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The Correlation of Promoter Polymorphism and Expression of Androgen Receptor Gene with Hypospadia Incidence

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Authors' contributions

This work was carried out in collaboration between all authors. Author YZ designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors Yanwirasti, Jamsari and IW managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

Article Information

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ABSTRACT

Introduction: Hypospadia is one of the most common congenital abnormalities, yet the exact cause remains unknown. Androgen Receptor (AR) is suspected to cause hypospadias. **Methods:** As many as 49 post-operative prepuces of hypospadia patients and 49 normal prepuces from elective circumcision were recruited. Materials of this study were prepuces of hypospadia patients and normal children's prepuces. The prepuces were collected from residual tissue of the patients who underwent operation. The operation was a standard procedure done by urologist in the hospital.

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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.

Results: 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 was correlated with hypospadia incidence.

Keywords: Hypospadia; polymorphisms; Androgen Receptor (AR); gene expression.

1. INTRODUCTION

Hypospadia is one of the most common congenital abnormalities, yet the exact cause remains unknown. Androgen Receptor (AR) is suspected to cause hypospadia. This study prospectively examined polymorphism and expression levels of the AR in 49 hypospadia sample's tissues, and then compared with 49 normal penis skin tissues, collected from elective circumcision. Prepuces tissues were collected during operation. PCR-sequencing was used to examine AR polymorphism and qPCR to examine gene expression. In this study 49 hypospadia patients and 49 elective circumcision patients (as control) were underwent the operation at the age from 10 to 14 years old. The most frequent hypospadia type was distal hypospadia. 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 was correlated with hypospadia incidence.

1.1 Background

Hypospadia is one of the most frequent male congenital abnormalities. Hypospadia incidence was reported 3-4 per 100.000 live births [1]. Hypospadia incidence was found higher in Rotterdam Child Health Care Centre in 2000, those were 53 cases from 7.292 male babies (0.73%) [2]. Hypospadia patients in RSUP Ciptomangunkusumo Jakarta from 2002-2008 were 139 children [3]. A study from January 2011 to September 2012 in Sardjito Hospital Yogyakarta showed there were 60 cases of hypospadia [4]. According to a study conducted by Pande et al. there were 61 hypospadia cases from 2011-2014 [5]. While Bavu et al. found 15 hypospadia cases from 2009-2011 [6]. Data from medical records from the Urology Department M Djamil Hospital in the last 5 years (2010-2014), showed that hypospadia repair operation had been performed 95 times, with average of 19 patients per year [7].

Until now, certain etiology of hypospadia is not clearly known. Researchers are still expecting genetic factors, endocrine, and environmental factors as the etiological factors [8,9,10]. Van der Zanden et al. reviewed some articles and concluded that there was correlation between gene mutation and polymorphism with hypospadia incidence [9].

Several studies regarding genetic markers to predict the etiology of this hypospadia using microarray analysis have been reported. The authors used hypospadia patients' prepuces and compared them with normal prepuces collected patients who underwent from elective circumcision. Some genes showed a strong correlation with hypospadia incidence based on protein expression analysis and mRNA expression. Those genes include ATF3, zinc finger protein 36 (ZFP36), connective tissue factor (CTGF), and cysteine-rich growth angiogenic inducer 61 (CYR61). Genetic factor together with environmental exposure during urethral development in the uterus is believed to be the cause of hypospadia [11,12].

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

2. MATERIALS 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 hypospadia patients and 49 normal patients who underwent elective circumcision from January 2014 to September 2016. Hypospadia 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.

This study used post-operative prepuces of hypospadia patients and normal prepuces from elective circumcision patients as control. Materials of this study were prepuces of hypospadia 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 was 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.

3. RESULTS

Average age of patients operated was between 10-14 years olds and the most frequent type of hypospadia 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 hypospadia and control patients who were sequenced, the mutation rate was 0%.

