


# Molecular and cytogenetic analysis of infertile Hakka men with azoospermia and severe oligozoospermia in southern China

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## Abstract

**Objective:** To determine the prevalence of chromosome abnormalities and azoospermia factor (AZF) microdeletions in Hakka men with infertility in southern China.

**Methods:** Hakka male patients, who received clinical counselling for infertility between August 2016 and October 2017, and fertile male controls, were enrolled into this retrospective study. Patients diagnosed with infertility and controls underwent cytogenetic analysis by standard G-banding; AZF microdeletions were examined by multiplex polymerase chain reaction and capillary electrophoresis.

**Results:** Out of 918 male patients who received fertility counselling, 57 were diagnosed with infertility due to azoospermia or severe oligozoospermia. Of these infertile patients, 22.81% (13/57) carried chromosome abnormalities, with 47, XXY being the most common abnormal karyotype. In addition, 36.84% (21/57) presented with Y chromosome microdeletions, most

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frequently in the complete AZFc and partial AZFc region. Duplication of the AZFc region was found in three patients. No AZF microdeletions were found in 60 fertile male controls.

**Conclusion:** The high AZF microdeletion frequency in the current Hakka population suggests that AZF microdeletion analysis is essential in fertility screening, and combined with cytogenetic analysis, may influence the choice of assisted reproductive techniques and reduce the risk of inherited genetic disease.

### Keywords

Male infertility, Y chromosome, karyotype, AZF microdeletions, azoospermia, oligospermia, Hakka population

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## Introduction

Infertility can be defined as a failure of fertilization in a couple after a year of regular unprotected sex,<sup>1</sup> and about half of infertility in couples is caused by male factors, either acting alone or in combination with female factors.<sup>2</sup> Major genetic causes of male infertility are chromosomal abnormalities and Y chromosome microdeletions.<sup>3</sup>

Cytogenetic analysis can be used to detect numerical and structural chromosomal abnormalities,<sup>4</sup> and is recommended by the American Urological Association and European Academy of Andrology guideline in all men with a motile sperm cell count <5 million and who are considered to have non-obstructive azoospermia.<sup>5</sup> Y chromosome microdeletions in the azoospermia factor (AZF) region cannot be observed cytogenetically, however, some studies suggest that a Y chromosome microdeletion test should be offered to men with non-obstructive azoospermia or severe oligospermia (total motile sperm count <5 million),<sup>6</sup> while other studies recommended this test should be carried out in men with total motile sperm count <10 million.<sup>7</sup> The AZF locus includes three subregions, namely, AZFa, AZFb and AZFc, which are commonly found in the q11.23 band of the Y

chromosome and are recognized as being important for spermatogenesis.<sup>8,9</sup> Microdeletions in these regions have been shown to have adverse effects on spermatogenesis,<sup>3</sup> thus, AZF microdeletions are most commonly seen to occur in patients with azoospermia or severe oligospermia.<sup>10</sup>

With the development of assisted reproductive technology, infertile males with Y chromosome microdeletions are able to produce offspring through intracytoplasmic sperm injection.<sup>11</sup> However, the potential risks of passing genetic abnormalities to their offspring are increased.<sup>12</sup>

The primary purpose of the current study was to assess the prevalence and major types of chromosomal abnormalities among infertile Hakka male patients with azoospermia or severe oligospermia, and to emphasize the need for genetic consultation and examination prior to providing fertility therapy using assisted reproductive technology in Hakka male patients in southern China.

## Patients and methods

### Study population

This observational study included Hakka male patients receiving clinical counselling

for infertility who were referred to the Centre for Reproductive Medicine of Meizhou People's Hospital (Huangtang Hospital), Meizhou Academy of Medical Sciences, Meizhou Hospital Affiliated to Sun Yat-sen University, China between August 2016 and October 2017, including patients diagnosed with azoospermia or severe oligozoospermia. A control group of male volunteers, who had previously produced offspring, were also recruited from the Centre for Reproductive Medicine of Meizhou People's Hospital (Huangtang Hospital). The study was performed in accordance with the Declaration of Helsinki, and was approved by the Ethics Committee of the Meizhou People's Hospital (Huangtang Hospital), Meizhou Academy of Medical Sciences, Meizhou Hospital Affiliated to Sun Yat-sen University. All study participants provided written informed consent.

