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·*Original Article*·

Routine screening for classical azoospermia factor deletions of the Y chromosome in azoospermic patients with Klinefelter syndrome

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Abstract

Aim: To evaluate the occurrence of classical azoospermia factor *(AZF*) deletions of the Y chromosome as a routine examination in azoospermic subjects with Klinefelter syndrome (KS). **Methods:** Blood samples were collected from 95 azoospermic subjects with KS (91 subjects had a 47,XXY karyotype and four subjects had a mosaic 47,XXY/46, XY karyotype) and a control group of 93 fertile men. The values of testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) were measured. To determine the presence of Y chromosome microdeletions, polymerase chain reaction (PCR) of five sequence-tagged site primers (sY84, sY129, sY134, sY254, sY255) spanning the *AZF* region, was performed on isolated genomic DNA. **Results:** Y chromosome microdeletions were not found in any of the 95 azoospermic subjects with KS. In addition, using similar conditions of PCR, no microdeletions were observed in the 93 fertile men evaluated. The level of FSH in KS subjects was higher than that in fertile men (38.2 ± 10.3 mIU/mL *vs*. 5.4 ± 2.9 mIU/mL, $P < 0.001$) and the testosterone level was lower than that in the control group (1.7 ± 0.3 ng/mL *vs*. 4.3 ± 1.3 ng/mL, $P < 0.001$). **Conclusion:** Our data and review of the published literature suggest that classical *AZF* deletions might not play a role in predisposing genetic background for the phenotype of azoospermic KS subjects with a 47,XXY karyotype. In addition, routine screening for the classical *AZF* deletions might not be required for these subjects. Further studies including partial *AZFc* deletions (e.g. *gr/gr* or *b2/b3*) are necessary to establish other mechanism underlying severe spermatogenesis impairment in KS. *(Asian J Androl 2007 Nov; 9: 815–820)*

Keywords: Y chromosome; chromosome deletion; Klinefelter syndrome; azoospermia

1 Introduction

Infertility is a major health problem that affects approximately 15% of the population of reproductive age; a male factor can be identified in approximately half of these cases [1]. Moreover, a significant proportion of infertile males are affected by either oligospermia or azoospermia. These conditions have many causes, such as varicocele, infection, cryptorchidism, endocrinological disorders or obstruction/absence of a seminal pathway [2]. However, up to 66% of all infertile men have idiopathic azoospermia or severe oligospermia [3, 4].

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Because infertility is largely a result of impaired gametogenesis, in which a number of genes participate, it is reasonable to predict that deletions in spermatogenic genes result in impaired spermatogenesis, leading to infertility. Therefore, evaluation of microdeletions might be useful for identifying molecular defects of the Y chromosome.

Microdeletions of the Y chromosome represent the most frequent cause of male infertility, and are responsible for 10%–15% of cases of azoospermia or severe oligozoospermia. Such deletions have been localized to one or more loci referred to as azoospermia factors (*AZF*) *a*, *b* and *c*. These three non-overlapping regions are mapped within intervals five and six of the Y chromosome and lie within Yq11.21. to Yq11.23 [5]. Deletions have been shown to more frequently involve the deleted in azoospermia (*DAZ*) gene in *AZFc*, and are associated with both azoospermia and severe oligospermia; however, men with deletions involving the proximal regions (*AZFa* and *AZFb*) have also been shown to present with azoospermia or severe oligospermia [2, 6, 7]. Since 1995, based on the results of a large number of studies, Y chromosome microdeletion screening has become part of the routine diagnostic work-up for severe male factor infertility [8, 9]. The screening for Yq deletions has provided an etiology for spermatogenic disturbances, and has also provided a prognosis for testicular sperm retrieval based on the type of deletion. Assisted reproductive techniques have offered an efficient therapy for men bearing Y microdeletions; however, this genetic defect is then transmitted to male offspring in cases of successful reproduction.

Klinefelter syndrome (KS) is the most common sex-chromosome abnormality in men, and results in testicular failure, variable degrees of androgen deficiency and infertility. It affects approximately one in 500 newborn boys and accounts for up to 11% of azoospermic men [10]. KS results from an extra X chromosome in male karyotypes (47,XXY) or a combination of normal and extra X karyotypes (mosaic pattern, 47,XXY/46,XY) in somatic and germ cells. Most men with KS have a nonmosaic karyotype; only about 10% of men are mosaic.

