Molecular genetic analysis of 1,980 cases of male infertility

MEIMEI FU, MEIHUAN CHEN, NAN GUO, MIN LIN, YING LI, HAILONG HUANG, MEIYING CAI and LIANGPU XU

Medical Genetic Diagnosis and Therapy Center, Fujian Maternity and Child Health Hospital, College of Clinical Medicine for Obstetrics and Gynecology and Pediatrics, Fujian Medical University, Fujian Key Laboratory for Prenatal Diagnosis and Birth Defects, Fuzhou, Fujian 350001, P.R. China

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Abstract. The present study aimed to investigate the occurrence of chromosomal karyotype abnormalities and azoospermia factor (*AZF*) microdeletion on the long arm of the Y chromosome (Yq) in infertile men, and to determine their association with infertility to ultimately improve clinical outcomes in these patients. A total of 1,980 azoospermic and oligospermic men from the outpatient department of the Fujian Maternity and Child Health Hospital (Fuzhou, China) were recruited between January 2016 and December 2019. Peripheral blood was used for karyotype analysis; *AZF* microdeletion analysis of the Yq was performed using capillary electrophoresis. Among the 1,980 patients, 178 had chromosomal abnormalities (9.0%; 178/1,980), of whom 98 had an abnormal number of chromosomes. Among the abnormal karyotypes, the most common was 47, XXY (80/178; 44.9%). *AZF* microdeletion on the Yq occurred at a rate of 10.66% (211/1,980); the most common type was the *AZFb/c* deletion (sY1192; 140/211; 66.4%). The present findings showed that karyotype abnormalities and *AZF* gene microdeletion are important drivers of male infertility. Specifically, men with Yqh- and del(Y)(q11) had a higher risk of *AZF* microdeletion. These results suggested that patient treatment could be personalized based on routine molecular genetic analysis, which could further alleviate the economic and emotional burden of undergoing redundant or ineffective treatments.

Correspondence to: Professor Meiying Cai or Professor Liangpu Xu, Medical Genetic Diagnosis and Therapy Center, Fujian Maternity and Child Health Hospital, College of Clinical Medicine for Obstetrics and Gynecology and Pediatrics, Fujian Medical University, Fujian Key Laboratory for Prenatal Diagnosis and Birth Defects, 18 Daoshan Road, Gulou, Fuzhou, Fujian 350001, P.R. China
E-mail: 22234534@qq.com
E-mail: xiliangpu@fjmu.edu.cn

Abbreviations: AZF, azoospermia factor; Yq, long arm of the Y chromosome

Key words: male infertility, chromosomal abnormality, *AZF* microdeletion, oligospermia, cytogenetic analysis

Introduction

Male infertility is becoming an increasingly serious medical concern; 15% of adult couples experience infertility, with male infertility occurring in nearly half of the cases worldwide (1-3). The etiology of infertility is complex and involves various factors, including genetics, endocrine diseases, immune dysfunction, reproductive tract infections or abnormalities, and sexual dysfunction, among other factors. Patient genetics is a particularly important factor in male infertility, contributing to ~20% of all cases (3). However, the roles of genetic factors in male infertility are unclear. Chromosomal abnormalities are detected in 2.2-33% of men suffering from infertility (4). Azoospermia factor (*AZF*) is a gene associated with spermatogenesis that is located in region 1 band 1 of the long arm of the Y chromosome (Yq11). *AZF* microdeletion occurs in 6.6% of patients with azoospermia and severe oligozoospermia (5-7). Chromosomal abnormalities and *AZF* microdeletion are important genetic factors associated with male infertility, and are among the most common genetic abnormalities observed in clinical cases. Further etiological examinations are needed to improve clinical treatment and guide the application of assisted reproductive technologies to ultimately reduce the emotional and economic burden of patients. The present study conducted karyotype and molecular genetic analyses in 1,980 infertile men to elucidate the occurrence of chromosomal abnormalities and *AZF* microdeletions.

