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Asian J Androl 2008; 10 (6): 873–881
DOI: 10.1111/j.1745-7262.2008.00436.x

AJA

·*Original Article*·

The prevalence of azoospermia factor microdeletion on the Y chromosome of Chinese infertile men detected by multi-analyte suspension array technology

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Abstract

Aim: To develop a high-throughput multiplex, fast and simple assay to scan azoospermia factor (*AZF*) region microdeletions on the Y chromosome and establish the prevalence of Y chromosomal microdeletions in Chinese infertile males with azoospermia or oligozoospermia. **Methods:** In total, 178 infertile patients with azoospermia (non-obstructed), 134 infertile patients with oligozoospermia as well as 40 fertile man controls were included in the present study. The samples were screened for *AZF* microdeletion using optimized multi-analyte suspension array (MASA) technology. **Results:** Of the 312 patients, 36 (11.5%) were found to have deletions in the *AZF* region. The microdeletion frequency was 14% (25/178) in the azoospermia group and 8.2% (11/134) in the oligospermia group. Among 36 patients with microdeletions, 19 had deletions in the *AZFc* region, seven had deletions in *AZFa* and six had deletions in *AZFb*. In addition, four patients had both *AZFb* and *AZFc* deletions. No deletion in the *AZF* region was found in the 40 fertile controls. **Conclusion:** There is a high prevalence of Y chromosomal microdeletions in Chinese infertile males with azoospermia or oligozoospermia. The MASA technology, which has been established in the present study, provides a sensitive and high-throughput method for detecting the deletion of the Y chromosome. And the results suggest that genetic screening should be advised to infertile men before starting assisted reproductive treatments. *(Asian J Androl 2008 Nov; 10: 873–881)*

Keywords: Y chromosome microdeletion; azoospermia factor; male infertility; multi-analyte suspension array (MASA)

1 Introduction

Infertility, which is defined as an inability to conceive or produce an offspring, is affecting about 15% of all the couples attempting to generate pregnancy. In the case of infertility, approximately 50% of the cases can be attributed to male factors [1]. It is reported that nearly 20% of all infertile men have idiopathic azoospermia or oligozoospermia (< 20 × 10^6 spermatozoa/mL). Deletions of the azoospermia factor (*AZF*) of the Y chromosome are associated with severe spermatogenic failure and represent the most frequent molecular genetic cause of azoospermia and severe oligozoospermia [2]. These microdeletions were mapped to three non-overlapping regions, named as *AZFa*, *AZFb*, and *AZFc*, at Yq11.22-23

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Received 2008-03-16 Accepted 2008-06-01

of the Y chromosome. Different candidate genes had been identified in *AZF* loci. In these cases, the molecular extensions of the most commen *AZF* microdeletions are already known as they result from intrachromosomal recombinant mechanisms and the gene content is also established, but the function of these genes is poorly understood.

Recently, infertility treatment has been performed by intracytoplasmic sperm injection (ICSI) and *in vitro* fertilization (IVF) techniques. However, deletions on the Y chromosome might be spread to the male offspring, causing the persistence of infertility problems in the next generations. Therefore, it is necessary for infertile men with azoospermia and severe oligozoospermia to have been screened for Y chromosome microdeletions before ICSI/IVF.

Currently, the most common method used to detect microdeletions of *AZF* on the Y chromosome is based on multiplex polymerase chain reaction (PCR) and conventional agarose electrophoresis. However, there are some limits to the application of the conventional methods, mainly owing to their low specificity, inaccuracy and time-consuming procedure [3]. So a more precise methodology is necessary to efficiently pursue diagnostic studies for male infertility. Here we present a novel and rapid method to scan for *AZF* region microdeletions of the Y chromosome in Chinese infertile males with azoospermia or oligozoospermia using multi-analyte suspension array (MASA) technology.

The molecular test developed uses Luminex (Luminex Corporation, San Diego, CA, USA) xMAP technology, a flow cytometer that allows simultaneous identification of the *AZF* microdeletion by mixing different sets of microspheres that contain specific capture probes derived from target sequence-tagged site (STS) markers in the *AZF* region. This technology permits the simultaneous detection of 100 analytes by combining 100 different sets of microspheres in a single reaction. Since each microsphere set is internally dyed with two spectral fluorochromes of different intensities, their unique spectral emissions are recognized by a red laser. On hybridization, the biotinylated amplicon bound to the surface of the microsphere is recognized by a green laser that quantifies the fluorescence of the reporter molecule (streptavidin R-phycoerythrin) [4]. Therefore, with this method, seven STS markers, including sY84 for *AZFa*, sY127 and sY134 for *AZFb*, sY254 and sY255 for *AZFc*, sex-determining region Y (SRY or sY14) and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene can be detected simultaneously in a single well.

