Down-regulation of FGF10 in hypospadias prepuce

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Research Article

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Abstract

Background: Fibroblast growth factors (FGF) expressed in the genital tubercle, especially FGF8 and FGF10, may be implicated in the morphogenesis of external genitalia. We investigated the expression of FGF8 and FGF10 in hypospadias prepuce to confirm any correlation.

Methods: Hypospadias was classified according to the location of the external meatus as coronal/subcoronal (mild; n=7), midshaft (moderate; n=6), penoscrotal (severe; n=3). Prepuce tissue specimens obtained intraoperatively from hypospadias patients (H; n=16), phimosis patients (P; n=8) between 2010 and 2016 were examined immunohistochemically. Quantification of immunostaining was performed using imageJ®. The protein expression of FGF10 in each group was examined by Western blot analysis.

Results: Mean ages at surgery were 3.9 and 4.5 years for H and P, respectively. Both FGF8 and FGF10 were expressed in the upper dermis, especially the papillary layer. FGF8 was similar, but FGF10 was down-regulated in H in proportion to severity (P: 13.08% versus mild H: 7.28%, moderate H: 5.01%, severe H: 1.38%). The FGF10 protein amount results showed FGF10 was down-regulated in H (phimosis 0.91, mild 0.99, moderate 0.65, severe 0.48).

Conclusion: The significant down-regulation of FGF10 identified may contribute to further understanding the etiology of hypospadias and may have some possible applications for treating hypospadias.

Introduction

Hypospadias results from failure of the formation or fusion of the urethral folds in the male fetus and affects 1 in 200–300 boys. It is thought to be due to a combination of genetic and environmental factors [1]. However, despite being such a common congenital anomaly, its exact etiology has yet to be determined. From a detailed report on the mechanism by which the human male urethra is formed [2] and optical projection tomography of the development of the male urethra from 6.5 to 16.5 weeks gestation, progression of the urethral meatus from the scrotal folds at 6.5 weeks gestation to a terminal position on the glans at 16.5 weeks gestation, it is known that the edges of a wide open urethral groove best seen from 9.5 to 13 weeks gestation, proceed to fuse from proximal to distal to form a tubular urethra. By 13 weeks gestation, the urethral groove is within the glans penis and the tubular urethra is completely formed within the shaft of the penis. While androgens have been reported to play a central role during these changes [3, 4], it is difficult to believe that androgens alone could induce and control the complex mechanisms involved. Advances in molecular biology suggest that genetic factors may also be involved, and that some derangement in their expression may contribute to the development of hypospadias.

Recent studies have hinted that fibroblast growth factors (FGF) expressed in the genital tubercle, especially FGF8 and FGF10, may play a pivotal role in the morphogenesis of external genitalia [5, 6, 7]. In fact, FGF10 knockout mice develop with severely malformed external genitalia [8, 9], but there are no

equivalent data for human subjects. We investigated the expression of FGF8 and FGF10 in hypospadias prepuce to improve understanding of its etiology.

Materials And Methods

Subjects

In selecting the subjects for this series, we randomly chose 16 hypospadias patients without cryptorchidism, and 8 phimosis patients with no history of balanoposthitis or advanced preputial fibrosis treated at our institution between 2010 and 2016. Severity of hypospadias was classified according to the location of the external meatus; coronal/subcoronal (mild H; n=7), midshaft (moderate H; n=6), or penoscrotal (severe H; n=3) [Table 1]. One case of severe H had an incidental chromosomal anomaly.

Table 1
Subject data

	Severe hypospadias	Moderate hypospadias	Mild hypospadias	Phimosis
n	3*	6	7	8
Mean age (range) at surgery (years)	3.3 (2.2–4.3)	4.3 (3.1–5.5)	3.9 (2.2–8.8)	4.5 (2.0-8.5)
FGF8	2.42%	2.07%	2.14%	2.10%
FGF10	1.38%	5.01%	7.28%	13.08%
*46XY/45X chromosomal mosaic (n = 1)				

Tissue specimens

Full thickness prepuce tissue specimens were obtained intraoperatively during repair surgery in H and during circumcision in P. All specimens were fixed in formalin and embedded in paraffin, then sectioned transversely at a thickness of 3 μm.

