

**THE CORRELATION OF PROMOTER POLYMORPHISM AND EXPRESSION OF
ANDROGEN RECEPTOR GENE WITH HYPOSPADIA INCIDENCE**

ABSTRACT

Introduction: Hypospadias is one of frequent congenital abnormalities, yet the certain etiology has not been found thus far. Along with the development of molecular biology and microarray analysis, hypospadias is now related with abnormalities of genetic polymorphism and expression. Gene, such as Androgen Receptor (AR) is expected to correlate with hypospadias incidence

Methods: This study used post-operative prepuces of hypospadias patients and normal prepuces from elective circumcision patients as control. Materials of this study were prepuces of hypospadias patients and normal children's prepuces. The collected prepuces were residual tissue of the operation undergone to the subject. The operation was a treatment done by urology surgeons and was a standard procedure for this case in the hospital. Ethical problem of this study which possibly could appear is the utilization of residual prepuces as study material. So that, explanation to patient's parents about purpose, advantage, and disadvantage of the study was necessary. All cost needed to examine AR gene polymorphism and expression was borne by authors. Confidentiality of the study is guaranteed.

Result: The result of this study showed no AR polymorphism found in experimental samples neither in control samples. AR gene over-expression was found in experimental samples, and it was statistically significant ($p < 0.05$). From this study, we found that AR gene over-expression is correlated with hypospadias incidence

Hypospadias is one of frequent congenital abnormalities, yet the certain etiology has not been found thus far. Along with the development of molecular biology and microarray analysis, hypospadias is now related with abnormalities of genetic polymorphism and expression. Gene, such as Androgen Receptor (AR) is expected to correlate with hypospadias incidence. This study prospectively examined polymorphism and expression level of AR in 49 hypospadias sample's tissues, and then compared with 49 normal penis skin tissues, taken from elective circumcision. Prepuce tissue was collected during operation. PCR-sequencing technique was used to examine AR polymorphism and qPCR technique to examine gene expression. The study subjects were 49 hypospadias patients and 49 elective circumcision patients (as control) who underwent the operation at the age from 10 to 14 years old, with the most frequent hypospadias type was distal hypospadias. The result of this study showed no AR polymorphism found in experimental samples neither in control samples. AR gene over-expression was found in experimental samples, and it was statistically significant ($p < 0.05$). From this study, we found that AR gene over-expression is correlated with hypospadias incidence.

Keyword : Hypospadias, Polymorphism, Androgen Receptor (AR) gene

BACKGROUND

Hypospadias is one of frequent male congenital abnormalities. Hypospadias incidence was reported 3-4 per 100.000 live births.¹ This case was reported around 0.3-1% of live births in America.^{2,3,4} Hypospadias incidence was found higher in Rotterdam Child Health care centre in 2000, those were 53 cases from 7.292 male babies (0.73%).⁵ Hypospadias patients in RSUP Ciptomangunkusumo Jakarta from 2002-2008 were 139 children.⁶ A study from January 2011- September 2012 in Sardjito Hospital Yogyakarta showed there were 60 cases of hypospadias.⁷ According to a study conducted by Pande et al., there were 61 hypospadias cases from 2011-2014 (Pande et al., 2015),⁸ while Bayu et al., found 15 hypospadias cases from 2009-2011 (Bayu et al., 2014).⁹ Data from Urology Department medical record of M Djamil Hospital in last 5 years (2010-2014), showed that hypospadias repair operation has 95 times been performed, with average 19 patients per year.

Until now, certain etiology of hypospadias is not clearly known yet. Researchers are still expected genetic factors, endocrine, and environmental factors as the etiological factors.^{10,11,12} Van der Zanden et al. reviewed some articles and concluded that there was correlation between gene mutation and polymorphism with hypospadias incidence.¹¹

Polymorphism in some genes have been studied, such as Fibroblast growth factor 8 (FGF8), fibroblast growth factor receptor 2 (FGFR2), AR, ATF3, SRD5A2, estrogen receptor 1 (ESR1), ESR2, mastermind-like domain containing 1 (MAMLD1), and diacylglycerol kinase kappa (DGKK).¹³

There are many studies about genetic marker to predict the etiology of this case using microarray analysis. They used hypospadias patient's prepuces and compared them with normal prepuces collected from patients who underwent elective circumcision. Some genes are expected to have strong relation hypospadias incidence based on protein expression analysis and mRNA expression. Those genes include ATF3, zinc finger protein 36 (ZFP36), connective tissue growth factor (CTGF), and cysteine-rich angiogenic inducer 61 (CYR61). Theory trusted to explain hypospadias incidence today is genetic factor combined with environmental exposure during urethral forming in the uterus.^{14,15}

This study aimed to analyse the correlation between AR gene promoter polymorphism and AR gene expression with hypospadias incidence.

