ORIGINAL ARTICLE

Investigation of Azoospermia Factor (AZF) microdeletion of hypospadia patients in Indonesian population

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ABSTRACT

Background: Hypospadia, a midline fusion defect of the male ventral urethra, is disorder of male external genital development occurring 0.7 – 4.5 per 10.000 live births. Hypospadia patients might have fertility problem and genetic factor couldbe involved on this aspect. Microdeletion of the Y chromosome, notably in Azoospermia factor region (AZF) have been observed in some patients with cryptorchidism and severe defects of spermatogenesis.

Aim: This study aimed to investigate microdeletions of AZF region in patients with hypospadia as a potential predictor factor for infertility. AZF amicrodeletion was associated to sertoli cell syndrome, while AZF bmicrodeletion lead to maturation arrest at the spermatocyte stage and AZF cmicrodeletion caused defect in sperm production.

Methods: Total of 60 isolated hypospadia patients who admitted to CEBIOR were analyzed for AZF microdeletions during period of 2008 – 2016. DNA samples were analyzed by PCR-screening using several sequences-tagged-site (STS) markers from different region of the AZFa, AZFb, AZFc on Yq chromosome and SRY on Yp as internal control.

Results: Out of 60 analyzed cases (mean age 5.66 years), 3 (5%) patients showed microdeletion of AZF regions and only detected in AZFa region. No deletion was observed in AZFb and AZFc region. In addition, used as internal control, there no SRY gene microdeletion was found.

Conclusions: AZF microdeletions analysis canbe used as an infertility potential prognostic predictor in hypospadia patients and become important leading of genetic counseling related to possibility of infertility in the future.

Keywords: Azoospermia Factor (AZF), Microdeletions, Hypospadia, Indonesia

INTRODUCTION

Hypospadias is a congenital abnormality of the urogenital tract in which the external urethral meatus is ectopically located over ventral aspect of penis. It is the second most prevalent congenital disorder in boys after cryptorchidism and the most frequent malformation of the penis[1]. Recent study from China population showed increasing trend in hypospadia, occurred in 0.7 – 4.5 per 10.000 live births[2]. However the true prevalence of hypospadia worldwide were difficult to estimate because there was wide variation of prevalence according to countries and ethnicity[3]. Hypospadias becomes an important health issue and its relation with long-term sexual and reproductive implication. There is a considerable number of patient suffering from functional difficulties that can affect urinary and sexual function[4].

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Parents usually bring their children at an early age because their children have abnormalities in their genitals. The boys cannot stand to urinate so that they may be teased by their peers. Moreover, it has been realized that fertility becomes substantial concern in patients with hypospadia. Previous study have shown that patients with hypospadia tend to have fewer children than the unaffected population[5]. Decreased semen quality represent reduction of paternal fertility was shown in a group of father of boys with hypospadia[6]. As the child grows into adulthood, future fertility status becomes important issue frequently raised by patient and family. Gene mutations, environment and lifestyle could be the contributing factors influencing inter-related pathways related to male reproductive disorders[7,8].

Copy-number variations (CNV) of the Y chromosome, notably in Azoospermia factor region (AZF) have been observed in some patients with cryptorchidism and infertility, represent the main risk factor of spermatogenic failure (SF) in humans. Microdeletion in this region causes defect in spermatogenesis leading to development of azoospermia and oligozoospermia[10,11]. Three major loci have been identified in the AZF region called AZFa, AZFb and AZFc regions and contain genes

that play role in spermatogenesis process[12]. Since hypospadias is frequently associated with cryptorchidism and severe defects of spermatogenesis, there is possibility that AZFmicrodeletion in the patients may occur in this clinical setting.

In this study, we examined the frequency of microdeletion in the AZF gene in hypospadia patients. To investigate the possibility of infertility in the future, we studied a group of 60 patients with isolated hypospadias. We evaluated the Ychromosome focusing on the YqAZF regionusing a set of 8 specific sequence-tagged sites (STS).

MATERIAL AND METHODS

Clinical data: Patients with 46, XY isolated hypospadia were referred to the Center for Biomedical Research (CEBIOR), Faculty of Medicine, Diponegoro University (FMDU), Semarang, Indonesia. The medical ethics committee of the Dr. Kariadi Hospital / FMDU approved this study, and informed consent was obtained from all participants, as well as their parents or guardians for patients below 18 years old, prior to their participation in this study. Referral and data collection took place between 2008 – 2016.

Subject: A total of 60 patients with 46,XYisolated hypospadiawith range of age 0-25 years were included in this study. Patients with cytogenetically detectable chromosomal aberrationswere excluded. None had a family history of disorders of sex development.

