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ORIGINAL ARTICLE ***Andrology***

Molecular basis of non-syndromic hypospadias: systematic mutation screening and genome-wide copy-number analysis of 62 patients

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STUDY QUESTION: What percentage of cases with non-syndromic hypospadias can be ascribed to mutations in known causative/candidate/susceptibility genes or submicroscopic copy-number variations (CNVs) in the genome?

SUMMARY ANSWER: Monogenic and digenic mutations in known causative genes and cryptic CNVs account for >10% of cases with non-syndromic hypospadias. While known susceptibility polymorphisms appear to play a minor role in the development of this condition, further studies are required to validate this observation.

WHAT IS KNOWN ALREADY: Fifteen causative, three candidate, and 14 susceptible genes, and a few submicroscopic CNVs have been implicated in non-syndromic hypospadias.

STUDY DESIGN, SIZE, DURATION: Systematic mutation screening and genome-wide copy-number analysis of 62 patients.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The study group consisted of 57 Japanese and five Vietnamese patients with non-syndromic hypospadias. Systematic mutation screening was performed for 25 known causative/candidate/susceptibility genes using a next-generation sequencer. Functional consequences of nucleotide alterations were assessed by *in silico* assays. The frequencies of polymorphisms in the patient group were compared with those in the male general population. CNVs were analyzed by array-based comparative genomic hybridization and characterized by fluorescence *in situ* hybridization.

MAIN RESULTS AND THE ROLE OF CHANCE: Seven of 62 patients with anterior or posterior hypospadias carried putative pathogenic mutations, such as hemizygous mutations in *AR*, a heterozygous mutation in *BNC2*, and homozygous mutations in *SRD5A2* and *HSD3B2*. Two of the seven patients had mutations in multiple genes. We did not find any rare polymorphisms that were abundant specifically in the patient group. One patient carried mosaic dicentric Y chromosome.

LIMITATIONS, REASONS FOR CAUTION: The patient group consisted solely of Japanese and Vietnamese individuals and clinical and hormonal information of the patients remained rather fragmentary. In addition, mutation analysis focused on protein-altering substitutions.

WIDER IMPLICATIONS OF THE FINDINGS: Our data provide evidence that pathogenic mutations can underlie both mild and severe hypospadias and that *HSD3B2* mutations cause non-syndromic hypospadias as a sole clinical manifestation. Most importantly, this is the first report documenting possible oligogenicity of non-syndromic hypospadias.

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Key words: copy-number / hypospadias / mutation / polymorphism / susceptibility

Introduction

Hypospadias is a relatively common form of 46,XY disorders of sex development (DSD) observed in ~4–40 per 10 000 live births (Kurahashi *et al.*, 2004; Nassar *et al.*, 2007; Blaschko *et al.*, 2012). Hypospadias occurs either as an isolated anomaly or as a component of congenital malformation syndromes (Wu *et al.*, 2002; Kurahashi *et al.*, 2004). Although non-syndromic hypospadias is a multifactorial disorder induced by both genetic and environmental factors, this condition can also take place as a result of single gene mutations (Kurahashi *et al.*, 2004; Wang *et al.*, 2004; Chen *et al.*, 2007; Köhler *et al.*, 2009). Previous studies revealed familial aggregation of non-syndromic hypospadias (Schnack *et al.*, 2008; van Rooij et al., 2013). In most cases, familial hypospadias is equally transmitted from the paternal and maternal sides of the family and shows similar recurrence risks between the brothers and sons of patients, indicating a significant role of single gene mutations in the development of the disease (Schnack *et al.*, 2008).

In 2012, van der Zanden *et al.* (2012) reviewed 162 prior studies and listed 15 causative genes and three candidate genes for this condition. They also introduced 49 polymorphisms in 13 genes associated with disease risk, together with one susceptibility gene *CYP1A1* whose risk allele is yet to be determined. To date, however, there is no single report of systematic mutation analysis of the causative/candidate/susceptible genes. Likewise, while a small number of submicroscopic copy-number variations (CNVs) have been identified in patients with non-syndromic hypospadias (Tannour-Louet *et al.*, 2010), genome-wide copy-number analysis has been performed only in exceptional cases. Thus, the contribution of single gene mutations and submicroscopic CNVs to the etiology of non-syndromic hypospadias remains unknown.

The aim of this study was to clarify the frequency and type of genetic defects in patients with non-syndromic hypospadias. This study consisted of systematic mutation screening using next-generation sequencing (NGS) technology and cytogenetic analyses using comparative genomic hybridization (CGH) and fluorescence *in situ* hybridization (FISH).

Materials and Methods

Patients

A total of 57 Japanese and 5 Vietnamese patients with hypospadias participated in the study (Table I). All patients were referred to our clinics because of hypospadias. Patients with additional clinical features except for cryptorchidism and micropenis and those with cytogenetically detectable chromosomal abnormalities were excluded from this study. The 62 patients had no family history of 46,XY DSD. One of the 62 patients (case 18) was born to consanguineous parents. Hospital records of genital features at birth were obtained for 49 patients. Eleven patients manifested relatively mild hypospadias with the urethral opening at the anterior portion of the penis, while 14 and 24 patients presented with moderate (middle) and severe (posterior) hypospadias, respectively. Cryptorchidism and micropenis were observed in 5 and 11 patients, respectively.

