Y-Chromosomal Microdeletion in Idiopathic Azoospermic and Severe Oligozoospermic Indonesian Men

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ABSTRAK

Tujuan: *mendeteksi mikrodelesi kromosom Y pada pria dengan azoospermia atau oligozoospermia berat menggunakan multipleks PCR.* ***Metode:*** *kami menggunakan 2 multipleks PCR untuk mengamplifikasi regio AZF pada 71 pria. Kriteria inklusi adalah pria azoospermia atau oligozoospermia berat, dengan atau tanpa kelainan motilitas atau morfologi sperma, FSH meningkat atau normal, LH dan testosteron normal, dan tidak ada tumor testis atau kelainan lainnya. Lima pria normal berpartisipasi sebagai kontrol.* ***Hasil:*** *delesi parsial AZFa ditemukan pada 11 pria (15,49%), delesi komplit AZFb pada 1 pria (1,4%), dan delesi komplit AZFc pada 1 pria (1,4%). Tipe delesi tidak spesifik juga terdeteksi, yaitu delesi gen DBY pada 2 pria (2.81%), dan delesi parsial AZFa dan AZFb pada dua pria (2.81%). Pada pria kontrol tidak ditemukan delesi. Tipe delesi AZFa dan AZFb menunjukkan gangguan spermatogenesis pada sebagian tubulus, yaitu spermatogenesis berhenti pada fase spermatosit, sedangkan delesi gen DBY menunjukkan gambaran sel sertoli saja (SCO) pada semua tubulus.* ***Kesimpulan:*** *frekuensi delesi parsial AZFa relatif tinggi pada penelitian ini. Tipe delesi menunjukkan gambaran gangguan spermatogenesis yang berbeda pada histopatologi testis.*

Kata kunci: *azoospermia, infertilitas pria, mikrodelesi, multipleks polymerase chain reaction, kromosom Y.*

ABSTRACT

Aim: *to detect Y-chromosomal microdeletion in Indonesian men with azoospermia or severe oligozoospermia using multiplex PCR.* ***Methods:*** *we performed 2 multiplex PCR amplifications of the Azoospermia Factor (AZF) region in 71 men. Criteria for including a patient were fulfilled if they presented with azoospermia or severe oligozoospermia, with or without additional abnormalities of sperm motility or of head morphology, raised or normal levels of FSH, normal levels of LH and testosterone, and with no evidence of testicular tumors or other abnormalities. Five men participated as control persons.* ***Results:*** *partial deletion of AZFa was found in 11 men (15.49%), complete deletion of AZFb in 1 man (1.4%), and complete deletion of AZFc in 1 man (1.4%). The unspecific type of deletion was also detected, including the DBY gene in 2 men (2.81%), and partial deletion of both AZFa and AZFb in 2 men (2.81%). No AZF deletion was observed in the control probands. Related to the type of deletion, the AZFa and AZFb deletion showed spermatogenesis arrest in most tubules, while deletion of the DBY gene is associated with the sertoli cell only (SCO) syndrome.* ***Conclusion:*** *the frequency of partial deletion of AZFa was found to be relatively high in our center. The type of deletion is associated with the testicular histology.*

Keywords: *azoospermia, male infertility, microdeletion, multiplex polymerase chain reaction, Y chromosome.*

INTRODUCTION

Infertility is a major health problem that affects 10-15% of couples who are unable to have children. Infertility in men is known to be the cause of around 50% of these cases.[1,2] One of the causes of infertility is azoospermia varying between 10-15% in infertile men.[3]

With the development of assisted reproductive techniques, especially intra cytoplasmic sperm injection (ICSI), infertile men may well have a chance to become a father. However, several current researches suggest that as many as 10-15% of men with azoospermia or severe oligozoospermia have a genetic abnormality.[4] The two most common causes of genetic abnormality in infertile men are Klinefelter syndrome (47 XXY) and microdeletion of AZF region.[5] Therefore, it is highly recommended that microdeletion analysis is performed in those men with azoospermia or severe oligozoospermia prior to the TESE/ICSI procedure. The examination covers the diagnosis, management and prognosis of symptoms.[2,6]