MATERIAL AND METHODS

Design of this study is cross sectional study and we used chi square test and Mann-Whitney non parametric test for statistical analysis. Samples were collected from prepuce

tissues of 49 hypospadia patients and 49 normal patients who underwent elective circumcision from January 2014 to September 2016. Hypospadia patients with testicles undescended and micropenis were excluded from the study samples. PCR-sequencing technique was used to examine AR polymorphism and qPCR technique to examine gene expression.

Table 1. Primers Androgen Receptor (AR) gene

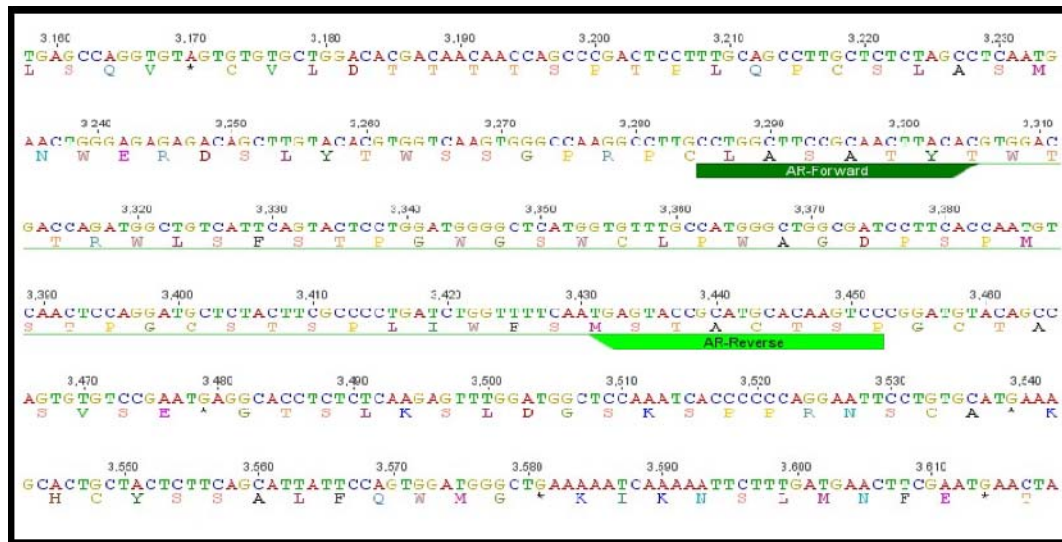
Primers Design	Primers sequence	Length (bp)
AR-Forward	5'-CCCGATCTATCCCTATGAC-3'	19
AR-Reverse	5'-AGCTGCTAAAGACTCGGAG-3'	19



Picture 1. Primers attachment of AR gene on genomic sequence of Androgen Receptor gene (NG_009014.2)

Table 2. Primers AR qPCR gene

Primers Design	Primers sequence	Length (bp)
AR-Forward	5'-CCTGGCTTCCGCAACTTACAC-3'	21
AR-Reverse	5'-GGACTTGTGCATGCGGTACTCA-3'	22



Picture 2. Primers attachment of androgen receptor qPCR on sequence of Androgen Receptor mRNA (NM_000044.3)

This study used post-operative prepuces of hypospadias patients and normal prepuces from elective circumcision patients as control. Materials of this study were prepuces of hypospadias patients and normal children's prepuces. The collected prepuces were residual tissue of the operation undergone to the subject. The operation was a treatment done by urology surgeons and was a standard procedure for this case in the hospital. Ethical problem of this study which possibly could appear is the utilization of residual prepuces as study material. So that, explanation to patient's parents about purpose, advantage, and disadvantage of the study was necessary. All cost needed to examine AR gene polymorphism and expression was borne by authors. Confidentiality of the study is guaranteed.

This is a voluntary experiment. Study subject had rights to refuse of being involved in this study. Patients who refused to be involved in this study will still be treated properly according to hospital standard procedure. Parents who were willing to be involved in this study must sign informed consent. This study was conducted after obtain ethical clearance from research ethical committee of Medical Faculty of Andalas University, padang.

RESULT

Average age of patients operated was between 10-14 years old with most frequent type of hypospadias was distal type. Result of the study showed no AR polymorphism found in experimental samples neither in control samples. We found AR gene over-expression in experimental samples and it was statistically significant ($p = 0.001$).

Result of AR gene sequencing and blasting showed there were no mutation in every sample. In other words, from all hypospadia patients who were sequenced, the mutation rate was 0%.

As well as control patients, sequencing analysis was also performed and no AR gene polymorphism found in control patients who underwent elective circumcision.

From qPCR data, AR gene expression level obtained from hypospadia samples was 34.913.906 with the average of expression level was 356.264, compared to standard value. Analysis of correlation between AR gene expression with hypospadia incidence, was found statistically significant where p value was 0.001.

