ORIGINAL ARTICLE

TWENTY-FOUR GENES ARE UPREGULATED IN PATIENTS WITH HYPOSPADIAS

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ABSTRACT

Hypospadias is a congenital hypoplasia of the penis, with displacement of the urethral opening along the ventral surface, and has been reported to be one of the most common congenital anomalies, occurring in approximately 1:250 to 1:300 live births. As hypospadias is reported to be an easily diagnosed malformation at the crossroads of genetics and environment, it is important to study the genetic component in order to elucidate its etiology. In this study, the gene expression profiles both in human hypospadias tissues and normal penile tissues were studied by Human Gene Expression Array. Twenty-four genes were found to be upregulated. Among these, *ATF3* and *CYR61* have been reported previously. Other genes that have not been previously reported were also found to be upregulated: *BTG2*, *CD69*, *CD9*, *DUSP1*, *EGR1*, *EIF4A1*, *FOS*, *FOSB*, *HBEGF*, *HNRNPUL1*, *IER2*, *JUN*, *JUNB*, *KLF2*, *NR4A1*, *NR4A2*, *PTGS2*, *RGS1*, *RTN4*, *SLC25A25*, *SOCS3* and *ZFP36* ($p <0.05$). Further studies including genome-wide association studies (GWAS) with expression studies in a large patient group will help us for identifying the candidate gene(s) in the etiology of hypospadias.

Keywords: Hypospadias; Gene expression; Microrarray.

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INTRODUCTION

Hypospadias is a congenital hypoplasia of the penis, with displacement of the urethral opening along the ventral surface, often associated with dorsal hooded foreskin and chordee [1]. The anatomical location of the misplaced urethral meatus determines the severity of this anomaly with the severity increasing from distal to proximal [2]. Hypospadias has also been reported to be one of the most common congenital anomalies, occurring in approximately 1:250 to 1:300 live births [3].

Although, the etiology of hypospadias remains unknown, a genetic component in the transmission of this birth defect has been suggested so it seems to be multifactorial [4]. In addition, 30.0% of severe hypospadias can be attributed to defects in the synthesis of testosterone or adrenal steroid hormones, receptor defects, syndrome-associated hypospadias, chromosomal anomalies, and/or defects in other genetic factors [2]. Thus, hypospadias may be a highly heterogeneous condition subject to multiple genetic and environmental factors [5].

With regard to molecular biology and microarray technology, it appears that hypospadias is potentially related to disrupted gene expression [6]. Previous studies revealed candidate genes including *WT1*, *SF1*, *BMP4*, *BMP7*, *HOXA4*, *HOXB6*, *AR*, *FGF8*, *FGFR2*, *HSD3B2*, *SRD5A2*, *ATF3*, *MAMLD1*, *MID1*, *BNC2*, *ESR1*, *ESR2*, *ATF3*, *DGKK*, *CYP1A1*, *GSTM1*, *GSTT1*, *CTGF*, *CYR61* and *EGF* [1]. Also, in order to study the epigenetic modification
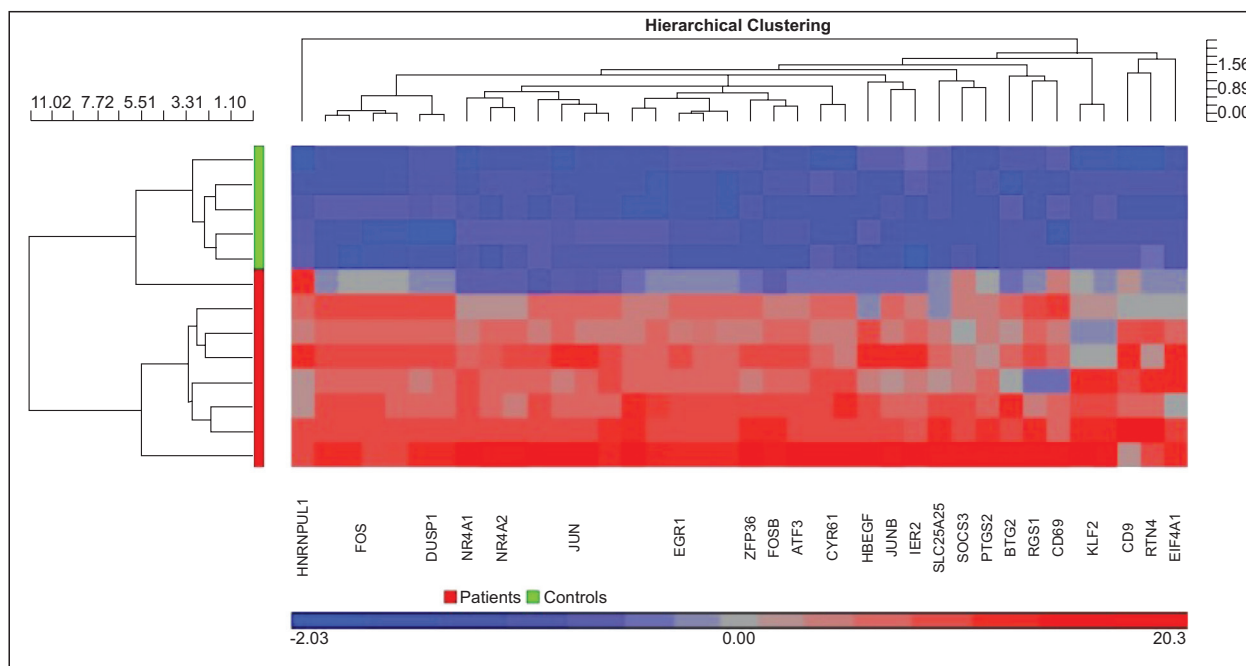
Figure 1. Gene tree of the top 24 upregulated genes whose expression significantly changed greater than 2-fold between hypospadias and control groups.