KS is the most common chromosomal abnormality associated with male infertility and azoospermia. The mechanism by which the chromosome abnormality leads to the spermatogenic defect remains unknown. Studies aiming to define the predisposing genetic background for the KS phenotype have not been successful. Several investigators have hypothesized that Y chromosome deletions might affect the phenotypic expression of KS in regard to spermatogenesis. However, there have been very few studies on Y chromosome microdeletions in subjects with KS and the reports have been conflicting regarding the occurrence of microdeletions [11–15]. The aim of the present study was to evaluate the occurrence of classical *AZF* deletions of Y chromosome in azoospermic subjects with KS, and to determine whether routine screening for this examination would be useful in the clinical setting.

2 Materials and methods

2.1 Study population

From September 2002 to December 2005, male subjects with primary infertility attending the fertility clinic were enrolled in the present study. The study protocol was approved by the Institutional Review Board and informed consent was obtained from all subjects. Each subject provided a detailed family, occupational and reproductive history. A general physical examination with particular attention to the scrotal contents, including measurement of the testicular volume, using an orchidometer, was performed. Semen analysis was performed twice according to the World Health Organization Guidelines [16]. Hormonal assays that reflected activity of the spermatogenic axis (follicle-stimulating hormone [FSH]) and the androgenic axis (testosterone and luteinizing hormone [LH]) were drawn.

Cytogenetic analysis was performed on peripheral lymphocytes that were cultured for 72 h. Karyotypes were analyzed by GTG banding (G-bands by trypsin using Giemsa). For each case, at least 30 metaphase spreads were examined.

Finally, based on cytogenetic analysis, 95 azoospermic subjects with KS and a control group of 93 fertile men with a normal 46,XY karyotype were evaluated. Of all the subjects with KS, 91 subjects had a 47,XXY (pure or nonmosaic) chromosomal pattern and 4 had a 47,XXY/46,XY (mosaic) chromosomal pattern.

2.2 Genomic DNA preparation

Genomic DNA was obtained from peripheral lymphocytes using the QIAamp Blood Kit (QIAZEN, Chatsworth, TN, USA) or the Aquapure Genomic DNA Isolation Kit (Bio-Rad, Hercules, CA, USA). After 10 mL of peripheral blood was collected in an EDTA vacuum

tube, procedures were performed as recommended by the manufacturer.

2.3 Polymerase chain reaction

A set of five Y specific sequence-tagged sites (STS, accessed from Bioneer, Seoul, Korea) were amplified using genomic DNA isolated from the subjects by polymerase chain reaction (PCR). According to the recommendations of the European Academy of Andrology guidelines [17], the representative STS spanning the *AZF* region were selected for use; a total of five loci, sY84 (*AZFa* region), sY129, sY134 (*AZFb* region), and sY254, sY255 (*AZFc* region) covering the euchromatic region between Yp 11.31 and distal Yq11.23 were examined. The sequences of one set of primers for each gene are shown in Table 1. Of these, sY254 and sY255 are encoded in the region of the *DAZ* gene in subinterval 6 of Yq11. As an internal control, coamplification of the sex-determining gene SRY and the autosomal gene GAPDH were routinely performed on all genomic DNA samples. PCR reactions were performed in 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 μmol/L $MgCl_2$, and 200 μmol/L dNTPs containing 100 ng of genomic DNA, 4–20 μmol/L of each primer, and 0.3 units of Taq polymerase in a final volume of 20 μL. After an initial denaturation step at 94ºC for 2 min, the cycle parameters were: 35 cycles at 94ºC for 40 s, 58ºC for 80 s and 72ºC for 60 s. This protocol was followed by the final extension step at 72ºC for 10 min. The reaction products were then analyzed by electrophoresis at 76 V on 2%–4% agarose gels (Sigma Chemical, St. Louis, MO, USA) containing ethidium bromide (0.1 mg/mL), and visualized under ultraviolet light. A positive control (sample from a normal fertile male) and two negative controls (normal female sample and every constituent except DNA), were included in every PCR assay. We confirmed a deletion at the loci studied when the product of the expected size was not obtained after at least three PCR experiments with a single primer pair. P value < 0.05 was used to define statistical significance. Results are presented as the mean ± SD unless otherwise indicated.

3 Results

The clinical and seminal parameters of the subjects are summarized in Table 2. The mean age of the KS subjects and normal fertile men were 32.9 ± 3.9 years and 33.5 ± 4.9 years, respectively. The level of FSH in KS subjects was higher than in fertile men (38.2 ± 10.3 mIU/mL *vs*. 5.4 ± 2.9 mIU/mL, $P < 0.001$) and the testosterone level was lower than in the control group (1.7 ± 0.3 ng/mL *vs*. 4.3 ± 1.3ng/mL, $P < 0.001$).

