—Original Article—

Exploring disease-specific methylated CpGs in human male genital abnormalities by using methylated-site display-amplified fragment length polymorphism (MSD-AFLP)

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Abstract. The incidence of male reproductive system disorders, especially hypospadias, has been increasing in developed countries since the latter half of the 20th century. Endocrine-disrupting chemicals from the environment are considered to be involved in hypospadias onset through epigenetic alterations. This pilot study aimed to explore disease-specific methylated CpGs in human patient samples using the methylated-site display-amplified fragment length polymorphism (MSD-AFLP) technique developed by our research group [1]. We compared clinical samples from hypospadias and phimosis patients. Foreskin and blood samples were collected from one- to two-year-old patients with hypospadias (N = 3) and phimosis (N = 3) during surgical treatment. MSD-AFLP analysis showed significantly decreased CpG-methylation levels of genes such as *MYH11* and increased CpG-methylation levels of genes such as *PLA2G15* in hypospadias patients. Hierarchical clustering analysis showed that genes with significantly altered CpG levels were more markedly altered in DNA from blood than from foreskin. Because of the small number of samples, further investigation is necessary to elucidate the association between variations in CpG levels in foreskin and blood DNA and male genital abnormalities. However, our MSD-AFLP method appears to be a useful tool for exploring disease-specific methylated-CpGs in human epidemiological studies.
Key words: DNA methylation, Environmental chemicals, Hypospadias, Phimosis

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Since the second half of the 20th century, the incidence of abnormal male reproductive and genital disorders, such as hypospadias, has been increasing in developed countries such as the United States, Australia, and various European countries [2–4]. Exposure to endocrine-disrupting chemicals in the environment like phthalate, heavy metals, and polychlorinated biphenyl (PCB) has been suspected to underlie this increase [3]. Although genetic abnormalities are required for the onset of these disorders, there is still no consensus about whether genetic mutations or epigenetic changes induced by environmental stimuli are the dominant cause [4].

Genes involved with male hormones, such as androgen receptor

(*AR*) and 5α-reductase type 2 (*SRD5A2*), are associated with the onset of hypospadias as indicated by genetic polymorphisms [5–7]. In addition, the causative gene of hypospadias, *MAMLD*, was discovered when it was found that *MAMLD* possesses a nonsense mutation in a 46, XY disorder of sexual development (DSD) [8, 9]. Several *MAMLD1* mutations have been shown to cause hypospadias owing to the production of dysfunctional proteins or unstable mRNAs [6].

Multiple studies on epigenetics of hypospadias have been carried out recently. A previous study showed that methylation levels of DNA in the upstream region of the *AR* transcription start site are higher and AR mRNA expression levels are lower in foreskin from hypospadias patients than in healthy individuals [7]. DNA methylation and mRNA expression levels in foreskin from hypospadias and phimosis patients have also been compared [8]. Although AR mRNA expression levels were significantly lower in hypospadias patients than in control phimosis patients, bisulfite genomic sequencing could not detect any methylated CpGs in the CpG-islands examined [8]. Although the methylation levels of the *SRD5A2* promoter region shows no significant difference between hypospadias and phimosis patients, a

negative correlation was detected in hypospadias patients between the DNA methylation level of the SP1 site of the *SRD5A2* minimum promoter and the mRNA expression level of CYP1 family members (CYP1A1 and CYP1B1) Phimosis samples lack such a correlation, implicating chemical exposure as a cause of hypospadias [8].

The Illumina Infinium® Human Methylation 450 BeadChip, which was generated for genome-wide DNA methylation analysis and customized for human samples, was employed by other group previously [9]. DNA methylation levels of CpG islands in foreskin samples from hypospadias patients were compared to those of healthy donors and are not different for *AR, SRD5A2,* and *MAMLD1* but are significantly different for *SCARB1* and *MYBPH*.

Previously, our research group developed the methylated-site display-amplified fragment length polymorphism (MSD-AFLP) technique, which is an affordable and highly sensitive methylation profiling system [1]. In this pilot study, we apply genome-wide DNA methylation analysis to foreskin and blood samples of hypospadias and phimosis patients and attempt to identify the candidate regions for disease-specific CpG-methylation underlying abnormal male genital disorders.

