Phenotype-Genotype and Pedigree Analysis of Isolated Hypospadias Patients

by Achmad Zulfa Juniarto

PHENOTYPE-GENOTYPE AND PEDIGREE ANALYSIS OF ISOLATED HYPOSPADIAS PATIENTS

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ABSTRACT

Objective: *Hypospadias is a malformation in urethra which has many range of severity. A patient with Isolated hypospadias (IH), a mild disorder of sex development (DSD) has a hypospadias phenotype only. Hypospadias is considered as multifactorial disease in which genetic factors contribute to its development. Chromosome analysis in DSD including hypospadias is conducted for gender assignment and other possible genetic contributions. This analysis solely could not elucidate all genetic causes of hypospadias. Polymorphism of V89L in SRD5A2 is suggested as one of genetic risk factors of hypospadias. To determine the genetic risk factor and pattern of inheritance, a good pedigree construction is required.* ***Material & methods:*** *35 eligible subjects with IH admitted to Center for Biomedical Research (CEBIOR) during 2012-2015 were randomly selected. 35 normal male as control were included in this study. Data on three generation pedigrees were collected from medical records in 35 affected subjects. Chromosome analyses were done by using G-banding technique. Polymorphism analysis of V89L in SRD5A2 gene was done using PCR-RFLP technique in all samples.* ***Results:*** *From the 35 affected subjects, the most frequent phenotype was penile hypospadias (47%), a pair of twins were monozygotic and one had a cousin diagnosed with urogenital abnormalities (i.e micropenis and chordae). All subjects had 46,XY chromosome. No chromosomal aberration was found. No positive correlation between polymorphism of V89L in SRD5A2 and risk of hypospadias (PR of CC+CG vs GG=1.0, 95% CI: 0.342−2.921, p value=1.0).* ***Conclusion:*** *The pedigree data from our study implies tendency of genetic involvement in hypospadias cases. There were no chromosomal aberrations in hypospadias cases. The finding on polymorphism of V89L in SRD5A2 gene does not support that of previous studies.*

Keywords: *Pedigree, hypospadias, risk factor, SRD5A2.*

ABSTRAK

Tujuan: *Hipospadia merupakan penyakit multifaktorial yang melibatkan faktor genetik dan memiliki derajat keparahan bervariasi. Isolated hypospadias (IH) termasuk kelainan ringan Disorder of sex development (DSD) yang hanya ditemukan fenotipe hipospadia saja. Analisis kromosom pada DSD termasuk hipospadia bertujuan untuk menentukan gender dan mencari kemungkinan faktor genetik yang berkontribusi. Analisis kromosom saja tidak dapat menyingkirkan semua penyebab genetik dari hipospadia. Polimorfisme V89L gen SRD5A2 diusulkan sebagai salah satu faktor risiko genetik hipospadia. Untuk menentukan faktor risiko genetik dan pola penurunan penyakit, penyusunan pedigree yang baik diperlukan.* ***Bahan & cara:*** *Sejumlah 35 subjek IH yang datang ke Center for Biomedical Research (CEBIOR) selama 2012-2015 dipilih secara acak. Sebanyak 35 laki-laki normal dilibatkan sebagai kontrol. Data pedigree tiga generasi didapatkan dari rekam medis pada 35 subjek IH. Analisis kromosom dilakukan dengan teknik G-banding. Analisis polimorfisme V89L gen SRD5A2 dikerjakan menggunakan tehnik PCR-RFLP pada semua sampel.* ***Hasil:*** *Dari 35 subjek IH, fenotip terbanyak adalah hipospadia penile (47%). Sepasang anak kembar monozigot ditemukan menderita hipospadia dan sepupu dari satu subjek IH memiliki kelainan urogenital lain (micropenis dan korda). Semua sampel memiliki kromosom 46,XY. Tidak ditemukan aberasi kromosom di semua sampel. Tidak ditemukan hubungan positif antara polimorfisme V89L gen SRD5A2 dan risiko hipospadia (PR of CC+CG vs GG=1.0, 95% CI: 0.342−2.921, p value=1.0).* ***Simpulan:*** *Data pedigree studi ini menunjukan kecenderungan keterlibatan faktor genetik pada hipospadia. Tidak ditemukan aberasi kromosom pada kasus hipospadia. Temuan polimorfisme V89L gen SRD5A2 studi ini tidak mendukung penelitian sebelumnya.*