2 Materials and methods

2.1 Patients and sample collection

In total, 312 infertile men with a normal 46,XY karyotype including 178 with azoospermia (non-obstructed) and 134 with severe oligozoospermia (semen count less than 5×10^6/mL, non-obstructed) aged from 16 to 48 years were recruited from the Affiliate Hospital of Sichuan Genitalia Hygiene Research Center (Chengdu, China) between June 2005 and August 2006. All patients underwent semen analysis at least twice. In addition, 40 fertile age-matched men with a 46,XY karyotype and one normal woman with a 46,XX karyotype were selected as controls. Informed consent was required from each subject to participate in the present study.

2.2 DNA isolation

Genomic DNA was isolated from peripheral blood lymphocytes by phenol-chloroform extraction.

2.3 Primers selection and multiplex PCR

Primers covering only hot spot regions were chosen according to previous reports [5–7]. A series of five STS from the *AZF* region on the long arm of the Y chromosome were used to detect microdeletions. These STS included sY84 for *AZFa*, sY127 and sY134 for *AZFb*, sY254 and sY255 for *AZFc*. In addition, testing for the Yp was performed using sY14, an STS located within the gene SRY (internal positive control) (Table 1). Fertile male and female samples were used as positive and negative controls. The GAPDH gene was used to detect the validity of the templates. Seven pairs of primers were amplified in a single reaction.

Multiplex PCR amplifications were carried out in a total volume of 25 μL buffered solution containing about 200 ng of genomic DNA, 800 μmol/L deoxyribonucleotide triphosphates (dNTP), 1.5 mmol/L Mg^{2+}, 10 pmol/L of each primer and 2.5 U *Taq* polymerase. The cycling conditions were as follows: 94ºC for 10 min followed by 94ºC for 30 s, 55ºC for 30 s and 72ºC for 45 s for 35 cycles, with a final extension at 72ºC for 5 min.

2.4 Coupling of oligonucleotide probes

The 5' amino-modifier C-12-linked oligonucleotide probes (Table 2) corresponding to seven STS, respectively,

Table 1 Seven sequence-tagged sites (STSs) primer sequences for polymerase chain reaction (PCR) amplification. *: 5'-biotin-labeled Y deletion primer. SRY, sex-determining region Y; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

STSs	Region	Primer sequence 5' → 3'	PCR products (bp)
SRY	Yp	CGCATTCATCGTGTGGTCTC*	228
		TTCTTCGGCAGCATCTTCG	
sY84	AZFa	TGTAGGCATGCTCAGGCAAG*	156
		ACTACCTGGAGGCTTCATCA	
sY127	AZFb	AGCACCCACTGGAATCTACC*	195
		CATGGCTACACAGACAGGGA	
sY134	AZFb	GTCTGCCTCACCATAAAACG*	301
		ACCACTGCCAAAACTTTCAA	
sY254	AZFc	GGGTGTTACCAGAAGGCAAA*	238
		TTATCCCCGAATGACCAGCAG	
sY255	AZFc	GTTACAGGATTCGGCGTGAT	123
		CTCGTCATGTGCAGCCAC	
GAPDH	12 chr	GCTAGGAAGGACAGGCAACT*	178
		AGGACGGGAAACTCAAACTA	

Table 2. Oligonucleotide probes used for the multiplex polymerase chain reaction (PCR) products. STSs, sequence-tagged sites; SRY, sex-determining region Y; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

STSs	Probe sequences 5' → 3'
SRY	TGCTGATCTCTGAGTTTCG
sY84	AAGGGTCCCACTGAATCTCC
sY127	CAAGGACTTTCACAAACATCTG
sY134	CCTCTTTCAGTCACAGAACG
sY25	CCATTGTTCATGATGTATGT
sY255	CCAAACACTGAAACCTACCT
GAPDH	TCAGAAATTAACTGGACAGG

were coupled to carboxylated beads by a carbodiimide-based coupling procedure. For each combination of probe and bead set, 2.5 million carboxylated beads were suspended in 25 μL of 0.1 mol/L 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 4.5. Probe oligonucleotides (400 pmol) and 200 μg of *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC) were added and thoroughly mixed with the beads. Incubation was performed in the dark under agitation for 30 min and was interrupted by a thorough mixing step after 15 min. The addition of EDC and incubation steps were repeated twice, and the coupled beads were finally washed once with 0.5 mL of 0.02% Tween-20 and once with 0.5 mL of 0.1% sodium dodecyl sulfate before being stored in 100 μL Tris-EDTA buffer at 4ºC in the dark.