Table 1. Primer sequences of the Y-chromosome sequence-tagged sites analyzed. STS, sequence-tagged site.

STSs	Forward	Reverse
sY84	AgAAgggTCTgAAAgCAggT	gCCTACTACCTggAggCTTC
sY129	AgCTTCAggAggTTCAAAAC	AAgTgggACCTAAgCTACgA
sY134	gTCTgCCTCACCATAAAACg	ACCACTgCCAAAACTTTCAA
sY254	gggTgTTACCAgAAggCAAA	gAACCgTATCTACCAAAgCAgC
sY255	gTTACAggATTCggCgTgAT	CTCgTCATgTgCAgCCAC

Table 2. Clinical features of patients. Data represent mean ± SD.

Feature	Klinefelter syndrome (n = 95)	Control (n = 93)	P value
Age (years)	32.9 ± 3.9	33.5 ± 4.9	0.138
Testicular volume (cm^3)	4.4 ± 2.0	20.2 ± 4.2	< 0.001
Semen analysis			
Volume (mL)	2.8 ± 1.2	2.9 ± 1.4	0.167
Sperm concentration (10^6/mL)	0	68.6	< 0.001
Hormone level			
Luteinizing hormone (mIU/mL)	13.5 ± 5.4	3.0 ± 1.3	< 0.001
Follicle-stimulating hormone (mIU/mL)	38.2 ± 10.3	5.4 ± 2.9	< 0.001
Testosterone (ng/mL)	1.7 ± 0.3	4.3 ± 1.3	< 0.001

Y chromosome microdeletions using a total of five representative STS spanning the *AZFa*, *AZFb* and *AZFc* loci were not found in any of the 95 azoospermic subjects with KS. In addition, using similar conditions of PCR, none of the 93 fertile men showed any Y chromosome microdeletions.

4 Discussion

The etiology of the defects of spermatogenesis in KS might involve many factors that remain to be defined: the underlying mechanisms of testicular degeneration are poorly understood. Many hypotheses regarding the underlying mechanism of depletion of germ cells in Klinefelter men have been reported and include insufficient supernumerary X-chromosome inactivation, Leydig cell insufficiency and disturbed regulation of apoptosis of Sertoli and Leydig cells. Lee *et al*. [11] suggest that X chromosome over-dose might interfere with the function of the Y chromosome in non-mosaic type. In contrast, altered dosage of some genes on the X chromosome might affect the development and/or degeneration of germ cells in men with 47,XXY. However, at present, the exact mechanism remains unclear. To date, little data is available on the frequency of Y chromosome microdeletions in subjects with KS. There are conflicting reports on the occurrence of Y chromosome microdeletions in subjects with KS (Table 3). Tateno *et al*. [12] failed to find microdeletions of the *DAZ* or *YRRM* genes in 21 nonmosaic KS subjects with (n = 1) and without (n = 20) spermatogenesis. In another study, Y chromosome microdeletions were observed in one of nine (11.1%) subjects with idiopathic azoospermia using 60 STS, whereas no deletions were found in subjects with the non-mosaic type of KS. However, others have reported a low incidence of Y chromosome microdeletions. In an independent study of the prevalence of Y chromosome microdeletions in 186 oligospermic and azoospermic men opting for intracytoplasmic sperm injection, only one man belonged to the Klinefelter mosaic category and also had *AZFc* microdeletions [14]. In another screening study for Y chromosome microdeletions in 226 Slovenian subfertile men, it was observed that five subjects had low-level mosaicism 46,XY/47,XXY (abnormal karyotype < 2.5%) and of these, only one subject had an *AZFc* microdeletion [15]. However, because of the small number of subjects analyzed in the three studies, no firm conclusion could be drawn regarding this issue. Recently, Y chromosome microdeletions using 19 sets of primers in 4 of 14 azoospermic KS subjects studied was reported [13]. All 4 cases with microdeletions were of mosaic type (a 47,XXY/46,XY pattern in three subjects and a 46,XY/47,XXY/48,XXXY/48,XXYY pattern in one subject), whereas 11 subjects without microdeletions were all nonmosaic type 47,XXY. Although the present study showed that no microdeletions were present in four subjects with mosaic KS, further studies are needed to clarify the association between the mosaic pattern and microdeletions. Based on the findings of the present study and review of published reports it appears that classical AZF deletions might not play a role in predisposing genetic background for the phenotype of azoospermic KS subjects with a 47,XXY. 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 [19, 20]. In the present study, the *AZFc* subdeletions were not evaluated because they had not yet made an issue of the subdeletions when this study was designed. Therefore, we could not get the results of subdeletions. Additional further studies would resolve this issue.

