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OBM Genetics

Original Research

Molecular Study on Y Chromosome Microdeletion in Male Infertility: A Cross-Sectional Design in Indonesian Men

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Academic Editor: Ivan Y Iourov

Collection: Genetic Testing

OBM Genetics
2024, volume 8, issue 1
doi:10.21926/obm.genet.2401216

Received: October 09, 2023
Accepted: February 21, 2024
Published: February 27, 2024

Abstract

Y chromosome microdeletions (YCMs) are one kind of genetic disorder that contributes to male infertility. This study aims to determine the profile of YCMs in the infertile male population in Indonesia. This cross-sectional study was conducted by identifying YCMs testing data on 49 infertile male patients identified with azoospermia and oligoasthenoteratozoospermia (OAT) based on their sperm analysis, who visited andrology

polyclinics in several hospitals in Yogyakarta Province between March 2021 to August 2022. Study participants underwent YCMs testing at the Molecular Medicine and Therapy Research Laboratory, Muhammadiyah University of Yogyakarta, Indonesia, using the Polymerase Chain Reaction (PCR) method according to the procedures established by the laboratory. Four out of 49 (8.2%) participants were identified to have YCMs with deletions in the Azoospermia Factor C (AZFc) subregion. Two participants identified with YCMs had cryptozoospermia in their sperm analysis. Hormonal examination showed variable results in 4 participants, including hypergonadotropic, hypogonadism, and normogonad. All participants in the study identified with YCMs showed a deletion in the AZFc subregion. This type of deletion is different from previous studies in Indonesia, so broad examinations of infertile male patients are required to figure out the deletion profile in a larger population of Indonesian sterile males.

Keywords
Y chromosome microdeletions; male infertility; AZF; azoospermia; oligoasthenoteratozoospermia

1. Introduction

Infertility is defined as the inability of a couple to achieve a clinical pregnancy after one year of regular unprotected sexual intercourse [1]. It is estimated to affect 10-15% of couples globally [2]. The study stated that male factors contributed to 20-70% of infertility cases [3-5]. Sperm analysis and hormonal examination are two essential examinations to support the diagnosis of male infertility [6]. Sperm analysis examination and the interpretation of the results are based on the World Health Organization (WHO) manual procedure for examining and processing human semen, released in 2021 [7]. Oligozoospermia and azoospermia are known to be underlying conditions that contribute to 90% of male infertility cases [8, 9].

Oligoasthenoteratozoospermia (OAT) is a combination of conditions of oligozoospermia (sperm count <15 million/mL) which is subdivided into mild to moderate (5-20 million/mL) and severe (<5 million/mL), asthenozoospermia (progressive motile spermatozoa <32%), and teratozoospermia (normal forms of spermatozoa <4%) [10, 11]. OAT can be idiopathic (iOAT) when there are no possible causes found through physical and hormonal examination, with a prevalence of 30% in all OAT men [12, 13]. Azoospermia is a condition where no spermatozoa cells are found in semen samples through two separate examinations and are estimated to affect 1% of males globally and 10%-15% of all male infertility cases [14, 15]. Azoospermia is divided into two categories, obstructive azoospermia (OA) and nonobstructive azoospermia (NOA), based on the obstruction found in the vas deferens [16].

Infertility is a complex reproductive problem due to the involvement of genetic and environmental factors in its development [17]. Genetic disturbances on the Y chromosome are known to have a critical role in the development of male infertility, especially the presence of microdeletions on the Y chromosome (Y chromosomal microdeletions/YCMs) [18-20]. YCMs are found in 10-15% of azoospermia patients and 5-10% of severe oligospermia patients. This genetic

disorder usually occurs in the azoospermia factor (AZF) locus in the Yq11.23 band and is divided into three subregions: AZFa, AZFb, and AZFc [19, 21-23].