2.5 Hybridization assay and bead analysis

Following PCR amplification, 2.5 μL of each reaction mixture was transferred to a tube containing 33 μL of 1.5 × tetramethylammonium chloride (TMAC) hybridization solution and a mixture of 2000 probe-coupled beads of each set. TE buffer (14.5 μL) was added, followed by gentle mixing with a pipette. The mixture was heated to 95ºC for 10 min. Then hybridization was performed at 55ºC for 15 min. Subsequently, 25 μL of 10 μg/mL streptavidin-R-phycoerythrin diluted with 1 × TMAC hybridization solution was added to each tube and mixed. The mixture was incubated at 55ºC for 5 min. Beads were analyzed for internal bead color and R-phycoerythrin reporter fluorescence on a Luminex 100 analyzer. The median reporter fluorescence intensity (MFI) of at least 100 beads was computed for each bead set in the sample.

3 Results

3.1 Assay optimization

Final assay conditions used for the present study were established after systematic variation of the following parameters: coupling procedure of probes to beads (EDC and probe input); temperature, salt concentration and hybridization conditions; Strep-PE concentration; incubation time for staining; concentrations of different blocking substances in the washing buffer; and number of

wash cycles and wash buffer composition (see Materials and methods).

3.1.1 Optimization of hybridization time

To identify the optimum hybridization time for multiplex PCR products with bead-conjugated specific probes, we incubated samples of the mixture for 10, 15, 20, 25 and 30 min in 55℃. The results (Figure 1) at sequential time points indicated that 15 and 20 min showed similar optimal levels of MFI for the detection of microdeletions. Therefore, we have chosen the earlier time point (15 min) for the detection of microdeletion assay.

3.1.2 Optimization of single and multiplex PCR detection

Single (one set of primer) and multiplex PCR (five mixed sets of primer) were performed using the same STS marker primers (SRY, GAPDH, sY84, sY127, sY134, sY254 and sY255).

In a comparison between single and multiplex detection MFI, the MFI of five STS markers (SRY, GAPDH, sY84, sY127, sY134, sY254 and sY255) in single detection were slightly higher than those in multiplex detection (Figure 2). However, there is no deterrent in multiplex detection that prevents it from detecting distinct deletions or presence of STS markers on the Yq chromosome, and this form of detection provides the means by which to measure multiple analytes simultaneously, potentially saving time and use of expensive resources. Therefore, we have decided to pursue the multiplex PCR detection system for Yq microdeletion analysis in infertile males.

3.1.3 Verifying the reproducibility of the assay

To verify the reproducibility of the hybridization assay, multiplex PCR products were used in the experiments. These products were hybridized to the probe-coupled bead mix. Every experiment was repeated five times, and the results were recorded and analyzed by the software SPSS version 11.0 (SPSS, Chicago, IL, USA) and Origin 6.0 (Microcal Software, Northampton, MA, USA). The results are shown in Table 3.

3.1.4 Determining the specificity of the assay

To determine the specificity of the assay, seven probes (SRY, GAPDH, sY84, sY117, sY134, sY254 and sY255) were coupled individually to define bead sets (7-plex) and hybridized to 2.5 μL of singleplex and multiplex PCR products, respectively. The results showed

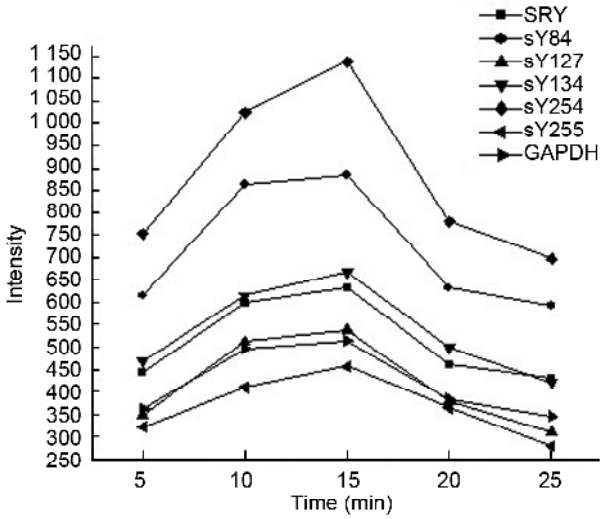

Figure 1. Mean fluorescence detection of polymerase chain reaction (PCR) products for five different lengths of hybridization time using the liquid microbead array, in triplicate. SRY, sex-determining region Y; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

