AZFa Candidate Gene Deletions in Taiwanese Patients with Spermatogenic Failure

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Background and purpose: Deletions of the azoospermia factor subregion a (AZFa) genes in proximal Yq11 are not frequently reported. The majority of AZFa deletions are thought to be associated with more severe testicular phenotypes, such as Sertoli cell-only syndrome. There is a lack of data on AZFa gene deletions in East Asian populations. In this study, we investigated the deletion status of AZFa genes in Taiwanese men with spermatogenic failure.

Methods: One hundred and eighty-three consecutive men with severe oligozoospermia or non-obstructive azoospermia were enrolled in this study. Genomic DNA was extracted from peripheral blood samples and polymerase chain reaction (PCR) was performed using primers specific to four AZFa genes: AZFaT1, DFFRY, DBY, and UTY. Sequence-tagged site markers (sY740, sY630, sY86, sY85, sY87, sY709, and sY88) were used to define the position of deletions. One hundred and twenty fertile men with normal spermatogenesis were enrolled as controls.

Results: Of the 183 patients, two showed single AZFa gene deletions, resulting in an overall frequency of 1.1%. One of these two patients had DFFRY deletion and the other had DBY deletion; their testicular phenotypes were Sertoli cell-only syndrome and hypospermatogenesis, respectively. Neither patient had deletions extending from AZFa through AZFb or AZFc.

Conclusion: Our results suggest that AZFa gene deletion is infrequent in Taiwanese patients with severe oligozoospermia or non-obstructive azoospermia.

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Microdeletions in the long arm of the human Y chromosome (Yq11) are associated with spermatogenic failure. From the deletion patterns, the three recurrently deleted non-overlapping subregions on Yq11 have been designated as azoospermia factors a, b, and c (AZFa, AZFb, and AZFc) [1]. Deletions in the AZFc region involving the DAZ (deleted in azoospermia) gene are the most frequent finding in patients with oligozoospermia or azoospermia [1–6]. Deletions in AZFb occur less commonly, and most of these are associated with deletion in the AZFc region [7]. Deletions in the AZFa region are the least commonly reported and are considered to be associated with a more severe testicular phenotype, ie, Sertoli cell-only syndrome (SCO) [8, 9]. The AZFa interval has been estimated to span 800 kilobases (kb) of DNA, and four candidate genes for the AZFa phenotype have been isolated [10, 11]. It is still unclear whether the AZFa phenotype is caused by the loss of one or more of these genes. DFFRY (Drosophila fat facets related Y), later named USP9Y, is located in interval 5C and expressed in a wide range of tissues. DFFRY and its X-homologue, DFFRX, might be involved in maintaining male germ cell development [10]. Loss of the DFFRY results in depopulation of germ cells rather than their complete absence [8, 10]. Distal to DFFRY, two other genes, DBY (DEAD/H box polypeptide, Y chromosome) and UTY (ubiquitously transcribed tetratricopeptide repeat gene, Y chromosome), were mapped to interval 5C/D [12]. Deletion of DBY alone or in combination with UTY has been observed in a subset of patients with spermatogen-

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esis defects [5]. DBY and UTY also have X-homologues (DBX and UTX) and their transcripts are identified in a wide range of tissues. Recently, an anonymous expressed sequence tag (AZFaT1) has been mapped proximal to DFFRY [13]. It is also transcribed in a wide range of tissues. Deletion of DFFRY and/or AZFaT1 is associated with oligozoospermia, and additional loss of DBY with a more severe SCO phenotype [13].

We previously tested four sequence-tagged site (STS) markers (sY81, sY82, sY84, and sY88) in 94 Taiwanese men with non-obstructive azoospermia, and no deletion of these four markers was detected [6]. One possible explanation for this is that the STSs were not adequately selected in this interval and small deletions could not be detected. In this study, we selected STSs within the AZFa subregion, including markers specific to the AZFaT1, DFFRY, DBY, and UTY genes, to determine the deletion status in Taiwanese patients with spermatogenic failure.

