COMPARISON OF ANDROGEN AND ESTROGEN RECEPTORS' EXPRESSION IN DARTOS TISSUE OF BOYS WITH AND WITHOUT HYPOSPADIAS

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ABSTRACT

Objective: *This study aims to investigate the characteristics of androgen receptors (AR), estrogen receptor 1 (ER1) and estrogen receptor 2 (ER2) expression in dartos tissue of patients with congenital hypospadias, compared to normal penis.* ***Material & Methods:*** *We harvested 63 dartos tissue consisting of 53 congenital hypospadias that underwent urethroplasty (20 distal and 33 proximal) and 10 normal penis that underwent circumcision as controls from September 2017 to September 2018. The expressions of AR, ER1, and ER2 were measured using Quantitative Real-Time PCR (qPCR). All data were analyzed by Prism 7, and one-way ANOVA tests were used to compare gene expressions between the groups.* ***Results:*** *The mean age was 68.99 (± 45.5) and 65.6 (± 25.8) months in boys with and without hypospadias, respectively. The expression of mRNA AR was decreased in proximal (6.26 ± 2.30) and distal hypospadias (6.43 ± 2.22) compared to controls (9.69 ± 1.10), which were statistically significant ($p=0.0001$ and $p<0.0001$, respectively). We found a statistically significant difference of ER1 expression compared to controls ($p=0.0064$). The expression of ER2 was significantly increased in distal (21.03 ± 5.00) and proximal hypospadias (25.21 ± 8.06) groups compared to controls (11.80 ± 2.49) ($p<0.0001$). There was no statistically significant mean difference in mRNA ER1 expression ($p=0.65$).* ***Conclusion:*** *The repressed AR and elevated ER mRNA as shown in our study may suggest that defects in those receptors' interaction and/or balance may contribute to hypospadias and penile curvature condition. Further studies are needed to evaluate any gene-related problems in hypospadias.*

Keywords: *Hypospadias, androgen receptor, estrogen receptor, dartos tissue.*

ABSTRAK

Tujuan: *Penelitian ini bertujuan untuk mengetahui karakteristik reseptor androgen (AR), reseptor estrogen 1 (ER1) dan reseptor estrogen 2 (ER2) pada jaringan dartos pasien hipospadia kongenital, dibandingkan dengan penis normal.* ***Bahan & Cara:*** *Kami mengumpulkan 63 jaringan dartos yang terdiri dari 53 hipospadia kongenital yang menjalani urethroplasty (20 distal dan 33 proksimal) dan 10 penis normal yang menjalani sirkumsisi sebagai kontrol dari September 2017 hingga September 2018. Ekspresi AR, ER1 dan ER2 diukur menggunakan Quantitative Real- Waktu PCR (qPCR). Semua data dianalisis dengan Prism 7, dan tes ANOVA one-way yang digunakan untuk membandingkan ekspresi gen antar kelompok.* ***Hasil:*** *Rerata usia adalah 68.99 (± 45.5) dan 65.6 (± 25.8) bulan pada anak laki-laki dengan dan tanpa hipospadia. Ekspresi mRNA AR menurun pada hipospadia proksimal (6.26 ± 2.30) dan distal (6.43 ± 2.22) dibandingkan dengan kontrol (9.69 ± 1.10), yang secara statistik signifikan ($p=0.0001$ dan $p<0.0001$, masing-masing). Kami menemukan perbedaan ER1 yang signifikan secara statistik dibandingkan dengan kontrol ($p=0.0064$). Ekspresi ER2 meningkat secara signifikan pada kelompok hipospadia distal (21.03 ± 5.00) dan proksimal (25.21 ± 8.06) dibandingkan dengan kontrol (11.80 ± 2.49) ($p<0.0001$). Tidak ada perbedaan rata-rata yang signifikan secara statistik dalam ekspresi mRNA ER1 ($p=0.65$).* ***Simpulan:*** *AR yang ditekan dan ER mRNA yang meningkat seperti yang ditunjukkan dalam penelitian kami mungkin menunjukkan bahwa cacat pada interaksi dan / atau keseimbangan reseptor tersebut dapat berkontribusi pada hipospadia dan kondisi kelengkungan penis. Studi lebih lanjut diperlukan untuk mengevaluasi masalah terkait gen pada hipospadia.*

