

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

ABSTRACT

Introduction: Hypospadias is one of the most common congenital abnormalities, yet the exact cause remains unknown. Androgen Receptor (AR) are suspected to cause hypospadias

Methods:

As many as 49 post-operative prepuces of hypospadias patients and 49 normal prepuces from elective circumcision were recruited. Materials of this study were prepuces of hypospadias patients and normal children's prepuces. The prepuces were collected from residual tissue from subjects who underwent operation. The operation was a standard procedure for this case in the hospital and done by urologist.

Utilization of residual prepuces as study material could be an ethical issue. Therefore, 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 was guaranteed.

Result: The result of this study showed no AR polymorphisms in experimental and control group. AR gene over-expression was found in experimental group, and it was statistically significant ($p = 0.001$). From this study, we found that AR gene over-expression is correlated with hypospadias incidence

Hypospadias is one of the most common congenital abnormalities, yet the exact cause remains unknown. Androgen Receptor (AR) is suspected to cause hypospadias. This study prospectively examined polymorphism and expression levels of the AR in 49 hypospadias sample's tissues, and then compared with 49 normal penis skin tissues, collected from elective circumcision. Prepuces tissue were collected during operation. PCR-sequencing was used to examine AR polymorphism and qPCR 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. The most frequent hypospadias type was distal hypospadias. No AR polymorphisms were found in the experimental and control group. AR gene over-expression was found in the experimental group, and it was statistically significant ($p = 0.001$). From this study, we found that AR gene over-expression is correlated with hypospadias incidence.

Keyword : Hypospadias, Polymorphisms, Androgen Receptor (AR), Gene expression

BACKGROUND

Hypospadias is one of the most frequent male congenital abnormalities. Hypospadias incidence was reported 3-4 per 100.000 live births.¹ 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 to 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 2011-2014⁸. While Bayu et al., found 15 hypospadias cases from 2009-2011.⁹ Data from medical records from the Urology Department M Djamil Hospital in the last 5 years (2010-2014), showed that hypospadias repair operation had been performed 95 times, with average of 19 patients per year.

Until now, certain etiology of hypospadias is not clearly known. Researchers are still expecting 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

Several studies regarding genetic markers to predict the etiology of this hypospadias using microarray analysis have been reported. The authors used hypospadias patients' prepuces and compared them with normal prepuces collected from patients who underwent elective circumcision. Some genes showed a strong correlation with 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). Genetic factor together with environmental exposure during urethral development in the uterus is believed to be the cause of hypospadias.^{14,15}

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

MATERIAL AND METHODS

Design of this study was cross sectional. Chi square test and Mann-Whitney non parametric test were used for statistical analysis. Samples were collected from prepuce tissues of 49 hypospadias patients and 49 normal patients who underwent elective circumcision from January 2014 to September 2016. Hypospadias patients with undescended testicles and micropenis were excluded. PCR-sequencing was used to examine AR polymorphism and qPCR was used to examine gene expression through the absolute quantification method.

Table 1. Primers Androgen Receptor (AR) gene

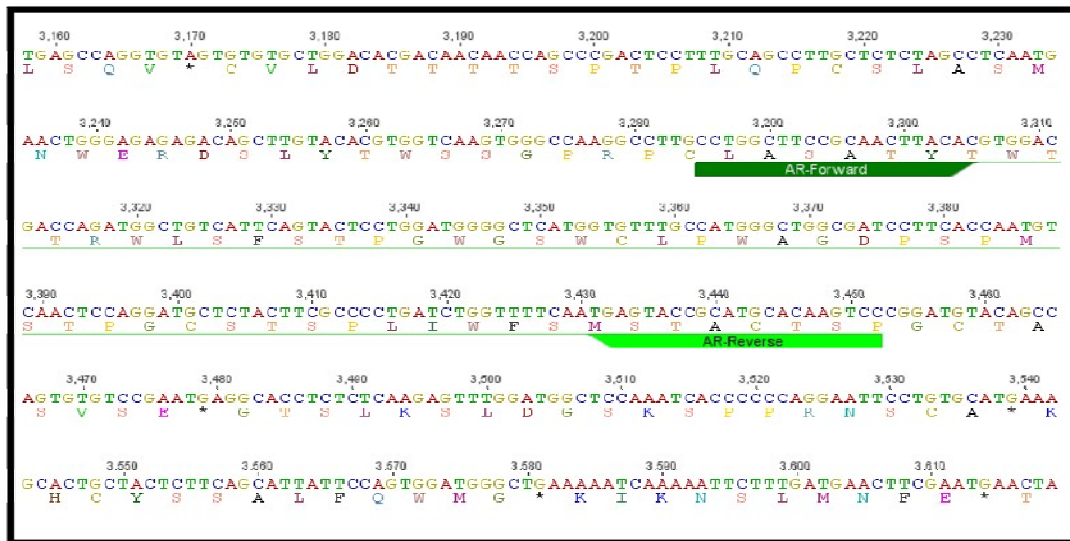
Primers Design	Primers sequence	Length (bp)
AR-Forward	5'-CCCGATCTATCCCTATGAC-3'	19
AR-Reverse	5'-AGCTGCTAAAGACTCGGAG-3'	19