Immunohistochemistry

Immunohistochemical staining was used to determine the expression of FGF8 and FGF10 in each group using avidin-biotin-peroxidase complex (ABC) staining system (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). Firstly, deparaffinization was performed, and then blocking was performed with a methanol solution containing 0.3% H2O2. Sections were incubated overnight at 4°C with each of the following primary antibodies; rabbit anti-FGF8 (ab203030, 1:100) and rabbit anti FGF10 (ab80064, 1:50) (Abcam, Cambridge, UK). Sections were then washed in PBS and incubated with anti-Rabbit biotinylated secondary antibody (1:300) (Vector Laboratories, USA) for 30 minutes at room temperature. After

washing, sections were treated with streptavidin 1:300 (DAKO, USA) for 30 minutes at room temperature. Color development was performed with 3,3'-Diaminobenzidine (DAB) (Dojindo, Japan). All sections were counterstained with hematoxylin and examined under light microscopy (x20). FGF8 and FGF10 expression was determined by calculating the mean number of FGF positive cells in each of 5 randomly chosen 100μm x 100μm sample areas, blindly. Results were expressed as a percentage mean of the 5 sample areas. Quantification of immunostaining was performed using imageJ® (Wayne Rasband, National Institutes of Health; NIH). A color deconvolution function was used to extract the brown part of the original image and FGF8 and FGF10 distribution was converted to red with a color threshold tool set to 150. Comparison of each group was conducted by Tukey's multiple comparisons test. A p value of < 0.05 was considered to be statistically significant.

Immunoblot analysis

The prepuce specimen collected by surgery was frozen as it was with liquid nitrogen. One tablet /10 ml of protease inhibitor was added to T-PER Tissue Protein Extraction Reagent (Thermo Scientific product number: 78510). Per 1 g of tissue weight, 20 ml of T-PER Reagent was added and homogenized on ice. After centrifugation at 4 ° C for 5 minutes, the precipitate was discarded, the supernatant was transferred to a new tube, and the protein was extracted. The extracted protein sample was adjusted to 1: 1 with sample buffer (Wako 193-11032). The adjusted sample was reduced and denatured at 90–95 ° C for 5 minutes, and then cooled on ice. 10 μl of sample solution was applied to each well of SDS gel (Mini-PROTEAN TGX Precast Gels 12% Bio-RAD Cat. # 456–1046). Electrophoresis was performed at 0.025 A for 1 hour. Before electrophoresis was completed, the nitrocellulose membrane (BioTrace NT nitrosellulose 30 cm X 3 M Roll, LOT # 25026688) and the filter were immersed in Western Blot B solution. After completion of the electrophoresis, the gel was immersed in Western Blot B solution.

Semi-dry method was carried out and two filters, gel, membrane, two filters were stacked in order from the bottom, and transferred for 1 hour at 0.15 A. After dipping in 0.1% BSA, the mixture was reacted at room temperature for 30 minutes for blocking. The membrane was washed with TBST for 2 minutes, twice in total. In the primary antibody reaction, an anti-FGF10 antibody (abcam, ab 71794) diluted 1: 5000 in TBST and an anti-β-actin antibody (mouse monoclonal antibody) diluted 1: 200 were reacted at 4 ° C overnight. The membrane was washed three times with TBST for 10 minutes. In the secondary antibody reaction, each secondary antibody diluted to 1: 5000 in TBST was added and allowed to react at room temperature for 1 hour. The secondary antibody of anti-FGF antibody is Peroxidase AffiniPure Goat Anti-Rabbit IgG (H + L) (Jaxon Immuno Research 111-035-144). Peroxidase Goat Anti-Mouse IgG (H + L) (Jaxon Immuno Research 115-035-003) was used as a secondary antibody for anti-β-actin antibody. The membrane was washed three times with TBST for 10 minutes. Signal was detected by reacting with Immunostar Zeta (Wako, 297-72403) for 3 minutes. Excess detection reagent was removed and the membrane was covered with wrap. Finally, we photographed the membrane and observed it. Results were expressed as the ratio of target density to the density of β-actin expression.