Kata Kunci: *Hipospadia, reseptor androgen, reseptor estrogen, jaringan dartos.*

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INTRODUCTION

Hypospadias is a common congenital disorder in male genitalia.[1] The incidence of hypospadias approximately one in 250 newborns.[2] In patients with hypospadias there is a failure or blockage in the normal development of the urethral, foreskin, and ventral aspect of the penis during urethral tube closure at the genital tubercle (gestational weeks 8-16) accompanied by various anatomical defects, e.g., penile curvature (chordee), dorsal hooded or penoscrotal transposition.[3] The varying degrees of prepuce and spongiosum deficiency will defect penile and urethral development.[4]

Both management and classification in hypospadias are based on the location of the urethral meatus, which is divided into distal-anterior hypospadias (located on the glans, or distal shaft of the penis) and proximal-posterior (penoscrotal, scrotal, or perineal).[5] Currently, the etiology of hypospadias is still unclear, with a debate at the crossroads of genetics, endocrine, and environmental mechanisms.[6]

Androgen and estrogen hormones play an essential role in the development of genital organs, including the formation and shape of the penis. Recent evidence suggests that epigenetic disruption to those hormones' receptors, called the androgen receptors (AR) and estrogen receptors (ER), may lead to hypospadias.[7] However, conflicting results were reported about those expressions in previous studies comparing hypospadias and normal penes.[8-9] These observations lead to the question about what exactly is the role of those hormones' receptors in patients with hypospadias.

Particular attention was addressed to the progress in detecting the exact molecular events required for normal development of the external genitalia.[7] Here, we statistically compared the AR and ER mRNA levels in dartos tissue of normal boys and different severities of hypospadias.

OBJECTIVE

This study aimed to investigate the expression characteristics of AR and ER in dartos tissue of patients with congenital hypospadias, compared with healthy children.

MATERIAL & METHODS

We designed a cross-sectional study including 63 dartos tissue (DT) consisting of 53 congenital hypospadias that underwent urethroplasty (20 distal and 33 proximal hypospadias) and 10 normal penes that underwent circumcision from September 2018 to September 2019 in our centers. The control samples were randomly collected from boys with non-malformed penes, age ranging from six months to six years, who underwent elective circumcision with the underlying DT.

Hypospadias subjects were eligible if boys, ranging from six months to six years old, had the urethral orifice abnormally located along the ventral penis and requiring surgery, having no history of previous reconstruction surgery, with no significantly related anomaly suggesting that it was part of a syndrome. In the hypospadias group, we harvested tissue during chordee correction by unelastic dartos tissue excision1, as suggested by previous studies that congenital penile abnormality has association with dartos tissue.[10-12]

The hypospadias types were determined after the penile degloving procedure, with the patient positioned prone and the meatus position was examined relative to the upper pubic bone. Proximal hypospadias was defined as the urethral meatus which is located maximally below or equal to the pubic level after the penile degloving procedure, while distal hypospadias was defined as above that level.[13]

The degree of penile curvature was done intraoperatively while artificially erected, determined by placing a goniometer in the angle formed by the intersection of two imaginary lines running parallel to each of the two bent portions of the shaft. Persistent curvature of $< 30°$ after degloving and transection of the urethral plate was defined as mild curvature. When the curvature remained persistent within the range of 30° and 60° even after the transection of the urethral plate, it was classified as moderate hypospadias. If the persistent curvature remained $> 60°$ after transection of the urethral plate, it was classified as severe penile curvature.[1]

The plates were measured during the surgery at the widest area of the urethra (in mm). The narrow plate was determined as the width of <8mm.[14] The sample size was calculated with the following formula = $(Z1\text{-}\alpha/22\ SD2)\div d2$.[15] Recognizing the 95% Confidence Interval (CI) and 20% permissible error, the estimated sample size was required to include 61 subjects. However, the researchers decided to include 63 boys, since the Z1-α/2-standard normal variate (at 5% type 1 error ($p<0.05$) is 1.96 and at 1% type 1 error ($p<0.01$), it is 2.58). As

common with the previous studies, p-values are considered significant below 0.05; hence, 1.96 was utilized in the formula.

We addressed age as the potential confounding factor. Thus, we designed the study with the randomization of subject's recruitment, restriction of subjects' enrollment to a specific age, and matching the compared groups according to their similar age group.

We performed qPCR examination in the Pathology Anatomy Laboratory, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada. The FavorPrepTM Tissue Total RNA Mini Kit (FAVORGEN Biotech Corp. Pingtung County, Taiwan) was used for isolation of total RNAs according to the manufacturer's protocol. DNA was extracted using FavorPrep Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp.) The thermal cycles were as follows: initial denaturation at 94 °C for 1 min and 30 sec, followed by 5 cycles at 94 °C for 20 sec, 5 cycles at 64 °C for 5 sec and 5 cycles at 72 °C for 5 sec. Then, the process continued with 40 cycles at 94 °C for 5 sec, 40 cycles at 64 °C for 5 sec, 40 cycles at 72 °C for 5 sec. These procedures produced a DNA fragment in length of 185 bp.