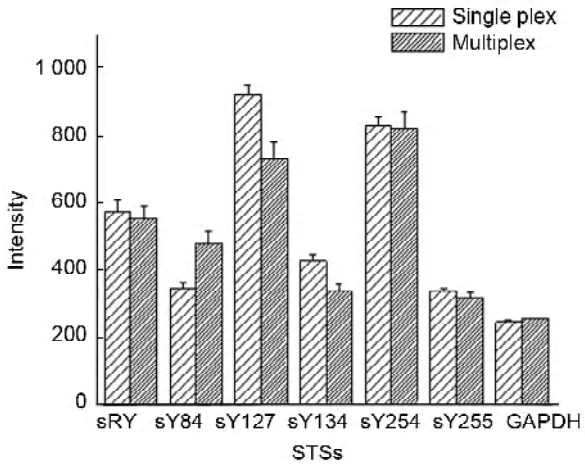

Figure 2. Comparison of the sequence-tagged site (STS) marker detection sensitivity in single and multiplex polymerase chain reaction (PCR) formats using the microbead array. SRY, sex-determining region Y.

that the assay was highly specific (Table 4).

3.1.5 Analysis of data validity

Microdeletion can be easily measured by comparing the MFI score with the MFI scores of positive and negative controls. From multiplex bead array screening, we determined a cut-off value of 100 MFI for the STS.

Table 3. Repeat assay result of multiplex polymerase chain reaction (PCR) products. SRY, sex-determining region Y; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Probes	Mean fluorescence intensity (n = 5)	Standard deviation (SD)	Coefficient of variation (CV) (%)
SRY	552.5	37.9	6.9
sY84	479.8	37.3	7.8
sY127	732.3	48.0	6.6
sY134	339.2	21.0	5.2
sY254	823.1	49.0	6.0
sY255	318.8	19.0	6.0
GAPDH	256.9	12.2	4.7

Table 4. Specificty of seven specific probes in the multiplex bead array assay. PCR, polymerase chain reaction; SRY, sex-determining region Y; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

PCR products	SRY	sY84	sY127	sY134	sY254	sY255	GAPDH
SRY	584	12	10	15	19	18	22
sY84	7	743	20	18	17	21	9
sY127	10.5	29	498	13	13.5	14.5	19
sY134	11	30.5	12	635.5	18	10.5	17
sY254	12.5	12.5	21	11	1 082	17	11.5
sY255	27	10	14.5	10	17	425	23
GAPDH	24	17	13	15	16	8	486.5
Multiplex products	654.5	945	480	382	902	359	398
Negative control	12	11	17.5	20	27	15	31

Table 5. Relative prevalence of deletions in AZFa, b, and c regions in infertile men with azoospermia or oligozoospermia.

AZF region	Azoospermia (n = 178)	Oligozoospermia (n = 134)	Deletion rate (%)
AZFa	sY84 (4)	sY84 (3)	2.2 (7/312)
AZFb	sY127/sY134 (3)	sY127 (3)	1.9 (6/312)
AZFc	sY254/sY255 (13)	sY254/sY255 (4)	6.1 (19/312)
	sY254 (1)	sY254 (1)	
AZFb+c	sY127/sY254 (2)		1.3 (4/312)
	sY127/sY134/sY254/sY255 (2)		
Total	25	11	11.5 (36/312)

3.2 Application of the assay in detecting male infertility

Screening for *AZF* microdeletions by multiplex PCR-MASA was performed in a total of 312 patients, including 178 infertile patients with azoospermia and 134 infertile patients with oligozoospermia who had a normal karyotype as well as 40 fertile man controls. As shown in Table 5, 36 (11.5%) of 312 patients were found to have deletions in the *AZF* region. The microdeletion frequency was 14% (25/178) in the azoospermic group and 8.2% (11/134) in the oligospermic group. Among 36 patients with microdeletions, 19 had deletions in the *AZFc* region, seven had deletions in *AZFa* and six had deletions in *AZFb*. In addition, four patients had both *AZFb* and *AZFc* deletions. No deletion in the *AZF* region was found in the 40 fertile controls. Some examples are shown in Figure 3.