To date, Y-chromosomal microdeletion screening has been performed in azoospermic and severe oligozoospermic men with differing results.[7-9] It has been proposed, however, that patients with microdeletion involving AZFa and b should not undergo the Testicular Sperm Extraction (TESE)/ICSI procedure. The microdeletion detection method was established some time ago, in 1990, presenting inconsistent results due to the widely differing sequences in the Y chromosome and the many homologous locations. Therefore, Simoni et al.[8] published a paper on the analysis of Y chromosome microdeletion with appropriate internal control in standardized multiplex Polymerase Chain Reaction (PCR) format as the methodology advocated by the European Academy of Andrology (EAA)/European Molecular Genetics Quality Networks (EMQN).[8]

So far, microdeletion analysis is still uncommon and not routinely carried out as a diagnostic procedure in patients undergo TESE/ICSI in Indonesia. Research has been conducted by Suryandari DA et al.[10], who found that the frequency of microdeletions in Indonesian men with azoospermia was 5.7%. The prevalence is apparently less than the prevalence obtained from a number of similar studies performed in different countries (10-15%).[8] This fact might be due to the difference of genetic backgrounds in Indonesia, as compared to other countries, including the genetic causes of azoospermia. Another possibility might be due to differences in the methodology used. Hence, we performed Y- chromosomal microdeletion screening based on the EAA/EMQN method in idiopathic azoospermic or severe oligozoospermic patients presenting at our urologic clinic between 2010 and 2012.

METHODS

Seventy one patients from the Urologic Clinic of Cipto Mangunkusumo Hospital between April 2010 and April 2011 were clinically evaluated and found to have azoospermia or severe oligozoospermia (≤5 million/ml). Consecutive sampling method was used for recruiting the patients. Each patient was questioned and examined for testicular volumes, varicoceles, potential epididymal or prostate abnormalities, potential testicular tumors, seminal analysis and hormonal profile. An informed consent was obtained from all patients after approval local ethics committee (132/PT02.FK/ETIK/2010).

Criteria for including a patient in this study were fulfilled if they presented with azoospermia or severe oligozoospermia, with or without additional abnormalities of sperm motility or of head morphology, raised or normal levels of FSH, normal levels of LH and testosterone, and with no evidence of testicular tumors or other abnormalities. This patient group was asked to present an analysis of the respective cytogenetic chromosomes in order to exclude chromosome abnormalities associated with infertility. Only 2 patients gave their consent to undergo cytogenetic analysis.

Five fertile males joined this study as normal control persons and 5 females as control persons for DNA contamination during the whole procedure. We randomly chose men of our clinic member who have normal sperm analysis and hormone parameter to be a fertile control. The mean age is 33 SD 4.2 years. The five fertile men and 5 females, from our clinic member, gave their consent to be part of the research.

Genomic DNA Isolation from Blood Samples and Testicular Testis Biopsy

Genomic DNA was extracted from blood samples (3 ml) from all patients and control persons. Testicular testis biopsy was carried out following consent from those patients at the same time of TESE procedure in our clinic. The tissue samples of testicular biopsy was placed in a tube with Bouin's solution and sent to Department of Biology Faculty of Medicine Universitas Indonesia for full report of spermatogenesis activity. This samples also placed in formalin solution 70% and sent to pathologic anatomical analysis for potential of tumors.

Multiplex PCR Assay for AZF Microdeletion and Interpretation

Two multiplex PCR reactions (A and B) were used to identify deletions in AZFa, b and c regions. Both multiplex reactions contained 5 fragments, 3 fragments marking the AZF loci and 2 fragments the ZFY and SRY gene sequence. ZFY and SRY genes were used as a control. Twenty five μL PCR reaction solution was made up of 5 μL KAPA 10x buffer (containing MgCl2 15mM), 0.5 μL10mM dNTP, 0.1μL KAPA Taq Polymerase enzyme, 0.25 μL 10x primer mix (2 μM each primer), 0.8 μL DNA samples, and H2O up to 25 μL. 10X Primer Mix A and B is made by mixing each primer according to the multiplex A and B. The primer mix solution was then stored at 4°C temperature. All primer sequences are according to Foresta C, et al.[11]