Semen specimens, acquired after 3–7 days of abstinence from sexual activity, were sent at room temperature to the andrology laboratory of the Centre for Reproductive Medicine of Meizhou People's Hospital and analysed for several clinical parameters, measured according to the 2010 criteria of the World Health Organization.<sup>13</sup> Additionally, if no sperm were found following examination of the samples under the microscope, the samples were centrifuged at  $3000 \times g$  for 15 min at room temperature, and the precipitate was re-examined.

Patients were diagnosed with infertility if they were found to have severe oligozoospermia ( $\leq 5 \times 10^6$  sperm cells/ml) or azoospermia (no sperm observed following centrifugation of semen samples obtained more than twice at intervals of 1–3 weeks).<sup>13</sup> Cytogenetic analysis and AZF microdeletion tests were conducted for all infertile male patients and controls.

Patients with normal sperm concentration, abnormal endocrinology, seminal

duct obstruction, gonad anomalies, varicocele, cryptorchidism, orchiditis, ionizing radiation exposure, testicular trauma or other possible causes of male infertility were excluded from the study.

### *Cytogenetic analysis*

Peripheral blood (2.0 ml) was collected from all patients into aseptic 30 U/ml heparin vacutainer tubes and 0.5 ml blood of each sample were transferred into two sterile tubes with lymphocyte culture medium (Guangzhou Baidi Biotech, Guangzhou, China) and cultured for 72 h at 37°C (shaken twice daily). Three hours before the end of the culture, two drops of 20 ug/ml colcemid (Baidi Biotech, Guangzhou, China) was added to each tube. Microscopy images of karyotype analysis were captured using a ZEISS Axio Imager.Z2 system (ZEISS, Hensoldt Wetzlar, Germany). Karyotypes were analysed using standard G-banding and reported in line with the International System for Human Cytogenetic Nomenclature 2009.<sup>14</sup> A total of 20 GTG-banded metaphases were routinely counted with five metaphases analysed on each slide. The analysis was performed by two laboratory technicians (HMW and XWD) with qualifications in prenatal diagnosis.

### *Molecular analysis*

Genomic DNA was extracted from 200 µl venous blood in EDTA-coated tubes using QIAamp DNA blood Mini kit (Qiagen, Duesseldorf, Germany) according to the manufacturer's instructions. Multiplex polymerase chain reaction (PCR) analysis of AZF microdeletions was performed as recommended by the European Academy of Andrology and European Molecular Genetics Quality Network,<sup>15</sup> using a Y chromosome deletion kit (Microread, Beijing, China) according to the

manufacturer's instructions, including sequence-tagged sites: sY84, sY86 (AZFa); sY127, sY134, sY1161, CDY2, SCMY (AZFb); sY157, sY254, sY255, sY1191, DAZ, and CDY1 (AZFc). The SRY gene (located on the y chromosome) and ZFX/ZFY gene (located on the X and Y chromosome) were used as internal controls.

Multiplex PCRs were performed for each sample using the Y chromosome microdeletion detection kit (Microread) with BioRad DNA amplification system (Bio-Rad, CA, USA), according to the manufacturers' instructions, with the following amplification conditions: 5 min initial denaturation at 95°C, and 30 cycles of 60 s denaturation at 94°C, 60 s extension at 60°C and 90 s elongation at 72°C, followed by a final annealing process of 60 min at 60°C. PCR products were added to a mix containing ROX 500 probes (ROX fluorescence-labelled DNA fragment) and formamide, then denatured at 95°C for 3 min and placed on ice for 3 min. PCR products were then sequenced using an Applied Biosystem PRISM 3730xl Analyzer (Applied Biosystems, Foster City, CA, USA). No-template and female blood samples were used as negative controls, and a male blood sample was used as a positive control.

### Statistical analyses

Data are presented as *n* (%) prevalence or mean  $\pm$  SD, and were statistically analysed using SPSS software, version 20.0 (SPSS Inc., Chicago, IL, USA). Between-group differences in frequencies were evaluated using  $\chi^2$ -test and a *P* value <0.05 was recognized as being statistically significant.