Kata kunci: *Pedigree, hipospadia, faktor risiko, SRD5A2.*

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INTRODUCTION

Hypospadias is the most common malformation in the TIP of urethra of male babies which can result in difficulty of gender assessment. It is characterized by the ostium urethra externum (OUE) located in anterior part of penis instead of in the TIP of penis.[1] Phenotype of hypospadias is classified into 3 types based on the location of urethra (Sheldon & Duckett, 1987): anterior (OUE located in glanular, subcoronal, and distal penis), middle (OUE located in mid shaft penis and proximal penis), and posterior (OUE located in penoscrotal, scrotal, and perineal).[2] Meanwhile, IH is a malformation which is characterized by hypospadias as a phenotype without other concomitant abnormality. Severe hipospadia (OUE located in posterior) is categorized as DSD phenotype based on Chicago classification.[3] Phenotype of hypospadias which involved other malformation such as micropenis, scrotum bifid, or undescensus testiculorum is included in DSD. In case of DSD, chromosomal analysis is done in order to assess X and Y chromosome for gender confirmation.[4] Chromosomal analysis in DSD case including hypospadias could not elucidate any genetic abnormalities since chromosomal analysis cannot detect abnormalities less than 4 Mbp. Molecular analysis is warranty to be done.

Up to now, the etiology of hypospadias is not well understood yet and thought to be a multifactorial disease in which genetic factor might play role in the development.[5] In the case of searching the etiology, scientists were focusing on the genetic factor that might be the cause of this disease, since the occurrence tends to increase each year.[6] Mutations in several genes have been identified to be involved in sex differentiation. One of the candidates of genes that affect genetic susceptibility for this disease is SRD5A2 gene. Steroid 5-alpha reductase type 2 is a gene which is located in chromosome 2 (2p23) and consists of 5 exons and 4 introns. It encodes steroid 5-alpha reductase, alpha polipeptida 2 protein which has 254 amino acids that were predominantly expressed in prostatic tissue. This protein has two domains which are testosterone binding domain and NADPH cofactor-binding domain in N-terminal region. Any mutation located either in exon 1 or 5 affect testosterone binding region while mutations that affect the NADPH binding is more varied and commonly mapped between exon 3 and 4.[7] SRD5A2 encodes a microsomal protein called steroid 5-alpha reductase, alpha polypeptidea 2. This protein is an enzyme which converts testosterone (T) into the more potent dihydrotestosterone (DHT) in steroid metabolism.[8] Polymorphism in SRD5A2 particularly c.265G>C (CGT>CCT) leads to amino acid change from valine to leusin (V89L). This change was shown to reduce 5-alpha reductase activity by approximately 30% and resulted in lower dihydrotestosterone concentration thus it was associated with the increasing risk of hypospadias.[9-11] Several studies in Asia showed a positive association between this polymorphism and hypospadias.[9,12,13] A study in India indicated a strong association of this polymorphism with hypospadias with OR 2.4, 95% CI 1.2-4.6, $p<0.05$.[9] There is lack of study about genetic analysis of SRD5A2 in Indonesia. One study of patients with 5-alpha reductase deficiency led to the discovery of 2 new mutations in pGly34fs and c.699-1G>T.[14]