To determine the validity of the MASA results, all samples were detected by classic PCR and identified by

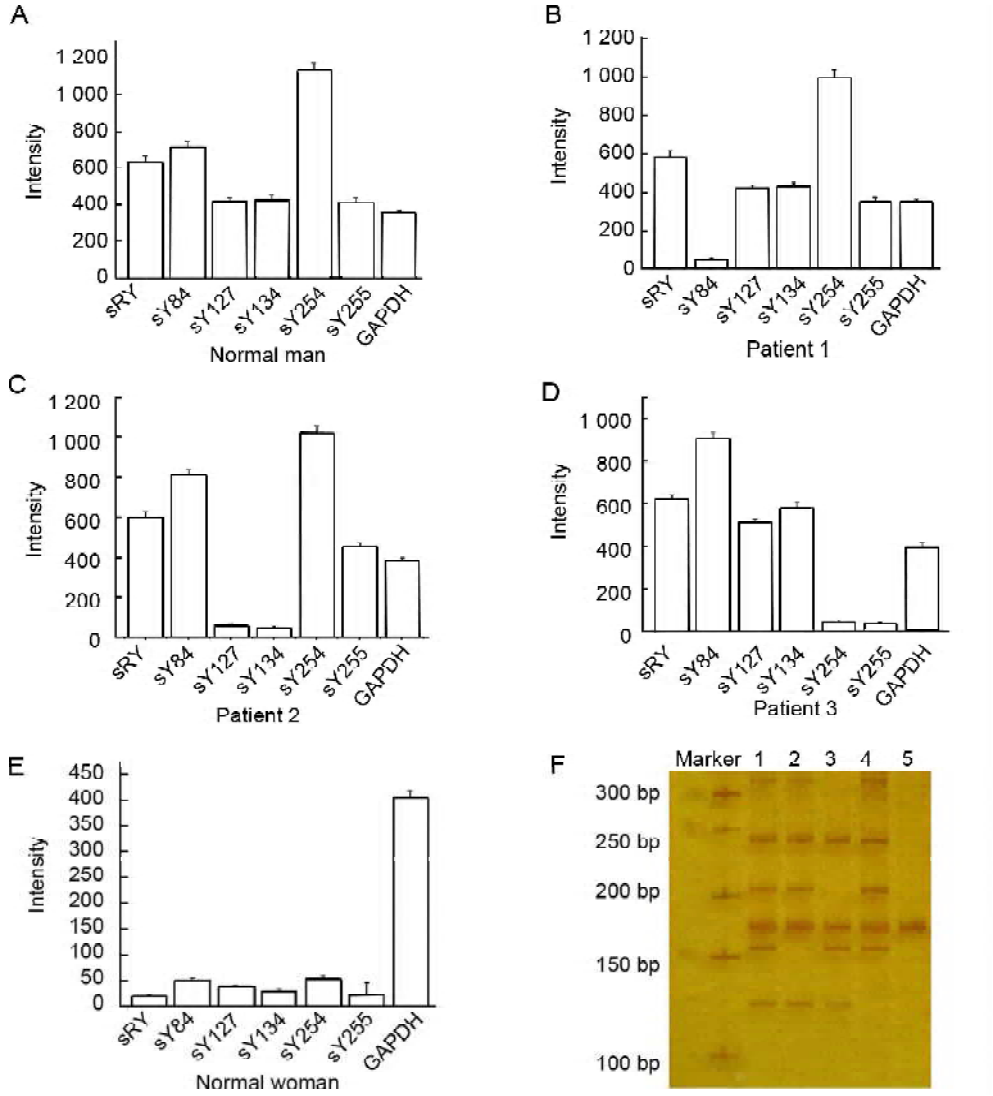

Figure 3. Some samples detected by the multi-analyte suspension array and results of multiplex polymerase chain reaction (PCR) by polyacrylamide gel electrophoresis (PAGE). It is consisted of Figure 3A–F. (A): DNA from normal man; (B): DNA from patient with sY84 microdeletion; (C): DNA from patient with sY127 and sY134 microdeletions; (D): DNA from patient with sY254 and sY255 microdeletions; (E): DNA from normal woman; (F): Image of polyacrylamide gel electrophoresis. Lane marker: 50 bp ladder; lane 1: normal man; lane 2: patient with sY84 microdeletion; lane 3: patient with sY127 and sY134 microdeletions; lane 4: patient with sY254 and sY255 microdeletions; lane 5: normal woman.

polyacrylamide gel electrophoresis (PAGE). The results were coincident with those detected by MASA technology.