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

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

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

Table 1. Primers Androgen Receptor (AR) gene

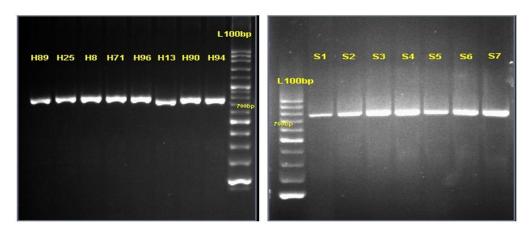
CTTA	4.000	4,010	4,020 TETETETET	4.030	4.040	4,050 CACCTITACA	4,060	4.070	4.080 CCACAÁAAGA	4,090	4.100	4,110 CATCCTCTAC	4.120 occroactor	4,130 CEATCAAGAG	4.140 C ANÍA CA	4.150 ACCCTATATA	4,160	4,170	4.18
TAAA	4.190	4,200	4,210	4.220	4.230	4,240	4,250	4.260	4,270	4,290 0101464100	4,290	4.300	4,310 GTGTTTACCY	4,320 ICTTOTCTOD	4.330	4340	4,350	4.360	4.37
AATTO	4.380 CCASACAOS	4.390	4,400	4.410 TATAAATCAC	4.420	4,430 T0000¢T0A0	4,440		4,460 caatoccaco erAR-F net		4,480 TATCCCTATC	4.490	4.500	4.510	4.520 reoctroote	4.530 ATOOCTTOCT	4,540	4,550	4.56
CTCC	4.570 CATCTICCC	4,580	4,590	4.600	4.610	4.620 CAODTATTCC	4.630	4.640	4.650 001000000000	4.660	4.670	4.680	4.690	4,700	4.710	4.720	4,730 cccrolcocc	4,740	4,75
LAROC/	4.760	4,770	4,780	4.790 A000ctanao	4,800 CTAOCCTCTC	4,810 стосстсое	4.820 CCACOCTOCO	4.830	4.840	4,850 occactagoc	4,860	4,870	4,880	4.890	4.900	4910 004445040	4,920	4.930	4.94
	4.950	4.960	4.970	4.980	4.990 CTCCCA0COC	5,000 eccertée AG	5.010 ATCCC0000A	5.020 CCANCTTIC	5.030	5.040	5.050	5.060	5.070 0400¢04C40	5.080	5.090	5.100	5.110	5.120	5.13 00ACOCA
CACIO	5,140 CEASCECEA	5,150	5,160	5.170 AACOCCTCTT	5,180	5,190	5,200	5.210				5.250	5.260	5,270	5,280	5,290	5,300	5,310	5,32
ecce	5.330 CEACCETIIC	5,340	5,350 reccccatet	5.360 rererecede	5.370 AGETGÉCTEA	5.380	5.390 CTCA0ĊCAAC	5.400 ccccctcacc		5,420 CCACCOCCC		5.440 ITCO/CCCA0	5.450 COCTOCCAOC	5,450	5.470	5.480 CTCCCTTTIN	5.490 CTOCOACCOO	5.500	5.51 CTUCACA
TOCAN	5.520	5.530	5,540 ASOCOÁCTOD	5.550 MACCOCTT	5.560	5,570	5.580 CCTOTTA	5,590 octocheses	5,600	5.610	5,620	5,630 СТССАССТСС	5,640	5,650 CECACCCCCA	5,660	5.670	5.680	5,690	5,70
	5.710	5.720	5,730	5,740	5,750	5,760	5,770	5,780	5,790	5.800	5.810	5.820	5.830	5.840	5.850	5.860	5.870	5.880	5.89

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

rimers design	Primers sequence	Length (bp)
R-Forward	5'-CCTGGCTTCCGCAACTTACAC-3'	21
R-Reverse	5'-GGACTTGTGCATGCGGTACTCA-3'	22
3,160 3,170	3,180 3,190 3,200 3,210	3,220 3,230
TGAGCCAGGTGTAGT	TGTGTGCTGGACACGACAACCAACCAGCCCGACTCCTTTGCAGC	CCTTGCTCTCTAGCCTCAATG
3,240 3	3,250 3,260 3,270 3,280 3,290	3,300 3,310
AACTGGGAGAGAGAGAG	1 1 1 1 1	TTCCGCAACTTACACGTGGAC
NWERD	SLYTWSSGPRPCLA	S A T Y T W T AR-Forward
		AR-FOIWard
3,320	3,330 3,340 3,350 3,360	3,370 3,380
		CTGGCGATCCTTCACCAATGT
0.000	a 40a a 40a a 40a	2.472
3,390 3,400 CAACTCCAGGATGCT	3,410 3,420 3,430 3,440 FCTACTTCGCCCCTGATCTGGTTTTCAATGAGTACCGCATGC	3,450 3,460
S T P G C	S T S P L I W F S M S T A C	TSPGCTA
	AK-Reverse	
3,470 3,4	480 3,490 3,500 3,510 3,520	3,530 3,540
AGTGTGTCCGAATGA	AGG CACCTCTCTCAAGAG TTTGGATGG CTCCAAATCACCCCCC	CAGGAATTCCTGTGCATGAAA
and the second se	I have a second s	The second se
AGTGTGTCCGAATGA SVSE	AGGCACCTCTCTCAAGAGTTTGGATGGCTCCAAATCACCCCCC G T S L K S L D G S K S P P	CAGGAATTCCTGTGCATGAAA R N S C A K
AGTGTGTCCGAATGA SVSE 3.550	AGG CACCTCTCTCAAGAG TTTGGATGG CTCCAAATCACCCCCC G T S L K S L D G S K S P P 3.580 3.570 3.580 3.590	CAGGAATTCCTGTGCATGAAA R N S C A * K 3.000 3.010
AGTGTGTCCGAATGA SVSE 3.550	AGGCACCTCTCTCAAGAGTTTGGATGGCTCCAAATCACCCCCC G T S L K S L D G S K S P P	CAGGAATTCCTGTGCATGAAA R N S C A K

Table 2. gPCR primers for the AR gene

Picture 2. Primers binding on the Androgen Receptor mRNA (NM_000044.3)



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

Table 3.	Characteristic	of subjects
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Ages (years)	Нур	ospadia	Control		
	f	%	f	%	
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	

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.