The amplified products were analyzed using 2% agarose gel electrophoresis with 0.5 µg/mL ethidium bromide staining and visualized under UV transillumination. All amplifications were conducted in triplicate. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as internal control. PCR products were measured using the Exicycler™ 96 Quantitative Real-Time PCR System (Bioneer, Daejeon, South Korea).

We used the Livak method for calculating gene expression.[16] Detailed information of the primer sequences is shown in Table 1.

Statistical comparisons were performed and charts produced by Prism 7 software (GraphPad Software, San Diego, CA, USA). The non-parametric data on clinical characteristics frequencies among the groups were compared using the Kruskall-Wallis test and Mann-Whitney U test. Correlations between AR and ER expression and clinical findings were assessed using the one-way ANOVA followed by Bonferroni post-hoc analysis. Values of $p < 0.05$ denoted a statistically significant difference.

RESULTS

The mean of age in control and hypospadias groups were 65.6 ± 25.8 (range 24–120) and 68.99 ± 45.5 (range 12–168) months, respectively. In the hypospadias groups (n = 53), we found most of the cases were proximal hypospadias (62%). The demographic data and clinical characteristics are shown in Table 2.

Quantification using qPCR was used to obtain the expressions of AR and ER in the collected dartos tissue. Lower expression of AR levels, with higher expressions of ER1 and ER2, were found in the hypospadias groups compared to controls, with AR and ER2 levels statistically significantly different with $p < 0.05$. (Fig 1 and Table 3).

AR expression was lower in proximal than distal hypospadias, but statistically not significant ($p=1.000$). If compared to controls, AR expressions were lower in both proximal and distal hypospadias, and statistically significant with $p=0.000$ and $p<0.0001$, respectively. The expression of ER1 in proximal hypospadias was lower compared to distal, but higher than normal penile, but not statistically significant ($p=1.000$ and 0.411, respectively). The mean expressions of ER2 in distal and proximal hypospadias were significantly higher compared to controls with $p<0.002$ and $p<0.000$, respectively. (Fig. 1 & Table 5).

Table 1. PCR primer sequences of androgen and estrogen receptors.

Gene	Primer sequences	Product size (bp)
AR	F: CCTGGCTTCCGCAACTTACAC	170
	R: GGACTTGTGCATGCGGTACTCA	
ER1	F: ATTTCTCTGCGCCCCTAGAC	137
	R: CATAGTGGTACCCAGACGCA	
ER2	F: TGAACTGGGCCCAGACTATG	85
	R: ATGGTACCCTGAGGCGTAGT	
GADPH	F: CATGTTCGTCATGGGTGTGAACCA	160
	R: AGTGATGGCATGGACTGTGGTCAT	

AR (Androgen receptor); ER1 (Estrogen receptor 1); ER2 (Estrogen receptor 2)

Table 2. Demographic and clinical characteristics of patients according to the groups.

Variable	Distal Hypospadias (n=20)	Proximal Hypospadias (n=33)	Normal penile (n=10)	*p-value**
Age (months)				
X ± SD	53.99 ± 41.87	83.99 ± 45.83	65.6 ± 25.8	0.0879
Penile torsion				
Yes	0 (0)	7 (11.1)	0 (0)	< 0.001
No	20 (31.7)	26 (41.3)	10 (15.9)	< 0.001
Penile Curvature				
Mild	3 (4.8)	13 (20.6)	0 (0)	< 0.001
Moderate	6 (9.5)	6 (9.52)	0 (0)	n.s
Severe	11 (17.5)	14 (22.2)	0 (0)	< 0.001
No	0 (0)	0 (0)	10 (15.9)	< 0.001
Urethral plate				
Narrow	10 (15.9)	15 (23.8)	0 (0)	< 0.001
Wide	10 (15.9)	18 (28.6)	0 (0)	< 0.001
Normal	0 (0)	0 (0)	10 (15.9)	< 0.001
Penoscrotal transposition				
Yes	2 (3.2)	5 (7.9)	0 (0)	< 0.001
No	18 (28.6)	28 (44.4)	10 (15.9)	< 0.001
Management				
First	15 (23.8)	19 (30.1)	0 (0)	< 0.001
Redo	5 (7.93)	14 (22.2)	0 (0)	< 0.001
Circumcision	0 (0)	0 (0)	10 (15.9)	< 0.001

