

**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. ~~Along with the development of molecular biology and microarray analysis, hypospadias is related with abnormalities of genetic polymorphism and expression. Promoter polymorphisms and genetic dysregulation of the Gene, such as~~ Androgen Receptor (AR) ~~are~~ suspected to cause ~~hypospadias~~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 ~~found in experimental group neither in and~~ control groups. 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. ~~Along with the development of molecular biology and microarray analysis, hypospadias is related with abnormalities of genetic polymorphism and expression. Promoter polymorphisms and genetic dysregulation of the Gene, such as~~ Androgen Receptor (AR) is suspected to cause hypospadias. This study prospectively examined polymorphisms 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 tissues were collected during operation. PCR-sequencing ~~technique~~ was used to examine AR polymorphisms 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 n~~No AR polymorphisms ~~was were~~ found in ~~the~~ experimental ~~group neither in and~~ control groups. 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), ~~G~~gene expression

BACKGROUND

Hypospadias is one of the most frequent male congenital abnormalities. Hypospadias incidence was reported 3-4 per 100.000 live births in Italy,¹ and 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 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 2011-2014 (Pande et al., 2015),⁸ while Bayu et al., found 15 hypospadias cases from 2009-2011 (Bayu et al., 2014).⁹ Data from medical records of 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 mutations and polymorphisms 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 were many studies about several studies regarding genetic markers to predict the etiology of this case 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 were expected to have shown 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 factors together with environmental exposure during urethral forming development in the uterus is believed to be the cause of hypospadias.^{14,15}

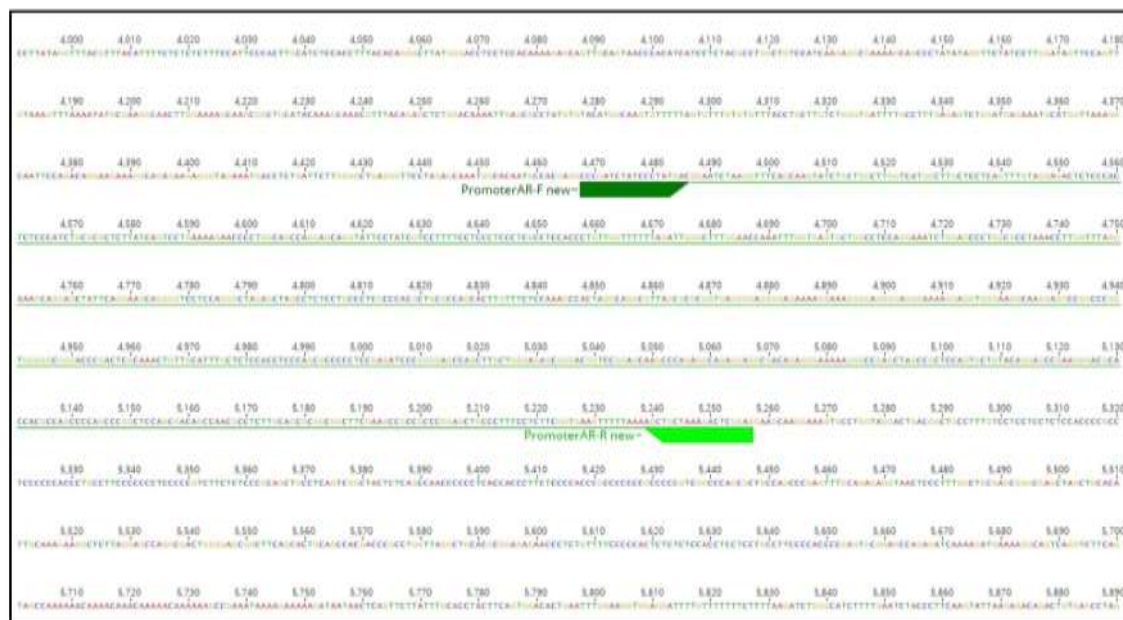
This study aimed to analyse the correlation between AR gene promoter polymorphisms and AR gene expression with hypospadias incidence.

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 technique was used to examine AR polymorphisms and Real-Time absolute-qPCR technique was used to examine gene expression through the absolute quantification method.

Table 1. Primers Androgen Receptor (AR) gene

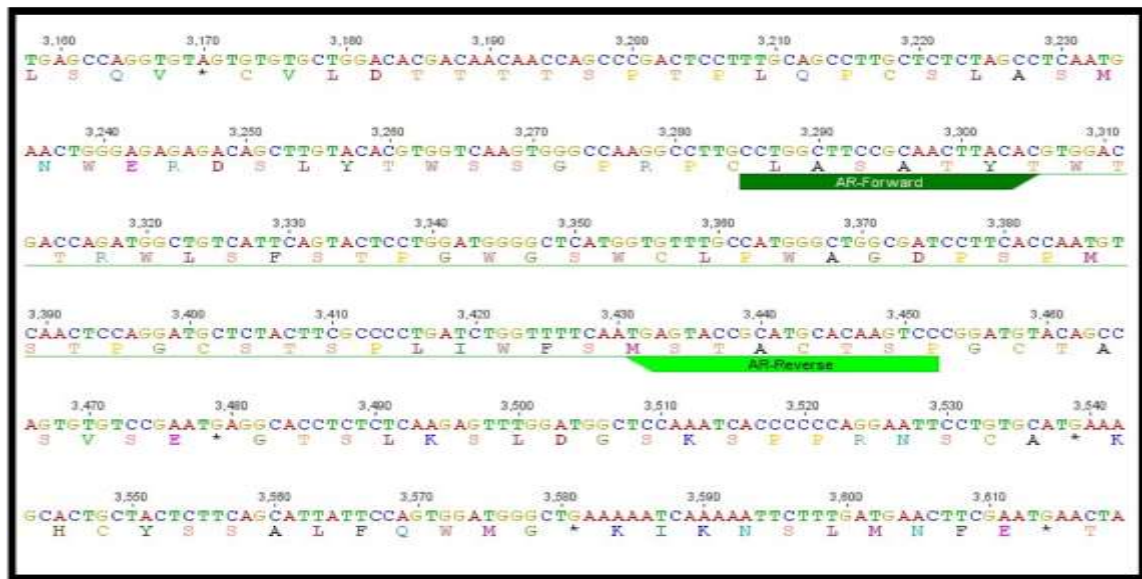
Primers Design	Primers sequence	Length (bp)
AR-Forward	5'-CCCGATCTATCCCTATGAC-3'	19
AR-Reverse	5'-AGCTGCTAAAGACTCGGAG-3'	19



