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Early detection of Y chromosome microdeletions in infertile men is helpful to guide clinical reproductive treatments in southwest of China

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Abstract

The microdeletions of azoospermia factor (AZF) genes in Y chromosome are greatly associated with male infertility, which is also known as the second major genetic cause of spermatogenetic failure. Accumulating studies demonstrate that the different type of AZF microdeletions in patients reflect different clinical manifestations. Therefore, a better understanding of Y chromosome microdeletions might have broad implication for men health. In this study, we sought to determine the frequency and the character of different Y chromosome microdeletion types in infertile men in southwest of China.

In total, 1274 patients with azoospermia and oligozoospermia were recruited in southwest of China and screening for Y chromosome microdeletions in AZF regions by multiplex polymerase chain reaction.

The incidence of AZF microdeletions in southwest of China is 12.87%, which is higher than the national average. Further investigations unveiled that azoospermia factor c (AZFc) is the most frequent type of all the AZF microdeletions. Additionally, the number and also the quality of sperm in patients with AZFc microdeletion is decreasing with the age. Therefore, it is conceivable that the early testing for Y chromosome microdeletions in infertile men is crucial for fertility guidance.

The early detection of Y chromosome microdeletions in infertile men can not only clearly explain the etiology of oligzoospermia and azoospermia, but also help for the clinical management of both infertile man and his future male offspring.

Abbreviations: AZF = azoospermia factor, E = estradiol, EAA = European Academy of Andrology, EMQN = European Molecular Genetics Quality Network , FSH = follicle-stimulating hormone, gDNA = genomic DNA, ICSI = intracytoplasmic sperm injection, LH = luteinizing hormone, PCR = polymerase chain reaction, STSs = sequence-tagged sites, SYR = sex-determining region of the Y chromosome, T = testosterone, TESE = testicular sperm extraction, Yp = short arm, Yq = long arm, ZFX = zinc finger protein, X-linked, ZFY = zinc finger protein, Y-linked.

Keywords: AZF, azoospermia, male infertility, oligozoospermia, Y chromosome microdeletion

1. Introduction

Infertility is a global health problem caused by multiple factors and affects approximately 10% to 15% of couples worldwide.[1] It has been estimated that almost half of infertility is due to male infertility, and the genetic factors, especially the microdeletions of Y chromosome might be closely associated with male infertility.[2–4] It is reported that nearly 10% to15% infertile men are suffered from the microdeletion caused spermatogenetic failure.[3] As one of the most frequent genetic cause of male infertility, the screening of Y chromosome microdeletions has attracted increasing attention in recent years. Not only to diagnose the etiology of oligzoospermia and azoospermia, but also provide the patients with precious time for timely and appropriate assisted reproductive therapy.[5]

The Y chromosome is one of the sex-determining chromosomes, which consists of a short (Yp) and a long (Yq) arm.[6] It is reported that the majority of Y chromosome microdeletions are occur in the regions of long arm.[4] In addition, these microdeletions are involved in spermatogenesis progress and meanwhile affect the development of testis as well, therefore leading to azoospermic or oligozoospermic manifestation in patients.[7–9] Accumulating studies have proved that the microdeletion of azoospermia factor (AZF) in Y chromosome can be used to diagnose microdeletion caused infertility.[10–12] Clinically, there are 3 important nonoverlapping regions in AZF gene, including azoospermia factor a (AZFa), azoospermia factor b (AZFb), and azoospermia factor c (AZFc).[13,14] These 3 regions correspond to 5 microdeletion patterns: AZFa, AZFb, AZFc, AZFb + c and AZFa + b + c.[12,14] It is reported that AZFc is the most frequent deletion type among all the microdeletions, followed by AZFb and AZFa.[12] Generally, AZF microdeletions are too small to be detected by karyotyping. However with the development of molecular biology technology, now AZF microdeletions can be clearly identified by multiplex polymerase chain reaction (PCR) method within a short time.[15]