Subjects and Methods

From January 1997 to December 2000, 183 consecutive men presenting with severe oligozoospermia or non-obstructive azoospermia in our urology clinic were enrolled in this study. One hundred and twenty fertile men with normal spermatogenesis were enrolled as controls. All patients underwent a comprehensive examination, including a detailed history, physical examination, a minimum of two consecutive semen analyses, and endocrinology profiles including luteinizing hormone, follicle-stimulating hormone (FSH), prolactin, and testosterone. Severe oligozoospermia was defined as a sperm count of less than 5×10^6/mL. Patients highly suspected of having non-obstructive azoospermia were advised to undergo bilateral testicular biopsies. Non-obstructive azoospermia was defined as spermatogenic defects in the testicular biopsy (such as hypospermatogenesis, maturation arrest, and SCO), elevated serum FSH concentration, total testicular volume less than 30 mL, or none of the other diagnoses applicable [14]. All patients had a normal 46, XY karyotype. Yq euchromatin microdeletion tests were performed simultaneously in all patients using 27 STS markers (including RBM and DAZ genes in AZFb and AZFc, respectively) as described previously [6].

DNA samples and polymerase chain reaction analysis

Genomic DNA was extracted from peripheral blood samples using a Puregene DNA isolation kit (Gentra, Minneapolis, MN, USA). The polymerase chain reaction (PCR) was performed using primer sets for AZFaT1, DFFRY-1, DBY-1, and UTY genes (Table 1). PCR amplification failures for these genes were further confirmed by at least two more amplification failures by single PCR, and amplification failures using another set of gene-specific primers (DFFRY-2 and DBY-2). In cases with deletions, other STS markers (sY740, sY630, sY86, sY85, sY87, sY709, and sY88) were used to define the position of deletions (Table 1, Fig. A). All STSs were derived from data in previous reports [8, 11–13, 15, 16]. sY14 was specific to the SRY (sex-determining region Y chromosome) gene (interval 1A) and was used as an internal control for each assay. PCR amplification was carried out under the following conditions: a final volume of 20 μL contained 0.2 μM of each primer, 1 x PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl), 2.5 mM $MgCl_2$, 200 μM of each dNTP, 100 ng genomic DNA, and 0.5 unit of Taq DNA polymerase (Gibco/BRL, Rockville, MD, USA). Initial denaturation was at 95°C for 5 minutes and was followed by 30 cycles of amplification — 94°C denaturation for 1 minute, 58°C (for DFFRY, DBY, AZFaT1, sY87) or 60°C (for UTY, sY740, sY630, sY86, sY85, sY709) annealing for 1.5 minutes, 72°C extension for 1 minute — and a final extension at 72°C for 5 minutes. The reaction products were fractionated on 10% acrylamide gels (for UTY, DBY-1, sY630, sY709), 1.5 % agarose gels (for DFFRY-1, DFFRY-2, AZFaT1, sY86, sY85, sY87), or 1.2% agarose gel (for DBY-2). The PCR products were made visible with ethidium bromide. Each assay incorporated three samples from controls: a genomic DNA sample from a normal fertile man, a genomic DNA sample from a normal fertile woman, and a PCR mixture containing no DNA (blank control).

Results

Among the 183 patients, severe oligozoospermia was diagnosed in 45 and non-obstructive azoospermia in 138. Among the patients with non-obstructive azoospermia, 72 had undergone testicular biopsies, which revealed hypospermatogenesis in 28, maturation arrest in 16, and SCO in 28.