Materials and Methods

Reagents

The reagents and materials used in this study are as follows. T4 DNA ligase and the restriction enzymes *Hpa* II, *Msp* I, *Sbf* I, and *Stu* I were from New England Biolabs (Ipswich, MA, USA). The AllPrep DNA/RNA mini kit was from QIAGEN (Hilden, Germany). RNA*later*™ Stabilization Solution and oligonucleotides were from Operon (Alameda, CA, USA) and streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin) were from Dynal (Oslo, Norway). TITANIUM Taq DNA polymerase and TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) were from Takara Bio (Kusatsu, Shiga, Japan). POP-7™ polymer, GeneScan™ 500 LIZ® Size Standard, and the BigDye® Terminator v3.1 Cycle Sequencing Kit were from ThermoFisher Scientific (San Diego, CA, USA). LightCycler® 480 SYBR Green I Master was from Roche Diagnostics GmbH (Mannheim, Germany).

Ethics approval and consent to participate

Sample collections and analysis were carried out as part of a study approved by the ethics committees of Nagoya City University's Graduate School of Medical Sciences (Committee's Reference Number, 65), the National Center for Child Health and Development (Committee's Reference Number, 512), and the Graduate School of Medicine and Faculty of Medicine, The University of Tokyo (Committee's Reference Number, G10027).

Participants and sample collection

After obtaining informed consent from parents, foreskin samples were collected during surgical treatment of one- to two-year-old hypospadias patients (N = 3) and phimosis patients (N = 3) at Nagoya City University. The samples were preserved by immersion in RNA*later*. Blood and urine samples were also collected from the same patients.

MSD-AFLP

Genomic DNA was isolated using the AllPrep DNA/RNA Mini Kit. The protocol of MSD-AFLP was modified for application to the human genome. Genomic DNA was digested with restriction enzymes *Sbf* I and *Msp* I then ligated to adapter A (Upper, b-TCC GAC TGG TAT CAA CGC AGA GTA CTA GAG TTG CA; b = 5'-biotinylation; Lower, p-ACT CTA GTA CTC TGC GTT GAT ACC AGT CGGA; p = 5'-phosphorylation). The ligated DNA fragment was then digested with methylation-sensitive *Hpa* II and ligated to adaptor B (Upper, AAT GGC TAC ACG AAC TCG TTC CAT GAC C; Lower, p-CGT GTC ATG AAC CGA GTT CGT GTA GCC ATT), at which point pre-PCR was performed using adapter-specific primers (Forward, AAT GGC TAC ACG AAC TCG TTC CAT GAC ACG G; Reverse, TCC GAC TGG TAT CAA CGC AGA). The resulting amplicons (the MSD library) were amplified by selective PCR using 256 different primer pairs (Forward, f-AAT GGC TAC ACG AAC TCG TTC CAT GACA II INN; f = 5'-6-carboxyfluorescein (6-FAM) conjugation; Reverse, AGA GTA CTA GAG TTG CAG GNN). The products of selective PCR were electrophoresed using a capillary sequencer to obtain an AFLP chart. CpG positions in the human genome were predicted using the human module of the computer simulation system Genome DNA Fragment Database (GFDB), described in our previous study [1, 10]. The frequencies of SNPs that are likely to affect MSD-AFLP analysis of restriction enzyme (*Msp* I) sequences and affect two bases in selective primers were identified using information from a public database (dbSNP ver 142). Data analysis was performed using GeneMapper® *ID* Software v3.7 (ThermoFisher Scientific) and HiAL version 5.2 software (Maze, Tokyo, Japan) as previously described [1].

Nucleotide sequence analysis

Analysis of nucleotide sequences to determine the CpG locations and cis-element motifs around target CpGs was performed using Genetyx ver 11 (Genetyx, Tokyo, Japan). Briefly, a minimum 200-bp sequence showing GC content > 50% and CpG observed/expected > 0.6 was classified as a CpG island. Neighboring regions were classified based on their distance from the CpG island, with distances of 0–2 kbp, 2–4 kbp, and > 4 kbp from a CpG island labelled CpG island shores, CpG island shelves, and Open Seas, respectively. Motif analysis was performed using Genetyx commands based on David Ghosh's Transcription Factor Database (TFD) in the National Library of Medicine [11].