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As far as we know, the different regions of AZF microdeletion in patients reflect different clinical manifestation.[13] It is reported that the type of AZF microdeletions has been proposed as a potential influence factor for testicular sperm extraction (TESE).[5] For example, the deletion of entire AZFa region will definitely lead to severe azoospermia and sertoli cell-only syndrome.[15–17] Indeed, AZFa plays a key role in the spermatogenic process, especially regulates the early stage of spermatogonial proliferation and also the survival of germ cells.[18] Therefore, the assisted productive treatment of intracytoplasmic sperm injection (ICSI) is not recommended for patients with entire AZFa deletion.[19] Additionally, the deletion of AZFb in patients will cause the absence of postmeiotic germ cells at the spermatocyte stage.[16,20] In this case, ICSI is only recommended for the patients with partial AZFb microdeletion, since none of the mature spermatozoa can be found in patients with complete AZFb microdeletion.[5,21] Moreover, the deletion of AZFc region is associated with variable clinical phenotypes and can be found in either oligozoospermia or azoospermia men.[22,23] It is generally accepted that the AZFc region is essential to complete the spermatogenic process, and the deficiency of AZFc will result in hypospermatogenesis.[24,25] These information implies that the patients with AZFc deletion may have sperm in the ejaculate, however the numbers and also the quality of sperm are declining.[26,27] Hence, the assisted reproductive treatment method of semen cryopreservation in early adulthood is highly recommended for patients with AZFc microdeletion.[5] In brief, the type of AZF microdeletions can provide a lot of effective information for clinical diagnosis and management of male infertility. While screening for these AZF microdeletions, we may timely choose appropriate medical and surgical treatments for infertile man.[28] Until now the exact role of AZF regions in male infertility has not yet been comprehensively confirmed, therefore more data needs to be collected for clinical research and application.

In this study, we aimed to determine the character and the consequence of different Y chromosome microdeletions types in infertile men in southwest of China. In addition, we will figure out the relationship between the regions of AZF deletion and clinical phenotype, and therefore avoid unnecessary medical treatments and reduce the economic burden on patients as well as the society. A better understanding of AZF microdeletions and also its associated phenotype is crucial for designing specific and effective assisted reproductive method for infertile men, and furthermore benefit for both infertile man and his future male offspring.

2. Methods

2.1. Patients

A retrospective study was performed with 1274 azoospermic or oligozoospermic men aged between 16 and 45 years old from southwest of China. All the inspections were performed in the West China Second University Hospital from July 2016 to July 2018. Semen samples were obtained in the period of 2 to 7 days after ejaculatory abstinence, and analyze according to the World Health Organization guidelines.[29] Oligozoospermia was diagnosed with a sperm count $<20 \times 10^6$/ml. This study was approved by the Reproductive Medicine Ethics Committee of West China Second University Hospital, Sichuan University, and all patients signed informed consents of this study before semen analysis.

2.2. AZF microdeletion analysis

Fresh peripheral blood (3 ml) were obtained and stored for AZF microdeletions detection. Firstly, the genomic DNA (gDNA) was isolated using the commercial kit (Tiangen Biotech Co, Ltd, Beijing, China). And then, following the recommendations of European Academy of Andrology (EAA) and European Molecular Genetics Quality Network (EMQN), 50 ng gDNA was used to test the classical AZF microdeletions by multiplex PCR method (Tegen Biotech Co, Ltd, Shanghai, China). Each gDNA sample was subjected to 2 multiplex PCR reactions as described previously.[15] In summary, the kit has an ability to detect 6 sequence-tagged sites (STSs), including AZFa (sY84, sY86), AZFb (sY127, sY134), and AZFc (sY254, sY255). Sex-determining region of the Y chromosome (SRY) and Zinc finger protein X-linked/Y-linked (ZFX/ZFY) were used as internal control.