### Results

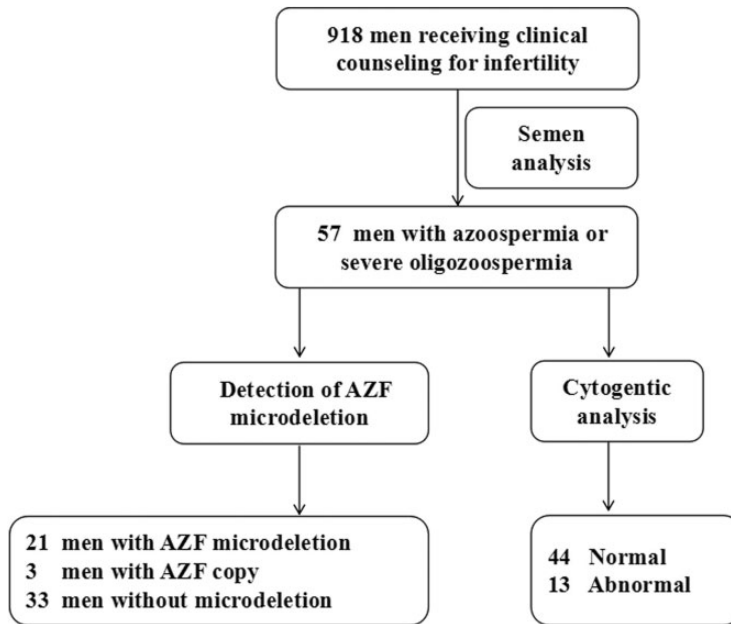
A total of 918 male patients who were receiving clinical counselling for infertility were enrolled into the study, and of these, 57

were defined as having infertility due to azoospermia or severe oligozoospermia. A total of 60 male volunteers who had previously produced offspring were recruited as controls. The 57 infertile male patients and 60 controls were assessed by cytogenetic and molecular analysis for karyotype and AZF microdeletions, respectively (Figure 1).

The mean age was  $28 \pm 5$  years in patients with azoospermia (*n* = 33),  $27 \pm 4$  years in patients with severe oligozoospermia (*n* = 24) and  $31 \pm 5$  years in controls (*n* = 60). Karyotyping revealed that 22.81% (13/57) of males diagnosed as infertile had chromosome abnormalities, including 30.30% (10/33) of males with azoospermia and 12.50% (3/24) of males with severe oligozoospermia (Table 1). No chromosome abnormalities were found in the control group.

The most frequent abnormal karyotype was 47, XXY (*n* = 8) followed by two patients with 46, XY (Y<G), one patient with 46, XX, one patient with 46, X, Yqh+ and one patient with 46, X, del(Y)(q11.23). No statistically significant differences in abnormal karyotype were found between patients with azoospermia compared with severe oligozoospermia ( $\chi^2 = 2.501$ , *P* = 0.200) (Table 1). Representative Y chromosome G-banding patterns found during karyotype analyses are shown in Figure 2.

Multiplex PCR to assess AZF microdeletions in 57 Hakka male patients diagnosed as infertile revealed deletions in the AZFa, AZFb, AZFc, AZFb+c, AZFa+b+c, partial AZFc, and partial AZFb/c loci, and duplication of AZFc (Table 2). The proportion of infertile males found to have AZF deletions was 36.84% (21/57) overall, with a frequency of 33.33% (11/33) in male patients with azoospermia, and 41.67% (10/24) in male patients with severe oligozoospermia. There was no statistically significance difference in terms of frequency of AZF microdeletions between the two groups ( $\chi^2 = 0.415$ ,



**Figure 1.** Flow chart showing selection and cytogenetic and molecular analyses for karyotype and AZF microdeletions in male patients diagnosed with infertility associated with azoospermia or oligozoospermia

**Table 1.** Clinical features regarding sperm count and chromosome abnormalities shown by analysis of karyotype in Hakka male patients diagnosed with infertility

Karyotype	Clinical group		Cases with AZF microdeletion
	Azoospermia n = 33	Severe oligozoospermia n = 24	
47, XXY	7 (21.21)	1 (4.17)	0
46, XX	1 (3.03)	0 (0.00)	1
46, XY(Y<G)	1 (3.03)	1 (4.17)	1
46, X, Yqh+	0 (0.0)	1 (4.17)	1
46, X, del(Y)(q11.23)	1 (3.03)	0	1
Total	10	3	4