In the conclusion, we reported that there is correlation between AR gene over-expression with hypospadia incidence, and the difference was statistically significant with p value 0.001.

Table 3. Characteristic of Subjects

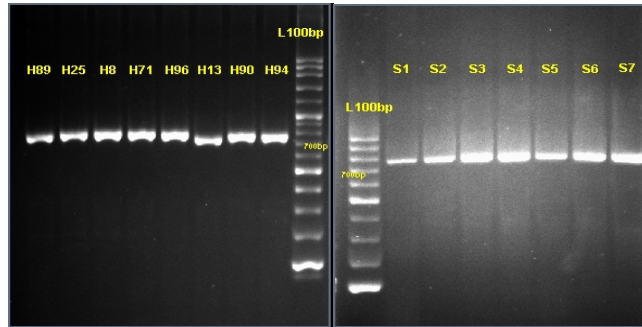
	Hypospadia		Control	
	f	%	f	%
Ages (years)				
0-4	8	16,3	3	6,1
5-9	18	36,8	9	18,4
10-14	20	40,8	34	69,4
15-19	1	2	2	4,1
>20	2	4,1	1	2
Total	49	100	49	100

Table 4. Characteristic of hypospadia types

Type of hypospadia	Hypospadia	
	f	%
Proximal	6	12,2
Distal	43	87,8
Total	49	100

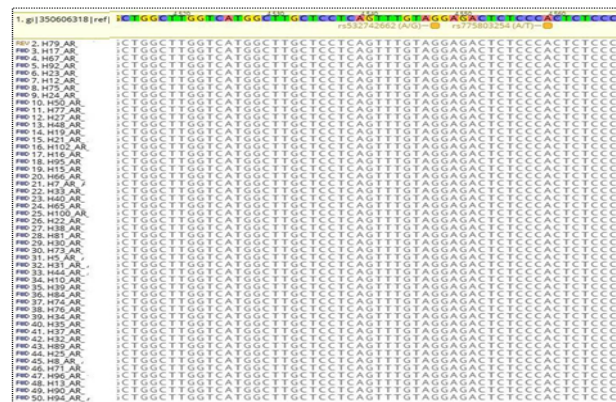
Table 5. The Correlation of Androgen Receptor (AR) gene expression with hypospadia incidence

Group	Expression of AR gene		p
	Mean	Standard Deviation	
Hypospadia	356.264,43	443.012,17	0.001
Control	116.271,29	182.688,08	

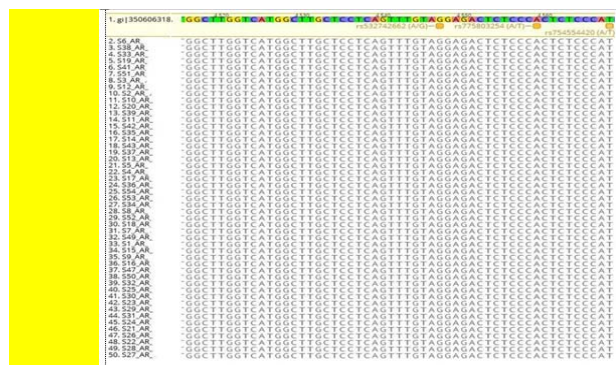


Picture 3. Electrophoresis result of Androgen receptor gene PCR product in hypospadias and control (790 bp)

The Androgen receptor product was observed with electrophoresis using Agarose gel 1,5% which had been coloured with Gelred DNA colouring and checked with GelDoc.

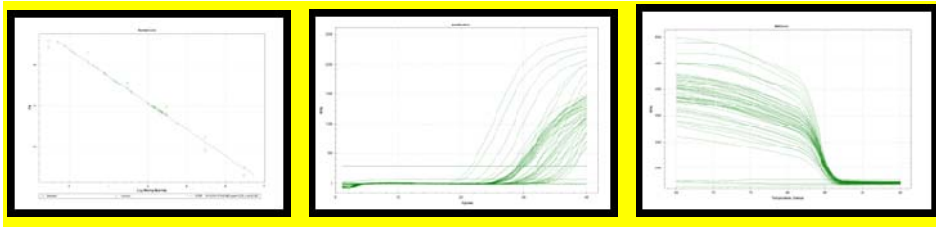


Picture 4. Multiple alignment Androgen receptor gene in hypospadias



Picture 5. Multiple alignment Androgen receptor gene in Control

From this study, we didn't found any mutation from all of the sampel and control



Picture 6. Standard Curve of Androgen receptor gene Expression

From the picture above, we can see that androgen receptro gene expression is same as the standard curve, and from qPCR, we get the total androgen receptor gene expression is 34.913.906 with the mean expression is 356.264 if compared with standard.

