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·*Original Article*·

Uniform deletion junctions of complete azoospermia factor region c deletion in infertile men in Taiwan

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Abstract

Aim: To determine the deletion junctions of infertile men in Taiwan with azoospermia factor region c (*AZFc*) deletions and to evaluate the genotype/phenotype correlation. **Methods:** Genomic DNAs from 460 infertile men were examined. Bacterial artificial chromosome clones were used to verify the accuracy of polymerase chain reaction. Deletion junctions of the *AZFc* region were determined by analysis of sequence-tagged sites and gene-specific markers. **Results:** Complete *AZFc* deletions, including *BPY2*, *CDY1* and *DAZ* genes, were identified in 24 men. The proximal breakpoints were clustered between sY1197 and sY1192, and the distal breakpoints were clustered between sY1054 and sY1125 in all but one of the 24 men. The testicular phenotypes of men with complete *AZFc* deletion varied from oligozoospermia, to hypospermatogenesis, to maturation arrest. **Conclusion:** We identified a group of infertile men with uniform deletion junctions of *AZFc* in the Taiwan population. Despite this homogeneous genetic defect in the *AZFc* region, no clear genotype/phenotype correlation could be demonstrated. *(Asian J Androl 2006 Mar; 8: 205–211)*

Keywords: azoospermia factor; *BPY2*; *CDY1*; deleted in azoospermia; Y chromosome

1 Introduction

Deletions in the azoospermia factor (*AZF*) region on the Y chromosome have been considered one of the major genetic causes of male infertility. Based on observations of recurrent, non-overlapping deletion patterns, it was proposed that multiple genes might be implicated in spermatogenesis defects. These genes are located in the proximal, middle and distal subregions of Yq11, designated *AZFa*, *AZFb* and *AZFc*, respectively [1]. Among the three non-overlapping *AZF* loci, deletions occur most commonly in *AZFc*. *AZFc* deletion accounts for 10–15% of men with non-obstructive azoospermia and 5–10% of those with oligozoospermia [1–6].

Recently, several deletion mechanisms have been proposed in these *AZF* regions [7–11]. The deletion in the *AZFa* region is believed to be the result of recombination between homologous retroviral sequence blocks, HERV15yq1 and HERV15yq2, leading to the deletions of both *USP9Y* and *DBY* genes [9]. For the deletions in-

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volving the *AZFb+c* regions, three types of deletion models have been identified: palindrome P5 to the proximal arm of palindrome P1; palindrome P5 to the distal arm of palindrome P1; and palindrome P4 to the distal arm of palindrome P1 [10, 11]. For the *AZFc* region, a more detailed physical map has been constructed by Kuroda-Kawaguchi *et al*. [7]. By sequencing highly overlapping bacterial artificial chromosome (BAC) clones derived from a single man, the regional structure and repetitive sequence composition spanning the entire *AZFc* were determined. They found that *AZFc* of the Y chromosome is made up almost entirely of long direct and inverted repeats, or amplicons, and is particularly susceptible to deletions. Homologous recombination between amplicons b2 and b4 is probably the most common cause of *AZFc* deletion [7]. It is noteworthy that partial *AZFc* deletion might cause spermatogenic failure, and several types of partial *AZFc* deletions have been proposed and designed as gr/gr, g1/g2, b1/b3 , b2/b3, rg/gr, g1/g3 and b3/b4 deletions [12, 13]. The gr/gr, g1/g2 and b1/b3 deletions, which remove deleted in azoospermia 1 (*DAZ1*) and deleted in azoospermia 2 (*DAZ2*) copies, were associated with varied degrees of spermatogenic impairment [12, 13].