Materials and methods

Ethics approval and consent to participate. The research protocol was approved by the ethics committee of Fujian Maternity and Child Health Hospital (Fuzhou, China; approval no. 2014042); written informed consent was obtained from all patients.

Clinical data. In total, 1,980 men with azoospermia and oligospermia (age, 21-56 years; median age, 34 years) were recruited from the outpatient department of Fujian Provincial Maternity and Children's Hospital between January 2016 and December 2019. Patients were included if they were male and diagnosed with infertility, and excluded if their infertility was caused by varicocele, cryptorchidism, vas deferens obstruction, retrograde ejaculation or urogenital tract infection.

Table I. Classification of the chromosomal abnormalities in 178 infertile men via karyotyping using peripheral blood.

Classification	Karyotype	Number	Percentage (%)
Numerical abnormalities	47, XXY	80	44.9
	47, XYY	4	2.2
	48, XXYY	4	2.2
	45, X/46, XY	7	4.0
	47, XY, +mar	3	1.7
Structural abnormalities	46, X, inv(Y)(p11.2q11.2)	10	5.6
	46, X,del(Y)(q11)	12	6.7
	45, XY,rob(13;14)(q10;q10)	6	3.4
	45, XY,rob(14;21)(q10;q10)	4	2.2
	46, XY,t(6;20)(q21;p13)	1	0.5
	46, XY,t(1;11)(q24;q25)	1	0.5
	46, X,t(Y;15)(q12;p11.3)	2	1.1
	46, XY,t(4;9)(q25;q33)	1	0.5
	46, XY,t(1;7)(q22;p15)	1	0.5
	46, XY,inv(9)(p12q13)	5	2.8
Polymorphisms	46, X,Yqh+	13	7.3
	46, X,Yqh-	12	6.7
Sex reversal syndrome	46, XX	12	6.7
Total		178	100

Routine semen analysis was performed according to the World Health Organization diagnostic criteria (8). All patients had been cohabiting with their partners for >1 year and were not using any form of contraception.

Karyotype analysis. Peripheral blood (2 ml) from each patient was collected into anticoagulant tubes containing heparin. It was cultured for 72 h (Peripheral blood lymphocyte culture medium, Qingdao Leifer Biological Co., Ltd.; 37˚C).Colchicine (0.04 ml; 100 μg/ml) was added and the cells were further cultured for 20 min. KCl (0.075 mol/l) low exosmotic solution was added (8 ml) for another 30 min of incubation, and methanol and acetic acid were prepared at a ratio of 3:1. After pre-fixation at room temperature for 5 min, refixation with methanol-glacial acetic acid and centrifugation at 1,000 x g for 10 min at 24˚C, the supernatant was discarded and the pellet prepared. Chromosomes and G-bands were prepared for trypsin digestion, Giemsa dye staining. An image analysis system, including an automatic scanning chromosomal microscope (CytoVision GSL-120; Zhejiang Bosheng Biotechnology Co., Ltd.), was used to scan the samples. For each case, 20 karyotypes were counted and five karyotypes were analyzed. The C- and N-bands were redisplayed as needed. Counting and analysis were repeated for cases in which abnormalities were detected.

Molecular genetic analysis for detection of AZF microdeletion on the long arm of the Y chromosome (Yq). Peripheral blood (2 ml, in EDTA-K2 anticoagulant tubes) was collected from each patient. Total DNA was extracted using a Blood & Cell Culture DNA Maxi kit(Qiagen GmbH). An *AZF* detection kit (cat. no. 20173403324; including 15 sequence tag sites: sY84, sY86, sY88, sY82, sY1064, sY1065, sY127, sY134, sY105, sY121, sY254, sY255, sY153, sY1192 and sY160, using the SRY and ZFAX/Y sites as internal controls; Yaneng Biotechnology) was used according to the manufacturer's instructions. The amplification conditions were as follows: UNG enzyme reaction at 50˚C for 10 min, pre-denaturation at 95˚C for 15 min, denaturation at 94˚C for 30 sec, 58˚C for 60 sec, 72˚C for 60 sec, totaling 35 cycles, and a final extension at 72˚C for 10 mi. Electrophoresis conditions were 2% Agar, 100 V, 40 min.