Ethics

Written consent to participate in this study was obtained for all subjects. This study was approved by the Juntendo University School of Medicine Ethics Committee.

Results

Mean ages at surgery were 3.9 and 4.5 years for H and P, respectively. Both FGF8 and FGF10 were expressed in the upper dermis, especially the papillary layer. FGF10 was significantly down-regulated in H, and the down-regulation was proportional to severity (P: 13.08% versus mild H: 7.28%, moderate H: 5.01%, and severe H: 1.38%) (p<0.05) [Fig. 1]. On the other hand, there were no significant differences in expression of FGF8 [Fig. 2] with a low level of FGF8 present in all groups. The protein expression of FGF10 down-regulation in these hypospadias and phimosis samples was confirmed by Western blot analysis. These results were expressed as the ratio of FGF10 protein amount per β-actin (phimosis 0.91, mild 0.99, moderate 0.65, severe 0.48). The ratio of the expression level of FGF10 was the average of two samples in each group, and the proportion of FGF10 protein tended to be low in the serious group [Fig. 3] .

Discussion

FGF are essential signaling molecules for embryogenesis [10-14]. FGF8 and FGF10 are closely linked to embryonic development, especially to formation of limbs and of the genital tubercle at an early, androgen independent stage in embryonic development via the regulation of SHH [14]. FGF signaling has been shown to work directionally and reciprocally across epithelial-mesenchymal borders, prompting the induction of overlying epithelial differentiation by mesenchymal FGF expression [15]. Although FGF10, via its receptors FGFR1/2b, is linked to a lot of signaling pathways, many of its known implications in human pathology are connected to differences in expression of the molecule partly regulated by circular RNAs [16].

In a recent mouse study, expression of FGF8 was seen outside the urogenital sinus epithelium in early embryogenesis, and expression gradually came to be confined to the distal urethral epithelium [6,7]. FGF8 expression gradually weakens and begins to be replaced by FGF10 as the reproductive nodule mesenchyme differentiates. Initial extension of the genital tubercle progresses by a series of processes. It is thought that prepuce formation will also progress as the external genitalia extend. Therefore, suppression of FGF8 or FGF10 in the distal urethral epithelium, which is important for the initial elongation of the genitalia, could conceivably cause hypospadias, with severity being correlated with the degree of suppression. This is exactly what we identified in this study; i.e., decreased expression of FGF10 with increasing severity of hypospadias. Of note is the seemingly stable expression of FGF8 identified in all groups. This hints that FGF8, which is expressed in early embryogenesis, may not be a causal factor for hypospadias per se; rather, it is a precursor for FGF10 and it is FGF10 that may cause hypospadias by affecting migration of the urethral meatus or elongation of the external genitalia or both.

Studies using various animal models of hypospadias have also shown the importance of malformations of the genital tubercle. Unlike FGF8, which plays an early role in genital tubercle formation, FGF10 and its receptor (FGFR2 IIIb) appear to be involved in a hormone dependent phase [9]. In FGF10 deficient mutant male mice, ventral fusion of the urethral plate fails, resulting in a deformity consistent with hypospadias [8]. However, FGF10 deficiency appears to be incompatible with life, because FGF10 deficient mutant male mice die immediately after birth. On autopsy there is no evidence of lung, salivary gland, or kidney development and absence of definitive limbs. Studies of the mouse homolog suggest that FGF10 is required for embryonic epidermal morphogenesis including brain development, lung morphogenesis, and initiation of limb bud formation and that FGF10 signaling is required for epithelial branching. Therefore, all development reliant on morphogen induced branching such as occurs in the lungs, skin, ears, salivary glands, and urethra require constant expression of FGF10. Furthermore, there is a strain of mice with loss of FGF10 expression in the urethra and phenotypic hypospadias [9], suggesting that FGF10 may also be sensitive to androgen stimulation.

