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Male infertility in Northeast China: molecular detection of Y chromosome microdeletions in azoospermic patients with Klinefelter's syndrome

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ABSTRACT. The prevalence of microdeletions of azoospermia factor (AZF) among azoospermic Klinefelter's syndrome (KFS) patients shows conflicting data. We aimed to detect this frequency in a Northeast Chinese population, and to investigate the possible association between AZF microdeletions and KFS by comparison with previous conflicting reports. Eighty men affected with KFS and a random healthy control group comprising 60 fertile men and women were recruited. AZF microdeletions were detected by multiplex polymerase chain reaction using 9 specific sequence-tagged sites. Karyotype analyses were performed on peripheral blood lymphocytes using standard G-banding. Finally, azoospermia was confirmed in 77 men affected with KFS and no AZF microdeletions were found. Karyotype analysis revealed 1 patient with karyotype 47,XXY,inv (9) (p11, q13), and 2 with mosaic karyotypes (46,XX/47,XXY and 46,XY/47,XXY). All other patients had karyotype 47,XXY. Review of the literature showed that these results

were similar to those of other regions of Northeast Asia, but differed from those obtained from Caucasian populations. Our results supported the proposal that AZF microdeletions and KFS result from separate genetic defects. The prevalence of AZF in azoospermic KFS patients varies among populations, and it might result from genetic drift or selective pressure. These results suggest that routine screening for classical AZF microdeletions among infertile azoospermic men with a 47,XXY karyotype might not be necessary in Northeast Chinese individuals. However, it remains imperative for patients considering assisted reproductive treatments, particularly for those with mosaic karyotypes.

Key words: Y chromosome; AZF microdeletion; Azoospermia; Klinefelter's syndrome

INTRODUCTION

The Y chromosome contains several spermatogenesis-related genes and plays an important role in male germ cell development (Li et al., 2008; Behulova et al., 2011). Microdeletions of azoospermia factor (AZF) loci on the long arm of the Y chromosome are a major cause of male infertility in populations throughout the world (Mitra et al., 2006; Li et al., 2008; Wang et al., 2010). As early as 1976, Tiepolo and Zuffardi followed by other authors postulated that genes and gene families located on locus 11 of the Y chromosome long arm (Yq11) were associated with male germ cell development (Ambasudhan et al., 2003; Wang et al., 2010; Behulova et al., 2011). It was later found that microdeletions of four subregions of the AZF region (AZFa, AZFb, AZFc, and AZFd) led to various types of spermatogenesis impairments, from teratozoospermia to infertility, with different population distributions depending on their Y haplogroup profile (Li et al., 2008; Wang et al., 2010). For example, microdeletions in AZFa led mostly to Sertoli cell-only syndrome, mutations in AZFb resulted in an arrest of spermatogenesis at meiosis I, and mutations in AZFc were associated to hypospermatogenesis with progression to severe azoospermia or oligospermia (Ferrás et al., 2004; Foresta et al., 2005; Zhou et al., 2006; Arruda et al., 2007; Wang et al., 2010; Behulova et al., 2011).

Klinefelter's syndrome (KFS) is the most frequent chromosomal anomaly observed among infertile men (Mitra et al., 2006; Choe et al., 2007; Wang et al., 2010; Behulova et al., 2011; Zhang et al., 2012). Although most men with KFS have the 47,XXY karyotype, some show the mosaic karyotype (KFM) in their somatic and germ cells (Mitra et al., 2006; Choe et al., 2007). Testicular failure, elevated level of serum gonadotropins, and infertility are some of the most important characteristics observed in these patients.

KFS has been shown to be the main factor of spermatogenic failure, and its prevalence has since been associated with microdeletions in Y chromosomal AZF regions (Ceylan et al., 2010); however, the role of KFS in the pathogenesis of spermatogenesis remains unclear (Amory et al., 2000; Choe et al., 2007; Behulova et al., 2011). Therefore, the association between KFS and AZF microdeletions has attracted the attention of many researchers.