Despite the highly polymorphic nature of this Y-chromosomal region, the *AZFc* deletion model suggested by Kuroda-Kawaguchi *et al*. [7] was based on DNA sequences of a single man. Although the polymorphic nature of the Y chromosome could be obviated by using genomic DNA from one man, the versatility of the proposed model should be tested in a different group. Given that *AZFc* deletion is one of the important genetic causes for male infertility, the determination of deletion junctions of the AZFc region in those patients would be of considerable value. Here, we collated the Y chromosome sequence-based map and designed gene-specific primers for deletion mapping analysis. Our goal was to define the extent of *AZFc* deletions in men in Taiwan with spermatogenic failure, and to address the genotype/phenotype correlation in men with *AZFc* deletion.

2 Materials and methods

2.1 Patients

From January 1997 to March 2004, DNA samples of 460 infertile men in Taiwan with oligozoospermia or non-obstructive azoospermia were collected for the study of genetic defects. Non-obstructive azoospermia was defined as spermatogenic defects on testicular biopsy or elevated serum follicle stimulating hormone (FSH) level, total testicular volume less than 30 mL, and no other applicable diagnosis. All infertile men underwent a comprehensive examination, including a detail history, physical examination, at least two consecutive semen analyses, endocrinology profiles (FSH, luteinizing hormone [LH], prolactin and testosterone), and chromosome analysis. This study had been approved by the National Scientific Council of Taiwan and the Institutional Review Board of National Cheng Kung University Medical Center. The study was designed in accordance with the Helsinki Declaration of 1975 on human experimentation. Informed consent was obtained from all enrollees.

2.2 Validation of DAZ-, BPY2- and CDY1-specific primers and polymerase chain reaction (PCR)

We designed a set of gene-specific primers for *CDY1*. The primers for *DAZ* and *BPY2* were the same as described elsewhere [5]. In order to verify the specificity of these primers, five BAC clones were used that contained a single copy of the *DAZ* (RP11-26D12), *CDY1* (RP11-497C14), *CDY2* (RP11-509B6), *BPY1* (RP11-264A13) or *BPY2* (RP11-86G22) genes. The clones were available from Pieter de Jong's BACPAC Resources Center (http://www.chori.org/bacpac). Genomic DNA and clone DNA were extracted using the standard methods. During PCR analysis, the sex-determining region Y (*SRY*) gene was used as an internal control for amplification failure. Each PCR reaction consisted of 0.12-0.50 μmol/L of primer, 1 × PCR buffer, 1.5 mmol/L $MgCl_2$, 200 μmol/L of each deoxyribonucleotide triphosphate, 150 ng of genomic DNA, and two units of Taq DNA polymerase (Perkin-Elmer Cetus, Emeryville, CA, USA) in a total volume of 20 μL. Thermocycling (OmniGene Thermal Cycler; Hybaid, Ashford, Middlesex, UK) consisted of 10 min at 95ºC for initial denaturation, followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 62ºC for 1 min, extension at 72ºC for 1 min, and a final extension at 72ºC for 10 min. The reaction products were fractionated on 2.5% agarose gels. The PCR products were made visible with ethidium bromide. For each assay, we incorporated the following controls: a genomic DNA sample from a normal fertile man; a genomic DNA sample from a normal fertile woman; a PCR mixture containing no DNA (blank control); and clone DNA. PCR amplification failures for these genes were further confirmed by at least two more amplification failures.

2.3 Determination of AZFc deletion junctions

According to the model proposed by Kuroda-Kawaguchi *et al*. [7], the proximal boundary of *AZFc* falls between sites sY1197 and sY1192, and the distal boundary of *AZFc* falls between sites sY1054 and sY1125. To determine the deletion junction, sY1197, sY1192, sY1054, and sY1125 were used. Site sY1196 is a marker proximal to sY1197, site sY1190 is a marker within *AZFc*, and site sY1201 is a marker distal to sY1125. The latter three markers were used for further confirmation of deletion junctions. The sequences of sequence-tagged site (STS) primers were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). The primer sequences and sizes of the amplified fragments are listed in Table 1. We analyzed the deletion junctions of *AZFc* in patients with interstitial *AZFc* deletion, which was defined as deletion of one of *BPY2*, *CDY1* or *DAZ* genes, or a combined deletion of at least two of these genes. The PCR conditions for STS markers were the same as those for gene-specific markers. Cases with *AZFc* deletion were also analyzed for the presence of sY159 and sY160 to exclude the possibility of Yq terminal deletion according to methods described previously [5].