*Kruskal-Wallis test
AR (Androgen receptor); ER1 (Estrogen receptor 1); ER2 (Estrogen receptor 2)

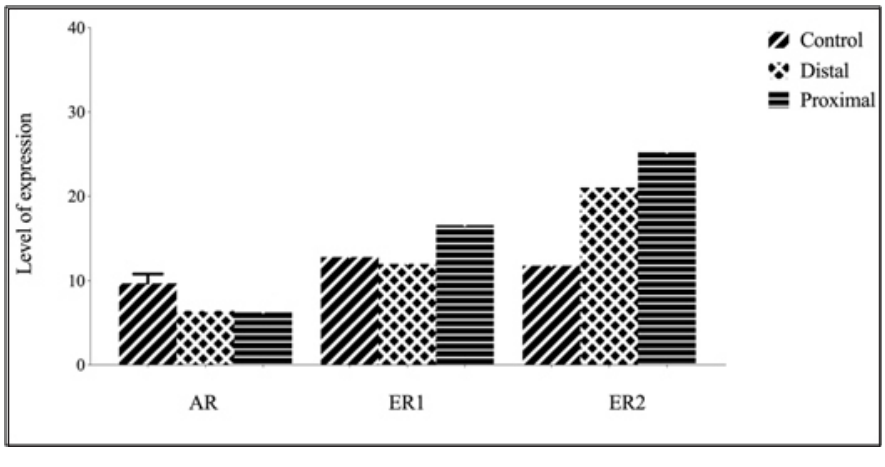

Figure 1. Comparison of AR, ER1, and ER2 mRNA levels among the group according to severity of hypospadias and normal group. mRNA expression were measured by quantitative PCR. GADPH was used as an internal control. Error bars represent standard deviation of the groups.

Table 3. Comparison of AR, ER1 and ER2 expression in hypospadias and normal penile.

	Hypospadias (n=53)	Normal penile (n=10)	*p*-value*
AR	6.49	9.51	< 0.001
ER1	17.14	13.02	0.1025
ER2	24.25	12.12	< 0.001

*Mann-Whitney U test, Data are presented as median.

Table 4. Comparison of AR, ER1, and ER2 expression in normal penile, distal and proximal hypospadias.

	Distal Hypospadias	Proximal Hypospadias	Normal penile	*p*-value*
AR	6.43 ± 2.22	6.26 ± 2.30	9.69 ± 1.10	< 0.001
ER1	17.32 ± 8.35	16.59 ± 6.88	12.82 ± 2.23	0.231
ER2	21.03 ± 5.00	25.21 ± 8.06	11.80 ± 2.49	< 0.001

*One-way ANOVA, Data are presented as mean.

Table 5. Post hoc analysis.

	Mean difference (95% CI)	*p*-value*
Androgen receptors		
Normal vs distal hypospadias	3.3 (1.22, 5.30)	0.001
Normal vs proximal hypospadias	3.4 (1.53, 5.34)	< 0.001
Distal vs proximal hypospadias	0.2 (-1.32, 1.66)	1.000
ER1		
Normal vs distal hypospadias	4.50 (-2.12, 11.12)	0.297
Normal vs proximal hypospadias	3.78 (-2.39, 9.94)	0.411
Distal vs proximal hypospadias	0.73 (-4.12, 5.57)	1.000
ER2		
Normal vs distal hypospadias	9.23 (2.94,15.53)	0.002
Normal vs proximal hypospadias	13.42 (7.55, 19.28)	< 0.001
Distal vs proximal hypospadias	4.18 (-0.42, 8.79)	0.087

*Post-hoc Bonferroni.

DISCUSSION

Hypospadias is a commonly occurring congenital disorder in male genital development, but its etiology remains poorly understood. One of the most widely believed causes is that it is a consequence of genetic susceptibility and environmental triggers. This could alter the androgen and estrogen hormonal balance, which is a crucial axis to harmonize the whole external genitalia development.[4]

The Androgen receptors (AR) play an important role in developing external sex organs through the binding of testosterone and dihydrotestosterone following secretion from the testis. AR is normally present in the developing human penis.[7] Previous studies documented hemizygous mutations in hypospadias patients, but the presence of varying polymorphisms in AR has yet to be proven, with some studies suggesting the longer AR gene CAG repeat polymorphisms were associated with the risk of hypospadias, and others have found it has an association with the length of GGN repeats.[3] Such documented polymorphisms may affect the transcriptional activity of AR and reduce the binding capacity to testosterone instead of the AR levels itself, as mice experiments demonstrated no significant association in alterations of AR mRNA expression and the hypospadias events.[17]