Familial clustering of hypospadias has been reported in 4-28% cases. About 7-9% fathers of patients with hypospadias have the same malformation. The risk increased among boys whose fathers had hypospadias as well. It was found that 28% of patients had at least one family member with hypospadias.[15] The familial occurrence of hypospadias indicates an important genetic component in the etiology of hypospadias, although family member may share environmental risk factor as well.[16] An increased risk for hypospadias among twins has been reported in some studies and the prevalence is higher among male-male twins than male-female twins.[17] Some studies have been described showing Mendelian trait, although the transmission is not simple but complex Mendelian inheritance pattern.[15] Pedigree construction particularly three generation pedigree might assess risk factor for inherited disease or multifactorial suspected disease with the role of genetic in the development.[18] Thus we know further about how the disease pattern occurred in a family whether it is due to genetic factor solely or environmental factor contribute to the developing disease.

OBJECTIVE

In this study, authors try to focus on phenotype and genotype of hypospadias which is shown by chromosome analysis and molecular analysis particularly V89L polymorphisms in SRD5A2 as one of suggested risk factor in hypospadias. Furthermore, authors would like to analysis

the pedigree data that found from the medical records of the patients.

MATERIAL & METHODS

Thirty five selected random sampling of IH subjects during 2012-2015 and 35 normal male subjects as control were included. The inclusion criteria of samples were IH patients (ethnic background was ignored) whose parents signed informed consent. Severe hypospadias phenotype accompanied by other genital malformation which was considered as DSD phenotype was excluded in this study. Three generation pedigrees were obtained from all 35 affected subjects. The age of patients ranged from 3 months to 14 years old. Normal male group were volunteers from the academic society of Faculty of Medicine Diponegoro University who reported had no history of hypospadias or other genital anomaly whose ages ranged from 22-53 years old. This study was done in Centre for Biomedical Research (CEBIOR) - Faculty of Medicine Diponegoro University, Semarang. All 35 affected subjects were referred by Urologist or Pediatricians for chromosome analysis prior to repair surgery. All of them were from Semarang and surrounding area.

Blood samples were collected into EDTA tube for molecular analysis and heparin tube for chromosomal analysis. Chromosomal analyses were done on the first visit to CEBIOR by using G-banding technique. DNA extraction was done using salting out method. PCR-RFLP to detect V89L polymorphisms were done in those samples.

PCR-RFLP technique was done using previously described technique.[19] Instead of using agarose gel electrophoresis, visualization of digestion product was done using PAGE (Polyacrilamide Gel Electrophoresis). GG or VV allele (wild type) were shown by 169bp, 105bp, and 64bp fragments size. CC or LL allele (mutant) was shown by 169bp, 105bp, and 83bp fragments size. Heterozygote GC or VL were shown by 169bp, 105bp, 83bp, and 64bp fragments size. Data of V89L polymorphism among isolated hypospadias patients and normal male control were then analyzed using SPSS for Windows version 17 to get the prevalence ratio (PR).

RESULTS

From 35 IH subjects, the mean age of admission was 65.5 months (5.4 years). The oldest age of admission was 14 years while the youngest age was 3 months. The most frequent phenotype of hypospadias was penile (47%), followed by penoscrotal (32%), scrotal (6%), perineal (6%), coronal (3%), glanular (3%) and 2 OUE/ostium urethra externum in 1 patient located on penile and penoscrotal.

Three generation pedigree from 35 family of cases revealed a pair of monozygotic twins with both affected hypospadias (penile and penoscrotal) as seen on figure 1A and one other affected family member (cousin) with urogenital anomaly (i.e micropenis and chordae) as seen on figure 1B. Pedigrees of monozygotic twin family were shown on figure 1. The rest 33 family pedigree found no consanguineous or other family member with the same symptoms.

