Investigation of *AZF* microdeletions in patients with Klinefelter syndrome

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ABSTRACT. We investigated azoospermia region microdeletions in male infertility patients with Klinefelter syndrome (KFS), as well as the association between azoospermia symptoms in patients with KFS and Y chromosome microdeletion polymorphisms. A total of 111 cases with male infertility confirmed to have KFS (47, XXY) and 94 fertile men were included in this study. Peripheral blood was drawn and DNA was extracted from these samples. Multiplex polymerase chain reaction was performed to screen the partial deletions of 25 sequence-tagged sites on the Y chromosome. In 111 cases with KFS, 1 case contained the *AZFb+d+c* deletion. The Gr/Gr deletion was identified in 12 KFS cases and 5 control cases. In addition, the b2/b3 deletion was identified in 13 KFS cases and 6 control cases. There were no significant differences in phenotype and genotype of the 2 partial *AZFc* deletions between patients and controls ($P > 0.05$). Our results suggest that patients with KFS may also have Y chromosome microdeletions to varying degrees

and that the gr/gr deletion and b2/b3 deletion may not play a role in the susceptible genetic background of azoospermia in patients with KFS in the Sichuan population.

Key words: *AZF* microdeletions; Klinefelter syndrome; Male infertility

INTRODUCTION

Infertility, which is defined as the inability to conceive an offspring, affects approximately 12-15% of all couples attempting to generate pregnancy worldwide. Approximately 30-40% of infertility cases can be attributed to male factors (Tüttelmann et al., 2007). Around 30% of male infertility cases may result from failed spermatogenesis caused by genetic alterations (McElreavey et al., 2006), chromosome abnormalities, and Y chromosome microdeletions, and are considered to be the most common causes of male infertility (Stouffs et al., 2008). Klinefelter syndrome (KFS) is the most common abnormal chromosome karyotype in male infertility, with a morbidity of approximately 1 in 500 newborn males (Tateno et al., 1999). Men with KFS typically have a 47, XXY chromosome karyotype; 15% are classified as mosaic, have a 46, XY/47, XXY chromosomal karyotype, and are characterized by azoospermia, seminiferous tubular dysgenesis, and elevated serum gonadotrophin concentrations (Kamischke et al., 2003).

Studies have shown that the azoospermia factor (*AZF*) locus on Yq11.23 is recurrently deleted in infertile males (Skaletsky et al., 2003). *AZF* can be further subdivided into 3 regions, including *AZFa*, *AZFb*, and *AZFc*. Each region contains several candidate genes involved in different stages of spermiogenesis, and deletions or mutations in these genes may cause spermatogenesis disorder, resulting in oligozoospermia or azoospermia (Pandey et al., 2010). In addition, some scholars identified a region known as *AZFd* between *AZFb* and *AZFc* (Kent-First et al., 1999).

Previous studies have examined Y chromosome microdeletions in patients with KFS, but the samples they examined or the sequence-tagged sites (STS) they selected were limited, and no statistical analysis was conducted for different *AZF* deletion types between case and control groups. The Y chromosome microdeletion polymorphism has not been shown to be a pathogenic factor of KFS or related to azoospermia symptoms in patients with KFS. We selected 25 STSs using larger case and control groups to investigate whether a relationship exists between different types of *AZF* deletions and azoospermia of KFS patients.

MATERIAL AND METHODS

Subjects

A total of 94 normospermic controls and 111 azoospermia patients whose chromosome karyotypes were confirmed as KFS (47, XXY) were included in this study. All subjects and controls were from Sichuan Province. Informed consent was obtained from all participants.

DNA extraction

Genomic DNA was extracted from peripheral venous blood using the Ezup Column Blood Genomic DNA Purification Kit (Sangon, Shanghai, China) according to the manufacturer

recommendations. Briefly, red blood cells were lysed and white blood cells pelleted, followed by white blood cell lysis. Proteinase K was added to remove proteins. DNA was then precipitated with 100% alcohol, washed, and re-suspended in Tris-ethylenediaminetetraacetic acid. DNA concentration was measured by spectrophotometric analysis at 260 nm. All DNA samples were preserved at -20°C.