3 Results

3.1 Validation of gene-specific primers

The specificity of primers for *BPY2*, *DAZ* and *CDY1* is demonstrated in Figure 1. Sites sY277 and sY283 were specific to the *DAZ* gene. By using both primers, *DAZ* could be amplified in a *DAZ*-containing clone and genomic DNA from normal men, but not in genomic DNA from female subjects. *BPY2* primer amplified a single band from a *BPY2*-containing clone and genomic DNA from normal men, but could not amplify a *BPY1*-containing clone, genomic DNA from female subjects. Similarly, *CDY1* primer amplified a single band from a *CDY1*-contaning clone, genomic DNA from normal men, but could not amplify a *CDY2*-containing clone, genomic DNA from female subjects.

Table 1. Primer set used for polymerase chain reaction (PCR) amplification of fragments to determine azoospermia factor region c (*AZFc*) deletion junctions. STS, sequence-tagged site.

Gene/STS	Primer	Sequence	Amplicon size (bp)
sY277 (*DAZ*)	Forward	5'-GGGTTTTGCCTGCATACGTAATTA-3'	275
	Reverse	5'-CCTAAAAGCAATTCTAAACCTCCAG-3'	
sY283 (*DAZ*)	Forward	5'-CAGTGATACACTCGGACTTGTGTA-3'	375
	Reverse	5'-GTTATTTGAAAAGCTACACGGG-3'	
BPY2	Forward	5'-ATGATAGTCGCGTCAGCT-3'	306
	Reverse	5'-GGGATTATCACATATTGCG-3'	
CDY1	Forward	5'-CTATTCTAAGAACTCTCCTAA AAC-3'	319
	Reverse	5'-CAGTCTGTTCTGCACCAGGA-3'	
sY1196	Forward	5'-GTTGGCAACTTGCACTGCT-3'	393
	Reverse	5'-CCTTTCCTCTCAAAGTCCCC-3'	
sY1197	Forward	5'-TCATTTGTGTCCTTCTCTTGGA-3'	453
	Reverse	5'-CTAAGCCAGGAACTTGCCAC-3'	
sY1192	Forward	5'-ACTACCATTTCTGGAAGCCG-3'	255
	Reverse	5'-CTCCCTTGGTTCATGCCATT-3'	
sY1190	Forward	5'-TTGTGAAATGGTGGTGATGG-3'	666
	Reverse	5'-CTGATTTGGAAACTCGTCCC-3'	
sY1054	Forward	5'-ACCTAAGGGAACCCAGGAGA-3'	340
	Reverse	5'-CGACACTTTTGGGAAGTTTCA-3'	
sY1125	Forward	5'-GTGGGGGTTTCACATTATGG-3'	283
	Reverse	5'-GGTCACAGACTCACATTTAAGCA-3'	
sY1201	Forward	5'-CCGACTTCCACAATGGCT-3'	677
	Reverse	5'-GGGAGAAAAGTTCTGCAACG-3'	