Of the 183 patients, two with azoospermia had deletions involving a single gene, a deletion frequency in the AZFa subregion of 1.1%. One of these patients had a DFFRY deletion with a histologic diagnosis of SCO, and the other had a DBY deletion with hypospermatogenesis. Two pairs of PCR analyses using another gene-specific primer (DFFRY-2 and DBY-2) confirmed the failure to amplify DFFRY or DBY in these

Table 1. Primer sets used for polymerase chain reaction

Gene/locus	STS size	Primer sequence	Reference
UTY	65 bp	GCATCATAATATGGATCTAGTAGG	
		GGAGATACTGAATAGCATAGC	Lahn et al [12]
DFFRY-1*	249 bp	GGCTGATATATGCTGGTACTTCATTCA	
		CAGTACTCAAAACAACACAG	Silber et al [15]
DFFRY-2*	345 bp	TTGTTACTTTTATAATCTAATGCTT	
		TAATTTATTACTTTACAGTCACA	Oefner et al (1999, unpublished)
DBY-1*	61 bp	AGTTCCCGCTATTCGGTCTCA	
		CCCTGAAGAGAAGCGAAAAA	Silber et al [15]
DBY-2*	689 bp	ATCGACAAAGTAGTGGTTCC	
		AGATTCAGTTGCCCCACCAG	Foresta et al [8]
AZFaT1*	250 bp	AGAGGACGGGTCTAATAGATC	
		CACGGACTCCAGGTGATGAG	Sargent et al [13]
sY740	100 bp	ATGACTGGCTGTCGGAGTTC	
		AAGCTCTGTGGGAATGGTTG	Sun et al [11]
sY630	184 bp	CCAGTCCTATTGGGTCAGGA	
		CAGGCAAGGATTCCATTTG	Sun et al [11]
sY86	318 bp	GTGACACACAGACTATGCTTC	
		ACACACAGAGGGACAACCCT	Vollrath et al [16]
sY85	369 bp	TGGCAATTTGCCTATGAAGT	
		ACAGGCTATTTGACTGGCAG	Vollrath et al [16]
sY87	252 bp	TCTGTTGCTTGAAAAGAGGG	
		ACTGCAGGAAGAATCAGCTG	Vollrath et al [16]
sY709	99 bp	CATGACTGTGCATTGTGCTATGC	
		GGTTGTGGGAGAAAACTCCC	Sun et al [11]

STS = sequence-tagged site. *Primer sequences designed by our laboratory according to genome data published in references.

two patients (Fig. B). Neither of these patients had deletions detected by screening for 27 STSs, including markers for AZFa loci and RBM1 and DAZ gene families [6]. In addition, no deletions of DBY or DFFRY genes were found in any of the control specimens.

Discussion

In this study, we found a low incidence (1.1%) of AZFa deletions in Taiwanese men presenting with spermatogenic failure. To date, fewer than 30 infertile patients have been reported as carrying deletions in AZFa loci [1, 5, 8–10, 13, 17, 18]. The reason for the rarity is unclear. It is well documented that breakage hotspots are clustered around areas with repetitive DNA sequences, and that deletions usually arise from unequal cross-over events during meiosis or mitosis [19]. Enriched repetitive DNA blocks in distal Yq11 may explain the more frequent deletions occurring in the AZFc region than the AZFa region [7, 20]. Recently, it was proposed that the combined deletion of DFFRY, DBY, and possibly AZFaT1 is caused by an intrachromosomal recombination between two long homologous retroviral sequence blocks, and that this event probably causes most AZFa deletions observed in men with SCO [17, 18, 21]. However, the deletions in the two patients in the present study were restricted to a single gene and, therefore, certainly could not have been caused by intra-chromosomal human endogenous retrovirus 15 (HERV15) recombination events. Thus, other molecular mechanisms must account for these deletions.