DNA extraction: Genomic DNA was obtained from peripheral EDTA-blood samples using salt extraction procedures.

Molecular Analysis: Microdeletion in AZF region were detected using polymerase chain reaction (PCR) technique as previously described[13]. Eight sequence tagged sites (STS) in the AZF region were used as markers for analysis of microdeletion. The STS markers were the zinc finger Y-chromosomal (ZFY) gene and the sex-determining region of the Y chromosome(SRY) geneincluded asinternal control (IC). Two multiple reactions were designed to screen the AZF regions, encompass multiplex A (sY86, sY127, sY254 for AZFa, AZFb and AZFc)and multiplex B (sY84, sY134, sY255 for AZFa, AZFb and AZFc) respectively. STS PCR multiplexing groups and their amplified fragments are shown in **Table 1**. PCR was carried out in 50 µl reaction mixture containing 200 ng (1 µl) of DNA, 37.8 µlmQ, 5 µl PCR buffer 10x + $MgCl_2$ 15 mM, 1 µldNTPs, 5 µlforward and reverse primer and 0.2 µlAmplitaq Gold Enzyme. The PCR conditions start with initiation at 94^0C for 3 minutes, denaturation at 94^0C for 30 minutes followed with annealing at 55^0C for 45 minutes, elongation at 72^0C for 45 minutes ended by last elongation step at 72^0C for 7 minutes and cooling to 10^0C.

Table 1. Sequence tagged sites (STS)-PCR locations and their amplified sizes[13]

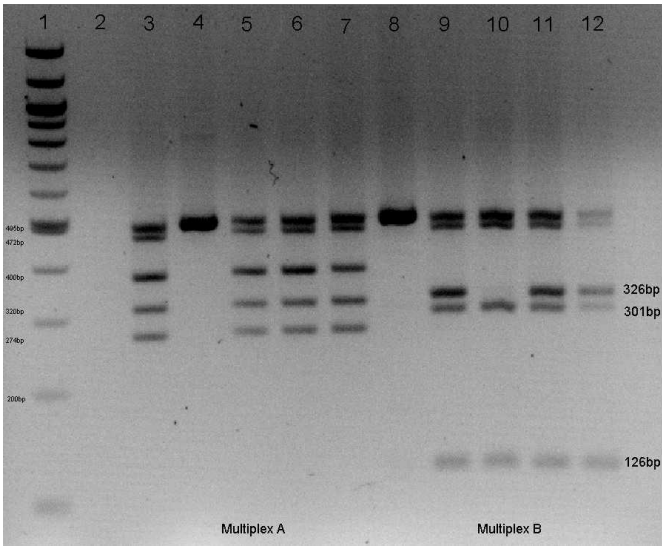
Name of STS	Region	Amplified fragments (Bp)	Sequence of the PCR primers
ZFY	IC	495	ZFY-F: 5'-ACC RCT GTA CTG ACT GTG ATT ACA C-3' ZFY-R: 5'-GCA CYT CTT TGG TAT CYG AGA AAG T-3'
SRY	IC	472	SRY-F: 5'-GAA TAT TCC CGC TCT CCG GA-3' SRY-R: 5'-GCT GGT GCT CCA TTC TTG AG-3'
sY84	AZFa	320	sY84-F: 5'-AGA AGG GTC TGA AAG CAG GT-3' sY84-R: 5'-GCC TAC TAC CTG GAG GCT TC-3'
sY86	AZFa	326	sY86-F: 5'-GTG ACA CAC AGA CTA TGC TTC-3' sY86-R: 5'-ACA CAC AGA GGG ACA ACC CT-3'
sY127	AZFb	274	sY127-F: 5'-GGC TCA CAA ACG AAA AGA AA-3' sY127-R: 5'-CTG CAG GCA GTA ATA AGG GA-3'
sY134	AZFb	301	sY134-F: 5'-GTC TGC CTC ACC ATA AAA CG-3' sY134-R: 5'-ACC ACT GCC AAA ACT TTC AA-3'
sY254	AZFc	400	sY254-F: 5'-GGG TGT TAC CAG AAG GCA AA-3' sY254-R: 5'-GAA CCG TAT CTA CCA AAG CAG C-3'
sY255	AZFc	126	sY255-F: 5'-GTT ACA GGA TTC GGC GTG AT-3' sY255-R: 5'-CTC GTC ATG TGC AGC CAC-3'

RESULTS

In this study 60 patients with isolated hypospadia were screened for AZF microdeletions in the Y chromosome. The average age of patients was 5.66 years and only 2 patients over 18 years old. Most of them come at pre-pubertal age. All 60 patients had no consanguinity and had no family history of disorders of sex development. Thirtysevenpatients manifested mild hypospadias with the urethral opening at the anterior portion of the penis, while twenty three patients presented withsevere

hypospadias respectively. None of the patients showed complete microdeletion of all AZF region. However, 3 patients had partial microdeletions (5%) in AZFa region. No deletion was observed in SRY gene. An example of PCR result patients with microdeletions in AZF gene region were given in Fig. 1.