For DNA amplification, the process begins with the initial activation at a temperature of 95°C for 2 minutes, followed by 35 cycles of denaturation (94°C) for 15 seconds, annealing at 58°C for 30 seconds, the elongation phase at 72°C for 10 minutes, and ending with the elongation phase end 72°C for 2 minutes. The cooling process was performed at 4°C. The 7 mL reaction products were separated by 2.5% of agarose gel at 50 volt for 3.5 hours. The PCR machine condition is based on the recommendation of KAPA 2GTM Fast Hot Start Ready Mix cycling parameters, with annealing temperature modification.

RESULTS

Seventeen out of 71 patients had microdeletion in their Y chromosome (23.94%). The mean age of evaluated patients was 35.98 SD 6.12 years. The partial deletion of AZFa is the most common type deletion (15.49%). The frequency of microdeletion is given in **Table 1**. The clinical characteristic of men with Y-chromosomal microdeletion is given in **Table 2**.

In the PCR reaction, partial deletion of AZFa is characterized by absence of one of the two

Table 1. The frequency and types of observed Y chromosome microdeletion in AZF regions

Type of microdeletion	Frequency, n (%)
Partial deletion of AZFa	11 (15.49)
Complete deletion of AZFb	1 (1.40)
Complete deletion of AZFc	1 (1.40)
Deletion of DBY gene exon 2	2 (2.81)
Partial deletion of AZFa and AZFb	2 (2.81)
Undetected microdeletion	54 (76)

Table 2. Clinical charateristics of men with Y-chromosomal microdeletions

ID	Semen analysis	Hormones			Types of microdeletion	Testicular histology
		FSH	LH	T		
RA	Azoospermia	6.6	NA	3.8	Partial deletion of AZFa	NA
ME	Azoospermia	5.6	2.6	5.6	Partial deletion of AZFa	NA
PU	Azoospermia	8.2	6.2	6.1	Partial deletion of AZFa	NA
RD	Azoopspermia	15.2	NA	3.3	Partial deletion of AZFa	MA
AP	Severe oligospermia	4.4	4.5	4.3	Partial deletion of AZFa	NA
MD	Azoospermia	7.5	4.4	7.1	Partial deletion of AZFa	NA
FN	Azoospermia	18.2	NA	2.6	Partial deletion of AZFa	MA
SY	Azoospermia	10.5	4.3	2.9	Partial deletion of AZFa	NA
LC	Azoospermia	9.8	NA	3.0	Partial deletion of AZFa	NA

Table 2. Clinical charateristics of men with Y-chromosomal microdeletions

ID	Semen analysis	Hormones			Types of microdeletion	Testicular histology
		FSH	LH	T		
DN	Severe oligospermia	5.6	NA	3.2	Partial deletion of AZFa	NA
TF	Azoospermia	4.4	NA	4.5	Partial deletion of AZFa	NA
WD	Azoospermia	30.5	8.8	5.2	Deletion of DBY gene exon 2	SCO
HN	Azoospermia	6.2	NA	6.8	Deletion of DBY gene exon 2	NA
AF	Azoospermia	25.6	NA	1.5	Complete deletion of AZFc	NA
YR	Azoospermia	8.4	NA	4.4	Complete deletion of AZFb	MA
HN	Azoospermia	4.4	NA	4.8	Partial deletion of AZFa and AZFb	NA
AP	Azoospermia	79	9.1	6.4	Partial deletion of AZFa and AZFb	NA

FSH=Follicle Stimulating Hormone (mIU/ml); LH=Leutenizing Hormone (mIU/ml); MA= maturation arrest; NA= not available; SCO= Sertoli-cell only; T=Testosterone (ng/ml)

primers, sY84 and sY86 (**Figure 1**). Complete deletion of AZFb is characterized by the absence of both primers, sY127 and sY134 (**Figure 2**).[8] Complete deletion in the AZFc region is characterized by the absence of primers, sY255 and sY254.