MSRE-PCR

The protocol for methylation-sensitive restriction enzyme de-pendent PCR (MSRE-PCR) is from [1] with modifications. Briefly, purified genomic DNA was divided into two portions. One portion was digested with the methylation-sensitive restriction enzyme *Hpa* II and the other was digested with *Stu* I, which should not cut the two PCR target sequences described below. The two resulting digested DNA samples were subjected to relative quantitative PCR measurements by a LightCycler480 (Roche Diagnostics GmbH) with locus-specific primers for Chr.5 156091371 (Forward, CTC AGG ATT GGC GTT TTC ATA; Reverse, CAA AAG AGC CCT ACC CTG ATT) and for Chr.14 62709877 (Forward, CAT GGA AAA CCC ACA CCA TTC; Reverse, GGA GCC TGA CAG CCT

TCT TTC). The ratio of target DNA amounts was determined using the $\Delta\Delta$Ct method in which the PCR efficiency is considered 2 and the methylation levels (expressed as % methylation) of *Hpa* II-CpG sites were calculated relative to those of *Stu* I-treated DNA, whose methylation level was set at 100%.

ICP-MS

Elemental concentrations in urine samples from hypospadias (N = 3) and phimosis patients (N = 3) were measured by inductively coupled plasma-mass spectrometry (ICP-MS). Analysis was performed by JAPAN TESTING LABORATORIES (Ogaki city, Gifu, Japan).

Statistical analysis

Differences in methylation levels between groups were analyzed by Student's *t*-test using Microsoft Office Excel 2013 (Microsoft, USA). CpG methylation levels were z-score normalized and subjected to hierarchical clustering analysis of methylation patterns utilizing Euclidean distance and using Cluster 3.0 (http://bonsai. hgc.jp/~mdehoon/software/cluster/software.htm#ctv) [12] and Java TreeView (http://jtreeview.sourceforge.net/) [13].

Results

Comparison of DNA methylation levels between foreskin and blood samples

MSD-AFLP showed numerous peaks representing methylated CpGs (Figs. 1a, b). A total of 47,838 CpGs were identified in all AFLP charts. As indicated by arrowheads, certain peaks show significantly different heights between foreskin and blood samples. Hierarchical clustering analysis of the methylation pattern reveals that two clusters for blood and foreskin samples were clearly distinct for both hypospadias and phimosis patients (Figs. 1c, d, e).

Comparison of foreskin DNA methylation levels between hypospadias and phimosis patients