In the present study, screening of Y chromosome microdeletion assay was performed in a clinical practice. The use of only one or two STS for each *AZF* region in this study might be considered a limitation of the study design. There is no consensus concerning which and how many loci should be analyzed; as has been reported, detected microdeletion frequencies are not dependent on the number of STS analyzed [21]. Several studies that use a variable number of STS have report that the prevalence of deletions does not increase with more STS used. Therefore, at present it appears that it is acceptable to use two or three STS markers for each *AZF* region, as is suggested by Simoni *et al.* [17]. In addition, the choice of STS/gene markers used to identify Yq deletions is unrestricted. In terms of the described Y-specific STS, different panels of STS may cause different results; this is because some show differences in their reported map locations, others have multiple loci, and several represent naturally occurring polymorphisms. Taking into consideration these patterns and the extent of published Yq deletion intervals, we selected the most widely used five STS that would unequivocally determine the presence or absence of Y-specific sequences across the three known *AZF* regions.

Some clinicians have performed genetic screening,

Table 3. Reported data of Y chromosome microdeletions in men with Klinefelter syndrome (KS) in five previously published studies and the present study.

References	Study population (*n*)	Number of analyzed STS/gene	Number of patients with microdeletions/ all KS patients (%)	Karyotype	KS patient with microdeletions		
					Semen analysis	STS and gene deleted	*AZF* deleted
Oliva, *et al.* [14]	Oligospermic and azoospermic patients (186)	12/0	1/2 (50%)	47,XXY[2]/46,XY[48]	Azoospermic	sY153, sY254, sY209, sY202, sY158, sY160	*AZFc*
Tateno, *et al.* [12]	Patients with nonmosaic KS (21: 20 azoospermic and 1 severe oligospermic patients)	29/2	0/21	—	—	—	—
Lee, *et al.* [11]	Korean idiopathic azoospermic (9) and nonmosaic KS patients (6)	56/4	0/6	—	—	—	—
Peterlin, *et al.* [15]	Slovenian sub-fertile men (226)	42/16	1/5 (20%)	Low-level mosaicism 46,XY/47,XXY (abnormal karyotype < 2.5 %)	Azoospermic	sY153, sY152, sY220, sY155, sY147, sY242, sY156, sY254, sY157, sY202, sY243, sY158	*AZFc*
Mitra, *et al.* [13]	Azoospermic patients with KS (14: 7 nonmosaic and 7 mosiac types)	16/3	4/14 (28.6%)	46,XY(60%)/47, XXY(40%)	Azoospermic	sY86, sY746, sY113, sY118, sY127	*AZFa*, *AZFb*
				46,XY(80%)/47, XXY(20%)	Azoospermic	sY86, sY746, sY127	*AZFa*, *AZFb*
				46,XY(85%)/47, XXY(15%)	Azoospermic	sY84, sY86, sY746, sY127, sY134	*AZFa*, *AZFb*
				47,XXY(87%)/46,XY(7%)/ 48,XXY(3%)/48,XXXY(3%)	No emission	sY84, sY86, sY746, sY127	*AZFa*, *AZFb*
Current study	Azoospermic patients with KS (95: 91 nonmosaic and 4 mosiac as a 46,XY/47,XXY pattern)	5/0	0/95	—	—	—	—

including karyotyping and an assessment of Y chromosome microdeletions simultaneously to save time for the couples. The reasoning behind this is that KS has a considerably different clinical presentation and in practice is difficult to distinguish from other causes of hypergonadotrophic hypogonadism. Although we agree with this opinion in part, the work up for infertility should proceed step by step. We suggest that the chromosomal analysis should be performed first in azoospermic men with a suspected KS. And then further genetic screening is indicated. Moreover, clinicians need to consider minimizing costs to reduce patient burden from unnecessary testing in fertility clinics. In our center, the cost is KRW116 448 (approximately $US124) for high-resolution chromosomal analysis and KRW 64 420 (approximately $US69) for microdeletions of *AZF*.

In conclusion, classical *AZF* deletions might not play a role in predisposing genetic background for the phenotype of azoospermic KS subjects with a 47,XXY. In addition, routine screening for the classical *AZF* deletions might not be required for these subjects. Further studies, including partial *AZFc* deletions (e.g. *gr/gr* or *b2/b3*), might be necessary to establish other mechanisms underlying severe spermatogenesis impairment in KS. We expect that the recent progress in genomic analysis of the X and Y chromosome as well as improved understanding of the regulation of gene expression will lead to a better understanding of the mechanisms involved in germ-cell depletion.

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