Picture 1. Primers binding on the genomic sequence of the Androgen Receptor (NG_009014.2)

Table 2. qPCR Primers for the AR gene

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



Picture 2. Primers binding on the 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 prepuces. The collected prepuces were residual tissue from the subjects who underwent operation. The operation was a standard procedure for this case in the hospital and done by the urologist. One ethical problem of this study which possibly could appear was the utilization of residual prepuces as study material. So, explanation to patients' parents about purpose, advantages, and disadvantages of the study was necessary. All expenses needed to examine AR gene polymorphisms and expression were borne by authors. Confidentiality of the study is guaranteed.

This was a voluntary experiment. Study subjects had the rights to refuse 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 ethical clearance was obtained from the research ethical committee of the Medical Faculty of Andalas University, Padang.

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96 RESULTS

Average age of patients operated was between 10-14 years olds and the most frequent type of hypospadias was distal type. No AR polymorphisms were found in experimental group and control groups. We found over-expression of the AR gene in the experimental group and it was statistically significant ($p = 0.001$).

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

AR gene expression levels in hypospadias samples was significantly higher than control samples ($p = 0.001$).

In the conclusion, there was a significant correlation between AR gene over-expression with hypospadias incidence.

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Table 3. Characteristic of Subjects

	Hypospadias		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

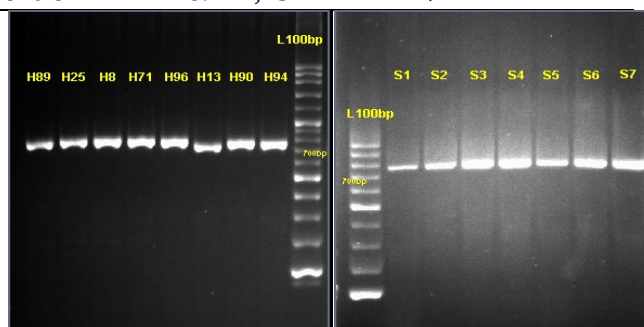
>20	2	4,1	1	2
Total	49	100	49	100

Table 4. Characteristic of hypospadias types

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

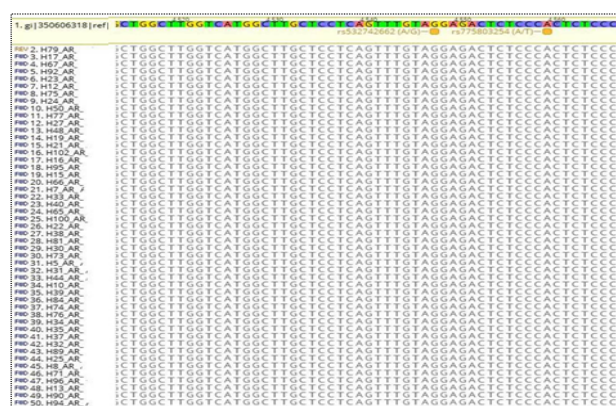
Table 5. The Correlation of Androgen Receptor (AR) gene expression with hypospadias incidence