Fig. 2a shows representative MSD-AFLP peak charts showing differences in methylation levels of foreskin DNA between hypospadias and phimosis patients. Hierarchical clustering was performed for all 47,750 CpGs detected, but no clusters were observed (Fig. 2b). Furthermore, statistically significant differences in methylation levels between hypospadias and phimosis patients were detected in 27 CpGs (P < 0.001), which are presented as dots in a volcano plot in Fig. 2c (13, higher in hypospadias patients; 14, higher in phimosis patients). Table 1 summarizes information about the five CpGs showing large differences between hypospadias and phimosis samples. In particular,
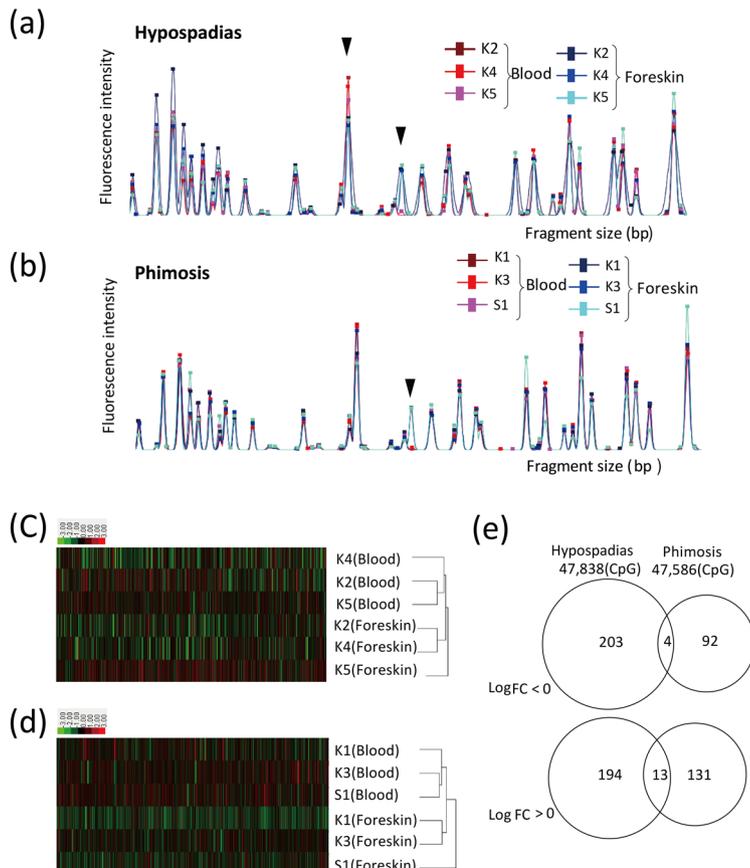

Fig. 1. DNA methylation levels in foreskin and blood determined by MSD-AFLP. (a) Representative AFLP charts of three hypospadias patients. Six electropherograms are shown (foreskin, N = 3; blood, N = 3). K2, K4 and K5 indicate hypospadias patients. Arrowheads indicate fragments showing significant differences in methylation levels between foreskin and blood samples. (b) Representative AFLP charts of three phimosis patients labelled K1, K3, and S1. (c) Hierarchical clustering of DNA methylation patterns in hypospadias samples. (d) Hierarchical clustering of DNA methylation patterns in phimosis samples. (e) Venn diagrams showing differences in methylation levels between blood and foreskin DNA. The numbers in circles indicate the number of CpGs in blood and foreskin DNA of hypospadias and phimosis patients (P < 0.001). Upper diagram shows the lower methylation levels of CpGs in blood DNA (logFC < 0). Lower diagram shows the higher methylation levels of CpGs in blood DNA (logFC > 0). FC describes the ratio of signal level in blood to foreskin samples.
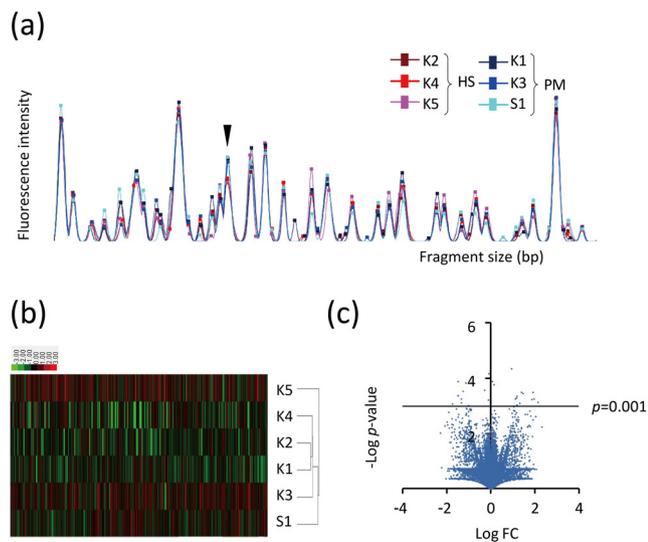
(a)

(b)

(c)