2.3. Real-time PCR

Each 25 ul reaction consist of 22.5 ul PCR Mix and 2.5 ul gDNA. Generally, the PCR Mix include Taq DNA polymerase, uracil-N-glycosylase, PCR reaction buffer, dNTP, Mg^{2+}, primers, and probes. All reactions were run on an ABI 7500 real-time PCR system (Life Technologies, CA) using the following cycling parameters: 50°C for 2 minutes; 95°C for 5 minutes; 38 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds; and a final elongation step of 72°C for 5 minutes. Usually, 1 sample required 2 PCR reactions simultaneously. In reaction A, the 4 fluorescent dyes of FAM, VIC, ROX, and Cy5 were used to detect the 4 gene sites including SRY, sY84, Y127, and Y255, respectively. While in reaction B, the 4 fluorescent dyes of FAM, VIC, ROX, and Cy5 were used to detect the 4 gene sites including ZFX/ZFY, sY86, sY134, and sY254, respectively. Results were considered positive when a clear amplification curve of the expected site was obtained ($Ct < 32$).

2.4. Statistical analysis

The SPSS statistical software was used to analysis. Student *t* test and Chi-square test were used to compare patterns of Y chromosome microdeletions in patients with azoospermia and oligozoospermia. Differences were considered to be statistically significant when $P < .05$.

3. Results

3.1. Amplification curves for different types of Y chromosome microdeletions

Multiplex PCR is a variant of PCR, which permit the simultaneous amplification of many targets in 1 reaction by using more than 1 pair of primers. In this study, 6 pairs of primers were used in 2 separate PCR reactions to check the AZF specific STSs makers, including AZFa (sY84, sY86), AZFb (sY127, sY134), and AZFc (sY254, sY255). Briefly in multiplex A reaction, we detected sY84, sY127, sY255, and the internal control SRY with fluoresces VIC, ROX, Cy5, and FAM separately. In multiplex B reaction, we detected sY86, sY134, sY254, and the internal control ZFX/ZFY with fluoresces VIC, ROX, Cy5, and FAM separately. As shown in Figure 1, the amplification curves represent 5 different types of Y chromosome microdeletions in patients, such as AZFa, AZFb, AZFc, AZFb + c, and AZFa + b + c. In this test, the gDNA form healthy male was