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

Table 2. qPCR Primers for the 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-binding of androgen-receptor qPCR on sequence-of-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's 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 that, explanation to patient's parents about purpose, advantages, and disadvantages of the study was necessary. All cost-expenses needed to examine AR gene polymorphisms and expression was/were borne by authors. Confidentiality of the study is guaranteed.

This was a voluntary experiment. Study subjects had the 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 procedures. 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.

RESULTS

Average age of patients operated was between 10-14 years old, and the with most frequent type of hypospadias was distal type. Result of the study showed no AR polymorphisms were found in experimental group neither in control groups. We found

AR gene over-expression of the AR gene in the experimental group and it was statistically significant (p = 0.001).

Results of AR gene sequencing and blasting showed there were no mutations in any sample. In other words, from all hypospadia and control 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 levels obtained from hypospadia samples was 34.913.906 with the average of expression level was 356.264, compared with standard value. Analysis of correlation between AR gene expression with hypospadia incidence was statistically significant with p value of significantly higher than control samples (p= 0.001).~~

In the conclusion, ~~we reported that~~ there was a significant 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

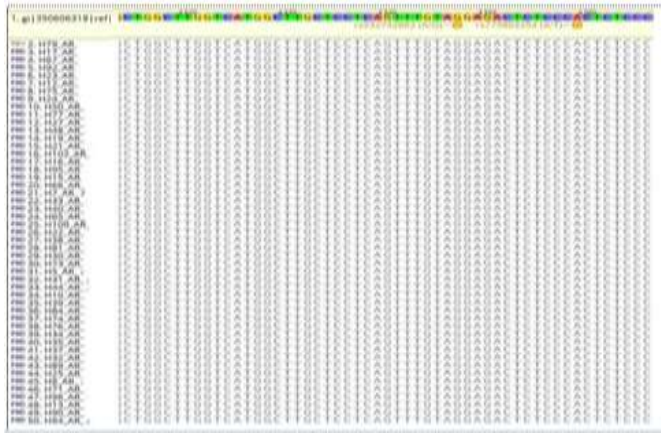
Group	Expression of AR gene		p
	Mean	Standard	

		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 hypospadia 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.

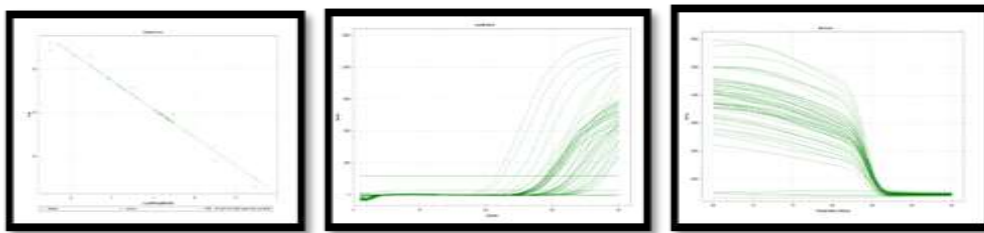


Picture 4. Multiple alignment Androgen receptor gene in hypospadia



Picture 5. Multiple alignment Androgen receptor gene in Control

From this study, we ~~didn't~~ did not find any mutation from any samples and control



Picture 6. Standard Curve of Androgen receptor gene Expression

From the picture above, we can see that androgen receptor 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 results of this study showed that the most common age group of hypospadias patients is in the age group of 10-14 years old, which ~~was as many as~~ included 20 ~~people~~ patients, the same amount with the control group. The youngest ~~age of~~ patient going through operation of hypospadias was 1.5 years old and the oldest ~~age~~ was 43 years old.

This was in accordance with a research conducted by Fariz in 2002-2008 for 116 patients who met 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 (56.3%) and 1 - 3 years old (-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 study of~~ 18 hypospadias patients, ~~and~~ found that ~~the patient with~~ distal-types of hypospadias ~~were~~ was present in 11 patients (61.1%) and the proximal-type ~~were~~ was present in 7 patients (38.9%).¹⁹

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

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

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

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

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

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

~~A statistical test could not be performed because there was no patient with positive mutation.~~

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

CONCLUSION

From this study we concluded that the distribution of promoter polymorphisms ~~and expression of on the~~ 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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