Fig. 2. Foreskin DNA methylation levels in hypospadias (HS) and phimosis (PM) patients. (a) Representative AFLP charts of six patients (HS, N = 3; PM, N = 3). K2, K4, and K5 are from hypospadias patients and K1, K3, and S1 are from phimosis patients. Arrowheads indicate fragments with different methylation levels between hypospadias and phimosis patients. (b) Hierarchical clustering analysis of DNA methylation of six foreskin samples. (c) Volcano plot showing the variation of methylation levels between the hypospadias and phimosis samples. The ratio of the mean signal level in hypospadias samples versus phimosis samples is presented as FC (fold change). The logarithmic value of methylation level fluctuations is indicated by the x-axis, and the logarithmic p-value obtained from comparison between hypospadias and phimosis samples by Student's *t*-test (N = 3) is indicated by the y-axis.
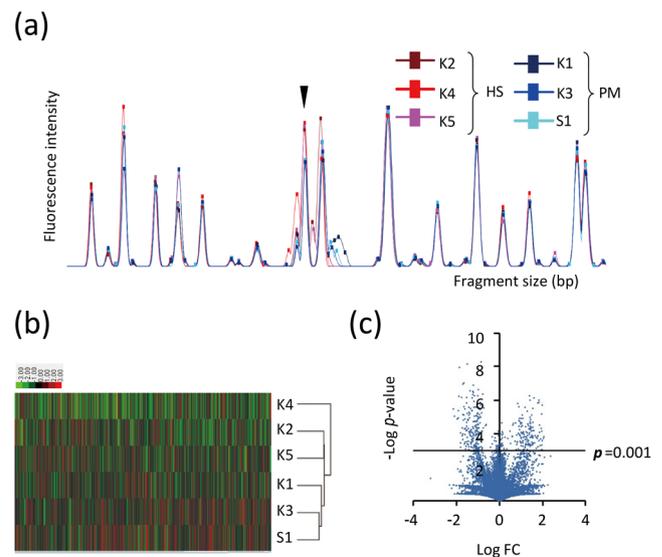
(a)

(b)

(c)

Fig. 3. Blood DNA methylation levels in hypospadias (HS) and phimosis (PM) patients. (a) Representative AFLP charts of six patients (HS, N = 3; PM, N = 3). K2, K4, and K5 are hypospadias patients and K1, K3, and S1 are phimosis patients. Arrowheads indicate fragments with different methylation levels in hypospadias and phimosis patients. (b) Hierarchical clustering analysis. (c) Volcano plot showing variations in methylation level between hypospadias and phimosis patients. The mean signal level present in hypospadias samples is divided by that of phimosis samples and is shown as FC. The logarithmic value of methylation level fluctuations is presented on the x-axis, and the logarithmic P-value obtained from comparison between hypospadias and phimosis samples by Student's *t*-test (N = 3) is presented on the y-axis.

Chr16 15950919 is in a CpG-island and located at –29 upstream of the TSS of the *MYH11* gene. This CpG element appears to be in the promoter of the *MYH11* gene. Three Metal Response Elements (MRE), whose consensus sequences are TGCRCNC (R = A/G; N = any nucleotide), are also located within a 1000-bp region centered on this CpG (Table 1).

Comparison of blood DNA methylation levels between hypospadias and phimosis patients

Fig. 3a shows representative examples of MSD-AFLP peak charts highlighting differences in methylation levels of blood DNA between hypospadias and phimosis patients. Hierarchical clustering was performed for all 47,889 CpGs detected in blood DNA. In contrast to foreskin DNA, the methylation patterns of blood DNA in the three

Table 1. CpGs showing significant differences in methylation levels between foreskin DNA samples from hypospadias and phimosis patients

Chr.	Position [a]	LogFC [b]	P-value [c]	Location [d]	Nearest gene [e]	Distance [f]	Representative motifs (Number) [g]
16	15950919	3.95	0.0005	Island	*MYH11*	–29	AP-2-CS6 (12), LBP-1-RS (8), TCF-1-CS (6), GCF-CS (5), NF-IL6-CS (3), MRE-CS2 (3)
9	138894756	3.91	0.0009	Open Sea	*NACC2*	+92,375	TCF-1-CS (10), bHLH-CS (7), GMCSF-CS (5), NF-IL6-CS (5), LBP-1-RS (4), γ-IRE-CS (4)
7	2577605	0.46	0.0002	Shelf	*C7orf27*	+5,064	γ-IRE-CS (9), TCF-1-CS (8), LBP-1-RS (5), bHLH-CS (5), ER-HALF-SITE (3), CTCF-RS (3)
1	25506531	0.21	0.0001	Open Sea	*RP4-706G24.1*	–28,099	γ-IRE-CS (9), α-INF2 (4), LBP-1-RS (3), bHLH-CS (3)
16	68292923	–0.42	0.0006	Open Sea	*PLA2G15*	+13,676	bHLH-CS (10), TCF-1-CS (10), γ-IRE-CS (9), LBP-1-RS (4)