of DNA methylation in hypospadias, genome-wide DNA methylation profiling was performed, and the *SCARB1* and *MYBPH* genes have been reported to be involved in the etiology of hypospadias [7].

As hypospadias is reported to be an easily diagnosed malformation at the crossroads of genetics and environment, it is important to study the genetic component in order to elucidate its etiology [5]. Thus, in this study, our aim was to study the gene expression profiles both in human hypospadias tissues compared with that in normal penile tissues.

MATERIALS AND METHODS

Patients. A total of eight patients with isolated distal (subcoronal) hypospadias (mean age 6.8; range 2-10 years) and five healthy circumcised controls (mean age 6.5; range 2-10 years) were enrolled in this study. The penile skin tissue specimens obtained at surgery during hypospadias repair or elective circumcision were divided into two groups: children with hypospadias ($n = 8$), and normal controls ($n = 5$). Informed consent was obtained from the parents/guardians of all the children.

Samples were flash frozen with liquid nitrogen and stored at –80 °C until further processing. The gene expression studies were assessed using

Genechip® Primeview™ Human Gene Expression Array (Affymetrix, Santa Clara, CA, USA) which contain more than 53,000 probes including over 36,000 transcripts and variants.

RNA Isolation. Samples were distrupted and powdered under liquid nitrogen with pestle and mortar. Tissues were transferred into microcentrifuge tubes and lysated with 1 mL of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). All samples were homogenized using Qiashredder (Qiagen, Valencia, CA, USA); 0.2 mL chloroform was added to homogenized samples and centrifuged at 12,000g for 15 min. Upper aqueous phase was transferred into a new microcentrifuge tube and isolation of the high-purity total RNA was perfomed using the RNeasy® Mini Kit (Qiagen) following the manufacturer's specifications. The quantity and purity of RNA was determined using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) at 260 nm.

Gene Expression Studies. Genechip® Primeview™ Human Gene Expression Array (Affymetrix) was used for gene expression studies. Five hundred ng of total RNA was reverse transcribed, amplified and biotin-labelled with Genechip 3'IVT (*in vitro* transcription) Express Kit (Affy-metrix) according to manufacturer's instructions. aRNAs were then purified with magnetic beads and after fragmenta-

Table 1. Significantly expressed genes that changed more than 2-fold between hypospadias and control groups.

Gene	Gene Title	Function	Fold-Change
FOS	FBJ murine osteosarcoma viral oncogene homolog	apoptosis, transcription	16.649
FOSB	FBJ murine osteosarcoma viral oncogene homology B	transcription	14.9028
ATF3	Activating transcription factor 3	transcription	13.2691
NR4A1	Nuclear receptor subfamily 4, group A, member 1	apoptosis, signaling	10.5824
PTGS2	Prostaglandin-endoperoxide synthase 2	metabolism	9.44542
DUSP1	Dual specificity phospatase 1	signaling	7.95108
SOCS3	Suppressor of cytosine signaling 3	signaling	7.24912
CD69	CD69 molecule	receptor activity	7.06541
CYR61	Cysteine-rich, angiogenic inducer 61	signaling	6.99616
JUN	Jun proto-oncogene	transcription	6.4233
EGR1	Early growth response 1	transcription	6.37844
NR4A2	Nuclear receptor subfamily 4, group A, member 2	signaling	5.27591
RGS1	Regulator of G-protein signaling 1	signaling	4.25487
HBEGF	Heparing-bindingEGF-like growth factor	signaling	3.61757
JUNB	Jun B proto-oncogene	transcription	3.57188
CD9	CD9 molecule	signaling	3.37889
RTN4	Reticulon 4	protein binding	3.21246
IER2	Immediate early response 2	transcription	3.05546
SLC25A25	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 25	transporter	2.92541
ZFP36	Zinc finger protein 36, C3H type, homology (mouse)	transcription	2.61352
KLF2	Kruppel-like factor 2 (lung)	transcription	2.39853
EIF4A1	Eukaryotic translation initiation factor 4A1	translation	2.36873
BTG2	BTG family, member 2	transcription	2.33987
HNRNPUL1	Heterogeneous nuclear ribonucleoprotein U-like 1	transcription	2.0158

tion of purified biotinylated aRNAs, samples were loaded to Genechips for subsequent hybridization. Afterwards, Genechips were washed and stained on the Fluidics station with specified protocol.