The unspecific types of deletion found in our study were the deletion of DBY gene exon 2 (**Figure 3**) and partial deletion in both of AZFa and AZFb. This kind of deletion has not yet been reported. In partial deletion of AZFa and AZFb, PCR reaction of multiplex B showed absence of sY84 and sY134. In multiplex A, all primers amplified the AZFs region.

Testicular Histology in Males with Microdeletion

We analyzed the testicular histology of 2 patients with partial deletion of AZFa, 1 patient with AZFb, and 1 patient with DBY gene exon 2. Patients with partial deletions of AZFa indicated divergent spermatogenesis activity between the tubules (**Figure 4**).

The testicular histology of patient with complete deletion of AZFb showed maturation arrest at the primary spermatocyte stage in all tubules (**Figure 5 left**). Patients with deletion of DBY gene exon 2 showed signs of SCO syndrome in the testicular histology (**Figure 5**

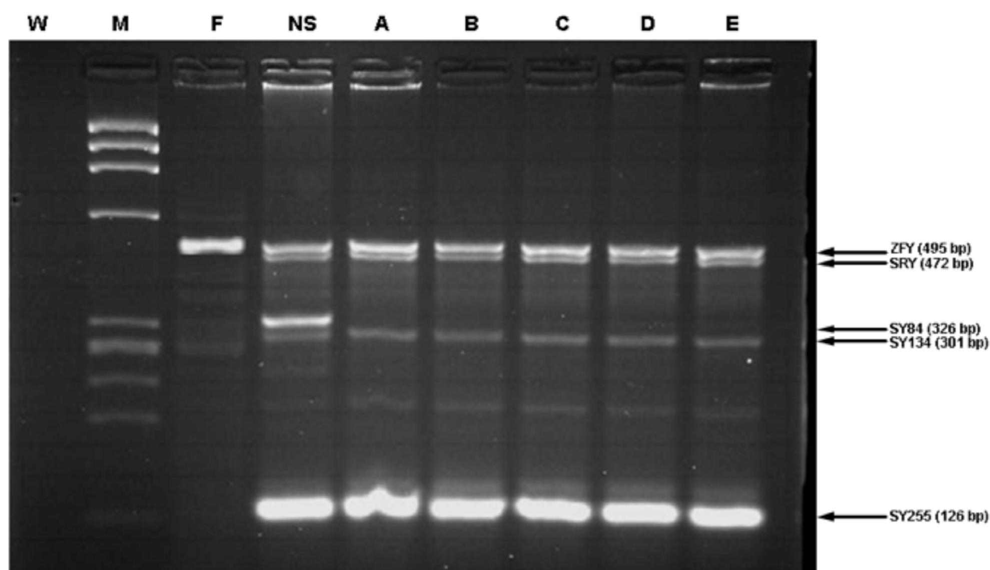

Figure 1. Identification of partial deletion of AZFa. Multiplex PCR B was performed to identify AZFa partial deletion. The absence of PCR fragment sY84 in patients' samples (line A-E) showed AZFa deletion. W=Water, M=Male, F=Female, NS= Male Normal Sample, A-E= Patients.