[a] Chromosomal position; [b] FC (Fold change, ratio of signal level in hypospadias sample to phimosis sample) ; [c] P-value from Student's *t*-test; [d] CpG locations (island, shore, shelf, open sea) identified using Genetyx ver. 11 software; [e] Name of gene nearest to the target CpG; [f] Distance from transcription start site (TSS); [g] Representative motif sequences within ± 0.5 kbp of the target CpG predicted by Genetyx ver 11.

phimosis patients were similar, despite the same pre-processing of MSD-AFLP data (Fig. 3b). Furthermore, statistically significant differences between methylation patterns of samples from hypospadias and phimosis patients (P < 0.001) were detected for 369 CpGs, which are presented as dots in a volcano plot in Fig. 3c. These results indicate that the number of CpGs showing significant differences in DNA methylation levels were much larger for blood DNA than foreskin DNA. Six CpGs that seems to be minimally affected by SNPs are listed as disease-specific methylated-CpGs (Table 2). Two CpGs, Chr5.156091371 (*SGCD1*) and Chr14.62709877 (14.7 kbp up-stream of AL390816.1), were analyzed by MSRE-PCR to validate the accuracy of MSD-AFLP. Although statistically significant differences were not detected for Chr5.156091371, the differences in methylation levels of both CpGs between the two patients as demonstrated by MSRE-PCR were similar to those shown by MSD-AFLP (Fig. 4).

Urinary elements

A total of sixty-three elements were analyzed by ICP-MS, 47 elements of which were below the detection limit in all patients, including lead and cadmium (Table 3). Even when detectable levels were observed, they were not statistically significantly different between the hypospadias and phimosis patients. In two hypospadias patients (K2 and K4), the potassium and rubidium concentrations were much higher than those in the other four patients.

Discussion

Endocrine-disrupting chemicals in the environment, including heavy metals, have been reported to cause epigenetic changes, diseases such as cancer, and reproductive and developmental abnormalities [14–16]. There are many reports describing the relationship between hypospadias and epigenetic changes, which appear to be caused by exposure to environmental endocrine-disrupting chemicals [8, 9, 14], therefore this study analyzed urinary elements of hypospadias patients. An epidemiological survey in India showed that the risk of hypospadias increases when the levels of cadmium and lead in blood are high [15]. However, in our study, blood levels of cadmium

and lead were below the detection limit. In addition, although no statistically significant differences in rubidium or potassium levels between hypospadias and phimosis patients were detected, rubidium and potassium levels were higher in two hypospadias patients than in other patients. Since it has been reported that hyperkalemia and hyponatremia may occur concomitantly in patients with hypospadias [16, 17], further investigation into potential causal relationships are necessary.

In this study, we attempted to use MSD-AFLP for analysis of clinical samples. The MSD-AFLP method detects genomic DNA fragments in regions from methylated *Hpa* II/*Msp* I sites to the nearest *Sbf* I site. Using this method, it is possible to detect a methylated *Hpa* II/*Msp* I site located within the boundary region of a CpG island. Unlike most unmethylated CpG islands, the methylation of a CpG island shore is known to be involved in tissue-specific expression of genes [18], therefore the CpGs detected in this study are likely important epigenomic information for disease-specific DNA methylation. Although it is difficult to precisely determine the total CpG methylation levels of *Msp* I/*Hpa* II sites, it is possible to estimate relative values from data sets obtained by MSD-AFLP. Average fluorescence intensities were tentatively converted to percent methylation by adjusting the average percent methylation of the peak from chr14.62709877 (N = 6) measured by MSRE-PCR (Fig. 4). Results from this estimation method indicate that the total CpG methylation levels in hypospadias blood DNA is 15.15 ± 0.15% and in phimosis blood DNA is 15.71 ± 0.06% (P = 0.0264, F = 0.275, two paired Student's *t*-test), indicating that the levels of *Msp* I/*Hpa* II site CpG methylation in hypospadias blood DNA are higher than those of phimosis blood DNA.