Previous studies reported that 30% of hypospadias cases might be related to genetic mutations conferring an increased risk of genitalia malformation.[18] One study found that AR levels were expressed higher in patients with severe hypospadias than mild hypospadias and controls[19], while probably the first finding exhibits the relationship of expressed AR in human tissue samples with the hypospadias severity. Remarkably, a newer study identified that hypospadias was rarely associated with mutated AR genes.[20] It was also suggested that ARs in patients with hypospadias were expressed similarly to controls.[21]

Although the expression of AR protein is tightly regulated at the transcription, translation, and post-translational control, it is the transcription step that is likely to have significant implications. The AR in protein and mRNA levels were ubiquitously overexpressed, suggesting transcriptional control of AR expression.[22] The increase of AR mRNA in our study appears to indicate the decreased ability to bind AR DNA, suggesting an AR signaling defect as

the hypothetical explanation of hypospadias possibly indicating further missing polypeptide-encoding. This is in accordance to the previous findings, where the AR were elevated in hypospadias patients, probably due to end-organ over expression.[8]

Previous studies have shown the positive influence of exogenous estrogen on the ontogeny of AR, demonstrated by the higher expression of AR in female than male mice after estrogen exposure to pregnant dams. The AR mRNA in the genital tubercle was expressed higher in estrogen-treated females than hypospadias estrogen-treated males.[17] These findings possibly happened in response to the small amount of available androgen, forcing the body to express more AR mRNA as the compensation mechanism. The up-regulated AR levels were expected to form complexes with any existing androgen firmly.

Skin tissues are widely known to possess a complex endocrine mechanism[21], including the expression and function of specific hormone receptors such as ERs that have been associated with skin tag lesions.[23] Because there is a deficiency of the urethral and ventral part of the penis skin in patients with hypospadias, attention has been drawn lately to expression and cellular provenance of ERs in this particular site.[24]

Our study demonstrates that normal dartos tissue in boys expressed the ER1 and ER2 mRNA. These results are in line with contemporary literature mentioning ER2 as the main receptor expressed in the penis.[25] In contrast to our findings, previous study noted in immunohistochemistry results that ERs were only weakly expressed or undetectable in hypospadias foreskin.[24] To a large extent, these findings suggest that ERs might play an integral role in developing normal human foreskin.

Our study aimed to evaluate whether there are expression divergences in ER variants that could enhance our knowledge in normal penis / urethral development. The present results suggest that changes in ER amounts may have occurred in normal and abnormal foreskin development. This finding is probably related to the skin tags presence in which the ER1 and ER2 expressions are significantly higher compared to normal skin[23], or the stratified ER1 and ER2 expressions which are found amongst histopathologically distinct subtypes in breast cancer.[26]

Normal human urethral development results from fusion of two epithelial surfaces in the urethral grooves along with epithelial seam remodelling.[4,27] The anomalies in hypospadias are either characterized by failure of urethral plate canalization, failure of urethral groove formation, failure of medial growth of the lateral edges of the urethral groove or failure of urethral folds's midline fusion.[28] Multiple studies have linked endocrine disruptors and ERs to hypospadias events.[6,25,29] Synthetic estrogen exposure in pregnant mice led to hypospadias in offspring, showing that antiandrogen hormones as the endocrine disruptors might be considered as a potential cause.[30] In a related study, the ER1 and ER2 was expressed in epithelial cells of the preputial lamina, canalization areas and remodelling and formation of the glandular urethral plate in utero.[31] This finding is concordant with our results, emphasizing the role of estrogen hormones and ERs in malformations of the penis.

The limitations of this study are mainly due to the few subjects with their heterogeneous characteristics. Moreover, we did not evaluate those hormonal expressions during a critical phase of phallic and urethral development. Further larger trials are needed in a well-defined homogeneous cohort to conduct structural analysis and evaluate the AR and ER mRNA levels as well as its potential association with the severity of hypospadias / penile curvature, and to clarify the possibility to measure fetal AR/ER levels during pregnancy and thereby corroborate these preliminary findings.

CONCLUSION

There is repression of AR and elevation of ER mRNA. This is supportive of possible hormonal signaling defect that leads to several biochemical aberrations, which have a significant role in penis development. The differences of mRNA AR, ER1 and ER2 expressions suggest that defects in those receptors' interaction and/or balance may contribute to hypospadias and penile curvature condition. Further studies are needed to evaluate any gene-related problems in hypospadias.

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