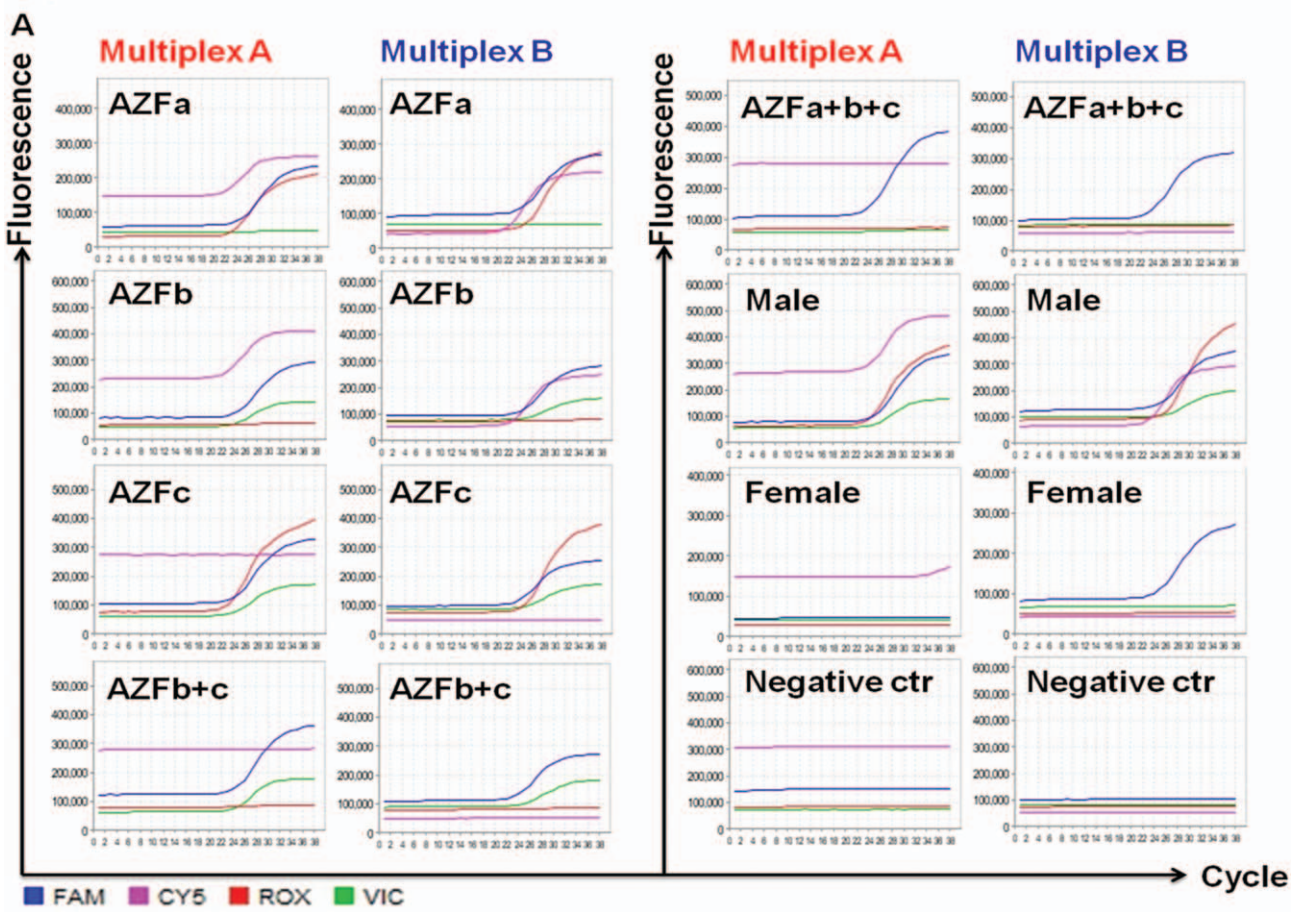


Figure 1. Y chromosome microdeletions detected by multiplex PCR using AZF specific STSs makers. (A) Multiplex PCR amplification results for various Y chromosome microdeletion types in multiplex A: SYR (FAM), sY84 (VIC), sY127 (ROX), sY255 (Cy5) and multiplex B: ZFX/ZFY (FAM), sY86 (VIC), sY134 (ROX), sY254 (Cy5). AZFa (sY84, sY86), AZFb (sY127, sY134), AZFc (sY254, sY255), AZFb + c and AZFa + b + c deletions were detected in different patients. gDNA from healthy male were used as the positive control. gDNA from female and water was used as the negative control. SYR and ZFX/ZFY were used as the internal reference. AZF = azoospermia factor, gDNA = genomic DNA, PCR = polymerase chain reaction, SYR = sex-determining region of the Y chromosome, ZFX = zinc finger protein, X-linked, ZFY = zinc finger protein, Y-linked.

used as positive control, and the ultrapure water was used as negative control. In addition, we also used the gDNA form healthy female to assess the sensitivity and the specificity of the multiplex PCR reaction system, this sample express the ZFX/ZFY only (Fig. 1).

3.2. The test of Y chromosome microdeletions is suitable for the childbearing age men with azoospermia or oligozoospermia

Out of the studied 1274 men with azoospermia or oligozoospermia, there were 164 patients had shown Y chromosome microdeletions (12.87%, Fig. 2A). Among these patients, age from 26 to 30 are the most common person who seeks for the Y chromosome inspection (56.1%, 92/164), followed by age 31 to 35 (18.29%, 30/164), age 21 to 25 (17.68%, 29/164), age 36 to 40 (6.1%, 10/164), age 41 to 45 (1.22%, 2/164), and age 16 to 20 (0.6%, 1/164) (Fig. 2B). Furthermore, 92.1% (151/164) of those with AZF microdeletions come to the hospital seeking for reproductive help, while 7.9% (13/164) just come for prepregnancy checkup (Fig. 2C). Therefore, the Y chromosome microdeletion is an important laboratory test for the male of childbearing age with azoospermia or oligozoospermia. The earlier we get the inspection, the more time we can save for the clinical infertility treatment.