STSs deletion detection

To screen for microdeletions in the *AZF* region of the Y chromosome, multiplex polymerase chain reaction (PCR) was performed to amplify 24 STSs in the *AZF* region; the sex-determining region of Y (SRY, sY14) was included as an internal control. All STSs selected are listed in Table 1. Twenty-five pairs of primers were used (**Table S1**). In addition, DNA from fertile male and female subjects were used as positive and negative controls, respectively. A blank control was also included in each reaction.

Table 1. STSs involved in this experiment.

	SRY	*AZFa*	*AZFb*	*AZFc*	*AZFd*
STS	SY14	SY84 SY86	SY121 SY124 SY127 SY133 SY134	SY142 SY143 SY157 SY242 SY254 SY255 SY277 SY1161 SY1191 SY1197 SY1201 SY1206 SY1258 SY1291	SY145 SY152 SY153

The PCR conditions were as follows: initial denaturation at 94°C for 5 min, 35 cycles for 30 s at 94°C, 30 s at 59°C, and 30 s at 72°C, with final extension at 72°C for 10 min. The PCR products were separated by electrophoresis on a 2% agarose gel stained with Dured and visualized using ultraviolet illumination. Failure of amplification for a given STS was confirmed 3 times by single PCR with an internal control and positive control.

Statistical analysis

The data were statistically analyzed using SPSS 13.0 (SPSS, Inc.; Chicago, IL, USA). P values, odds ratios (ORs), and 95% confidence intervals (95%CIs) were calculated; $P < 0.05$ was considered to be statistically significant.

RESULTS

In 111 KFS men studied, 28 (25.2%) showed the *AZF* deletion, 1 had discontinuous deletions of 13 STSs in *AZFb+d+c*, and the other 27 cases were partial *AZFc* deletions. In addition, 11 of the 94 (11.7%) controls showed partial *AZFc* deletions. The extent of the deletions is shown schematically in Table 2 and Figure 1.

Table 2. Y chromosome microdeletions data (N-Normal, K-KFS).

SY1161	SY1191	SY1291	SY157	*AZFb+c+d*
K62	N02, N37, N42, N53, N55, N68, K07, K16, K20, K28, K31, K61, K64, K68, K74, K76, K88, K95, K106	N39, N50, N61, N65, N75, K11, K22, K43, K49, K52, K56, K57, K77, K82, K83, K86, K87	K81	K04

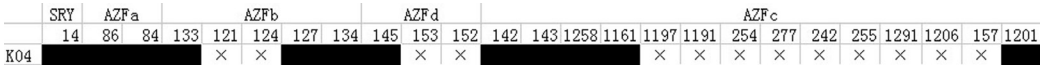

Figure 1. Y chromosome microdeletions data of the case K04 (x means this site deletion) with schematic diagram of *AZF* illustrating STS.

Classical *AZF* microdeletions

In the clinical diagnosis of Y chromosome microdeletions, according to the European Academy of Andrology/European Molecular Genetics Quality Network guidelines, 6 sites were selected for preliminary screening of *AZF* deficiency, including SY84/SY86 (*AZFa*), SY127/SY134 (*AZFb*), and SY254/SY255 (*AZFc*) (Simoni et al., 2004). In our study, we identified 1 case of KFS with deletions of SY254/SY255 (*AZFc*). No deletions of the 6 STSs were detected in the control group.

In addition to the 6 classical sites, we selected 3 STSs on *AZFb* (SY133, SY121, SY124), 3 STSs on *AZFd* (SY145, SY153, SY152), and 3 STSs on *AZFc* (SY277, SY242, SY157). Among these sites, the case with the SY254/SY255 (*AZFc*) deletion had deletions of SY121/SY124 (*AZFb*), SY153/SY152 (*AZFd*), and SY277/SY242/SY157 (*AZFc*) (Figure 1).

Partial *AZFc* deletions

Nine STSs on *AZFc* (SY142, SY143, SY1258, SY1161, SY1197, SY1191, SY1291, SY1206, SY1201) were screened for partial deletions of the *AZFc* region; the structure of *AZFc* is shown in Figure 2. The labels b1, b2, b3, and b4 represent *PRY2*; g1, g2, and g3 represent *BRY2*; r1, r2, r3, and r4 represent *DAZ*; and y1 and y2 represent *CDY*. Different *AZFc* partial deletions illustrate various losses of gene fragments.