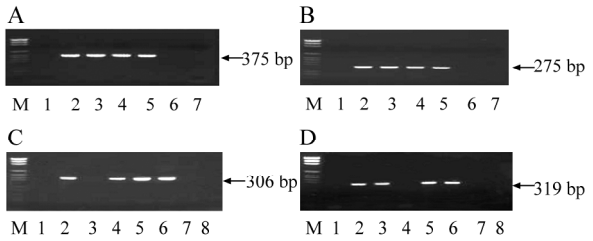

Figure 1. Verification of gene-specific primers for *BPY2*, *DAZ* and *CDY1* genes. (A): polymerase chain reaction (PCR) amplification of sY277. (B): PCR amplification of sY283. Sites sY277 and sY283 are specific to the *DAZ* gene. M, marker; 1, female control; 2, normal fertile male control; 3, *DAZ* clone; 4 and 5, infertile men without *DAZ* deletion; 6, infertile man with *DAZ* deletion; 7, blank control. (C): PCR amplifications of CDY1. M, marker; 1, female control; 2, normal fertile male control; 3, *CDY2* clone, 4, *CDY1* clone; 5 and 6, infertile men without *AZFc* deletion. 7, infertile man with *AZFc* deletion; 8, blank control. (D): PCR amplifications of BPY2. M, marker; 1, female control; 2, normal fertile male control; 3, *BPY1* clone; 4, *BPY2* clone; 5 and 6, infertile men without *AZFc* deletion; 7, infertile men with *AZFc* deletion; 8, blank control.

3.2 Uniform AZFc deletions and genotype/phenotype correlation

In this study, we identified 24 men with interstitial *AZFc* deletion. All of these men had a normal male karyotype. The deletion statuses of 24 men and their corresponding testicular phenotypes are shown in Figure 2. Twenty-three men revealed proximal breakpoints between sY1197 and sY1192 and a distal breakpoint between sY1054 and sY1125. Their testicular phenotypes were oligozoospermia in 11 men and azoospermia in 12 men. The sperm counts for the 11 oligozoospermic men varied from 8.8×10^6/mL to lower than 0.1×10^6/mL. Of the 12 men with azoospermia, eight underwent testicular biopsy. Their histologies were hypospermatogenesis in five men and maturation arrest in three men. Testicular histology was not available in the remaining four men. Patient TW158 revealed proximal breakpoints between sY1197 and sY1192 and distal breakpoints between CDY1 and sY1054. His testicular phenotype was oligozoospermia with a sperm count of 2×10^6/mL. All