All subjects either hypospadias or normal control had 46,XY karyotype. There is no numerical or structural chromosomal abnormalities found

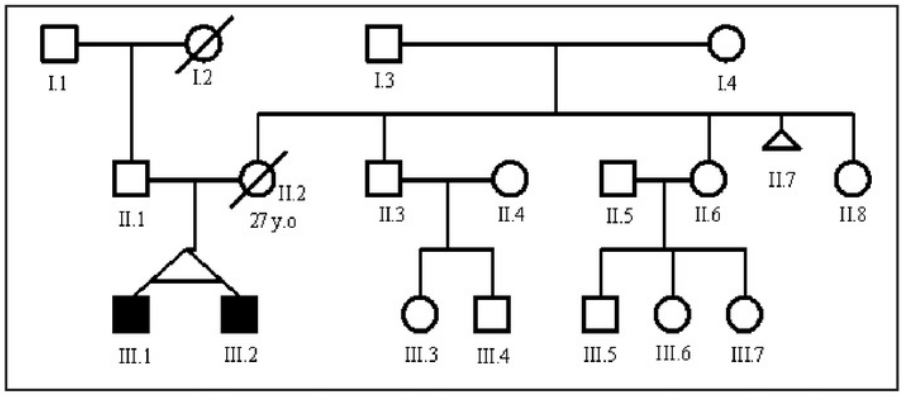

Figure 1A. A pedigree of affected twins (blocked square) with hypospadias (penile and penoscrotal).

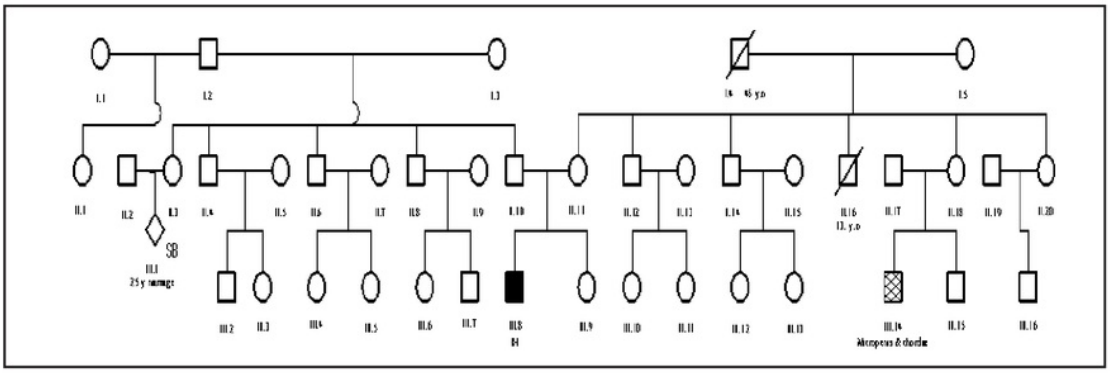

Figure 1B. A pedigree of family member of affected subject (cousin) had other urogenital anomaly (i.e micropenis and chordate). Blocked square indicated affected subject. Cross diagonal square indicated other urogenital anomaly.

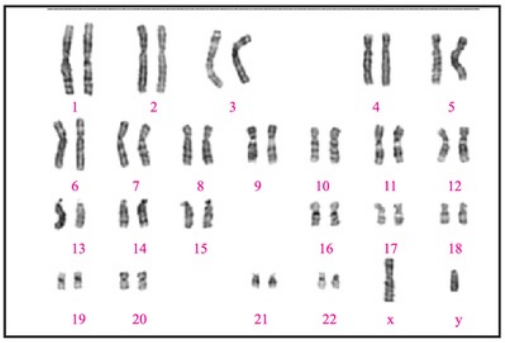

Figure 2. Karyotype of one of hypospadias subject. No chromosomal aberration was found.