Several methods in Assisted reproductive technology (ART), such as *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), and testicular sperm extraction (TESE), have been widely conducted around the world to overcome the problems of infertility among couples who wish to have a child. However, these methods are also thought to increase the risk of passing YCMs to their offspring. Therefore, it is essential to conduct a YCMs testing before teaching ART [24, 25]. WHO in 2021 also recommends that genetic and genomic examinations are necessary to be undertaken because chromosomal abnormalities (including microdeletions) and genetic mutations can be the underlying causes for various forms of problems in male infertility [7]. Detection of Y chromosome microdeletions has not been widely carried out and is not a routine examination in IVF laboratories in Indonesia. This study aims to determine the YCM phenomena in the Indonesian population. The difference in the prevalence of YCMs in each country is possible due to differences in the genetic profile of each individual. Therefore, YCMs must be detected to determine the gene profile present in infertile male patients in Indonesia.

2. Materials and Methods

2.1 Study Design

This is a cross-sectional descriptive study including YCMs testing results of 49 infertile male patients who visited andrology polyclinics in several hospitals in Yogyakarta Province between March 2021 to August 2022. The total sampling technique was used in this study by identifying YCMs examination data on all patients who met the study inclusion criteria within a specific period. Samples from normal fertile male respondents were used as positive controls in this study, while samples from female respondents were used as negative controls.

The inclusion criteria in this study were male patients with idiopathic infertility aged 20-50 years whose female partner had not obtained pregnancy after one year of regular unprotected sexual intercourse**.** Sperm analysis is carried out based on WHO 2021 guidelines, with normal values referring to the provisions of these guidelines. Males 20-50 years old having hormonal abnormalities, immunological disorders associated with male infertility, and incomplete examination data were excluded from this study. Patient examination data were collected after obtaining ethical approval from the Research Ethics Commission of PKU Muhammadiyah Gamping Hospital, Yogyakarta, Indonesia (148/KEP-PKU/VII/2022, dated July 22, 2022).

2.2 Y Chromosome Microdeletion Analysis by the Polymerase Chain Reaction (PCR)

YCMs testing was carried out at the Molecular Medicine and Therapy Research Laboratory, Muhammadiyah University of Yogyakarta, Indonesia, with the following procedure: Peripheral blood was taken from a venous vessel, which was then collected within a blood tube with an EDTA anticoagulant (Vaculab, China). Following the manufacturer's protocol, the blood samples were further processed using the gDNA extraction kit (Geneaid, Taiwan). The isolated and purified gDNA samples were then used as a PCR template for further target gene amplification. gDNA template was mixed with the 2X HS Red-Mix reaction solution (Bioline, UK), Nuclease Free Water (Promega,

USA), specific primer set (AZFa prox-2, AZFa dist-1, SY 127, SY 134, SY 254, and SY 255). In contrast, the sY1532 primer set was applied for Y-chromosome control gene (Table 1).

Table 1 Sequence-Tagged Site (STS) and primer sequences.

STS	Region	Primer sequence	Product size (bp)
sYprox2	AZFa	F: GGTTCCTGAACAGGGGACT R: GGCAGCAGAAGGGCCTCTC	220
sYdist1	AZFa	F: GGTTCCTGAACAGGGGACT R: GGCAGCAGAAGGGCCTCTC	390
sY127	AZFb	F: GGCTCACAAACGAAAAGAAA R: CTGCAGGCAGTAATAAGGGA	274
sY134	AZFb	F: ACCACTGCCAAAACTTTCAA R: GTCTGCCTCACCATAAAACG	301
sY254	AZFc	F: GGGTGTTACCAGAAGGCAAA R: GAACCGTATCTACCAAAGCAGC	350
sY255	AZFc	F: GTTACAGGATTCGGCGTGAT R: CTCGTCATGTGCAGCCAC	126
sY1532	SRY	F: TCCTTAGCAACCATTAATCTGG R: AAATAGCAAAAAATGACACAAGGC	167

PCR reactions were performed under 3 setting conditions according to the target gene. AZFa prox-2 gene was amplified under the initial denaturation at 94°C for 15 minutes, denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, extension of 65°C for 1 minute, and final extension of 65°C for 2 minutes. AZFa dist-1 gene was amplified under the initial denaturation of 94°C for 15 minutes, denaturation of 94°C for 1 minute, annealing of 48°C for 1 minute, extension of 65°C for 1 minute and final extension at 65°C for 2 minutes, and the settings for remaining targets SY 127, SY 134, SY 254, & SY 255 were applied by initial denaturation of 94°C for 15 minutes, denaturation of 94°C for 30 seconds, annealing of 51°C for 30 seconds, extension of 72°C for 15 minutes 30 seconds and 72°C final extension for 7 minutes. 25 times the total cycle number had been carried out for AZF gene detection. The amplification cycle number was determined to be 25 times. The amplification products were separated by using the agarose gel electrophoresis method. HPCR acyl mixed with loading dye (Genedirex, Taiwan) and electrophoresis on 3% agarose gel (NZYtech, Portugal) with 1X GelRed (Biotium, USA) had been applied before final visualization. Electrophoresis results were observed through UV transilluminators for further analysis.