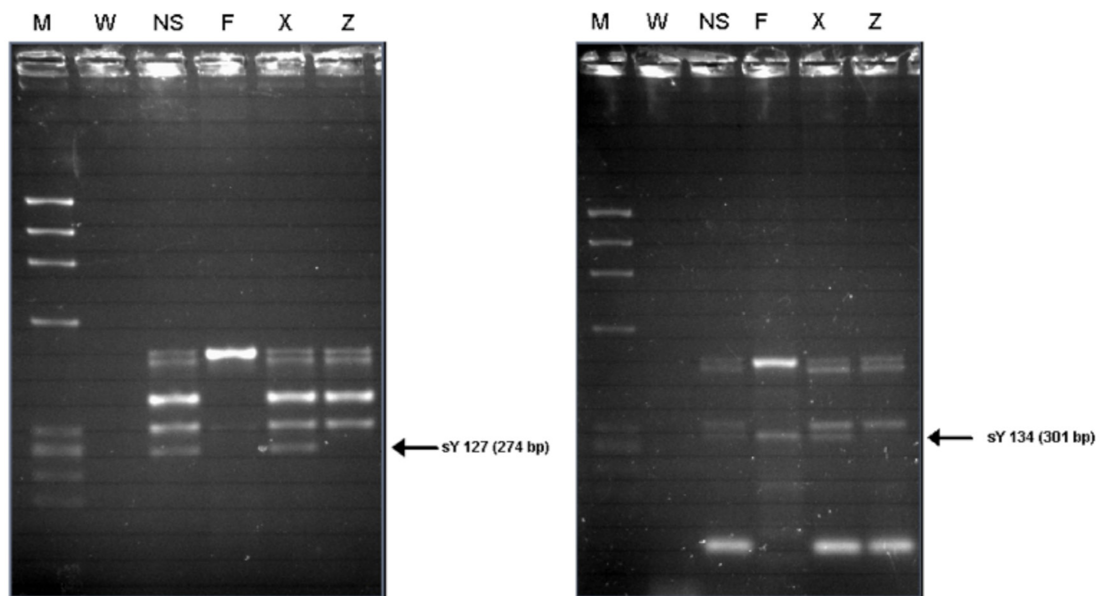

Figure 2. Identification of complete deletion of AZFb. The deletion was marked by the absence of sY127 and sY134 PCR fragments in sample Z. M=marker (Px174), W=water, NS=normal sample, F=female, X and Z= patient.

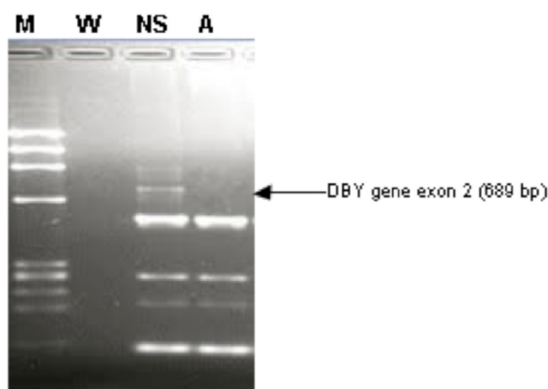

Figure 3. Identification of deletion of DBY gene. The deletion was marked by the absence of DBY gene exon 2 fragments in sample (arrow). M=marker, W=water, NS=normal sample, A=patient.

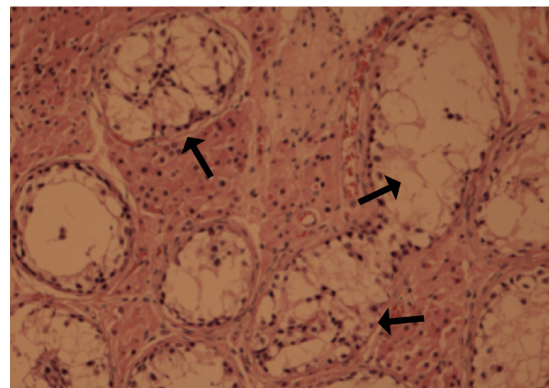

Figure 4. Testicular histology in patients with partial deletion of AZFa sequence. Shown is the divergent image of tubuli and sperm (indicated by the arrow).

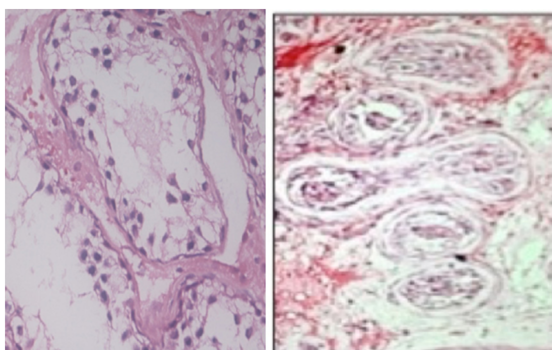

Figure 5. Testicular histological examination. Patient with complete deletion of AZFb showed spermatogenesis arrest at spermatocyte phase (left panel). Patient with complete deletion of DBY gene showed sertoli cells only syndrome (right panel).

right). Patients with non-specific deletion had not yet presented with a testicular histology.