3.3. AZFc is the most frequent type of Y chromosome microdeletions and half of those patients have potential to have the next generation

The occurrence of AZFc microdeletions was found at a rate of 62.20% (102/164) in all the patients with Y chromosome microdeletions (Fig. 3A). In fact, according to our retrospective study, AZFc is the most frequent type of Y chromosome microdeletions. The rate of other microdeletions are followed by AZFb + AZFc (25%, 41/164), AZFb (6.71%, 11/164), AZFa + AZFb + AZFc (25%, 6/164), and AZFa (2.44%, 4/164), respectively (Fig. 3A). In terms of the clinical manifestation of the patients with Y chromosome microdeletions, the different AZF deletion types reflect different clinical manifestation. We found that almost all of AZFa, AZFb, AZFb + c, and AZFa + b + c patients are manifested with azoospermia, however, 45.1% (46/102) AZFc patients are manifested with oligozoospermia (Fig. 3B). These results indicate that nearly half of the patients with AZFc deletion can have sperms through self-ejaculation, which is of great importance for subsequent reproductive

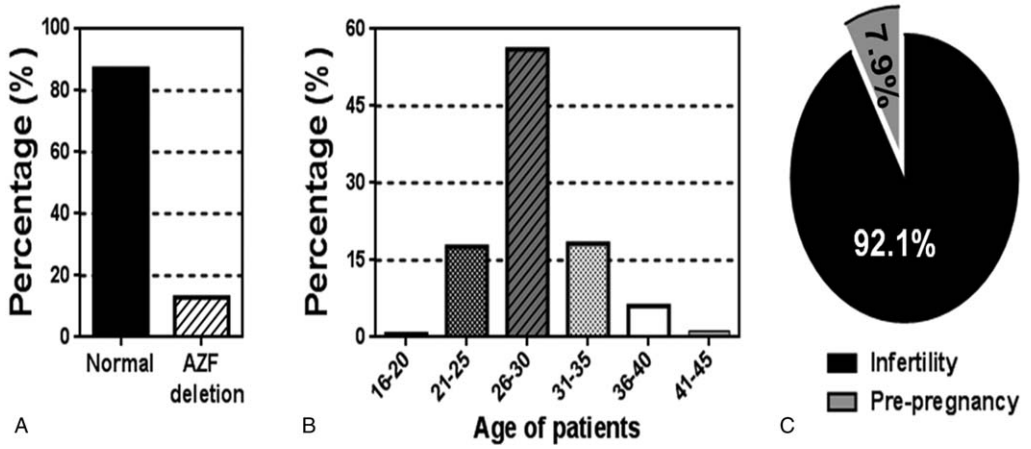


Figure 2. The test of Y chromosome microdeletions is suit for the childbearing age men with azoospermia or oligozoospermia. (A) The microdeletion of Y chromosome, including AZFa, AZFb, AZFc, AZFb+c, and AZFa+b+c, contribute about 12.87% of men with azoospermia or oligozoospermia (164/1274), therefore Y chromosome microdeletions are one of the main causes for male infertility. (B) Among the people who have AZF deletions, men in childbearing age (26–30) are the main group who seeking for the inspection of Y chromosome microdeletion (56.1%, 92/164). (C) In terms of the reasons for seeking Y chromosome microdeletion inspection, 92.1% (151/164) patients are due to the infertility problem, while 7.9% (13/164) patients are due to the prepregnancy checks. Therefore, the test of Y chromosome microdeletions is an important means to screen the cause of infertility in men with childbearing age and then help for guiding clinical reproduction treatments. AZF = azoospermia factor.

treatment. All in all, for the oligozoospermic patients with AZFc microdeletion only, they might have the next generation by the natural conception.