2.3 Statistical Analysis

Statistical analysis was not performed in this study. Collected research data is tabulated into a Microsoft Excel table (Microsoft Corp., Redmond, Washington, United States) and presented as a frequency distribution table.

3. Results

This study observed the YCMs testing on 49 infertile male patients who underwent consultation at the andrology polyclinic of several hospitals in Yogyakarta. Table 2 shows the number of patients screened for YCMs in relation to their sperm analysis results, as well as the number of YCMs detected in each category: 42% azoospermia, 8% cryptozoospermia, 2% oligozoospermia, 28% mild to moderate OAT, 8% severe OAT, and 10% extreme OAT.

Table 2 Frequency of YCMs in 49 patients with abnormal spermiogram.

Seminal parameters	Numbers screened [n (%)]	Numbers deleted [n (%)]
Azoospermia	21 (42)	1 (4.8)
Cryptozoospermia	4 (8)	2 (50)
Oligozoospermia	1 (2)	0 (0)
Mild to moderate OAT	14 (28)	0 (0)
Severe OAT	4 (8)	0 (0)
Extreme OAT	5 (10)	1 (20)

Four out of 49 infertile male patients (8.2%) were identified to have YCMs with deletions in the AZFc subregion (Figure 1).

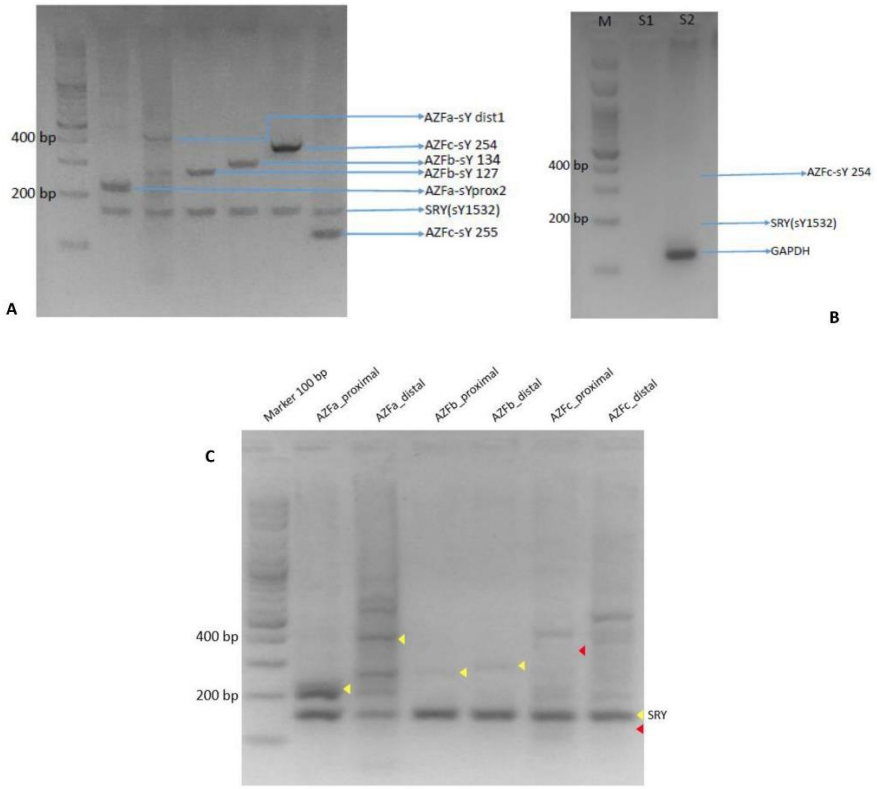

Figure 1 The agarose gel electrophoresis result was considered reliable since the SRYs gene targets were detected within each sample. a) Normal male sample as positive control; b) Female sample as negative control; c) Both AZFa and AZFb were detected approximately similar to expected product size as shown in Table 1 (AZFa: 220 and 390 bp, AZFb: 274 and 301 bp) and were pointed by yellow arrow-head marks. On the other hand, neither AZFc proximal nor distal targets were detected. The image above is the result of YCM testing on an AP patient, which shows the microdeletions at the same locus in 3 other patients.