Results

Cytogenetic analysis of infertile patients. Among the 1,980 cases evaluated, chromosome abnormalities were detected in 178 or 9.0% of cases (Table I). There were 98, 43, 25 and 12 cases with abnormal chromosome numbers, abnormal structures, polymorphisms and sex reversal syndrome, respectively. Among the 98 patients with abnormal chromosome numbers, 80 had Klinefelter syndrome; four had supermale syndrome; four had 48, XXYY; seven had 45, X/46, XY; and three had a marker chromosome. Among the 43 cases of abnormal chromosomal structures, there were 10 cases of Y inversion; 12 of 46, X, del(Y)(q11); 10 of Robertsonian translocation; six of balanced translocation; and five of 46, XY, inv(9)(p12q13). Among the 25 cases of polymorphisms, there were 12 cases of 46, X, Yqh-(Y≤21) and 13 of 46, X, Yqh+ (Y>18).47, XXY accounted for the largest proportion, also known as Klinefelter syndrome (Table I).

Comparison of AZF deletion rates in infertile patients. Among the 1,980 cases, *AZF* deletion on the Yq occurred in 211 or 10.6% of the cases. *AZFb/c* deletion was observed in 140 cases (7.1% of all *AZF* deletions); *AZFb/c+c* deletion in 38 (1.9%); *AZFa+b+c+b/c+Sy160* deletion in 12 (0.6%);

Table II. *AZF* gene microdeletion in 211 cases of Yq abnormalities.

Deletion type	Number	Constituent ratio (%)	Miss rate (%)	Clinical symptoms
AZFb/c	140	66.4	7.1	Oligospermia Asthenospermia
AZFb/c+c	38	18.0	1.9	Oligospermia Asthenospermia
AZFa+b+c+b/c+sY160	12	5.7	0.6	Azoospermia
AZFb+c+b/c	9	4.3	0.5	Azoospermia
AZFa	8	3.7	0.4	Azoospermia
AZFb	4	1.9	0.2	Azoospermia

AZF, azoospermia factor.

Table III. Cases with both chromosomal karyotype abnormalities and *AZF* deletion.

Complex chromosome abnormalities	*AZF* microdeletion	Number	Constituent ratio (%)
46, XX	*AZFa+b+c+b/c+*sY160	12	31.6
46, X,del(Y)(q11)	*AZFb+c+b/c*	7	31.6
	AZFb/c+c	5	
46, XYqh-	*AZFb/c*	4	18.4
	AZFb/c+c	2	
	AZFa	1	
45, X/46, XY	*AZFb+c+b/c*	2	7.9
	AZFb/c+c	1	
47, XXY	*AZFb/c*	2	5.3
46, XYqh+	*AZFb/c*	2	5.3

AZF, azoospermia factor.

AZFb+c+b/c deletion in nine(0.5%); *AZFa* deletion in eight (0.4%); and *AZFb* deletion in four (0.2%). The proportion of *AZFb/c* deletion was the highest and the proportion of *AZFb* deletion was the lowest (Table II).

Patients with infertility had both chromosomal karyotype abnormalities and AZF deletion. Among the 1,980 cases, 38 had both chromosomal karyotype abnormalities and *AZF* deletion, accounting for 1.9% of all cases. Among these, 12 had sex reversal syndrome, 46, XX, and deletion of *AZFa+b+c+b/c+sY160*; seven had 46, X, del(Y)(q11) and deletion of *AZFb+c+b/c*; and fivehead 46, X, del(Y)(q11) and deletion of *AZFb/c+c*. The present study observed four cases of 46, XYqh- and *AZFb/c* deletions; two of 46, XYqh- and *AZFb/c+c* deletions; one of 46, XYqh- and *AZFa* deletions; two of 45, X/46, XY and *AZFb+c+b/c* deletions; one of 45, X/46, XY and *AZFb/c+c* deletions; two of 47, XXY and *AZFb/c* deletions; and two of 46, XYqh+ and *AZFb/c* deletions. Sex reversal syndrome, 46, XX, and 46, X, del(Y)(q11) accounted for the same proportion of patients (Table III).