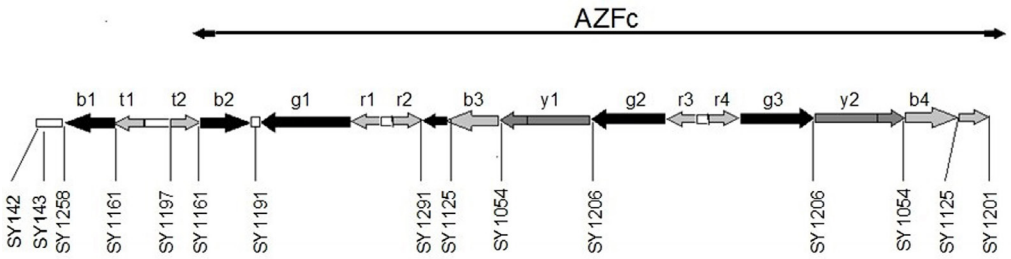

Figure 2. STSs and multicopy genes on *AZFc*.

Deletion of SY1291 indicated gr/gr deletions, except for classical microdeletions (K04), which were detected in 12 cases with KFS and 5 control subjects ($P > 0.05$); there was no statistically significant difference between the 2 groups. In addition, deletion of SY1191 indicated b2/b3 deletions, and except for case K04, this deletion was detected in 13 cases with KFS and 6 controls ($P > 0.05$); no statistically significant difference was observed between the

Table 3. Deletion of gr/gr and b2/b3.

	gr/gr	b2/b3
KFS cases	12 (10.9%)	13 (11.8%)
Controls	5 (5.3%)	6 (6.4%)
P value	P > 0.05	P > 0.05
Odds ratio	2.225	1.966
95%CI	(0.754-6.568)	(0.716-5.394)

2 groups. The detailed results are shown in Table 3.

DISCUSSION

The *AZF* region is important for male fertility because it contains genes required for spermatogenesis. Genes on *AZFa* are primarily involved in the proliferation of spermatocytes (Luddi et al., 2009). *DBY* is a candidate gene of *AZFa*, and loss of *DBY* can lead to a dramatic decrease in or even a complete lack of spermatogenic cells. The complete loss of *AZFa* usually manifests as Sertoli cell syndrome, the main feature of which is azoospermia. Previous studies have shown that the deletion of *AZFa* is relatively rare, accounting for approximately 1-5% of Y chromosome microdeletions (Vogh et al., 1996). No *AZFa* deletion was detected in this study.

Patients with *AZFb* deletions show spermatogenesis arrest during the spermatocyte stage; therefore, spermatogonia and primary spermatocytes are still visible in the testicle, but no sperm are observed. The main candidate gene of *AZFb* may be *RBMY*, the absence of which causes blockage of spermatogenic cells in the primary spermatocyte stage. Patients with *AZFb* deletion also show azoospermia (Simoni et al., 2004).

Few studies have been conducted to examine *AZFd*, and whether *AZFd* exists is controversial; no candidate gene has been identified in *AZFd*. Clinical symptoms of *AZFd* deletion are less clear than those of *AZFa/b/c* deletion, and patients with *AZFd* deletion typically show mild oligozoospermia or teratospermia (Maurer et al., 2001).

Deletions of *AZFc* are the most common deletions in clinical practice, which is an important cause of spermatogenic failure (Hopps et al., 2003). *DAZ* is known as the azoospermia deletion gene, the main candidate gene of which is *AZFc*. Although *DAZ* is not absolutely necessary for sperm formation, but the absence of *DAZ* is observed in approximately 10-15% of azoospermia patients and is the most frequent genetic factor in male infertility. In general, a proportion of patients with deletions of the *AZFc* region may still have sperm in the ejaculate and sperm within the testis on diagnostic biopsy or upon testicular sperm extraction; very few cases have shown that deletions of *AZFc* can be inherited (Pandey et al., 2010).