Separate to the activity of FGF10 is the process of urethral closure, already well known to be exquisitely responsive to the hormonal environment. It is well known that estrogenic and anti-androgenic compounds can induce hypospadias in humans and mice. In fact, the increasing incidence of hypospadias, particularly in developed countries, has led to the hypothesis that elevated exposure to estrogenic and anti-androgenic environmental factors may be etiologic [17]. This may include exposure to molecules that interfere with the synthesis, transport, and metabolism of endogenous hormones, such as xenoestrogens or endocrine disrupting compounds.

To the best of our knowledge, this is the first study to investigate the expression of FGF8 and FGF10 in human prepuce. From animal studies, suppression of FGF8 or FGF10, or both, or disruption of the transition from FGF8 to FGF10 have attracted attention as possible etiologic processes for the onset and severity of hypospadias. Development of the external genitalia is very complicated and while FGF10 is involved in fusion of the urethral folds and foreskin formation by controlling mesenchymal differentiation of the genital tubercle resulting in various phenotypes, the expression of FGF10 would appear to be important in humans although its role may not necessarily be similar to that in mice.

Cohn's extensive report [18] on the development of the external genitalia mentions the role of sex steroids in maturing the FGF10 mediated differentiation of the genital tubercle and that FGF8 is not involved in the development of the external genitalia. Our results confirm their findings based on animal experiments by identifying stable similar FGF8 expression in phimosis and hypospadias prepuce tissue and suppressed FGF10 expression correlated with severity in human hypospadias. A similar study to our current report in females would be valuable to establish FGF10 as the definitive causal factor of external genitalia malformation.

In conclusion, the significant down-regulation in FGF10 observed in human prepuce specimens from hypospadias patients in this study provides strong evidence that altered expression of FGF10 during embryogenesis prevents normal urethral development that in males manifests as hypospadias and

persists as a structural fault in hypospadias prepuce. Our study provides a valuable link between animal models of hypospadias and human hypospadias, enhancing the understanding of urethral formation and may have some possible application for developing new methods to detect or prevent hypospadias.

References

1. Baskin LS, Ebbers MB. (2006) Hypospadias: anatomy, etiology, and technique. J Pediatr Surg 41:463
2. Li Y, Sinclair A et al. (2015) Canalization of the Urethral Plate Precedes Fusion of the Urethral Folds during Male Penile Urethral Development: The Double Zipper Hypothesis. J Urol 193:1353
3. Hayashi Y, Kojima Y, Nakane A et al. (2003) A strategy for repairing moderately severe hypospadias using onlay urethroplasty versus onlay-tube-onlay island. Urology 61: 1019–1022
4. Hayashi Y, Kojima Y, Mizuno K, et al. (2007) Can a slit-like meatus be achieved with the V incision sutured meatoplasty for onlay island flap hypospadias repair? BJU International 9:1479–1482
5. Ana BM, Fredrik L et al. (2007) FGFR2, FGF8, FGF10 and BMP7 as candidate genes for hypospadias. Eur j Hum Genet 15:405–410
6. Haraguchi, R. et al. (2000) Molecular analysis of external genitalia formation: the role of fibroblast growth factor (Fgf) genes during genital tubercle formation. Development 127:2471–2479
7. Ogino Y, et al. (2001) External Genitalia Formation: Role of Fibroblast Growth Factor, Retinoic Acid Signaling, and Distal Urethral Epithelium. Ann NY Acad Sci 948:13–31
8. Yucel S, Liu W, Cordero D, Donjacour A, Cunha G, Baskin LS. (2004) Anatomical studies of the fibroblast growth factor-10 mutant, sonic hedge hog mutant and androgen receptor mutant mouse genital tubercle. Adv Exp Med Biol 545:123–148
9. Petiot A, Perriton CL, Dickson C, Cohn MJ. (2005) Development of the mammalian urethra is controlled by Fgfr2-IIIb. Development 132:2441–2450
10. Cohn M.J. et al. (1995) Fibroblast growth factors induce additional limb development from the flank of chick embryos. Cell 80:739–746
11. Yamasaki M. et al. (1996) Structure and expression of the rat mRNA encoding a novel member of the fibroblast growth factor family. J Biol Chem 271:15918–15921
12. Belluschi S. et al. (1997) Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. Development 124:4867–4878
13. Ornitz D.M. (2000) FGFs, heparin sulfate and FGFRs: complex interactions essential for development. Bioessays 22:108–112
14. Watson J, Francavilla C. (2018) Regulation of FGF10 signaling in development and disease. Front Genet 9:500.
15. Ornitz DM, Itoh N. (2001) Fibroblast growth factors. Genome Biol 2. reviews3005.
16. Dragomir M, Calin GA. (2008) Circular RNAs in cancer - lessons learned from microRNAs. Front Oncol 8:179.