Because of the rarity of AZFa deletions, it is difficult to characterize the roles of AZFa genes in human spermatogenic failure. In the present study, two patients had isolated deletions of DFFRY or DBY, the genes most frequently deleted in the AZFa region [5, 8, 9, 11]. The DFFRY gene might be involved in maintaining male germ-cell lineage, like its X-homologue (DFFRX), which Brown et al suggested is involved in oocyte development [10]. In addition, Sun et al reported a *de novo* mutation in USP9Y (also known as DFFRY) in 576 patients [11]. The 4-bp deletion in a splice-donor site caused skipping of an exon with resultant protein truncation. The phenotype of this mutation was azoospermia (SCO), suggesting the importance of the DFFRY gene in spermatogenic failure. A total of six patients with isolated deletion or mutation of the DFFRY gene have been reported; five of these

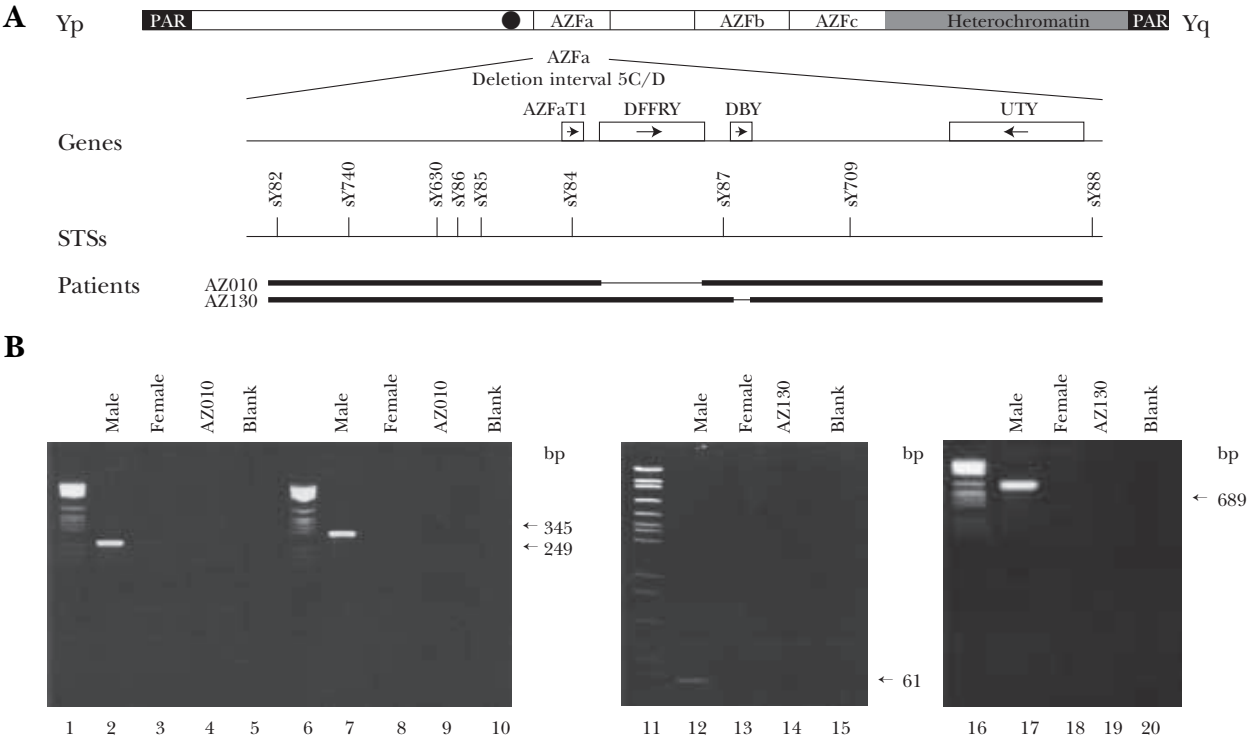

Figure. *A) The azoospermia factor subregion a (AZFa) of the human Y chromosome and the position of the four genes, AZFaT1, DFFRY, DBY, and UTY, and of the sequence-tagged site markers used in this study. B) Polymerase chain reaction (PCR) analysis for the patient with DFFRY deletion (lanes 1–10) and for the patient with DBY deletion (lanes 11–20). Lanes 2–5 represent PCR products of DFFRY-1; lanes 7–10 represent PCR products of DRRFY-2; lanes 12–15 represent PCR products of DBY-1; and lanes 17–20 represent PCR products of DBY-2. The DNA size maker used was pGEM (Promega, Madison, WI, USA).*

