Vogt et al. 1996). Many investigators have focused on the AZFb and AZFc regions, because the *RBM* and *DAZ* genes were identified on these regions (Ma et al. 1993; Reijo et al. 1995) and there have been many reports on the relationship between these genes and spermatogenesis. According to these reports, 3%–18% infertile men with azoospermia and severe oligozoospermia have microdeletions in the Y chromosome (Nagafuchi et al. 1993; Kobayashi et al. 1994; Reijo et al. 1995; Vogt et al. 1996; Najmabadi et al. 1996; Reijo et al. 1996; Stuppia et al. 1996; Simoni et al. 1997; Kremer et al. 1997; Vogt 1998; McElreavey and Krausz 1999). However, it is still not clear which gene or locus in the Y chromosome may be responsible for spermatogenesis.

In this study, we screened interval D16–22 (around the AZFc region), including *RBM* and *DAZ*, in infertile Japanese men, and identified microdeletions in 12 (7.6%) of 157 infertile men. We found no microdeletions in normal

Table 1. STS-PCR data of the 12 infertile men with microdeletions

	Patient number	STS marker in interval D16-22 of the Y chromosome															
		138	233	240	245	277	254	283	255	236	267	272	273	269	158	159	160
Azoospermia																	
A	401	+	+	−	+	+	+	−	+	+	+	+	+	+	+	+	+
AO	469	+	−	−	+	−	−	−	+	+	+	+	+	+	+	+	+
Oligozoospermia																	
OI	430	+	−	−	+	−	−	−	+	+	+	+	+	+	+	+	+
OI	389	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+
OI	342	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+	+
OI	191	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+
OI	157	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+
OII	30	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+
OII	40	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
OII	132	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
OII	390	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+
OIII	207	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+

STS—PCR, Sequence–tagged site—polymerase chain reaction
A, OA, OI, OII, and OIII, See text for definitions of these groups

Table 2. Hormonal data and sperm characteristics in infertile men with microdeletions

	Patient number	Sperm concentration ($\times 10^6$/ml)	Sperm motility[a] (%)	LH (IU/l)	FSH (IU/l)	Testosterone (ng/dl)
A	401	0	—	11.2	30.1	41
AO	469	0	—	3.1	12.7	260
OI	430	0.001	1	6.5	18.0	349
OI	389	0.001	12	5.3	27.6	538
OI	342	0.0001	0	9.2	11.0	537
OI	191	0.001	0	1.5	4.0	209
OI	157	0.001	1	NT	NT	297
OII	30	0.3	0	NT	NT	NT
OII	40	0.3	50	3.1	7.0	538
OII	132	0.3	50	7.4	12.5	541
OII	390	0.4	50	2.9	6.7	601
OIII	207	1	50	4.9	20.4	282

LH, Luteinizing hormone; FSH, follicle-stimulating hormone; NT, not tested
[a] Percentage of motile sperm

fertile men. Most investigators have demonstrated that the frequency of deletions of the Y chromosome in idiopathic infertile men was 3%–18% (Nagafuchi et al. 1993; Kobayashi et al. 1994; Reijo et al. 1995; Vogt et al. 1996; Najmabadi et al. 1996; Reijo et al. 1996; Stuppia et al. 1996; Kremer et al. 1997; Simoni et al. 1997; Vogt 1998; McElreavey and Krausz 1999). Our result (7.6%) was in agreement with these previous reports. However, Foresta reported a high incidence (37.5%) of microdeletions of the Y chromosome in idiopathic severe testiculopathy (Foresta et al. 1997). The reason for this difference may be that the number of subjects in the study of Foresta et al. was small and the subjects were selected mainly on the basis of histological findings, and not sperm concentration.

In our present study, oligozoospermic men were divided into three groups on the basis of sperm concentration. Microdeletions were found in ten infertile oligozoospermic men five in OI, four in OII, and one in OIII. The sperm concentration of the infertile man with microdeletion in OIII was 1×10^6/ml. However, no microdeletion was found in infertile men with sperm concentrations over 1×10^6/ml. In most of the previous reports, the sperm concentration of infertile men with microdeletions was also below 1×10^6/ml (Nagafuchi et al. 1993; Kobayashi et al. 1994; Reijo et al. 1995; Vogt et al. 1996; Najmabadi et al. 1996; Reijo et al. 1996; Stuppia et al. 1996; Simoni et al. 1997; Kremer et al. 1997). These results suggest that molecular analysis of the Y chromosome should be performed in infertile men who show a sperm concentration below 1×10^6/ml. As shown in Table 2, there was no correlation between the extent of microdeletions of the Y chromosome and sperm motility. Because microdeletions of varying extent were identified in two azoospermic and nine oligozoospermic men, as shown in Tables 1 and 2, no significant relationship between the extent of microdeletions and the severity of spermatogenic defects was identified. However, deletions in one common region around sY240, located in interval D21, was observed in 11 of the infertile men with microdeletions. This indicated that important genes involving spermatogenesis may be located around the region including sY240 in interval

D21. Different from our result, Stuppia et al. reported the presence of an oligozoospermia critical region within the interval D22 (Stuppia et al. 1996). Further analysis is now underway.

In regard to the endocrinological data, serum LH (1.5–9.2 IU/l) and serum FSH (4.0–27.6 IU/l) levels in oligozoospermic men with microdeletions were lower than those (LH, 11.2 IU/l and FSH, 30.1 IU/l) in the azoospermic man without obstruction with microdeletions. However, there was no significant difference in endocrinological data between oligozoospermic men with microdeletions and normal infertile men (data not shown). These results indicated no direct relationship between microdeletions of the Y chromosome and the endocrine system.

With the advances in artificial reproductive technology, microdeletions of the Y chromosome will be transmitted to male offspring, and these sons will be at risk of having the same infertility problem (Kremer et al. 1997; Thielemans et al. 1998; Kremer et al. 1998; Jiang et al. 1999). Actually, Kent-First et al. demonstrated that microdeletions were indeed transmitted to male offspring via intracytoplasmic sperm injection (ICSI) (Kent-First et al. 1996). We also found that microdeletions were transmitted to male offspring by ICSI (manuscript in preparation). This possibility should be conveyed to infertile couples with an explanation of the analysis of microdeletions of the Y chromosome. Because spermatogenesis is a process that produces gametes, any gene contributing to the quality and quantity of sperm has a chance of being incorporated on the Y chromosome. Therefore, much more analysis at the molecular level should be performed in order to elucidate the detailed mechanism of spermatogenesis.

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