4 Discussion

Although multiplex PCR is the most frequently used method in the detection of Y chromosome microdeletions, many problems remain in its practical application such as the specificity, sensitivity and throughput of the method. In general, the multiplex PCR method has a maximum of 1–2 h of running time for the separation of multiple bands, especially when the sizes of PCR products are close to each other. In addition, analysis of multiplex PCR products on an agarose gel based on molecular size and intensity is sometimes complicated when there are non-specific products if the primers for each STS are not

correct. So the result is often dependent on the experience of the investigator. In contrast, there are some benefits of suspension array technology, including rapid data acquisition, excellent sensitivity and specificity and multiplexed analysis capability. The MASA system incorporates 5.6 μm polystyrene microspheres that are internally dyed with two spectrally distinct fluorochromes. Using precise amounts of each of these fluorochromes, an array is created consisting of 100 different microsphere sets with specific spectral addresses. Each microsphere set can possess a different reactant on its surface. Because microsphere sets can be distinguished by their spectral addresses, they can be combined, allowing up to 100 different analytes to be measured simultaneously in a single reaction vessel. Also, the MFI of at least 100 beads is computed for each bead set in the sample, which means that each sample is detected at least 100 times. Therefore, this MASA technology might give more accurate results than gel electrophoresis analysis because of the sequence-specific hybridizations with numerical values. Additional benefits of the assay were a significant decrease in labor and turnaround time, flexibility to allow testing of 1 to 96 reactions without increase in labor time or cost per isolate, and it was less technically demanding. The cost per well (per patient) in reagents and consumables (DNA isolation, PCR, array microspheres, plasticware, *etc.*) is no more than USD4. And the detection can be completed in less than 30 min. This compares favourably with other commercially available *AZF* assays by gel analysis.

The basic components of nucleic acid detection methods are assay chemistry and analysis platform. Characteristic genotyping technologies include both solid phase (gels, DNA chips, glass slide arrays) and homogeneous solution assay formats (mass spectrometry, capillary electrophoresis). As compared to planar microarrays, suspension arrays have the benefits of convenience, low cost, statistical superiority, faster hybridization kinetics and more flexibility in array preparation. MASA technology is being used in a variety of applications, such as single nucleotide polymorphism (SNP) genotyping, genetic disease screening, gene expression profiling, human leukocyte antigen (HLA) DNA typing and microbial detection [4, 8].

Since 1976, when Tiepolo *et al.* [9] recognized that deletion in the long arm of the Y chromosome is associated with spermatogenic failure, there have been numerous studies on the association of *AZF* microdeletions with male infertility. The spermatogenesis locus *AZF* in Yq11 has been mapped to three non-overlapping regions designated as *AZFa*, *AZFb*, and *AZFc*. Many genes on the Y chromosome have been identified. It is currently accepted that *AZFa* contains two genes (USP9Y and DBY), *AZFb* contains eight protein-coding genes (CDY2, EIF1AY, HSFY, PRY, RBMYL1, RPS4YS, SMCY and XKRY) and *AZFc* contains five such genes (BPY2, CDY1, CSPG4LY, DAZ and GOLGA2LY), which are all transcribed in testicular tissue and, therefore, are all candidate genes for some functions in human spermatogenesis [10]. *AZF* microdeletions are caused by intrachromosomal recombination events between large homologous repetitive sequence blocks [11], and *AZFc* microdeletions are now recognised as the most frequently known genetic lesion causing male infertility.