DISCUSSION

Based on our study, the frequency of partial deletion of AZFa is relatively high. According to many international publications, most types of deletions are AZFc and only infrequently seen in the AZFa region.[8,12-16] In a study using the Indonesian population as probands, with different PCR method and primers, Suryandari DA, et al.[10] found AZFa and AZFb deletion in 2.8% and AZFc deletion in 2.8% patients

(n=35).10 They used duplex PCR, not multiplex, and sY14(SRY), sY84 (AZFa), RBMY1 (AZFb), sY 254 and sY255 (AZFc) as primers.

Deletion of the AZFa region is rare in all populations (about 2% in men with impaired spermatogenesis).[17] Therefore, it may be concluded that the literature, to date, presents very limited information on the AZFa region.

The possible explanation of high prevalence of partial AZFa microdeletion (sY84- absent, sY86-present) in our patients may be due to false positives of AZFa microdeletion caused by single-nucleotide polymorphism (SNP) as proposed by Qing Wu et al.[18] They found that there were single-nucleotide alteration of T to G in the target sequence of the reverse sY84 primer (rs72609647). This SNP is more prevalent in the Chinese ethnic group than other. They suggest that it will be necessary to confirm genotype this SNP if sY84 is absent in AZF screening, especially for patients in Chinese population.[18]

The sY84 and sY86 are the choice of primers for detecting AZFa microdeletion in Caucasian population to avoid polymorphism, according to EAA guidelines. However, The primers may not apply to other ethnic population.[18] Our patients were consisting of many ethnic groups, such as Javanese, Betawi, Batak, Minangkabau, and Chinese. We did not note some patients with Chinese ethnic group had this partial AZFa microdeletion. However, we cannot exclude the false positives of AZFa microdeletion caused by SNP in our samples because we did not sequence the sY84 locus. This can be considered as limitation of our study. Therefore, sequencing the genomic DNA sequence of sY84 will be needed in the future study to exclude this possibility of false positives results.

Partial AZFa deletions have been identified in some infertility clinics[19] not related to the pathology of testicular tissue, as suggested by Vogt et al.[20] Pathological examination of testicular tissue shows a varied picture of spermatogenetic activity. Testicular tissue biopsy performed in patients with partial deletion of AZFa often showed hypospermatogenesis, but not SCO.[11,20] In our study, the testicular histology of patients with partial deletion of AZFa was found to be similar to that in the literature, not demonstrating the SCO syndrome.

Complete deletions of AZFb showed a histological form of spermatogenesis arrest at primary spermatocyte. This result is consistent with the literature that complete deletion shows a picture of spermatogenesis arrest in all tubules.

We identified another type of deletion in our study, namely, deletion of DBY gene. Previous examinations using standard primers for the detection of AZFa, b and c regions showed no deletions.

To date, the candidate genes that regulate spermatogenesis are USP9Y (Ubiquitin-specific protease 9y copy) and DBY (Dead Box Y). Both of these genes are present in the AZFa region. The USP9Y is known to be dominant in the post-meiotic phase of spermatogenesis.[21]

Research on the DBY gene protein expression inferred that this protein is only found in human testicular tissue, and is dominant in the spermatogonia and spermatocytes leptoten. This fact demonstrates that the gene is functionally contributing to the process of cell differentiation in the pre meiosis phase. Therefore, DBY can now be considered as the primary regulator of spermatogenesis genes in the region AZFa.[22] The DBY genes often deleted in infertile patients may lead to severe damage to spermatogenesis, ending in a decrease in the number of germ cells (spermatogonia), or even the loss of testicular germ cells.[11] Our study offered comparable results to earlier studies that deletion of DBY played a specific role in spermatogenesis and gave the testicular pathology picture of SCOs.

CONCLUSION

The frequency of partial deletion of AZFa was found to be relatively high in our center. The type of deletion corresponds to the picture of testicular histology.

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