Fig. 1.Patients with microdeletion.Lane 1 size marker, lane 2 Blank.



Multiplex A: Lane 3 male control, lane 4 female control, lane 5 patient (proband), lane 6 and 7 are other patients that have normal alleles.
Multiplex B: Lane 8 male control, lane 9 female control, lane 10 patient (proband) showed **AZFa deletion**, lane 11 and 12 are other patients with normal alleles.

DISCUSSION

The results of AZF analysis showed that 3 out 60 patients had partialmicrodeletions in AZF region. The frequency of AZF microdeletion observed in this study was 5% among hypospadia patients. This is different from previous research which is only two studies about the frequency of AZF-linked CNVs in patients with hypospadias. Tateno et al.[14] and Castro et al.[15] performed STS-PCR assays on 44 and 20 hypospadiapatients, respectively, and found no AZF-linked deletions. Hypospadia as well as cryptorchidism, impaired spermatogenesis and testicular cancer were first described in 2011 as Testicular Dysgenesis Syndrome (TDS), asymptoms of one underlying entity that happened because ofdisturbed prenatal testicular development in intrauterine period.[16]The current understanding of TDS hypothesized that genetic and environmental factor such as estrogen disruptor has responsible for all four abnormalities.[17,18]Fetal disruption of endocrine balance and/or direct adverse effects to the testis may bring about genital birth defects, development of testicular cancer, or in the mildest cases only suboptimal sperm production capacity.[19]Therefore, it is important to evaluate hypospadia and its possibility of infertility in the future.

In the current study, the distribution of microdeletions was detected in AZFa region only. This result is dissimilar with previous research that indicate the prevalence of AZFc and AZF bmicrodeletions in azoospermic patients is higher compared to AZFa[10,11,20]. Microdeletion in AZFa region is rare and usually associated with complete absence of germ cells (Sertoli cell only [SCO] syndrome).[21]The genes have been found to be necessary for spermatogenesis in the AZFa locus include *USP9Y* (Ubiquitin specific peptidase 9, Y-linked), *DBY* (Dead box on Y) and *UTY* (Ubiquitously transcribed tetratricopeptide repeat gene, Y-linked)[22]. Complete and partial deletions of AZFa have been described. Complete deletions cause Sertoli cell-only syndrome and bilateral small-sized testes while partial deletion reported with involvement of *DBY* gene that plays a significant role in the pre-meiotic spermatogonia phase of spermatogenesis[23].

Limitation of the present study was in this study contains only eight STS to analyze the microdeletions. Advanced molecular analysis such as Next Generation Sequensing (NGS) or Whole Exome sequencing (WES)could be used to detect other CNVs in the Y chromosome that might be losing by this method. For further studies, sequence analysis on this result will be necessary to elaborate genotype-phenotype relationship in this region.

The infertility status in our patients was unknown because most of our samples consist of prepubertal children. Thus, long-term follow-up studies of these patients are important to clarify the relationship between AZF microdeletion on reproductive function in the future. Despite of only a few mutations were found, this study can be extended with more various hypospadias cases for predicting factor of infertility.

Transmission of Y chromosomes microdeletions become other issue in this study. Men with Y deletions are generally infertile and therefore, deletions cannot be transmitted to their offspring. As in vitro fertilization has become increasingly popular for treatment of severe male infertility, these techniques increase the risk of transmission *de novo* Y chromosome microdeletions to their male offspring.[23]Nevertheless, a rare caseof father-to-son transmission of Y-chromosome microdeletions have been reported and it described a natural vertical transmission of a partial AZFb over three generations.[24]To develop this study, segregation

analysis will be needed to ensure the transmission of AZF microdeletions between father and son. This familytracing investigation will determine the influence of genetic risks and family background on male reproductive function as basis for genetic counseling of these patients.

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Ethics approval and consent to participate: The medical ethics committee of the Dr. Kariadi Hospital/FMDU approved this study and informed consent was obtained from all participants and or their parents or guardians prior to their participation.

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