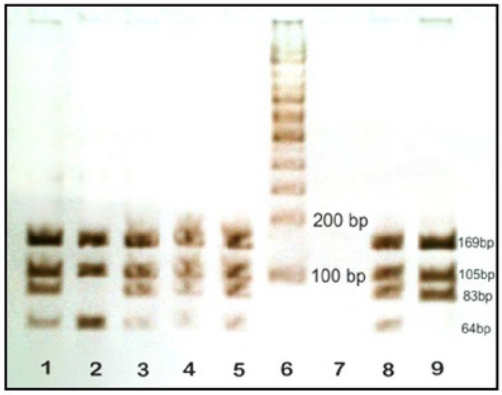

Figure 3. Visualization of RFLP using 10% polyacrilamide gel electrophoresis with silver staining. Lane 6: 100bp DNA ladder. Lane 7: Blank. Lane 2 represented wildtype allele (GG) that shown by fragment band 169bp, 105bp, and 64bp. Lane 1,3,4,5,8 represented heterozygote allele (CG) that shown by fragment band 169bp, 105bp, 83bp, and 64bp. Lane 9 represented mutant allele (CC) that shown by fragment band 169bp, 105bp, and 83bp.

in this study. Karyotype of one of the hypospadias subject was shown in figure 2.

Polymorphism of V89L of SRD5A2 was done using PCR-RFLP. Result of the investigation was shown in figure 3.

Allele C either homozygous form or heterozygous form were grouped as MUT (mutant allele), and homozygous GG were grouped as wildtype. There were no positive association between mutant allele CC+CG and risk of hypospadias (prevalence ratio/PR of CC+CG vs GG =1.0, 95% CI: 0.342 - 2.921, p value =1.0). Allele frequency of C and G in hypospadias sample and control group and frequency of C and G alleles of whole samples were represented in table 1.

Table 1. Frequency of C and G allele.

Allele	Frequency		Total Frequency
	Hypospadias	Normal	
C	0.26	0.25	0.51
G	0.24	0.25	0.49

DISCUSSION

The most frequent phenotype of hypospadias in this study were penile hypospadias (47%) which was considered as mild phenotype. This finding was different from that of India where the most frequent phenotypes were the severe forms (penoscrotal and scrotal) which were counted for 56.38% of total cases.[9] Penile hypospadias frequency which was considered as mild phenotype apparently was found to be predominant in the patients admitted to this study. The possible reason was the inclusion criteria used in this study which only includes patients with isolated hypospadias without other concomitant malformations. Severe phenotype of hypospadias was usually accompanied by other malformation and were categorized as DSD phenotype and had to be excluded from this study in order to attempt the homogeneity of samples.

Based on pedigree data, we found a pair of monozygotic twins affected with hypospadias in both of them. One of the twins had penile hypospadias which was considered as a mild phenotype while penoscrotal hypospadias in other twin was considered as a severe form. Previous study showed that monozygotic twin had a greater risk of hypospadias. The prevalence was higher in male-male twin than male-female twin.[17] A study suggested that dichorionic twins and monochorionic twins have an increased risk of developing hypospadias. However, the risk is fourfold higher in monochorionic twins.[20] Other study suggested that monozygotic twin has eightfold higher risk than singleton. It is possibly due to inadequacy of HCG (Human Chorionic Gonadotropin) requirement of 2 male fetuses.[17] The twins in this study have different degrees of severity. Data from medical record revealed that twin with mild phenotype had 2900 gram of birth weight while the birth weight of other twin was unclear. Data from alloanamnesis with the grandmother of the patients revealed that the birth weight of second twin was almost similar with the first one. Previous study of male-male twin with hypospadias and his normal pair showed that babies with hypospadias had lower birth weight than their normal pairs. The reasons might be due to placental insufficiency in supplying the needs of nutrition or gonadotropine hormone for two pairs of male gonad resulting in impairment of external genitalia development in the fetuses life.[7,21] Our finding of monozygotic twins who both are affected might indicate that hypospadias developing in that family involving genetic factor.