Previously, a CpG was found that is located on *SIGLEC12* and shows a lower level of methylation in blood DNA than foreskin DNA. SIGLEC12 belongs to the SIGLEC family, recognizes sialic acid-containing sugar chains in erythrocytes, and is expressed in macrophages [19]. This finding indicates that functional CpGs that may regulate gene expression can be detected by MSD-AFLP. In contrast to *SIGLEC12*, CpGs located on *GLI2*, *FOXQ1,* and *PKP3* had lower methylation levels in foreskin DNA than in blood DNA.

Table 2. CpGs showing significant differences in methylation levels between hypospadias and phimosis blood DNA samples

Chr.	Position [a]	LogFC [b]	P-value [c]	Location [d]	Nearest gene [e]	Distance [f]	Representative motifs (Number) [g]
5	156091371	0.42	0.0001	Open Sea	*SGCD1*	+337,197	bHLH-CS (6), GMCSF-CS (5), γ-IRE-CS (3), LBP-1-RS (3)
14	62709877	0.26	0.0006	Open Sea	*AL390816.1*	+125,802	TCF-1-CS (8), NF-IL6-CS (5), γ-IRE-CS (5), bHLH-CS (4), GMCSF-CS (3), LBP-1-RS (3)
9	27184052	−0.20	0.0005	Open Sea	*TEK-2*	+74,588	α-INF2 (4), γ-IRE-CS (4), NF-IL6-CS (4), TCF-1-CS (4), bHLH-CS (3)
19	35160325	−0.29	0.0007	Open Sea	*ZNK302*	−8,219	bHLH-CS (9), TCF-1-CS (7), γ-IRE-CS (5), GMCSF-CS (4), AP-2-CS6 (4)
5	64505996	−0.32	0.0005	Island	*CTD-2194F4.2*	+248	bHLH-CS (8), γ-IRE-CS (8), TCF-1-CS (7), LBP-1-RS (3), UCE2 (3), α-INF2 (3)
12	247895	−0.33	0.0005	Island	*IQSEC3*	+61,353	GCF-CS (8), UCE2 (8), AP-2-CS6 (7), bHLH-CS (6), SP1-CS (3), CTCF-RS (3), TCF-1-CS (3)

[a] Chromosomal position; [b] FC (Fold change, ratio of signal level in hypospadias sample to phimosis sample) ; [c] P-value from Student's *t*-test; [d] CpG locations (island, shore, shelf, open sea) identified using Genetyx ver 11 software; [e] Name of gene nearest to the target CpG; [f] Distance from transcription start site (TSS); [g] Representative motif sequences within ± 0.5 kbp of the target CpG predicted by using Genetyx.
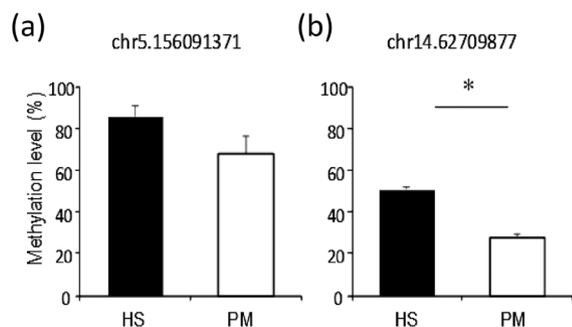
(a)

(b)

Fig. 4. Validation of methylation level differences detected by MSRE-PCR. Two CpGs (Chr5.156091371 and Chr14.62709877) were analyzed by MSRE-PCR as described in the Materials and Methods. HS, hypospadias samples (N = 3); PM, phimosis samples (N = 3). * P < 0.05 by Student's *t*-test.