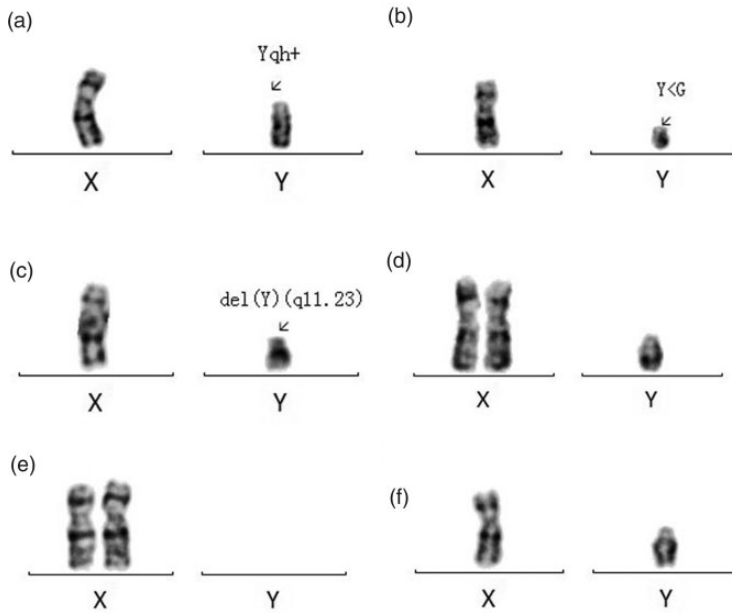
Data presented as n (%) prevalence of cases.

$P=0.585$ ) (Table 2). The most commonly detected microdeletions were within the partial AZFc region in male patients with azoospermia (15.15% [5/33]) and AZFc region in male patients with severe oligozoospermia (25.00% [6/24]). Duplication of the AZFc region was found in 3.03% (1/33) males

with azoospermia and in 8.33% (2/24) males with oligozoospermia.

## Discussion

Chromosomal anomalies have been shown to play an important role in male



**Figure 2.** Representative photomicrographs showing karyotype G-banding patterns in abnormal chromosomes from Hakka male patients diagnosed with infertility associated with azoospermia or oligozoospermia, and in normal male control chromosomes, showing: (a) Yqh+; (b) Y (Y<G); (c) del (Y) (q11.23); (d) XXY; (e) XX; and (f) XY (normal male karyotype)

**Table 2.** Azoospermia factor (AZF) microdeletions in Hakka male patients diagnosed with infertility associated with azoospermia or severe oligozoospermia, and in fertile male controls

AZF region	Sequence-tagged site deletions	Study group		
		Azoospermia n = 33	Severe oligozoospermia n = 24	Controls n = 60
AZFa	sY84, sY86	1 (3.03)	0 (0.00)	0 (0.00)
AZFc	sY157, sY254, sY255, sY1191, DAZ, CDY1	2 (6.06)	6 (25.00)	0 (0.00)
AZFb+c	sY127, sY134, sY1161, SMCY, sY157, sY254, sY255, sY1191, DAZ, CDY1	1 (3.03)	1 (4.00)	0 (0.00)
AZFa+b+c	sY84, sY86, sY127, sY134, sY1161, CDY2, SMCY, sY157, sY254, sY255, sY1191, DAZ, CDY1	1 (3.03)	0 (0.00)	0 (0.00)
Partial AZFc	CDY2/CDY1>1.5, DAZ/DAZL<1.5	5 (15.15)	3 (12.5)	0 (0.00)
Partial AZFb/c	sY1161, sY1191, DAZ/DAZL<1.5	1 (3.03)	0 (0.00)	0 (0.00)
Total		11 (33.33)	10 (41.67)	0 (0.00)

Data presented as n (%) prevalence.

infertility,<sup>16</sup> but the prevalence of these abnormalities has rarely been reported in the Hakka male population of China. In studies of different populations, the occurrence of chromosomal abnormalities in males has been reported to vary between 11 and 24% of patients with azoospermia<sup>17,18</sup> and between 2 and 16% of patients with oligospermia.<sup>19,20</sup> In the current study, chromosomal abnormalities were observed in 30.30% (10/33) of patients with azoospermia and 12.50% (3/24) of patients with severe oligospermia. Prevalence rates in the current study population were higher than those published for patients with azoospermia, but were within the published range for patients with oligospermia. Any differences between the current and previously published studies may reflect variations between the study populations or the patient selection criteria, such as the level of sperm impairment.