To date, conflicting results have been reported in regards to the prevalence of AZF microdeletions in KFS azoospermic patients. Some studies have shown an increased prevalence of classic AZF microdeletions in azoopspermic KFS patients, and suggested that partial AZFc

microdeletions might not play a role in laying the genetic background for KFS azoospermic phenotypes (Mitra et al., 2006; Hadjkacem-Loukil et al., 2009; Ceylan et al., 2010). However, several studies have found no association between these two genetic disorders (Tateno et al., 1999; Lee et al., 2000; Ambasudhan et al., 2003; Choe et al., 2007; Balkan et al., 2008; Behulova et al., 2011).

The aim of the present study was to detect the prevalence of AZF microdeletions in azoospermic patients with KFS in a Northeast Chinese population from Changchun. In comparing these results with previously published analyses, we investigated the possible association between AZF microdeletions and KFS.

MATERIAL AND METHODS

Patients

All primary infertile male patients (N = 4952) who sought help from our center at the First Hospital of Jilin University from September 2006 to October 2011 were considered for inclusion in this study. To rule out other anatomical causes of infertility, detailed medical histories, reproductive problems, occupation, and family backgrounds were collected on all patients along with their physical examination. Semen samples were obtained after a 7-day period of ejaculatory abstinence, and all analyses were performed according to World Health Organization (WHO, 1999) guidelines. Semen and urethral fluid were also collected for microbial infection testing. Diagnosis of azoospermia was only assigned following at least two semen analyses per patient. Blood samples were stored for further molecular studies and cytogenetic analysis. Patients with oligozoospermia were excluded from this study.

Finally, 80 males with KFS determined according to cytogenetic results were recruited for the study. A healthy control group comprised of 60 fertile unrelated men and women with normal karyotypes were included and underwent the same examinations and analyses as the men in the infertile group. Every male in the control group had fathered at least 1 child.

Molecular analysis

DNA isolation

Genomic DNA was isolated with the Tiangen blood DNA extraction mini kit (Tiangen Biotech Co., Ltd., Beijing, China) using peripheral blood lymphocytes according to the manufacturer protocol.

Selection of primers

Multiplex polymerase chain reaction (PCR) was used for all AZF microdeletion screenings. A series of 9 specific sequence-tagged sites (STSs) were selected for molecular genetic determination of microdeletions as follows: AZFa: SY84 and SY86; AZFb: SY127, SY134, and SY143; AZFc: SY157, SY254, and SY255; AZFd: SY152 (Wang et al., 2010). Human zinc-finger protein-encoding genes (*ZFX/ZFY*) located on the X and Y chromosomes were selected as internal control primers (SaoPedro et al., 2003; Arruda et al., 2007; Wang et al., 2010).

Multiplex PCR amplification was performed in a 10-μL reaction containing 200 ng genomic DNA, 1.5 mM Mg^{2+}, 800 μM deoxyribonucleotide triphosphates, 10 pM of each primer, and 2 U Taq polymerase. Thermocycling (Veriti Thermal Cycler 96-well, Alpha-SE, Applied Biosystems, USA) consisted of an initial denaturation step of 6 min at 94°C, followed by 35 cycles of 40 s at 94°C, 45 s at 55°C, and 60 s at 72°C, with a final extension step at 72°C for 6 min. Positive and negative controls of the 60 fertile men and women were used to ensure correct performance of the amplification reaction. Blank controls were also included to monitor sample contamination during the procedure. Finally, all PCR products were stored at 4°C before electrophoretic detection. Eight-microliter PCR products were then mixed with 1-2 μL 6X loading buffers and separated on 1.5% agarose gel (LP0028A, OXOID, UK) containing 0.5 μg/mL ethidium bromide at 100 V for 30 min. All samples with STS deletions were retested as described above for confirmation.