Two patients (SA and MY) were identified with cryptozoospermia in their sperm analysis, whereas NH and AP showed extreme OAT and azoospermia, respectively, in their sperm analysis examination (Table 3). Hormonal profiles in AP and SA (Table 3) showed hypergonadotropic results with FSH levels of 19.41 mUI/mL and 24 mUI/mL, respectively. The results of testosterone examination in AP patients showed levels below normal (1.91 ng/mL), which indicated the condition of hypogonadism. Hormonal examination results in the other patients (NH and MY) showed normal testosterone and FSH levels.

Table 3 Sperm analysis and hormonal profile of patients with microdeletion of AZFc subregion.

ID	Age/years	Semen analysis	Hormonal level Testosterone (2.27 ng/mL-10.30 ng/mL)	FSH (1.7 mUI/mL-12.0 mUI/mL)	Types of YCMs
NH	28	Extreme OAT	8.397	9	AZFc
AP	28	Azoospermia	1.91	19.41	AZFc

SA	48	Cryptozoospermia	4.22	24	AZFc
MY	32	Cryptozoospermia	3.44	6.55	AZFc

4. Discussion

We conducted an observational study on YCMs data of 49 infertile male patients for one year, and 4 male patients (8.2%) showed AZFc deletion. Y chromosome microdeletion is a male infertility factor and increases the risk of inheriting the chromosomal abnormalities in the male offspring [26]. In 2021, WHO recommends the examination of chromosomal abnormalities as an essential examination for identifying male infertility problems [7]. European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN) also recommend the YCMs testing in patients with azoospermia and severe oligozoospermia [27]. YCMs are the second cause of male infertility related to genetic disorders after Klinefelter syndrome, so its examination is highly recommended in patients with severe male infertility using the multiplex PCR technique as the gold-standard examination [27-30].

Colaco and Modi stated that approximately 7.5% Y chromosome microdeletion was identified among 40.000 Y chromosomes of infertile men with various demographic, cultural, and ethnic backgrounds [31]. The previous study conducted in Jakarta, Indonesia, on 71 men with azoospermia and severe oligozoospermia showed that 11 men (15.49%) had AZFa partial deletion, with the majority of semen analysis results being azoospermia [32]. The second study conducted in Semarang, Central Java, Indonesia, showed that 10 of 36 infertile male participants (27.78%) had YCMs, with the most common deletion occurring in AZFa (50%) [33]. A study by Hanizar and Hinting also showed the highest prevalence of deletion in the sY86 gene (AZFa subregion) occurring in all spermatozoa assay categories with low sperm quality and quantity [34]. This is in contrast to the results of our study, where 4 among 49 men with YCMs (8.2%) were identified as having deletions in the AZFc subregion with the most prevalence of semen analysis was cryptozoospermia (50%). This is unique because the same population in Indonesia has different characteristics of YCMs. Another research indicates that race and ethnicity might influence the YCMs subtype [31].

Our study's results align with a study conducted by Iijima, which stated that 12 out of 20 (60%) male patients with severe oligospermia had AZFc deletion and showed cryptozoospermia on the result of the sperm analysis [35]. The two patients with azoospermia and extreme OAT (Table 3) in this study also showed AZFc deletion. This result is similar to the survey conducted by Miraghazadeh et al., who stated that 9 of 90 (10%) patients with a positive microTESE outcome and 7 of 110 (6.3%) patients with a negative microTESE outcome showed AZFc partial microdeletion [36]. AZFc deletion in OAT is rarely reported, but there is one case report of a primary infertile male patient with 5 years of infertility who showed OAT in his sperm analysis and had complete AZFc deletion. This study stated a possible correlation between OAT, AZFc deletion, and oxidative stress, although the mechanism of their correlation is not yet clearly understood [37].