Ethical approval

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development and performed after obtaining written informed consent from the parents of patients.

Identification of nucleotide substitutions

Sequence analysis was carried out for 25 known causative/candidate/susceptible genes for non-syndromic hypospadias, i.e. *AR*, *ATF3*, *BMP4*, *BMP7*, *BNC2*, *CTGF*, *CYP1A1*, *CYR61*, *DGKK*, *EGF*, *ESR1*, *ESR2*, *FGF8*, *FGFR2*, *GSTM1*, *GSTT1*, *HOXA4*, *HOXB6*, *HSD3B2*, *HSD17B3*, *MAMLD1*, *MID1*, *NR5A1* (alias *SF1*), *SRD5A2*, and *WT1* (van der Zanden *et al.*, 2012). The coding regions of these genes were amplified from genomic DNA using the Haloplex Target Enrichment System (Design ID 02185-1348467147) (Agilent Technologies, Palo Alto, CA, USA), and were sequenced as 150 bp paired-end reads on a MiSeq sequencer (Illumina, San Diego, CA, USA). The average read depth of each amplicon was 115.0. Subsequently, nucleotide alterations in the samples were called by the Surecall system (Agilent Technologies) and SAMtools 0.1.17 software (http://samtools.sourceforge.net, 12 January 2015, date last accessed) (Li *et al.*, 2009). In the present study, we focused on non-synonymous substitutions in the coding regions and nucleotide changes at splice sites. Substitutions detected by NGS were confirmed by Sanger direct sequencing. The primers utilized in the present study are available upon request.

Characterization of nucleotide substitutions

Functional consequences of nucleotide alterations were predicted by *in silico* analyses. Single nucleotide polymorphisms (SNPs) with allele frequencies of >1.0% in the general population (dbSNP, http://www.ncbi.nlm.nih.gov/, 12 January 2015, date last accessed), except for those that have been reported as risk alleles (van der Zanden *et al.*, 2012), were excluded from further analyses. The effects of missense substitutions on protein function were predicted using Polyphen2 (http://genetics.bwh.harvard.edu/pph2/, 12 January 2015, date last accessed) (Adzhubei *et al.*, 2010), and those of intronic substitutions on splicing were assessed using Genome Project

Table I Nucleotide alterations identified in the present study.

Case[a]	Ethnic origin	Putative pathogenic mutation	Putative risk variant	Probable benign change	Copy-number alteration	Position of urethral opening[b]	Cryptorchidism	Micropenis
1	J	***AR* (p.S176R)**				Anterior	No	No
2	J	***AR* (p.A403V)**				No data	No data	No data
3	J	***AR* (p.R841S)**	*HSD17B3* (p.G289S)			Posterior	No	Yes
4	J	***AR* (delins[c])** *HOXB6* (p.S2N)	***MAMLD1* (p.N662S)**			No data	No data	No data
5	J	*BNC2* (p.M801R)				Posterior	No	No
6	V	***SRD5A2* (p.R227Q)**[d]	*HSD17B3* (p.G289S)			Posterior	No	Yes
7	V	***HSD3B2* (p.A10T)**	*SRD5A2* (p.R227Q)[d]			Posterior	Yes (right)	Yes
8	J		*HSD17B3* (p.G289S)	*CYP1A1* (p.T173R)	Y chromosome[e]	Posterior	No	No
9	J		***MAMLD1* (p.N662S)**			Anterior	No	No
10	J		*CYP1A1* (p.Q75P)			Middle	No data	No data
11	J		*CYP1A1* (p.A62P)			Middle	No	No
12	J		*BMP7* (p.T170M)			Middle	No	No
13	V		*HSD17B3* (p.G289S)			No data	No	No
14	J		*HSD17B3* (p.G289S)			Posterior	No	No
15	J		***HSD17B3* (p.G289S)**			Posterior	Yes	No
16	J		*HSD17B3* (p.G289S)			Posterior	No	No
17	J		*HSD17B3* (p.G289S)			Posterior	Yes (right)	Yes
18	J		*HSD17B3* (p.G289S)			Posterior	No	No
19	J		*HSD17B3* (p.G289S)			Middle	Yes (right)	Yes
20	J		*HSD17B3* (p.G289S)			Middle	No data	No data
21	J		***HSD17B3* (p.G289S)**			Middle	No	Yes
22	J		*HSD17B3* (p.G289S)			Anterior	No	No
23	J		***HSD17B3* (p.G289S)**			No data	No data	No data
24	J		*HSD17B3* (p.G289S)			No data	No data	No data
25	J		*HSD17B3* (p.G289S)			No data	No data	No data
26	J		*HSD17B3* (p.G289S) ***MAMLD1* (p.N662S)**			Middle	No	No
27	J		*HSD17B3* (p.G289S)	*BNC2* (p.M539V)		No data	No data	No data
28	J		*HSD17B3* (p.G289S)	*BNC2* (p.P614S)		No data	No data	No data
29	J		***MAMLD1* (p.N662S)**	*EGF* (p.S16R)		Posterior	No	No
30	J		*HSD17B3* (p.G289S)	*FGFR2* (p.M97V)		Anterior	No data	No data
31	J		*HSD17B3* (p.G289S)	*EGF* (p.S16R)		Middle	No	No
32	J		***MAMLD1* (p.N662S)**	*HSD3B2* (p.S284I) *EGF* (p.S16R)		Posterior	No	No