had SCO phenotypes and one had hypospermatogenesis [8, 9, 11]. DFFRY therefore appears to be a strong AZFa candidate. Foresta et al reported a relatively high prevalence (4.2%, 6/143) of *de novo* DBY deletions [8]. They also identified a testis-specific transcript of the DBY gene. Similarly, a total of six cases of isolated deletion of the DBY gene have been reported, and their phenotypes were SCO or hypospermatogenesis. These results also imply that DBY represents another strong AZFa candidate.

Definite genotype-phenotype correlation for patients with AZFa gene deletions still remains uncertain. Many authors have suggested that deletions in AZFa loci are commonly associated with SCO, and some authors have suggested that, as more genes are deleted, spermatogenesis defects may progress from hypospermatogenesis to SCO [5, 8, 9, 13]. Table 2 shows all relevant studies concerning AZFa gene deletions and corresponding testicular histology. Among 22 patients with SCO, Blagosklonova et al found four carrying deletions of the DFFRY gene [9]. Foresta et al reported nine patients with AZFa gene deletions [8], and Sargent et al reported four patients with AZFa gene deletions [13]. Kamp et al [17] and Sun et al [18] described six and two patients, respectively, with AZFa deletion breakpoints between two HERV15 sequence blocks (including AZFaT1, DFFRY, and DBY), and their testicular phenotype was SCO. These reports indicate that single or multiple deletions of AZFa genes result in hypospermatogenesis or SCO, but no specific genotype-phenotype correlation can be addressed. Because isolated UTY or AZFaT1 gene deletion has not been reported, the roles of these two genes as AZFa candidates remains obscure.

In conclusion, two of 183 Taiwanese men in this series had isolated deletions of AZFa genes. The frequency of AZFa gene deletions in this series of patients presenting with spermatogenic failure was thus 1.1%. None of the patients had deletions extending from AZFa through AFZb or AFZc. This study supports the hypothesis that DFFRY and DBY may have a role in human spermatogenesis. More studies are needed to elucidate the function of these two genes and the molecular mechanism by which their deletions arise.

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Table 2. Overview of the literature concerning the azoospermia factor subregion a (AZFa) gene deletions and corresponding testicular histology

Study	n	AZFa gene				Testicular histology
		AZFaT1	DFFRY	DBY	UTY	
Sargent et al [13]	1	–	–	+	+	Hypospermatogenesis
	1	–	–	–	–	Sertoli cell-only syndrome
	2	–	–	–	+	Sertoli cell-only syndrome
Foresta et al [8]	1	+	–	+	+	Hypospermatogenesis
	3	+	+	–	+	Hypospermatogenesis
	1	+	+	–	–	Hypospermatogenesis
	3	+	+	–	+	Sertoli cell-only syndrome
	1	+	–	–	+	Sertoli cell-only syndrome
Blagosklonova et al [9]	4	+	–	+	+	Sertoli cell-only syndrome
Sun et al [18]	2	–	–	–	+	Sertoli cell-only syndrome
Kamp et al [17]	6	–	–	–	+	Sertoli cell-only syndrome
Lin et al (2001)	1	+	–	+	+	Sertoli cell-only syndrome
	1	+	+	–	+	Hypospermatogenesis

+ = present; – = not present.