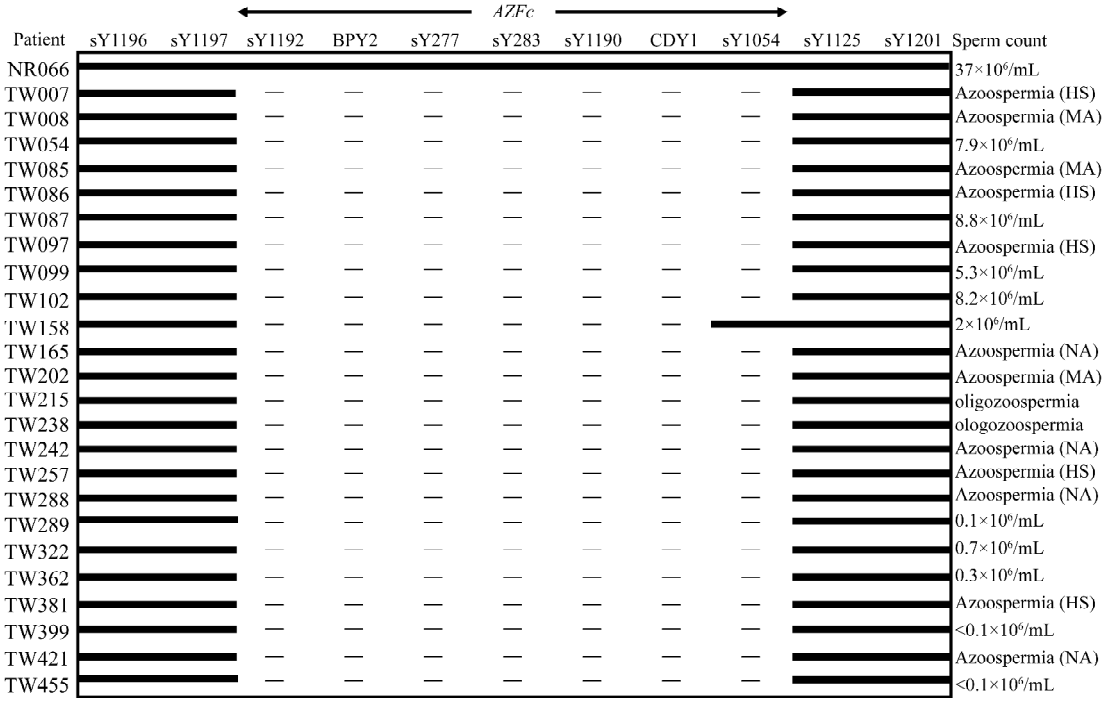

Figure 2. Maps of sequence-tagged site (STS) and gene deletions from a normal, fertile man (patient NR066) and 24 infertile men with azoospermia factor region c (*AZFc*) deletions on the Y chromosome. Sites sY277 and sY283 are specific to the *DAZ* gene. The proximal and distal boundaries of recurrent *AZFc* deletion located between sY1197 and sY1192 and between sY1054 and sY1125, respectively. In each map, the solid bars represent sites found to be present, and the dashes indicate sites to be deleted. HS, hypospermatogenesis; MA, maturation arrest; NA, not available; NR, normal fertile men; TW, infertile men in Taiwan.

24 men had completely lost the Y-chromosomal *BPY2*, *DAZ* and *CDY1* genes.

3.3 De novo AZFc deletion

The 24 men were routinely asked to assist in obtaining blood samples from their male relatives in order to determine the origin of genetic defects. The fathers or brothers of six of the 24 men provided their blood samples for PCR analysis. All of them had intact Y chromosome, indicating that these deletions had arisen *de novo*.

4 Discussion

Deletions of the *AZFc* region are the most common known cause of spermatogenic defect. Kuroda-Kawaguchi *et al*. [7] presented strong evidences that most *AZFc* deletions involve a 3.5 Mb segment, bounded by two 229 kb direct repeats. It was hypothesized that recurrent deletion of the *AZFc* region was caused by recombination between two direct repeats, b2 and b4, flanking the *AZFc* region. By examining the deletion boundaries of 48 infertile men with *AZFc* deletion, they found identical deletion junctions in 47 cases [7]. In the present study, we also identified a group of infertile men in Taiwan with uniform recurrent *AZFc* deletion. The proximal breakpoints were clustered between sY1197 and sY1192, and the distal breakpoints clustered between sY1054 and sY1125 in all but one of the 24 men. Both the proximal and distal breakpoints are identical to those described by Kuroda-Kawaguchi *et al*. [7]. The different distal breakpoint in patient TW158 may be due to simple nucleotide polymorphism; however, we were unable to perform sequence analysis because there was insufficient DNA from this patient.

There have been debates on the various types of AZFc deletions [5, 7, 14]. It is proposed that seven gene families, including *BPY2*, *DAZ*, *CDY1*, *CSPG4LY*, *GOLGA2LY*, *TTTY3* and *TTTY4*, are transcribed in the testis and located exclusively within the *AZFc* region [7]. According to this model, these gene families will be removed en block by a complete *AZFc* deletion [7]. However, Ferlin *et al*. [14] reported that some *AZFc*-deleted cases still retained the *CDY1* gene. In our previous report of 12 men with *DAZ* deletions, four had complete deletions of the *DAZ* gene cluster but at least one copy of the other two genes in the *AZFc* region (*BPY2* or *CDY1*) were retained [5]. With the new PCR protocol, *BPY2*, *DAZ* and *CDY1* genes were absent in all men with complete *AZFc* deletion, including the 12 men described in our previous report. Therefore, the four men with isolated deletion of the *DAZ* gene cluster described in our previous report should be re-categorized as complete *AZFc* deletions. The disparity stems from highly similar nucleotide sequences between the members of the gene families. For example, the coding regions of *CDY1* and *CDY2* are 99% identical in nucleotide sequences. Such a high degree of nucleotide similarity implies the importance of critical quality control for PCR analysis. The observation that *BPY2*, *CDY1* and *DAZ* genes are deleted simultaneously in cases with complete *AZFc* deletion is consistent with the model of Kuroda-Kawaguchi *et al*. [7]. In light of the uniform deletion pattern observed, other gene families located within the *AZFc* region, that is, *CSPG4LY*, *GOLGA2LY*, *TTTY3* and *TTTY4*, are believed to be deleted in our patients.