3.4. The early diagnosis of oligozoospermic patients with AZFc microdeletion is conducive to assisted reproductive treatment

As shown in Figure 4, among the 46 patients with AZFc microdeletion who are manifested with oligozoospermia, 28 patients have $0–3 \times 10^6$ sperm from 1 ejaculation, 10 patients have $3.1–20 \times 10^6$ sperm from 1 ejaculation, and 8 patients have more than 20×10^6 sperm from 1 ejaculation (Fig. 4A). Specifically, when compared to older patients (age 31–40), the younger patients (age 20–30) have significantly more sperm counts, which means that the older patients with AZFc microdeletion were eventually developed into azoospermia (Fig. 4B). In terms of the hormone level, the younger oligozoospermic patients (age 20–30) with AZFc microdeletion have higher expression level of testosterone and estradiol, and

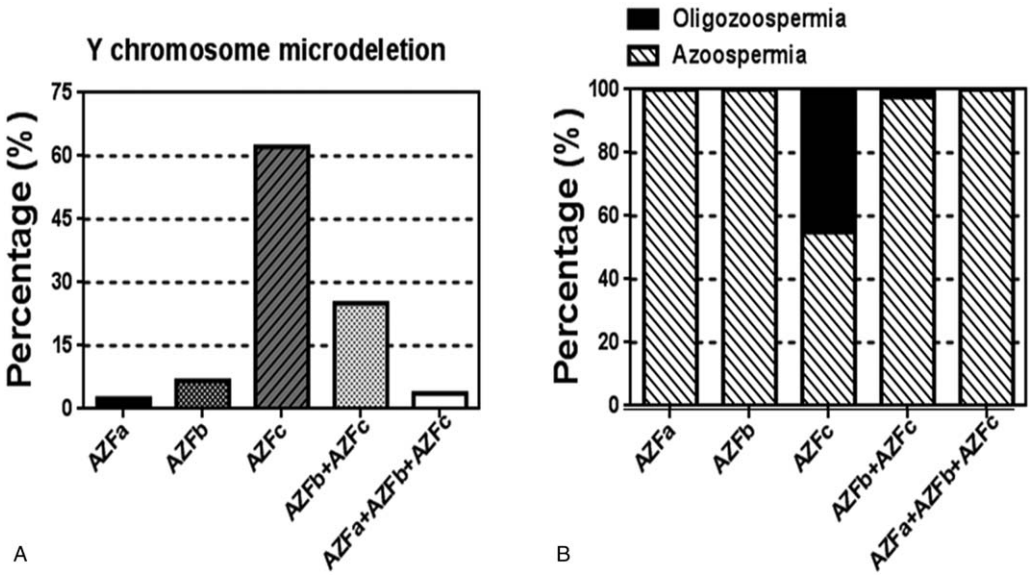
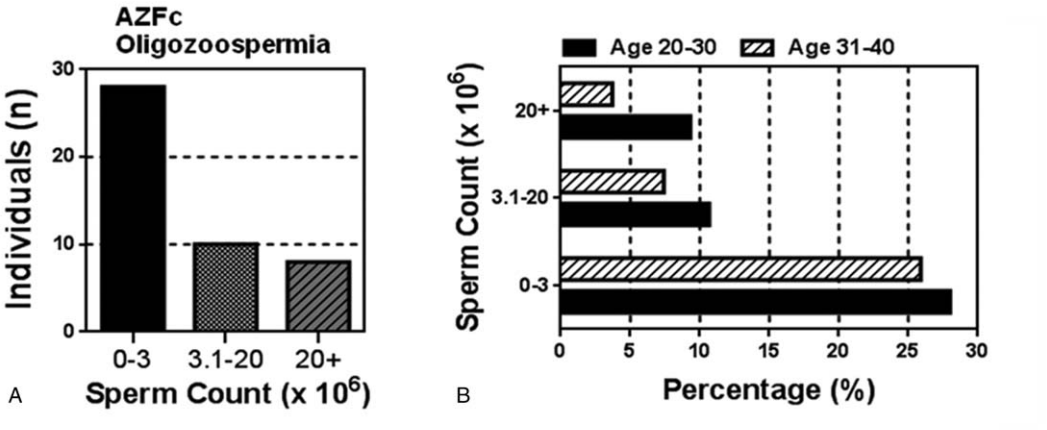