Table 3. Urinary concentrations of various elements as measured by inductively coupled plasma-mass spectrometry (ICP-MS)

Element	Detection limit (µg/ml)	Participant ID					
		HS			PM		
		K2	K4	K5	K1	K3	S1
Li	0.005	0.03	0.04	0.01	0.01	0.04	ND
Na	5.0	2500	2700	1600	1300	5100	3800
Mg	0.50	110	91	17	24	130	23
Si	5.0	16	28	–	–	18	–
P	0.50	890	900	150	130	1600	170
K	5.0	2900	3700	880	410	1100	450
Ca	5.0	140	23	–	12	150	110
Fe	0.050	–	–	0.06	–	–	–
Cu	0.005	0.02	0.01	–	0.33	0.01	–
Zn	0.50	1	1	–	–	–	0.6
Rb	0.005	3.1	4.3	0.73	0.29	1.4	0.47
Sr	0.050	0.18	–	1.73	–	0.15	0.11
Mo	0.005	0.19	0.18	0.04	0.01	0.29	0.01
Cs	0.005	0.01	0.02	1.73	–	0.01	–
Ba	0.005	0.01	–	–	–	–	–
W	0.005	0.01	–	–	–	–	–

Out of a total of 63 analyzed, the following 47 elements below were below the detection limit: Be, Al, Sc, Ti, V, Cr, Mn, Co, Ni, Ga, Ge, Y, Zr, Nb, Ru, Rh, Pd, In, Sn, Te, La, Ce, Pr, Nd, Sm, Eu, Gd, Yb, Dy, Ho, Er, Tm, Yb, Lu, Hf, Ta, W, Re, Ir, Pt, Au, TI, Bi, Th, U. –: Under detection limit.

GLI2 is involved in epithelial homeostasis [20]. FOXP1 regulates mucin expression in human male urogenital epithelium and is involved in the formation of hair follicles [21, 22]. PKP3 is synthesized in epithelial cells and is required for intercellular adhesion [23]. These genes thus may play an important role in epithelial morphogenesis. These findings also indicate that MSD-AFLP is capable of detecting differences in functional CpG methylation.

Through foreskin DNA analysis, a CpG likely located in the promoter region of *MYH11* shows a markedly higher methylation level in hypospadias patients than in phimosis patients. MYH11, also known as Myosin-11, is a biomarker of smooth muscle cell differentiation required for normal penis morphogenesis [24]. Exposure to estrogen-like substances causes downregulation of *MYH11* gene expression [24]. The hypermethylation of *MYH11* detected in this study may reduce the expression level of *MYH11*, resulting in the dysfunction of progenitor cells that regulate normal penis formation. *PLA2G15* transcription has been reported to promote epithelial mesenchymal transition (EMT) [25]. EMT is promoted by exposure to di-n-butyl phthalate (DBP), resulting in the onset of hypospadias [26]. The lower CpG methylation level of *PLA2G15* in hypospadias foreskin identified in this study may cause upregulation of *PLA2G15* transcription, leading to abnormal epithelial cell differentiation.

Human DNA methylation biomarkers have been the focus of much attention as for use as indicators in advanced diagnostics for lifestyle-related diseases, including cancer. Blood samples are considered suitable materials for exploring these biomarkers because of the minimal invasiveness of blood collection [27]. In this study, we found a higher number of CpGs with differential levels between hypospadias and phimosis patients in blood samples than in foreskin samples. These results indicate a larger number of DNA methylation biomarkers in blood than in other tissues. Although a more detailed analysis using a larger number of samples is necessary, MSD-AFLP can thus be a useful tool for epidemiological research.

In this study, we attempted to use MSD-AFLP to explore disease-specific methylated CpGs. Although this is considered a pilot study because of the small sample size, the CpGs with differential methylation levels detected in blood DNA seem implicated in hypospadias and phimosis development. Further investigations of disease-specific methylated CpGs related to male genital abnormalities are required using larger sample sizes.

Conflict of Interests: The authors declare that they have no competing interests.

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