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

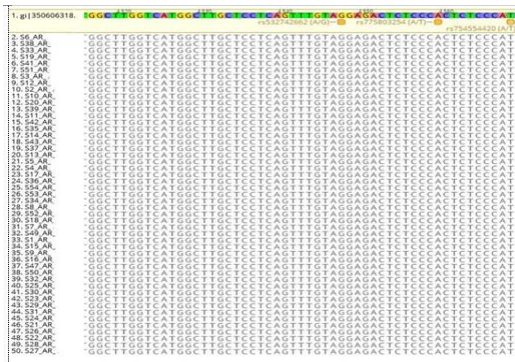


Picture 3. Electrophoresis of Androgen receptor gene PCR product in hypospadias and control (790 bp)

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



122 Picture 4. Multiple alignment Androgen receptor gene in hypospadia

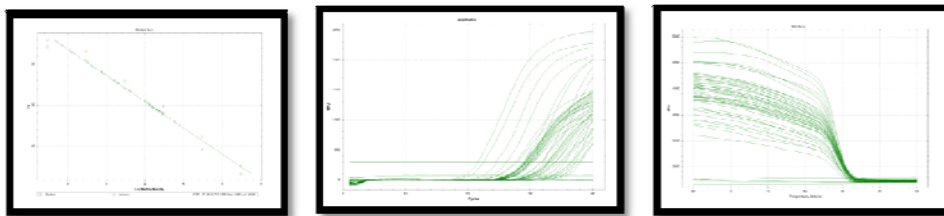


123

124 Picture 5. Multiple alignment Androgen receptor gene in Control

125 From this study, we did not find any mutation from any samples and control

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128 Picture 6. Standard Curve of Androgen receptor gene Expression

129 From the picture above, we can see that androgen receptor gene expression is same as
130 the standard curve, and from qPCR, we get the total androgen receptor gene expression is
131 34.913.906 with the mean expression is 356.264 if compared with standard.

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133 DISCUSSION

134 The results of this study showed that the most common age group of hypospadias
135 patients is in the age group of 10-14 years old which included 20 patients, the same amount
136 with the control group. The youngest patient going through operation of hypospadias was 1.5
137 years old and the oldest was 43 years old.

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

141 However, different results were obtained by Andika et al. who found the most
142 common age groups that came for treatment were 3 to 6 years old (56.3%) and 1 - 3 years old
143 (21.8%) .¹⁶

The results of this study were 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 was the distal-type as many as 43 patients (87.8%), while the proximal-type (penoscrotal, scrotal, and perineal) was only 6 patients (12.2%).

This was 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 was the midshaft-type of 33.3%.⁸ Similarly, a research conducted by Takahashi et al. in 2013 on a 18 hypospadias patients, found that distal-types of hypospadias was present in 11 patients (61.1%) and the proximal-type was present in 7 patients (38.9%).¹⁹

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

In this study, there were no AR gene mutations in both hypospadias and control group. Therefore, there was no relationship between AR gene promoter mutation and the occurrence of hypospadias. This study was consistent with the research of Radpour et al. who also found no AR gene mutations in hypospadias.²⁴

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

From this study, a significant relationship between AR gene expression and the occurrence of hypospadias was found, with p value <0,05. This was in accordance with a study conducted by Qiao et al., where they found an AR overexpression among patients with severe hypospadias. They also found higher levels of AR expression in mild hypospadias when compared with control group but it was not statistically significant.²⁸ This was in accordance with a literature stated that androgen signalling through AR greatly affects the normal growth of the penis, so a disturbance of androgen signalling will lead to various

disorders of the growth of external genitalia. The same result was also described by Manson et al. who stated that the AR gene plays an important role in male sexual differentiation by mediating the biological effects of gonadal androgens.²⁹ Increased AR expression increases the risk of malignant or early-onset prostate cancer, in which the lack of AR transcription factor activity increases the risk of demasculinization such as reduced sperm production, testicular atrophy, and infertility. Similarly, a study by Pichler et al. also found a meaningful correlation between AR overexpression with the occurrence of hypospadias.³⁰

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

Polymorphisms of AR promoter were not found in all hypospadias (0%). On the other hand, there was an increased level of AR expression in all hypospadias cases when compared with control group. 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.

In this study we did not explore other factors (like endogen and exogen factors) that could contribute to hypospadias incidence, we only investigated the androgen receptor gene, but we know there are so much genes that have a correlation to hypospadias and this could be a limitation of the study.

CONCLUSION

From this study we concluded that the distribution of promoter polymorphisms on androgen receptor gene were not different between hypospadias and control group, there was correlation between Androgen receptor gene expression with hypospadias incidence.

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