Discussion

A chromosome karyotype abnormality is one of the most common genetic indicators of male infertility (7). In the present study, abnormal karyotypes accounted for 9.0% of cases and the 47, XXY karyotype accounted for 44.9% of all abnormal karyotypes, which is consistent with the findings of previous reports (4,9,10). 47, XXY, also known as Klinefelter syndrome, is characterized by congenital testicular convoluted seminiferous tubule hypoplasia, spermatogenic tubule hyalinosis and fibrosis without spermatogenesis. The *SRY* gene on the Yq controls testicular development and the male phenotype; however, the function of the Yq is inhibited due to the additional X chromosome. This influences the development of the convoluted canal, hyaline degeneration and fibrosis in the spermatogenic tubule, and hyaline degeneration in the convoluted ducts, resulting in infertility owing to reduced sperm production (11). This syndrome is also associated with the failure of chromosome segregation during meiosis. 47, XYY accounted for 2.2% of abnormal karyotypes. Most patients with 47, XYY are tall, have a normal phenotype and are fertile; however, they exhibit personality and behavioral disorders (12). A few exhibit dysplasia of external genitalia, cryptorchidism and reduced fertility owing to inhibited Yq segregation during meiosis II (13). This results in the formation of 24, YY sperm, which forms a 47, XYY ovum on fertilization with a normal 23, X oocyte. In the present study, 48, XXYY accounted for 2.2% of the abnormal karyotypes. The XXYY genotype could have resulted from reduced chromosomal segregation during meiosis in the patients' parents. Severe fibrosis and

hyperplasia of the testicular tissue in these patients could have caused thickening of the nonspecific barrier and severe destruction of the blood-testicular barrier. These changes could lead to serious obstacles and pathological changes in the formation of spermatogenic cells, resulting in male infertility (14). The karyotype 46, X, inv(Y)(q11) accounted for 5.6% of abnormal karyotypes. Yq inversion can result in the loss of local genes associated with the development of male gonads, leading to gonadal dysplasia and infertility (15). In addition, Robertsonian translocation accounted for 5.6% of abnormal karyotypes. This phenotype has been associated with normal intelligence; however, semen examination has indicated oligospermia, asthenospermia and azoospermia. The oligospermia could be attributed to abnormal conjunctions during meiosis and the resulting inability to form gametes (16). Sex reversal syndrome accounted for 6.7% of abnormal karyotypes. The sex of these patients is inconsistent with their chromosome set owing to abnormal sex determination and differentiation. They do not have a normal Yq, but have an extra X chromosome, resulting in testicular tissue dysplasia, which influences sperm production (17).