The relationship between the *AZFc* deletion and testicular phenotypes is still not apparent, despite uniform deletion junctions in nearly all patients. Twenty-three men with uniform *AZFc* deletion junctions possessed different testicular phenotypes, ranging from moderate oligozoospermia (sperm count between 5.0×10^6/mL and 10.0×10^6/mL), to severe oligozoospermia (sperm count $< 5.0 \times 10^6$/mL), to azoospermia with hypospermatogenesis or maturation arrest. This finding is in agreement with previous studies that show that identical *AZFc* deletion might be associated with a different impairment of spermatogenesis and might not exclude the occurrence of spontaneous pregnancy [15, 16]. The implications of this finding are 2-fold. First, the human *AZFc* genes are not essential for sustained fertility. Some *AZFc*-deleted men will retain fertility and are capable of complete spermatogenesis. Second, the testicular phenotypes are modified by autosomal genes or other genetic backgrounds. The presence of the 45,X cell line illustrates the interactions between Y-linked sterile genes and other genetic backgrounds of patients. Mosaicism for 45,X cell lines might accentuate the severity of spermatogenic defects in men with *AZFc* deletions [17]. Autosomal genes have been shown to be associated with impaired production of human sperm [18]. Studies on how Y-linked sterility-associated genes interact with autosomal or X-chromosomal genes will provide further insight into the genetic pathways involved in human spermatogenesis.

The major obstacle in elucidating the roles of sterility-related genes stems from the complex and highly poly-

morphic genomic organization of the human Y chromosome. *AZFc* is almost entirely made up of long direct and inverted repeats (amplicons). Members of each amplicon family are more than 99% identical in genomic sequences, and such genomic structure is especially prone to amplification or deletion. It is highly likely that the amplicons vary in size or copy number within the population. Of the gene families located within the *AZFc* region, the *DAZ* gene cluster consists of four copies, the *BPY2* gene cluster consists of three copies, and the *CDY1* gene cluster consists of two copies [7]. However, the copy number of the *DAZ* gene may range from 4 to 7 according to different investigators [19, 20]. The copy number of *BPY2*, *CDY1* or other sterility-associated genes located within the *AZFc* region may vary significantly. It would be highly desirable to study the detailed organization of the *AZFc* region in different ethnic groups and in patients with spermatogenic defect.

Partial *AZFc* deletion cannot be detected by the protocol described in this study because PCR analysis only reveals "all or none" results. Partial deletion of the *DAZ* gene cluster has been reported to account for 5%–10% of cases of oligozoospermia and azoospermia [12, 13]. Likewise, partial deletion of BPY2 or CDY1 may be associated with various degrees of spermatogenic defect [18]. PCR amplification-restriction digestion assay, fiber fluorescence in situ hybridization, Southern blot analysis, or quantitative PCR will be helpful in detecting the copy numbers of Y-linked sterile genes and to delineate subgroups of patients with spermatogenic defect.

In conclusion, we identified a group of infertile men in Taiwan with complete *AZFc* deletion and uniform deletion junctions. Both proximal and distal deletion junctions were identical to the previous report by Kuroda-Kawaguchi *et al*. [7]. Despite a homogeneous genetic defect in the Y chromosome, no clear genotype/phenotype correlation could be demonstrated in patients with complete *AZFc* deletion.

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