Figure 3. AZFc is the most frequent type of Y chromosome microdeletions and nearly half of the patients with AZFc deletion can have sperms through self-ejaculation. (A) Among the patients who have the Y chromosome microdeletions, 62.20% are AZFc microdeletions (102/164), followed by AZFb+AZFc (25%, 41/164), AZFb (6.71%, 11/164), AZFa+AZFb+AZFc (25%, 6/164), and AZFa (2.44%, 4/164), respectively. Therefore, AZFc is the most frequent type of Y chromosome microdeletions. (B) In terms of the clinical manifestation of the patients with Y chromosome microdeletions, the different AZF deletion types reflect different clinical manifestation. Almost all of AZFa, AZFb, AZFb+c, and AZFa+b+c patients are manifested with azoospermia, however 45.1% AZFc patients are manifested with oligozoospermia (46/102). These data indicate that nearly half of the patients with AZFc deletion can have sperms through self-ejaculation, which is of great importance for subsequent reproductive treatment. AZF = azoospermia factor.



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AZFc_Hormone level					
Age/years	Sperm	T/(ng/mL))	E2/(pg/mL)	FSH/(IU/L)	LH/(IU/L)
20-30	Oligozoospermia	5.6±0.5	51.2±6.0	8.4±1.3	4.0±0.6
20-30	Azoospermia	3.5±0.4	34.9±2.0	14.1±1.4	5.1±0.6
31-40	Oligozoospermia	3.0±0.6	38.6±3.0	8.5±1.4	4.4±0.6
31-40	Azoospermia	2.7±0.4	35.0±2.0	9.2±1.4	4.8±0.6

T, testosterone; E, Estradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

Figure 4. The clinical characteristics of patients with AZFc microdeletion who are manifested with oligozoospermia. (A) Among the 46 patients with AZFc microdeletion who are manifested with oligozoospermia, 28 patients have 0–3 × 10^6 sperm from 1 ejaculation, 10 patients have 3.1–20 × 10^6 sperm from 1 ejaculation, and 8 patients have more than 20 × 10^6 sperm from 1 ejaculation. (B) Compare to older patients (age 31–40), the younger patients (age 20–30) have significantly more sperm counts. (C) In terms of the hormone level, the younger (age 20–30) oligozoospermic patients with AZFc microdeletion have higher expression level of testosterone and estradiol, and lower expression level of follicle-stimulating hormone and luteinizing hormone. These findings suggest that the early diagnosis of AZFc deletion is conducive to assisted reproductive treatment, especially in those patients with a certain amount of sperm. AZF = azoospermia factor.

lower expression level of follicle-stimulating hormone and luteinizing hormone (Fig. 4C). These findings suggest that the early diagnosis of AZFc microdeletion is conducive to assisted reproductive treatment, especially in those young patients with a certain amount of sperm.

4. Discussion

At present, the screening for Y chromosome microdeletions in azoospermic and oligozoospermic patients has already become a routine diagnostic test recommended by EAA and EMON.[14,16] Accumulating studies demonstrate that the deletion of AZFa, AZFb, and AZFc are the most common genetic microdeletions in Y chromosome for infertile male throughout the world.[8] However, the frequency and the pattern of Y chromosome microdeletions in infertile men are varying largely between different race and region, mainly because the Y chromosome microdeletion is a family of genetic disorders caused by missing genes in Y chromosome.[30] Therefore, to our knowledge it is important to understand the frequency and the pattern of Y chromosome microdeletion in people with certain race and birth region. It is reported that the frequencies of Y chromosome microdeletions are 5.42%, 8%, 7.7%, and 10.8% in Turkish, Iranian, Korean, and Chinese people, respectively.[27,31,32] It is worth mentioning that because of the China covers a large territory and has many ethnic groups, the frequency of AZF deletions also varies among Chinese in different regions. In our study, we assessed the frequency of Y chromosome microdeletions restrict in southwest of china. It has been shown that there are 164 patients out of 1274 infertile men in southwest china suffering from the AZF microdeletions, with a prevalence of 12.87%. While the remaining patients without AZF microdeletions may have other factors on infertility, such as unhealthy living habits, endocrine diseases, infections, environmental factors, and psychological stress. These data concluded that the frequency in southwest of China is higher than that the national average (10.8%).