A study by Kim et al. stated that male infertile patients with AZFc deletion showed low sperm numbers from both testicular tissue and ejaculation [38]. Spermatogenesis disorders are closely related to genetic factors, such as chromosomal abnormalities that can be identified through karyotype analysis and examination of Y chromosome microdeletions in the AZF region [39-41]. YCMs testing has not been widely conducted as an initial standard examination applied in the ART process in Indonesia. Conducting ICSI procedures using ejaculated sperm in the case of AZFc

deletion showed a more satisfactory outcome than using the testicular tissue sperm [38, 42]. A case report by Hu et al. stated that patients with deletion of AZFc conceived pregnancy after the ICSI procedure. There were no birth defects identified by prenatal diagnosis examination, but there was an increased risk of trisomy 21 syndrome on maternal serum examination in the second trimester [43]. A study by Xi et al. showed that male patients with AZF deletion who underwent ICSI procedures could conceive a good clinical pregnancy outcome. There were no differences in neonatal and perinatal outcomes in male patients with AZFc deletion and non-deletion [44].

The success of conceiving clinical pregnancy in the case of AZF deletion raises concerns about the risk of inheriting the disorder in the offspring. A previous study of 452 patients with NOA and severe oligozoospermia showed that 44 had AZF microdeletions. Tracing the family tree in 19 cases of AZFc deletion showed vertical transmission of AZFc deletion in 6 cases (31.6%). Subjects with vertical transmission of AZFc deletion from fertile fathers showed different effects on their fertility status [45]. Infertile men with AZF deletion who will undergo ART process are advised to take genetic counseling to anticipate the risk of AZF deletion vertical transmission in the next generation [35].

Testosterone and FSH hormonal profiles in four male infertile patients identified with AZFc deletion are shown in Table 3. One patient with azoospermia (Table 3) showed a testosterone level below the normal range value and an FSH level above the normal range. In comparison, the results of hormone examinations in SA patients (Table 3) with cryptozoospermia showed FSH levels above the normal range. Hormonal profiles in patients with YCMs in previous studies have shown various results. A prior study by Bahmanimehr et al. showed that FSH levels in male patients with YCMs were significantly higher than those without YCMs [46]. The results of our study are in line with the previous survey of Damdinsuren et al. who conducted research among 75 infertile Mongolian men. This study showed a higher FSH level in YCMs male patients than non-YCMs [47]. YCMs testing still needs to be carried out, considering that azoospermic patients with a microdeletion in the AZFc subregion can still be considered for micro-TESE [48].

5. Conclusions

The results of this study indicate that all participants identified with YCMs had deletions in the AZFc subregion. These results differ from several studies conducted on the Indonesian population. This study is very important to show the urgency of YCMs testing because this is not a routine laboratory examination in Indonesia due to limited resources in the laboratory, and not all infertility service units have the facilities to conduct this examination. We hope to identify more cases to be used as a basis for implementing a routine YCMs examination in infertility service units in Indonesia per WHO recommendations.

Acknowledgments

We are thankful to all the Molecular Medicine and Therapy Research laboratory (MMT Lab) staff who offered excellent technical help during the study.

Author Contributions

Conceptualization, D.M.R., I. S., A.A., A.W., and S. Methodology, D.M.R., I. S., A.A., A.W., and S. Software, A.A. Validation, D.M.R. Formal Analysis, D.M.R., A.A., and N.S. Investigation, D.M.R., and

A.A. Resources, D.M.R., and A.A. Data Curation, D.M.R., A.A., and N.S. Writing – Original Draft Preparation, D.M.R., A.A., and N.S. Writing – Review & Editing, D.M.R. and N.S. Visualization, A.A. Supervision, D.M.R. Project Administration, D.M.R. and N.S. Funding Acquisition, D.M.R., I. S., A.A., A.W., and S.

Funding

Research of Foreign Cooperation and International Publications (*Penelitian Kerja Sama Luar Negeri Dan Publikasi Internasional*), Universitas Muhammadiyah Yogyakarta, Yogyakarta, Indonesia.

Competing Interests

The authors have declared that no competing interests exist.

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