Chromosome analysis

G-banding was performed using cultured peripheral blood lymphocytes. After a 72-h incubation period, lymphocytes were cultured in RPMI-1640 (GIBCO, Invitrogen, USA), phytohaemagglutinin (Yihua Medical Technology, Shanghai, China), and fetal bovine serum (Dingguo Biotechnology, Beijing, China), followed by treatment with colcemid. G-banding of chromosomes in metaphase was then performed. At least 20 metaphases were analyzed per patient. Chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature (Mitelman et al., 1995; Zhang et al., 2012).

RESULTS

KFS was found in 80 patients. Seventy-five (87.5%) patients showed the 47,XXY karyotype, which was confirmed to be azoospermic by semen analysis. Three patients (3.75%) had mosaic karyotypes (Table 1), 1 had severe oligoazoospermia (46,XY/47,XXY), and the other 2 were rare variants of KFS with azoospermia carrying a 46,XX/47,XXY karyotype and an autosomal inversion 47,XXY,inv(9)(p11,q13).

Table 1. Distribution of karyotypes and semen analysis in infertile patients with Klinefelter's syndrome (KFS) in Northeast China.

Karyotypes	No.	KFS patients	
		Semen analysis	Number of microdeletions
47,XXY	75	Azoospermia	0
47,XXY	1	Severe oligozoospermia	0
47,XXY	1	Oligoasthenozoospermia	0
46,XY/47,XXY	1	Severe oligozoospermia	0
46,XX/47,XXY	1	Azoospermia	0
47,XXY,inv(9)(p11,q13)	1	Azoospermia	0

Seventy-seven KFS patients were confirmed to have azoospermia. Screening for Y chromosome microdeletions was performed on all of these patients using 9 specific STSs spanning AZFa, AZFb, AZFc, and AZFd. Interestingly, no patient showed Y chromosome AZF microdeletions (Table 1). As expected, all individuals in the fertile control group had normal karyotypes and no AZF microdeletions.

Table 2 shows a comparison of AZF microdeletion frequencies in azoopsermic KFS patients between this study and previous studies that used the same inclusion criteria. Consistent with results of the present study, none of the patients in northeast Asia (Japan, Korea and this study) carrying the same phenotype showed microdeletions.

Table 2. Comparison of the azoospermia factor (AZF) microdeletion frequency in azoospermic patients with Klinefelter's syndrome in this study and other studies.

Authors	Country	No. of loci tested	No.of patients	No. of patients with microdeletions	Frequency of AZF microdeletion	Deleted subregion
Hadjkacem-Loukil et al., 2009	Tunisia	Not known	9	6	67%***	AZFa AZFb AZFc AZFa+b+c gr/gr deletion
Ceylan et al., 2010	Turkey	7	14	5	35.71%***	AZFc
Balkan et al., 2008	Southeast Turkey	15	7	0	-	-
Behulova et al., 2011	Slovakia	6	12	0	0	-
Peterlin et al., 2002	Slovenia	48	5	1	20%	AZFc
Mitra et al., 2006	Northwest India	18	14	4	28.57%***	AZFa+b
Ambasudhan et al., 2003	Northeast India	35	8	0	0	-
Tateno et al., 1999	Japan	32	20	0	0	-
Lee et al., 2000	Korea	60	6	0	0	-
Choe et al., 2007	Korea	5	95	0	0	-
This study	North China	9	77	0	0	-

***P < 0.0001 (Fisher exact test between Northeast China and other studies).

Finally, Table 3 compares the number of STSs used to determine AZF deletions across studies. Although most studies used more STSs compared to the present study, increasing the number of primer sets used is not likely to increase the chances of finding microdeletions.

Table 3. Specific sequence-tagged sites (STSs) used in different studies for detecting azoospermia factor (AZF) microdeletion frequency in azoospermic patients with Klinefelter's syndrome.