DISCUSSION

The result of this study showed that the most common age group of hypospadias patients is in the age group of 10-14 years old which is as many as 20 people, the same amount of which in the control group. The youngest age of patient going through operation of hypospadias is 1.5 years old and the oldest age is 43 years old.

This is in accordance with a research conducted by Fariz in 2002-2008 for 116 patients who meet the inclusion criteria. The most common age groups in the study were 2-15 years old (79.3%), and under 2 years (18.2%).⁶

However, different results were obtained by Andika et al. who found the most common age groups that came for treatment were 3 to 6 years old with 56.3% and 1 - 3 years with 21.8%.¹⁶

The results of this study are not in accordance with the literatures stating that hypospadias correction should be done at the age of pre-school.¹⁷

In this study, the most common type of hypospadias were the distal-type which was as much as 43 patients (87.8%), while the proximal-type (penoscrotal, scrotal and perineal) was only 6 patients (12.2%).

This is in accordance with a research by Pande et al, who investigated hypospadias patients in Dr. M. Djamil Padang Hospital from January 2012 to January 2014 and found 44 patients with the most common type of hypospadias were the midshaft-type of 33.3%.⁸ Similarly, a research was conducted by Takahashi et al. in 2013 where they conducted a study of 18 hypospadias patients, and found that the patient with distal-types of hypospadias were 11 patients (61.1%) and the proximal-type were 7 patients (38.9%).¹⁹

171 These results are not in accordance with the research by Samtani et al. in 2010 in New
172 Delhi, India. The study was conducted on 80 patients with hypospadias and it was found that
173 the proximal-type was as much as 56.38 %, whereas distal-type was 43.62%, and the most
174 common type was the penoscrotal-type with 36 patients.²¹

175 In this study, there was no AR gene mutations found in both hypospadias and control
176 samples. This showed that there is no relationship between AR gene promoter mutation and
177 the occurrence of hypospadias. This study is consistent with the research of Radpour et al.
178 who also found no AR gene mutations in hypospadias.²⁴

179 Unlike a research by Adamovic et al. in 2012 who found a significant association
180 between AR gene mutation and the occurrence of hypospadias, with odds ratio 2 to 3 fold.²⁵
181 Also, different results were obtained by Borhani et al. in 2014 who conducted the study from
182 March 2012 to August 2012. The study found a new SNP that may play a role in causing
183 hypospadias.²⁶ This is also in accordance with a research by Aschim et al. who obtained a
184 correlation between GGN repeat length AR and the incidence of penile-type hypospadias and
185 cryptorchism.²⁷

186 From this study, a significant relationship between AR gene expression and the
187 occurrence of hypospadias was found, with p value <0,05. This is in accordance with a study
188 conducted by Qiao et al., where they found an AR overexpression in patients with severe
189 hypospadias. They also found higher levels of AR expression in mild hypospadias when
190 compared with controls but not statistically significant.²⁸ This is in accordance with a
191 literature stated that androgen signaling through AR greatly affects the normal growth of the
192 penis, so a disturbance of androgen signaling will lead to various disorders of the growth of
193 external genitalia. The same result was also described in the journal review of Manson et al.
194 who stated that the AR gene plays an important role in male sexual differentiation by
195 mediating the biological effects of gonadal androgens.²⁹ Increased AR expression increases
196 the risk of malignant or early-onset prostate cancer, in which the lack of AR transcription
197 factor activity increases the risk of demascolarization such as reduced sperm production,
198 testicular atrophy, and infertility. Similarly, a study by Pichler et al. also found a meaningful
199 correlation between AR overexpression with the occurrence of hypospadias.³⁰

200 Whereas different results were found in a research by Beleza-Meireles et al. who
201 found no significant association between AR expression and the occurrence of hypospadias,
202 either mild or severe hypospadias.³¹

Polymorphism of AR promoter was not found in all hypospadias (0%). On the other hand, there was an increased level of AR gene expression in all hypospadias when compared with controls. From the qPCR data, the level of AR expression of hypospadia gene was obtained with total of 34,913,906 and the average expression level was 356,264 as compared with the standard (hypospadias). From these results, there were 17 hypospadias patients (34.7%) whose AR gene expression values were above average, while the remaining 32 (65.3%) had an AR gene expression value below average hypospadia's patient.

A statistical test can not be performed because there is no patient has a positive mutation.

CONCLUSION

From this study we can get conclusion such as, the distribution of promoter polymorphism and expression of androgen receptor gene is not different in hypospadia and control, there is correlation between Androgen receptor gene with hypospadia incidence, and there is not correlation between promoter polymorphism and expression of androgen receptor gene in hypospadia incidence.

Limitation of this study is in this study we don't explore the other factor like endogenous and exogenous factor that can correlate to hypospadia incidence, and we only did a research at androgen receptor gene, as we know there are so much genes that suggested have a correlation to hypospadia.

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