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

From the Picture 6, 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.

Table 4. Characteristic of hypospadia types

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

Table 5. The Correlation of AndrogenReceptor (AR) gene expression withhypospadia incidence

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

4. 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 included 20 patients, the same amount with the control group. The youngest patient going through operation of hypospadias was 1.5 years old and the oldest 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%) [3].

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%) [13].

The results of this study were not in accordance with the literatures stating that hypospadias correction should be done at the age of preschool [14].

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

1.gi[350606318[ref]	ICTGGCTTGGTCATGGCTTGGCCCCCCGCGTCTGTGGGGGGGG
RIV 2. H79 AR	ICT66CTT66TCAT66CTT6CTCCTCAGTTT6TA66A6ACTCTCCCACTCTC
FID 3. H17 AR	ictggcttggtcatggcttgctcctcagtttgtaggagactctcccactctcc
PID 4. H67 AR	SCTGGCTTGGTCATGGCTTGCTCCTCAGTTTGTAGGAGACTCTCCCACTCTCC
FID 5. H92 AR	ictggcttggtcatggcttgctcctcagtttgtaggagactctcccactctc
FID 6, H23 AR	ictggcttggtcAtggcttgctcctcAgtttgtAggAgActctcccActctc
100 7. H12 AR	ictggcttggtcatggcttgctcctcagtttgtaggagactctcccactctc
NO 8. H75 AR	ictggcttggtcatggcttgctcctcagtttgtaggagactctcccactctcc
FID 9. H24 AR	ictggcttggtcatggcttgctcctcagtttgtaggagactctcccactctc
PED 10, H50 AR	ictggcttggtcatggcttgctcctcagttgtaggagactctcccactctcc
FED 11, H77 AR	ictggcttggtcatggcttgctcctcagtttgtaggagactctccccactctcc
PRD 12, H27 AR	ictggcttggtcatggcttgctcctcagtttgtaggagactctcccactetcc
PHD 13, H48 AR	ictggcttggtcatggcttgctcctcagtttgtaggagactctccccactctc
F80 14, H19 AR	ictggcttggtcatggcttgctcctcagtttgtaggagactctccccactctc
PED 15, H21 AR	ictggcttggtcatggcttgctcctcagtttgtaggagactctccccactctc
NO 16. H102 AR	ictggcttggtcatggcttgctcctcagttgtaggagactctcccactctc
NP 17, H16 AR	ICTGGCTTGGTCATGGCTTGCTCCTCAGTTTGTAGGAGACTCTCCCACTCTC
NO 18, H95 AR	ictggcttggtcatggcttgctcctcagtttgtaggagactctcccactctc
19. H15 AR	ICTGGCTTGGTCATGGCTTGCTCCTCAGTTTGTAGGAGACTCTCCCACTCTCC
10 20, H66 AR	ictggcttggtcatggcttgctcctcagtttgtaggagactctccccactctc
10 21, H7 AR 7	ICTGGCTTGGTCATGGCTTGCTCCTCAGTTTGTAGGAGACTCTCCCACTCTCC
10 22, H33 AR	ictggcttggtcatggcttgctcctcagtttgtaggagactctccccactctc
10 23. H40 AR	ICTGGCTTGGTCATGGCTTGCTCCTCAGTTTGTAGGAGACTCTCCCCACTCTC
10 24 H65 AR	ICTGGCTTGGTCATGGCTTGCTCCTCAGTTTGTAGGAGACTCTCCCACTCTC
10 25. H100 AR	ictggcttggtcatggcttgctcctcagtttgtaggagactctccccactctc
10 26. H22 AR	ictggcttggtcatggcttgctcctcagtttgtaggagactctccccactctc
10 27, H38 AR	ictggcttggtcatggcttgctcctcagtttgtaggagactctccccactctc
10 28. H81 AR	ictggcttggtcatggcttgctcctcagtttgtaggagactctccccactctc
10 29, H30 AR	letggettggtcatggettgeteeteagtttgtaggagaeteteecaetete
10 30, H73 AR	CTGGCTTGGTCATGGCTTGCTCCTCAGTTTGTAGGAGACTCTCCCACTCTC
10 30. H/3 AR	CTGGCTTGGTCATGGCTTGCTCCTCAGTTTGTAGGAGACTCTCCCCACTCTC
	CTGGCTTGGTCATGGCTTGCTCCTCAGTTTGTAGGAGACTCTCCCCACTCTCC
40 32. H31_AR_,	CTGGCTTGGTCATGGCTTGCTCCTCAGTTTGTAGGAGACTCTCCCACTCTC
40 33. H44_AR_	ICTGGCTTGGTCATGGCTTGCTCCTCAGTTTGTAGGAGACTCTCCCCACTCTC
40 34. H10 AR	ICTGGCTTGGTCATGGCTTGCTCCTCAGTTTGTAGGAGACTCTCCCCACTCTCC
40 35. H39_AR_	ICTGGCTTGGTCATGGCTTGCTCCTCAGTTTGTAGGAGACTCTCCCCACTCTC
90 36. H84_AR_	ictggcttggtcatggcttgctcctcagtttgtaggagactctcccactctcc
PED 37. H74 AR	
10 38. H76_AR_	
90 39. H34 AR	
#0 40. H35_AR_	
40 41, H37 AR	ICIGGCTIGGICAIGGCTIGCICCICAGITIGIAGGAGACICICCCACICIC
90 42. H32 AR	ICIGGCTIGGTCATGGCTIGCTCCTCAGTITGIAGGAGACTCICCCACTCICC
10 43. H89_AR_	ICIGGCTIGGICAIGGCTIGCICCICAGTIIGIAGGAGACICICCCACICIC
WD 44. H25_AR	ICIGGCIIGGICAIGGCIIGCICCICAGIIIGIAGGAGACICICCCACICIC
45. H8_AR_ /	ICTGGCTTGGTCATGGCTTGCTCCTCAGTTTGTAGGAGACTCTCCCACTCTCC
HED 46. H71_AR_	ICIGGCTIGGICAIGGCTIGCICCTCAGTIIGIAGGAGACICICCCACICICC
47. H96_AR_	ICTGGCTTGGTCATGGCTTGCTCCTCAGTTTGTAGGAGACTCTCCCACTCTCC
Pep 48. H13_AR_	ICTGGCTTGGTCATGGCTTGCTCCTCAGTTTGTAGGAGACTCTCCCACTCTCC
F80 49, H90 AR	ICTGGCTTGGTCATGGCTTGCTCCTCAGTTTGTAGGAGACTCTCCCACTCTCC
PBD 50, H94 AR +	ictggcttggtcatggcttgctcctcAgtttgtaggagactctcccactctcc