Deletion of the *AZFc* region can be divided into complete deletion of the *AZFc* region (b2/b4 deletion) and partial deletion. Partial deletions of the *AZFc* region have been reported to be a significant risk factor for male infertility. gr/gr deletion is the most common type of partial *AZFc* deletion; the length of the deleted DNA is 1.6 Mb, accounting for half of the *AZFc* gene content and involving 23 important genes, which may explain reduced sperm production (Repping et al., 2004). There are 3 types of gr/gr deletions: the g1/g2 deletion involving a missing a *DAZ* copy (*DAZ1/DAZ2*), whereas the r1/r3 and r2/r4 deletions involve *DAZ1/DAZ2* deletions, even more sophisticated copy deletions, and all 3 types contains deletion of SY1291. According to previous studies, the rate of gr/gr deletion may differ among population groups (Machev, 2004; de Carvalho et al., 2006; Wu et al., 2007; Shahid et al., 2011). The association between the gr/gr deletion and male infertility is not well understood. Machev et

al. (2004) found that in France, the deletion rate of gr/gr was 6% in male infertility patients and was 3.5% in controls, but no significant difference between groups was observed ($P > 0.05$). Chinese (Wu et al., 2007) and Japanese (de Carvalho et al., 2006) researchers reached the same conclusion, while studies in India revealed that the gr/gr deletion was a risk factor for spermatogenic failure (Shahid et al., 2011).

b2/b3 deletion is another common type of partial *AZFc* deletion; the length of the deleted DNA is 1.8 Mb, and the deleted DNA includes 12 testis-specific expression genes and transcripts. Deletion of SY1191 shows the b2/b3 deletion (Repping et al., 2004). The results of studies examining the association between b2/b3 deletion and male infertility also differ. Lynch et al. (2005) found no association between the b2/b3 deletion and spermatogenesis dysfunction among populations from Holland, Australia, and the USA, but a study of a Chinese population showed that the probabilities of b2/b3 deletions in infertile men and a normal control group were 8.9 and 3.2%, respectively, and there was a significant correlation between male infertility and congenital infertility in this population (Wu et al., 2007).

The proportion of KFS in patients with sex chromosome abnormalities is 10.0-38.5%, accounting for a considerable proportion in azoospermia patients. The mechanism of spermatogenesis disorder of KFS may result from an extra X chromosome, which affects testicular development, Leydig cell insufficiency, and regulation of apoptosis of Sertoli and Leydig cells (Visootsak and Graham, 2006). Among previous studies on KFS patients with Y chromosome microdeletions, some studies failed to identify microdeletions (Tateno et al., 1999; Lee et al., 2000; Choe et al., 2007). Other studies reported Y chromosome microdeletions in patients with KFS; Mitra et al. (2006) found that 4 of 14 KFS patients (18 STSs) showed microdeletions, including 3 cases with the *AZFa* deletion and 1 with the *AZFb* deletion. Ceylan et al. (2010) found that 8 of 14 Turkish KFS patients (6 STSs) had *AZFc* deletions, while no deletion was detected in the control group. Hadjkacem-Loukil et al. (2009) found that 6 of 9 KFS patients (19 STSs) showed *AZF* deletions, including a gr/gr deletion, indicating that partial deletion of *AZFc* may not play a role in susceptibility to KFS.

We examined Y chromosome microdeletions in KFS patients using 25 sets of primers and detected 28 cases with the Y chromosome microdeletion in 111 KFS patients and 1 case with discontinuous deletion of *AZFb+d+c*. Moreover, 13 cases had the b2/b3 deletion, 12 cases had the gr/gr deletion, 1 case had the SY1161 deletion, and 1 case had the SY157 deletion. These 27 cases include partial *AZFc* deletions. Six cases were found to have b2/b3 deletions and 5 cases had the gr/gr deletion in the control group, but there was no statistical difference between the 2 groups ($P > 0.05$). This result indicates that the gr/gr and b2/b3 deletions are not correlated with KFS in the Sichuan population. However, because the frequency distribution of b2/b3 and gr/gr deletions varies in different countries and populations, studies in other regions may show different results. In conclusion, based on previous reports and our study, Y chromosome microdeletions may not indicate a predisposed genetic background for KFS.

KFS patients are often classified as having azoospermia, but there are reports stating that some KFS patients can produce normal sperm (Gonsalves et al., 2005). In contrast, some patients may have no sperm in the semen, but a few sperm exit in the testis (Lanfranco et al., 2004). The ability to extract spermatozoa from the testes of patients with non-obstructive azoospermia followed by intracytoplasmic sperm injection offers an efficacious therapeutic approach for these patients to produce their own offspring. The possibility of using intracytoplasmic sperm injection with testicular spermatozoa has also been proposed in KFS

patients (Denschlag et al., 2004). Thus, KFS patients may also harbor Y microdeletions, and screening for deletions may be imperative for diagnostic work-up, particularly in subjects considering assisted reproductive techniques.

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Supplementary material

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