17. Bouty A. et al. (2015) The Genetic and Environmental Factors Underlying Hypospadias. Sex Dev 9(5):239–259
18. Cohn M.J. (2011) Development of the external genitalia: conserved and divergent mechanisms of appendage patterning. Dev Dyn.240(5):1108–1115

Figures

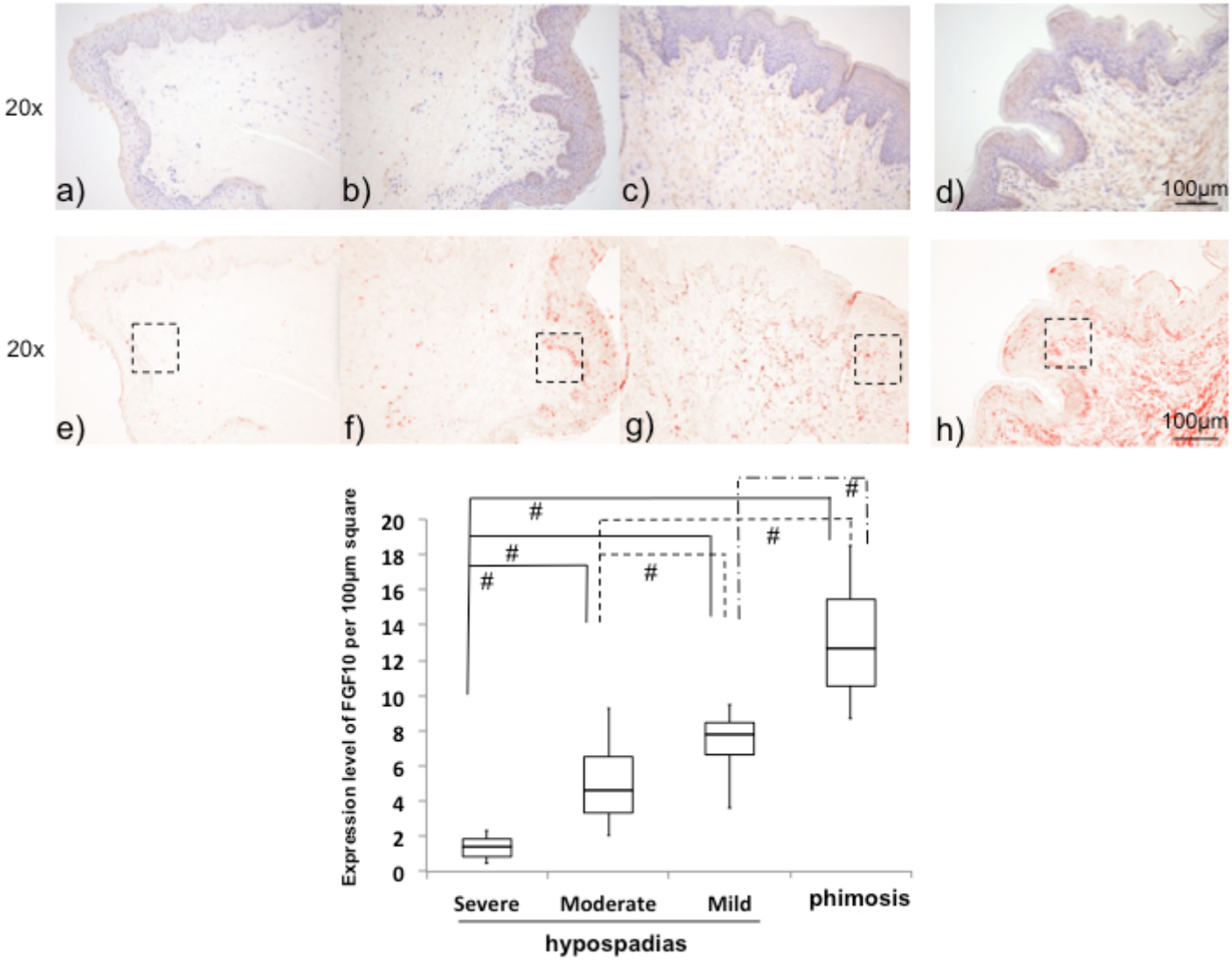

Figure 1

Immunohistochemical expression of FGF10

(a) severe hypospadias (b) moderate hypospadias (c) mild hypospadias (d) phimosis.

Conversion of FGF10 expression by imageJ®

(e) severe hypospadias (f) moderate hypospadias (g) mild hypospadias (h) phimosis.

Graph shows the statistical significance of differences in FGF10 expression.

$^{\#}p$ <0.05 between indicated samples.