Ferlin et al. (2006)<sup>21</sup> reported frequencies of Klinefelter's syndrome (XXY) in infertile male patients of approximately 10% and 5% in patients with azoospermia and severe oligospermia, respectively. Of the present 57 patients, 21.21% (7/10 with chromosome abnormalities) had Klinefelter's syndrome in the azoospermia group, while only 4.17% (1/3 with chromosome abnormalities) were found in the severe oligospermia group. Males with Klinefelter's syndrome are commonly diagnosed with azoospermia and are infertile.<sup>22</sup> Such patients may have offspring using intracytoplasmic sperm injection, however, their children may have the potential for increased risk of chromosomal abnormalities.<sup>23</sup>

Men with XX male syndrome can have a phenotypic resemblance to Klinefelter's syndrome with normal male genitalia, but they are sterile.<sup>24</sup> In present study, the frequency of XX males in patients with azoospermia was 3.03%, which was higher than the frequency of 1.36% previously reported

by Naasse et al.<sup>22</sup> This difference may be attributed to population variance and limited study subjects in the present study.

Infertile male patients with 46, XY (Y<G), 46, X, Yqh+, and 46, X, del(Y)(q11.23) were also revealed in the present study. The 46, XY(Y<G) and 46, X, Yqh+ karyotype are chromosome polymorphism variations that occur in the general population and have been regarded as normal for some time.<sup>25</sup> In the present study, however, the three patients found to have either the 46, XY(Y<G) or 46, X, Yqh+ karyotype were infertile, suggesting that polymorphisms in the Y chromosome may affect sperm production to varying degrees. There was one patient with azoospermia and the 46, X, del(Y)(q11.23) karyotype, supporting the evidence that deletions in the Y chromosome may lead to sterility.<sup>26</sup>

While Klinefelter's syndrome is closely related to male infertility, Y chromosome microdeletions also play an important role in infertility. In the present study, the frequency of AZF microdeletions was 36.84% (21/57) in patients with azoospermia or severe oligospermia. This frequency is higher than those reported in the literature of 5.08%<sup>27</sup> in Hong Kong, China, and 14.07%<sup>28</sup> in Northeast China. This difference may be due to several factors such as the sequence-tagged site markers used, sample size and different regions. The most common deletions in AZF regions found in the present study were complete and partial AZFc, followed by deletions in AZFb+c, AZFa, AZFa+b+c and partial AZFb/c.

Molecular analysis of the Y chromosome has shown that each sub-region of AZF plays a crucial role in the entire process of spermatogenesis.<sup>29</sup> Deletion of the AZFa region universally results in Sertoli cell-only syndrome and azoospermia, while deletion in the AZFb region affects spermatogenic disorders because of unmetabolized

metamerism.<sup>30</sup> Deletion of the AZFc locus primarily influences germ cell differentiation leading to severe oligozoospermia or azoospermia.<sup>31</sup> In the present study, deletion of the AZFa region was only observed in the azoospermia phenotype, while deletion of the AZFc region was detected in patients with severe oligozoospermia or azoospermia, which concurs with a previous report.<sup>9</sup> Furthermore, AZF microdeletions were observed in 30.76% (4/13) patients with chromosomal abnormalities and most were found in males with azoospermia, suggesting that the clinical manifestations of patients with both chromosomal abnormalities and AZF microdeletions are more serious.

In summary, the present study found that the prevalence of chromosomal abnormalities and AZF microdeletions in infertile Hakka men from southern China are marginally higher than previous reports in different regions, which may be due to the relatively small sample size, greater number of sequence-tagged site markers in the present study and the ethnicity of the populations. Carriers with chromosomal abnormalities and AZF microdeletions have the potential risk of passing genetic aberrations on to their descendants by intracytoplasmic sperm injection. The relatively high prevalence of genetic abnormalities in the present study indicates that male Hakka patients with azoospermia and severe oligozoospermia should be routinely screened for chromosomal abnormalities and Y chromosome microdeletions, and clinical counselling must be performed prior to the use of assisted reproductive technology in this population in southern China.

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### Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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