Y chromosome microdeletions have been of increasing interest to clinicians and scientists since ICSI was introduced to be the main treatment option for severe male factor infertility. The frequency of deletions was reported to be in the range of 0.7% to 34.5%, with an average frequency of 8.2% [1, 2]. In the present study, the frequency of *AZF* microdeletions was 11.5% (40/312) in infertile patients with azoospermia or oligozoospermia, which is little higher than the average value. Our data also revealed that there was a high prevalence rate of *AZF* microdeletion in severe oligozoospermic patients (8.2%) as well as in azoospermic patients (14%), which is not in agreement with some studies considering the low prevalence of *AZF* microdeletion in severe oligozoospermia [12–15]. These differences might be explained by selection criteria of the patients, methodological aspects, population/ethnic variances, particular Y chromosome haplotypes, genetic background and environmental influences. The microdeletions reported by other investigators in China [6] and other countries such as Turkey, France and Holland vary from our observations, because China is a country with a lot of minority groups. The discrepancy could also be related to the selection of patient groups with varied clinical criteria and the set of markers used.

In the present study, microdeletions in the *AZFc* region were the most prevalent (52.8%), followed by the *AZFa* (19.4%), *AZFb* (16.7%) and *AZFb/AZFc* combination (11.1%). However, the frequency of *AZFa* deletions was higher than other reports [16–18] and *AZFa* deletions were detected both in the patients with azoospermia and oligozoospermia. Although deletions occurring

in *AZFa* are mostly associated with Sertoli cell syndrome [19], oligozoospermia in our patients with *AZFa* deletions was not surprising. Although it has been reported that only complete *AZFa* deletion is associated with the absence of spermatozoa, there have been cases of spermatozoa retrieval with partial *AZFa* deletions [2, 19]. Furthermore, sY84 has been previously considered as a polymorphic locus of the Y chromosome that is not associated with sterility phenotypes in men [20]. More recently, the presence of sY84 false-positive results has been related to an alteration in a PCR primer of this STS marker [21]. In the research of Buch *et al.* [22], there was a patient, previously diagnosed in another laboratory as *AZFa* microdeleted (absence of *sY84* marker), who was checked using their real time PCR protocal. No microdeletions were observed in this patient using selected markers from the *AZFa* locus (sY81, sY82 and sY182). Increasing STS density with two additional *AZFa* STS markers, flanking *sY84* (DYS388 and sY745), failed to identify any *AZFa* locus alteration in the patient. So, in the beginning of our study, we planned to employ sY86 as the second STS marker for *AZFa* evaluation. Unfortunately, we did not find any probes specific to sY86, considering the conditions of multiplex PCR with another six STS primers. In addition, the results of the MASA could be confirmed by classic PCR analysis and polyacrylamide gel electrophoresis, including sY86. Of the seven samples with sY84 microdeletion detected by MASA, all were sY84 microdeletions and four of them were sY86 detected by PAGE.

To make the MASA more useful, we will optimize the STS marker and employ other STS markers that could help to detect false positives related to the sY84 marker.

In the present study, the deletion of *AZFc* was the most common *AZF* microdeletion in patients with azoospermia and oligozoospermia. The reason why the deletion of *AZFc* is more frequent is still not clear. One possible explanation is a repetitive sequence of the genes. Some candidate genes, like DAZ, are known to be repetitive on the Y chromosome. Infertility may be caused by the loss of the repetitive DAZ gene clusters [23, 24].

There are some familiarities between the research of Yeoma *et al.* [25] and our paper. Both studies used the same technology to detect *AZF* microdeletions. But there are still differences between their paper and ours. First, we employed different fluorochrome-labeling methods for the multiplex bead array system. In their paper, they used Cy3-labeled reverse primers to perform the PCR reaction. Instead, we employed the conventional streptavidin-R-phycoerythrin method. Although the Cy3 method can reduce the number of experimental steps, the conventional streptavidin–R-phycoerythrin method is five times higher in fluorescence intensity than Cy3 fluorescence intensity. Streptavidin-R-phycoerythrin is also more stable than Cy3. Also, we aimed to establish a high throughput sensitive method for detecting the deletion of the Y chromosome that is specific for Chinese infertile men. So we selected different STS markers and employed different probes based on the characteristic of the Chinese population. Although our methods are similar, the parameters of reaction are different owing to different primers and probes.

In conclusion, we developed a novel method to scan for *AZF* region microdeletions on the Y chromosome. Further, our data, based on a Chinese population, add to the evidence that there is a cause and effect relationship between Y chromosome microdeletions and azoospermia or oligozoospermia. It is suggested that the screening of Y chromosome microdeletion should be conducted in infertile men with azoospermia and oligozoospermia before ICSI/IVF.

Acknowledgment

This work was supported by the funds of Population and Family Planning Commission of Chongqing Municipality, China (No. 2001-06).

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Edited by Dr Ralf Henkel