STS	AZFa		AZFb			AZFc			AZFd	Other STSs used	Fresuency of AZF microdeletion
	SY84	SY86	SY127	SY134	SY143	SY157	SY254	SY255	SY152		
This study	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	0
Behulova et al., 2011	Y	Y	Y	Y	N	N	Y	Y	N	N	0
Mitra et al., 2006	Y	Y	Y	Y	Y	Y	Y	Y	N	sY746, sY153, sY148, sY158, sY160, sY118, sY113	28.57%
Choe et al., 2007	Y	N	N	Y	N	N	Y	Y	N	sY129	0
Tateno et al., 1999	N	N	N	N	N	N	Y	Y	N	28 other STSs	0
Lee et al., 2000	N	Y	N	Y	Y	Y	Y	N	Y	54 other STSs	0
Balkan et al., 2008	Y	N	Y	N	N	N	Y	Y	Y	sY81, sY82, sY142, sY164, sY257, sY145, sY153	0

Y = used; N = not used.

DISCUSSION

In the last decade, screening of AZF microdeletions in infertile men has become routine practice in both clinical and research study as deletions on the long arm of the Y chromo-

some are generally assumed to be responsible for defects in spermatogenesis. Microdeletions of the Y chromosome are the second most common cause of spermatogenic failure after KFS (Ceylan et al., 2010; Behulova et al., 2011). However, the frequency of AZF microdeletions in azoospermic males varies considerably across studies and populations (3-55%) (Foresta et al., 1998; Ambasudhan et al., 2003; Vogt et al., 2004; Behulova et al., 2011). Such a wide range of variation is likely not only due to the effects of geography, ethnicity, and environmental factors, but also to the choice of inclusion criteria and the number of STSs used in different laboratories (Ambasudhan et al., 2003; Mitra et al., 2006).

It is not well understood why chromosomal anomalies lead to impaired spermatogenesis (Amory et al., 2000; Choe et al., 2007; Behulova et al., 2011). Furthermore, the extra X chromosome seen among KFS patients has been shown to form a Barr body (a dense chromatin mass inside the nuclei of somatic cells), although the mechanism relating Barr body formation with testicular failure has not yet been elucidated. At present, it is generally accepted that the extra X chromosome observed in 97% of KFS cases results from sporadic chromosomal non-disjunction during parental gametogenesis (53% from sperm, 44% from egg), and that the remaining 3% of cases obtain the extra X chromosome as a result of postzygotic mitotic errors (Smyth and Bremner, 1998; Amory et al., 2000).

For the first time, this study analyzed the occurrence of AZF microdeletions in azoopsermic patients with KFS in Northeast China. There have been several conflicting reports about the association between KFS and AZF microdeletions (Ambasudhan et al., 2003; Ferrás et al., 2004; Foresta et al., 2005; Mitra et al., 2006; Arruda et al., 2007; Choe et al., 2007; Ceylan et al., 2010; Behulova et al., 2011). Accordingly, analysis and comparison with other studies of these associations is justified. In order to compare the results of this study with previous findings, the same inclusion criteria were utilized in this study as in other studies. Our comparison among similar studies (Table 2) revealed that no AZF microdeletions have been found among KFS patients with azoospermia in Northeast China (Changchun), and that although the number of patients enrolled in other studies was relatively small, most of the results were in agreement with our findings.

Based on this comparison, can we postulate that the occurrence of AZF microdeletions and KFS are not mutually causal? In general, patients with KFS and patients with AZF microdeletions are both commonly observed among infertile patients. The fact that Northeast Chinese/Asian KFS patients generally lack AZF microdeletions, as was shown in this study, might suggest that the occurrence of these two defects most likely represents two separate independent genetic events that can occur in the same individual by chance. This observation has also been illustrated in other studies (Lee et al., 2000; Plaseski et al., 2008; Behulova et al., 2011; Rajpert-De et al., 2011). Furthermore, it was proposed that Y chromosome deletions do not facilitate non-disjunction events in paternal sex chromosomes, and do not cause a gain of X chromosomes (Rajpert-De et al., 2011). This supports our proposal that Y chromosome deletions do not constitute a genetic factor linked to KFS.