Statistical Analysis. Signal intensities were acquired by Genechip Scanner 3000 7G (Affymetrix) to generate cell intensity files (CEL). Statistical analysis was performed using Partek Genomics Suite software (Partek Inc., St. Louis, MO, USA). Robust multi-array average (RMA) algorithm was used for data normalization. One-way analysis of variance (ANOVA) was used when >2 groups were compared, followed the by *t*-test. The statistical significance level was set at false discovery rate ((FDR) *p* <0.05 to minimize false identificaion of genes. Greater than 2-fold changes were analyzed for up or down regulated genes. Hierarchical clustering based on genes and samples was performed with Partek Genomics Suite Software (Partek Inc.).

RESULTS

Gene Microarray Analyses. A total of 24 genes were found to be upregulated (Figure 1). The upregulation of the *ATF3* and *CYR61* genes that were previosuly reported have been detected in hypospadias patients (*p* <0.05). Other genes that were not previously reported, were also found to be upregulated: *BTG2*, *CD69*, *CD9*, *DUSP1*, *EGR1*, *EIF4A1*, *FOS*, *FOSB*, *HBEGF*, *HNRNPUL1*, *IER2*, *JUN*, *JUNB*, *KLF2*, *NR4A1*, *NR4A2*, *PTGS2*, *RGS1*, *RTN4*, *SLC25A25*, *SOCS3* and *ZFP36* (*p* <0.05).

Cluster Analyses. The upregulated genes between hypospadias samples and normal controls were selected. Cluster analyses revealed several patterns of genes and included a number of transcription factors, signal pathways, cell cycle, metabolism, nuclear receptor family and structure proteins as well as growth factor receptors (Table 1).

DISCUSSION

Hypospadias has multifactorial origins that involve the actions of environmental factors with a genetic background [8]. The previous microarray studies indicated that, activating transcription factor 3 (*ATF3*), connective tissue growth factor (*CTGF*) and cysteine-rich, angiogenic inducer 61 (*CYR61*) genes were upregulated in hypospadias and all three genes were also estrogen-responsive [8-10].

The *ATF3* gene is upregulated in the skin of patients with hypospadias compared to normal prepuce. Also, *ATF3* expression at the mRNA level in fetal mouse tissues demonstrated that its mRNA is expressed significantly more in genital tubercles from fetal mice exposed *in utero* to estrogens than in those of unexposed fetal mice [4,10]. This gene has a role in suppression of cell cycling; therefore, it had been hypothesized that its role in hypospadias might be inhibition of cell growth in urethral formation. *ATF3* is upregulated in human and mouse hypospadiac tissues compared with control tissues, at both the mRNA and protein levels [8]. It has been suggested that *ATF3* may play a role in development of hypospadias as a result of exposure to estrogenic compounds [11]. Sequence variants of the *ATF3* gene may be involved in the genetic risk for hypospadias [12]. These genomic variants of *ATF3* have been reported to be present in 10.0% of patients with hypospadias [13]. In our study, we detected an upregulation of the *ATF3* gene by 13-fold in hypospadias tissues with respect to the controls.

The other genes that have been identified from a human microarray analysis study were *CTGF* and *CYR61*. These genes were both members of the cyclin gene family and might have roles in matrix remodeling through the activation of metalloproteinases [8,10]. Our study only revealed an upregulation of the *CYR61* gene by 5.8-6.0-fold.

Among the other 22 upregulated genes, several patterns of genes including apoptosis (*FOS*), apoptosis and signalling (*NR4A1*), metabolism (*PTGS2*), protein binding (*RTN4*), receptor activity (*CD69*), signalling (*DUSP1*, *SOCS3*, *NR4A2*, *EGR1*, *RGS1*, *HBEGF*, *CD9*), transcription (*FOSB*, *JUN*, *JUNB*, *IER2*, *ZFP36*, *KLF2*, *BTG2*, *HNRNPUL1*), translation (*EIF4A1*) and transporter activites (*SLC25A25*) were also assessed (Table 1).

With regard to the top upregulated genes, *FOS* and *NR4A1*, were shown to induce apoptosis (Table 1). Such expression of the *FOS* gene has been associated with apoptotic cell death, whereas the NR4A1 gene has also been reported to induce apoptosis [14,15]. These two apoptotic genes (*FOS*, *NR4A1*) have not been reported before.

It has been reported that apoptosis may induce external genitalia defects in fetal mouse [16]. The events leading to hypospadias formation had also been demonstrated to be associated with apoptotic and proliferative events in dorsal urethral epithelia and sinus cord [17]. However, Baskin *et al.* [18] indicated that hypospadias resulted from an arrest in urethral seam formation or seam remodeling but not by an epithelial apoptosis. Thus, the apoptotic genes need to be studied in a larger population.

In this study, we found a relation between hypospadias and the previously reported *ATF3* and *CYR61* genes. We also detected an upregulation of 22 genes in hypospadias patients that have not been reported before. Further studies including GWAS with expression studies in a larger patient group will help us to identify the candidate gene(s) in the etiology of hypospadias.

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