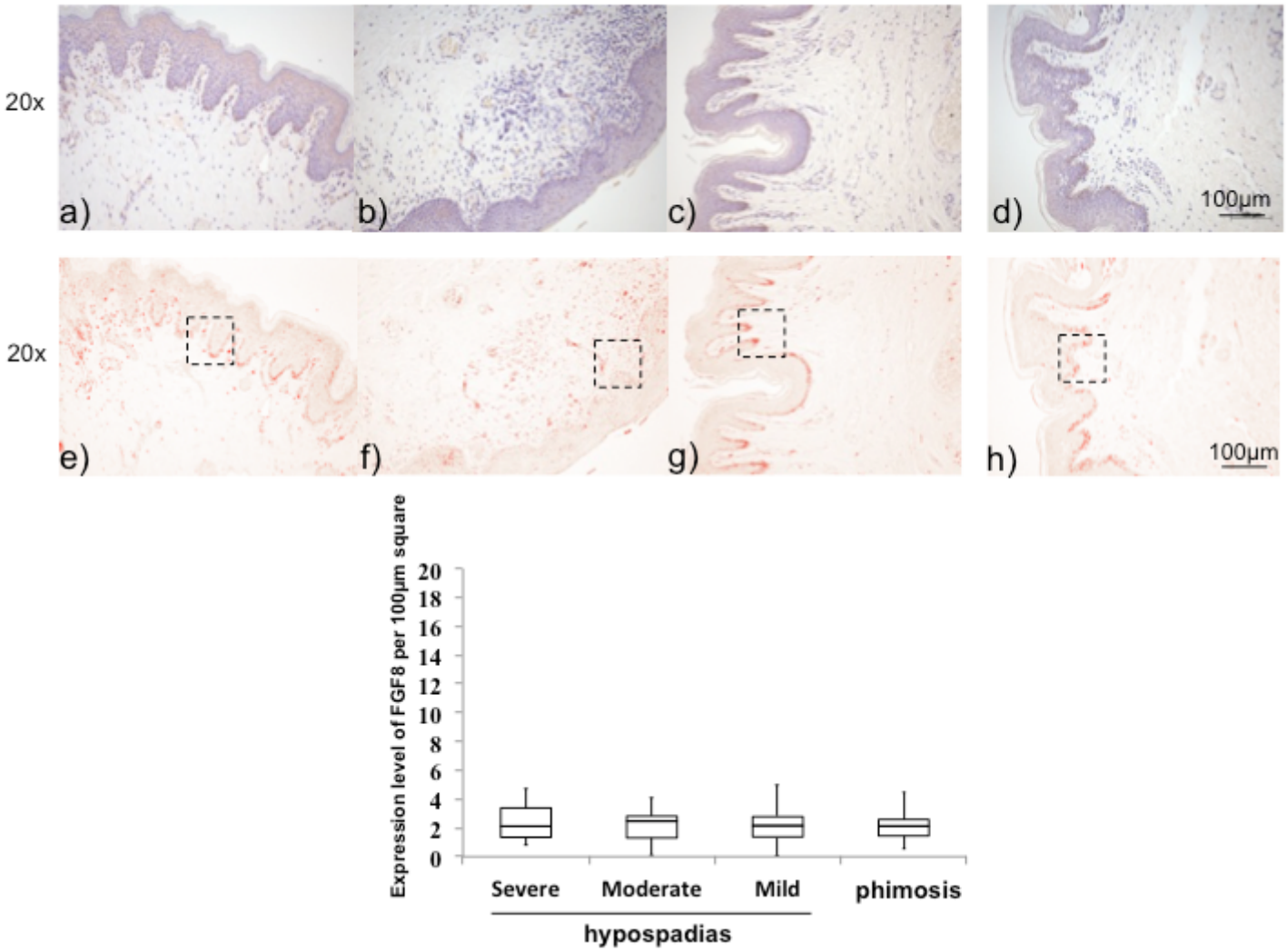

Figure 2

Immunohistochemical expression of FGF8

(a) severe hypospadias (b) moderate hypospadias (c) mild hypospadias (d) phimosis.

Conversion of FGF8 expression by imageJ®

(e) severe hypospadias (f) moderate hypospadias (g) mild hypospadias (h) phimosis.