AZF exists in the q11 region of the long arm of Yq, which consists of three non-overlapping regions, *AZFa*, *AZFb* and *AZFc*. The gene locus regulates spermatogenesis and the deletion of one or more regions can cause spermatogenic dysfunction, leading to asthenia and infertility. *AZF* microdeletions are common genetic causes of male infertility due to their associations with spermatogenic disorders (18). In the present study, *AZF* microdeletions were detected in 10.7% of the cases, which is consistent with results from several countries in Europe (8-18%) (19) and Southwest Asia (10-16%) (20,21). In the present study, the most common abnormality was the deletion of sY1192 in the *AZFb/c* region, accounting for 66.4% of all deletions. The second most common was the *AZFb/c+c* deletion (18.0%). sY1192 is located between *AZFb* and *AZFc*, and the deletion of sY1192 in the *AZFb/c* region occurs within the extended site near the *AZFc* region. This deletion has similar physiological relevance to that of the *AZFc* deletion, which is associated with various clinical manifestations; patients with this abnormality may exhibit normal spermatogenesis or oligospermia/azoospermia. In patients with oligospermia exhibiting the *AZFc* deficiency, sperm count decreases progressively, thus requiring the cryopreservation of semen to enable future utilization of intracytoplasmic sperm injection-assisted reproductive technologies (22). The present study found that 12 patients had *AZF* deletions (5.7% of all deletions), all of whom exhibited SRY-positive sex reversal syndrome (46, XX males).In these patients, testicular development was poor, testosterone level was reduced, and follicle-stimulating hormone and luteinizing hormone feedback was increased, suggesting hypogonadism and severe inhibition of spermatogenesis. *AZFa* deletion was relatively rare, accounting for 3.7% of all deletions. Patients with the *AZFa* deletion exhibited sterility owing to spermatogenesis disorders caused by Sertoli-cell-only and Klinefelter syndromes. In such cases, the only viable option for the couple is to use sperm from a donor. *AZFb* deletion accounted for 1.9% of all deletions and resulted in the retarded development of spermatogonia; the corresponding patients presented with aspermia, which is often accompanied by simultaneous deletion of other regions and results in diverse clinical manifestations (23). Among the 211 patients with the *AZF* deletion, 18% harbored abnormal karyotypes. This result may be underestimated as *AZF* microdeletion is difficult to detect using chromosomal karyotype detection. Therefore, future studies should employ alternative detection techniques to detect AZF in patients with the XY chromosomal karyotype.

Yq microdeletions were detected in seven of the 12 patients with 46, X, Yqh-(small Y), including four with *AZFb/c* deletions, two with *AZFb/c+c* deletions, and one with the *AZFa* deletion. The *AZFb/c* deletion was detected in two of the 13 patients with 46, X, Yqh+ (large Y). Therefore, the rate of *AZF* microdeletion was higher in men with the Yqh-chromosome, which is consistent with previous results (24). Indeed, infertility in patients with a large Yq may not be related to *AZF* microdeletion (25-27). The present study observed only few Yq length changes. The effects of this abnormality on male infertility should be analyzed with larger sample sizes, which will prove to be useful in guiding personalized treatment strategies. Among the 12 patients with 46, X, del(Y)(q11), seven had the *AZFb+c+b/c* deletion and fivehead the *AZFc+b/c* deletion, suggesting that these patients may be *AZF* deficient. Further molecular examinations should be performed to confirm these findings. Among the 80 cases of 47, XXY, the *AZF* gene was normal in 78 cases and the *AZFb/c* sY1192 deletion was detected only in two. There were no associations between azoospermia and *AZF* microdeletion on the Yq in patients with the 47, XXY karyotype. Therefore, *AZF* microdeletion analysis may not be useful in determining the etiology of infertility in patients with this karyotype. The key gene that determines the development of spermatogenic cells in the testicular curved spermatogenic cells is located in the q11 position of Yq. The deletion of this part may lead to the deletion or alteration of a series of genes related to spermatogenic function on Yq.

There are some limitations to the present study. First, the sampling principal employed may not guarantee a good reflection of the whole male infertile population. Secondly, the present study only reported the clinical results, but did not investigate the molecular mechanisms. There is scope for improving the molecular biological techniques used to further elucidate the molecular genetic mechanisms underlying male infertility.

In conclusion, chromosomal karyotype abnormalities and *AZF* microdeletions in the Yq are two major genetic factors related to male infertility. Regular cellular and molecular genetic examinations in men with infertility could help physicians determine the patient-specific etiology of infertility and promote the development of appropriate clinical treatments and effective assisted reproductive technologies to ultimately improve fertility and prevent birth defects.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MF, MYC and LX made substantial contributions to the conception and design of the study, acquisition of data, and analysis and interpretation of data. YL and NG participated in data collection and analysis. ML MHC and HH were involved in drafting the manuscript, acquisition of data and interpretation of data. MYC, MF and MHC confirm the authenticity of all the raw data. All authors read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All procedures involving human participants were performed in accordance with protocols approved by the ethics committee of Fujian Provincial Maternity and Children's Hospital (approval no. 2014042). Written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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