Three generation pedigree from medical record of one sample revealed cousin of the patient were affected with other urogenital anomaly. This occurrence might be a sporadic case and has no correlation with hypospadias in the sample. Previous study of 307 hypospadias samples found that recurrent risk estimation were about 11%, 9%, 2.2%, and 3%, respectively in younger brothers, fathers, uncles, and cousins of the affected subject.[22] The same shared of environmental exposure might be one of risk factor in familial hypospadias.[22]

From a good three generation pedigree construction, we can predict tendency of the types of multifactorial disease occurred in a family. Three generation pedigree could be a guide for clinician or genetic counselor in giving suggestion or counseling to patients about his disease and also for other family member who is suspected to have genetic abnormality. Thus all family members will get the benefit of good pedigree construction.

Chromosomal analysis in all subjects in this study turns out to be normal. No structural or numerical aberration was found. Chromosomal aberration in hypospadias particularly isolated hypospadias was rarely found. A study of chromosomal analysis in 21 hypospadias patients demonstrated that no chromosomal aberration was found and also none of Y chromosome appeared to be abnormal.[23]

However, chromosomal analysis could not elucidate any genetic abnormality. Molecular analysis is suggested to seek the involvement of genetic factor.

Molecular analysis of V89L polymorphism of SRD5A2 gene is one of our aims in this study. V89L polymorphism is located in exon 1. However, the exact mechanism of how this polymorphism contributes to the occurrence of hypospadias is poorly understood. This is a SNP where the nucleotide G changes to C in c.265 leading to amino acid change of valine to leusin.[11] The previous study about this polymorphism showed that this was related with the change of 5-alpha reductase enzyme activity in vivo, where V89 allele substantially encode higher enzyme activity than L89 allele because valine homozygous (VV) had nearly 30% 5α-androstane-3α,17β-diol-17β-glucoronide (AAG) serum level higher than LL homozygous. AAG is dihydrotestosterone metabolite which can be used to measure 5-alpha reductase activity.[11] However, mutations in SRD5A2 gene are usually found in severe cases of hypospadias together with other malformations such as micropenis, cryptorchidism and bifid scrotum. Only a few of isolated hypospadias cases were reported.[24]

According to the genetic analysis result, this study found that the association between V89L polymorphism and isolated hypospadias was still inconclusive (PR of mutant allele vs wildtype allele was 1, 95% CI: 0.342 - 2.921, p value=1.0). The study in Netherland with 620 hypospadias case samples and 596 controls showed an OR of CC genotype: 1.0 (ref), OR CG genotype: 1.0, 95% CI (0.8-1.3), OR GG genotype: 1.1, 95% CI (0.7-1.6).[8] The study in Netherland and this study could not replicate another study which showed positive association between C allele and the increasing risk of hypospadias.

This study showed different finding from that from Japan or India.[9,10] This might be caused by limited research subject in this study which not meets the ideal sample number for a polymorphism study. Different ethnicity might also contribute to this finding whether it reflects the real condition in Indonesian population or not.

CONCLUSION

The pedigree data from our study showed a tendency of genetic involvement in one family pedigree of hypospadias subject. Chromosomal analysis showed no numerical and structural abnormalities but it could not elucidate other genetic abnormalities in the patients. However, molecular analysis for curtained related genes is required to analyze possible single nucleotide polymorphism. Genetic analysis of polymorphism in SRD5A2 gene in this study could not replicate other study that showed positive association due to small sample size and different ethnicity.

Hormonal examination and environmental factor should be considered for further investigation of the role of this polymorphism. Three generation pedigree can guarantee a better result particularly if single gene mutation or familial hypospadias is suspected.

ACKNOWLEDGEMENT

This study was granted by Ministry of Health Republic Indonesia through RISBIN- IPTEKDOK 2015 grant (Contract no. 714A/UN-7/KS/2015). The authors would like to thank to Centre for Biomedical Research (CEBIOR) staff and DSD team Faculty of Medicine Diponegoro University / RSND for helping laboratory experiment and gaining data recording.

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GRADEMARK REPORT

FINAL GRADE

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GENERAL COMMENTS

Instructor

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