Graph shows the statistical significance of differences in FGF8 expression.

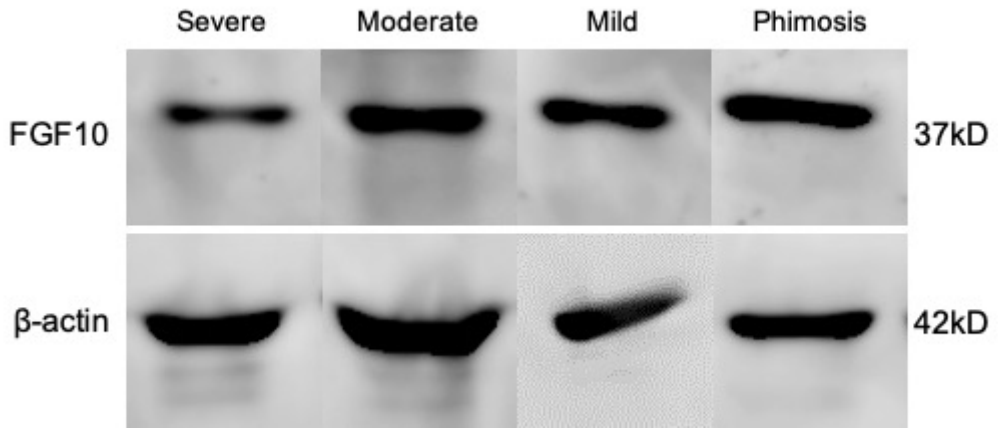

Figure 3

The protein expression of FGF10 in these hypospadias and phimosis samples by Western blot analysis. These results were expressed as the ratio of FGF10 protein amount per β-actin