As shown in Table 2, microdeletions have not been observed among azoospermic KFS patients from Northeast Asia, although they are commonly observed among west Eurasian, south Asian, and Caucasian populations. It is well accepted that genetic drift can cause significant variation in the distribution of genetic traits among populations around the world. Therefore, it is possible that the complete lack of microdeletions observed in our dataset is simply the result of drift. This type of population effect was recently shown to affect genetic

associations (Jin et al., 2003; Xue et al., 2006), indicating that Koreans appeared to be closely related to Manchurians, especially to the Harbin population and to Korean people living in the Changchun region where our data were collected. The genetic affinity or small genetic distance between these two population groups might explain the similar results obtained in this study with those of Lee et al. (2000) and Choe et al. (2007). Conversely, significant differences in results among studies are, in most cases, associated with the relatively large genetic distance between groups studied.

In addition, most AZF microdeletions found thus far have been observed in patients with KFM (Oliva et al., 1998; Oates et al., 2002; Peterlin et al., 2002; Mitra et al., 2006), but these findings remain debatable, as there is no evidence so far showing that KFM patients have a higher chance of also having an AZF microdeletion. In the present study, no microdeletion was detected in either of the two azoospermic patients with 46,XX/47,XXY and 47,XXY, inv(9)(p11, q13). However, these mosaic results must be interpreted with caution by clinical workers, and larger studies are necessary to shed new light on the association between AZF deletions and KFM in infertile patients.

Finally, despite debates about the sensitivity and specificity of multiplex PCR amplification, this method is generally accepted as the primary method of choice for AZF microdeletion detection. No consensus has yet been reached about the optimal number of STSs that should be used, or the specific loci that should be imperatively screened (Choe et al., 2007). Some authors have suggested that using more sets of primers increases the chances of detecting microdeletions (Thangaraj et al., 2003; Mitra et al., 2006). On the other hand, other studies have shown that the prevalence of microdeletions detected did not increase with increasing numbers of STSs used (Choe et al., 2007). In the present study (Table 3), we used 9 STSs, 6 of which were strongly recommended by the European Molecular Genetics Quality Network (EMQN) and the European Academy of Andrology (EAA). As shown in Table 3, although most studies have used more STSs, the same results were reached by all groups, with the exception of Mitra et al. (2006). Although a large number of STSs were used in Mitra et al.'s (2006) study, the STSs that determined deletions in their patients were obtained from sY86, sY746, sY113, sY118, sY127, sY84, sY746, and sY134, which could all be detected with the STS system that was used in the present study. These observations not only indicate that the use of more STSs is not an absolute requirement, but also that the absence of deletions in the Northeast Chinese patients was most likely associated with a population effect such genetic distance, genetic drift, selective pressure, linkage differences between loci, or isolation by distance.

CONCLUSIONS

AZF microdeletions were not found in azoospermic patients with Klinefelter's syndrome in a Northeast Chinese population. This study supports previous findings from Japan, Korea, and Northeast India, suggesting that Northeast Asian populations have a low incidence of AZF microdeletions among KFS patients. We propose that AZF microdeletions and KFS are separate genetic defects whose distribution profiles across different ethnic groups result from genetic drift, selective pressures, or other population effects. Larger studies including different populations and larger sample sizes will be needed to shed more light on the association between AZF deletions and KFM patients.

It was also shown that routine screening for classic AZF microdeletions in infertile azoospermic men with a 47,XXY karyotype might not be necessary in Northeast China. Nevertheless, we still recommend the determination of AZF microdeletions in patients considering assisted reproductive techniques, especially those with mosaic karyotypes.

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