In terms of the pattern of Y chromosome microdeletions in southwest of China, we found that AZFc is always the most common AZF microdeletions type in all the patients with Y chromosome microdeletion, with a frequency of 62.20% in our study. This rate consistent more or less with previous reports.[16,33] As mentioned before, the different type of AZF microdeletions in patients reflect different clinical manifestations. It is reported that the deletion of AZFa and AZFb in Y chromosome portends an exceptionally poor prognosis for sperm retrieval, whereas the majority of infertile men with AZFc deletion have sperm within the semen or testes available for use in IVF/ICSI.[16,34,35] Notably, in our study nearly half of the patients with AZFc microdeletion can have sperms through self-ejaculation and the numbers of the sperms are decreasing with the age. It is also worth mentioning that the most of the patients

diagnosed with Y chromosome microdeletions are aged from 26 to 30 (56.1%) in our study, they were seeking for the reproductive help in the hospital (92.1%). Therefore, these findings revealed that Y chromosome microdeletion is kind of important laboratory test suit for childbearing age men with azoospermia or oligozoospermia. The early diagnosis of Y chromosome microdeletions is conducive to assisted reproductive treatment, especially in those young patients with AZFc deletion.

As we all know, the genetic background might be important in IVF/ICSI outcomes. Therefore, the presence of genetic disease such as Y chromosome microdeletion does have an important infection in assisted reproductive medicine. Several investigations have shown that the azoospermia and oligozoospermia patients are candidate for ICSI or TESE.[15] In our study, we found that only 45.1% AZFc patients are manifested with oligozoospermia (46/102), and almost all the AZFa, AZFb, AZFb + c, and AZFa + b + c patients are manifested with azoospermia. Therefore, ICSI and TESE are not recommended for AZFa, AZFb, AZFb + c, and AZFa + b + c patients, because the chance to retrieve spermatozoa is close to 0. In this case, we sincerely advice all the infertile men to first undergo the screening for the Y chromosome microdeletion before being subjected to reproductive treatment. In this way, we can do the best to save the medical resources and reduce the burden of patients.

The present study has several limitations that must be considered. According to the recommendations of EAA and EMQN, we have checked 6 STSs, including AZFa (sY84, sY86), AZFb (sY127, sY134), and AZFc (sY254, sY255) in this study. However, a number of studies have revealed that a fourth AZF region exists between AZFb and AZFc, which we have termed AZFd. It is reported that the patients with AZFd microdeletions may present with mild oligozoospermia or even normal sperm counts associated with abnormal sperm morphology.[36] Since the clinical manifestations of AZFd microdeletion varies greatly, and the incidence of AZFd in patients is relatively low, we did not discuss AZFd in this study.

In conclusion, the detection of AZF microdeletions in Y chromosome has become the most important genetic test for male infertility problem throughout the world. A better understanding of AZF microdeletions, including the frequency and the characteristics, is of great significance for definitely diagnose the etiology of oligzoospermia and azoospermia, and also the clinical management of both infertile man and his future male offspring. AZFc is the most frequent type of all the AZF microdeletions. The oligozoospermic patients with AZFc microdeletion in our study will likely be able to have sperms through self-ejaculation, and therefore possibly have the next generation through IVF/ICSI. However, the quality and also the amount of sperm in AZFc microdeletion patients were decreasing with age. This suggests that the early diagnosis of Y chromosome microdeletions is conducive to guide reproductive treatment. The earlier we get the inspection, the more time we can save for the clinical infertility treatment.

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Project administration: Ting Liu.
Supervision: Yong-Mei Jiang.
Visualization: Ting Liu.
Writing – original draft: Ting Liu.
Writing – review and editing: Ting Liu, Yu-Xin Song, Yong-Mei Jiang.

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