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References

1. Vogt PH, Edelmann A, Kirsch S, et al: Human Y chromosome azoospermia factor (AZF) mapped to different subregions in Yq11. *Hum Mol Genet* 1996; 5:933–43.
2. Reijo R, Alagappan PK, Patrizio P, et al: Severe oligospermia resulting from deletions of azoospermia factor gene on Y chromosome. *Lancet* 1996;347: 1290–3.
3. Stuppia L, Gatta V, Calabrese G, et al: A quarter of men with idiopathic oligo-azoospermia display chromosome abnormalities and microdeletions of different types in interval 6 of Yq11. *Hum Genet* 1998;102: 566–70.
4. Foresta C, Ferlin A, Garolla A, et al: High frequency of well-defined Y-chromosome deletions in idiopathic Sertoli cell-only syndrome. *Hum Reprod* 1998;13:302–7.
5. Ferlin A, More E, Garolla A, et al: Human male infertility and Y chromosome deletions: role of the AZF-candidate genes DAZ, RBM and DFFRY. *Hum Reprod* 1999;14:1710–6.
6. Lin YM, Chen CW, Sun HS, et al: The Y chromosome microdeletion and its effect on reproductive decisions in Taiwanese patients presenting with non-obstructive azoospermia. *Urology* 2000;56:1041–6.
7. Vogt PH: Human chromosome deletions in Yq11, AZF candidate genes and male infertility: history and update. *Mol Hum Reprod* 1998;4:739–44.
8. Foresta C, Ferlin A, Moro E: Deletion and expression analysis of AZFa genes on the human Y chromosome revealed a major role for DBY in male infertility. *Hum Mol Genet* 2000;9:1161–9.
9. Blagosklonova O, Fellmann F, Clavequin M, et al: AZFa deletions in Sertoli cell-only syndrome: a retrospective study. *Mol Hum Reprod* 2000;6:795–9.
10. Brown GM, Furlong RA, Sargent CA, et al: Characterisation of the coding sequence and fine mapping of the human DFFRY gene and comparative expression analysis and mapping to the Sxrb interval of the mouse Y chromosome of the Dffry gene. *Hum Mol Genet* 1998; 7:97–107.
11. Sun C, Skaletsky H, Birren B, et al: An azoospermic man with a de novo point mutation in the Y-chromosomal gene USP9Y. *Nat Genet* 1999;23:429–32.
12. Lahn BT, Page DC: Functional coherence of the human Y chromosome. *Science* 1997;278:675–80.
13. Sargent CA, Boucher CA, Kirsch S, et al: The critical region of overlap defining the AZFa male infertility interval of proximal Yq contains three transcribed sequences. *J Med Genet* 1999;36:670–7.
14. Rowe PJ, Comhaire FH, Hargreave TB, et al: *WHO Manual for the Standardized Investigation and Diagnosis of the Infertile Couple*. 1st ed. Cambridge: Cambridge University, 1993:39.
15. Silber SJ, Alagappan R, Brown LG, et al: Y chromosome deletions in azoospermic and severely oligozoospermic men undergoing intracytoplasmic sperm injection after testicular sperm extraction. *Hum Reprod* 1998;13: 3332–7.
16. Vollrath D, Foote S, Hilton A, et al: The human Y chromosome: a 43-interval map based on naturally occurring deletions. *Science* 1992;258:52–9.
17. Kamp C, Hirschmann P, Voss H, et al: Two long homologous retroviral sequence blocks in proximal Yq11 cause AZFa microdeletions as a result of intrachromosomal recombination events. *Hum Mol Genet* 2000; 17:2563–72.

18. Sun C, Skaletsky H, Rozen S, et al: Deletion of azoospermic factor a (AZFa) region of human Y chromosome caused by recombination between HERV15 provirus. *Hum Mol Genet* 2000;9:2291–6.
19. Lupsky JR: Genomic disorders: structural features of the genome can lead to DNA rearrangements and human disease traits. *Trends Genet* 1998;14:417–22.
20. Yen PH, Allen E, Marsh B, et al: Cloning and expression of steroid sulfatase cDNA and the frequent occurrence of deletions in STS deficiency: implications for X-Y interchange. *Cell* 1987;49:443–54.
21. Blanco P, Shlumukova M, Sargent C, et al: Divergent outcomes of intrachromosomal recombination on the human Y chromosome: male infertility and recurrent polymorphism. *J Med Genet* 2000;37: 752–8.