Picture 4. Multiple alignment Androgen receptor gene in hypospadia

Picture 5. Multiple alignment Androgen receptor gene in control

hypospadias was the midshaft-type of 33.3%.[5] 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%) [15].

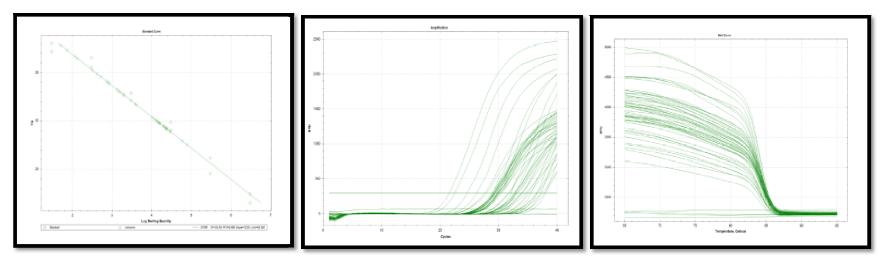
These results were not in accordance with the research by Samtani et al. in 2010 in New Delhi, India. The study was conducted on 80 patients with hypospadias and it was found that the proximal-type was as much as 56.38 %, whereas distal-type was 43.62%, and the most common type was the penoscrotal-type with 36 patients [16].

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 [17].

Unlike a research by Adamovic et al. [18] in 2012 who found a significant association between AR gene mutation and the occurrence of hypospadias, with odds ratio 2 to 3 fold. Also, different results were obtained by Borhani et al. [19] 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. 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 cryptochism [20].

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. [21] 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. 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 [22]. 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 correlation meaningful between AR overexpression with the occurrence of hypospadias [23].

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Picture 6. Standard curve of Androgen receptor gene expression

Whereas different result was found in a research by Beleza-Meireles et al. [24] who found no significant association between AR expression and the occurrence of hypospadias, either mild or severe.

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 hypospadia 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 hypospadia incidence, we only investigated the androgen receptor gene, but we know there are so much genes that have a correlation to hypospadia and this could be a limitation of the study.

5. CONCLUSION

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

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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