Continued

Table I *Continued*

Case[a]	Ethnic origin	Putative pathogenic mutation	Putative risk variant	Probable benign change	Copy-number alteration	Position of urethral opening[b]	Cryptorchidism	Micropenis
33	J		*HSD17B3* (p.G289S)	*HSD3B2* (p.R362W)		Anterior	No data	No data
34	J			*NR5A1* (g.IVS2-5G>A)		Posterior	No data	No data
35	J			*HOXB6* (p.P40S)		Posterior	No	Yes
36	J			***MAMLD1* (p.N675K)**		Posterior	No data	No data
37	J			*ESR2* (p.G67S)		Posterior	No	No
38	J			*EGF* (p.S16R) *BNC2* (p.I974V)		Middle	No data	No data

J, Japanese; V, Vietnamese.
Homozygous or hemizygous mutations/variations are boldfaced, and heterozygous substitutions are lightfaced.
[a]Cases 39–62 carried no nucleotide alterations in the target genes.
[b]Detailed clinical information was obtained only from 49 of the 62 patients.
[c]c.1995delTGAAGGCTATGAATGTCinsCAGAA; p.666delEGYECQinsRK.
[d]Homozygosity and heterozygosity of this mutation were described as a pathogenic defect and a disease-susceptible alteration, respectively.
[e]Copy-number gain of the region from Ypter to Yq11.223 and copy-number loss of the remaining Y chromosomal region.

Data (http://www.fruitfly.org/seq_tools/splice.html,12 January 2015, date last accessed) (Reese *et al.*, 1997). Nucleotide deletions and insertions in the coding regions were assessed as 'probably damaging'.

Nucleotide alterations were classified into the following three groups: (i) putative pathogenic mutations: mutations that have been associated with 46,XY DSD or hitherto unreported nucleotide changes in causative genes that were assessed as 'probably damaging' or 'possibly damaging' by *in silico* analyses; (ii) putative risk variants: previously reported risk SNPs or novel substitutions in susceptibility genes, or rare SNPs in causative genes that were assessed as 'probably damaging' or 'possibly damaging'; and (iii) probable benign changes: nucleotide substitutions in causative/susceptible/candidate genes that were assessed as 'benign'. To determine the possible association between the SNPs (putative risk variants and probable benign changes) and disease risk, we compared allele frequencies in the patient group with those in the male general population. In the SNP analysis, we focused on Japanese patients, for whom the allele frequencies in the general population were available in the public database (dbSNP, http://www.ncbi.nlm.nih.gov/,12 January 2015, date last accessed).

Statistical analysis

The statistical significance of the comparison of allele frequency in the patient group and the general population was evaluated using χ^2 and Fisher's exact probability tests.

Copy-number analyses

CNVs in the genome were screened by CGH using a catalog human array (8 × 60 k format, catalog number G4450A, Agilent Technologies), according to the manufacturers' instructions. In this study, we focused on copy-number alterations affecting genomic intervals larger than 1.5 Mb, which have a higher probability of being associated with disease phenotypes (Cooper *et al.*, 2011). We referred to the Database of Genomic Variants (http://projects.tcag.ca/variation/,12 January 2015, date last accessed) to exclude known benign variants. Genomic structures of CNVs were characterized by FISH analysis.

Results

Identification and characterization of nucleotide substitutions

Eight putative pathogenic mutations were identified in seven patients (Table I and Fig. 1). The eight mutations consisted of three hemizygous missense mutations and one hemizygous deletion/insertion in *AR*, one heterozygous missense mutation in *HOXB6*, one heterozygous missense mutation in *BNC2*, and apparent homozygous mutations in *SRD5A2* and *HSD3B2*. Of these, the *AR* mutation in case 3 and the *SRD5A2* mutation in case 6 were previously identified in patients with 46,XY DSD (Melo *et al.*, 2003 in which the p.R841S mutation in *AR* was described as p.R840S; Sasaki *et al.*, 2003; van der Zanden *et al.*, 2012), while the other mutations were first identified in the present study.

Putative risk variants were identified in 30 patients (Table I and Supplementary Table SI). These variants included three known risk alleles for hypospadias and/or micropenis: rs2066476 in *HSD17B3*, rs2073043 in *MAMLD1* and rs9332964 in *SRD5A2* (Sasaki *et al.*, 2003; Fukami *et al.*, 2008; Sata *et al.*, 2010; Kalfa *et al.*, 2011; van der Zanden *et al.*, 2012). The SNPs in *HSD17B3* and *MAMLD1* were identified in the Japanese patient group and the male general population at similar frequencies. We also identified a rare SNP in the causative gene *CYP1A1* which was shared by the Japanese patients and the male

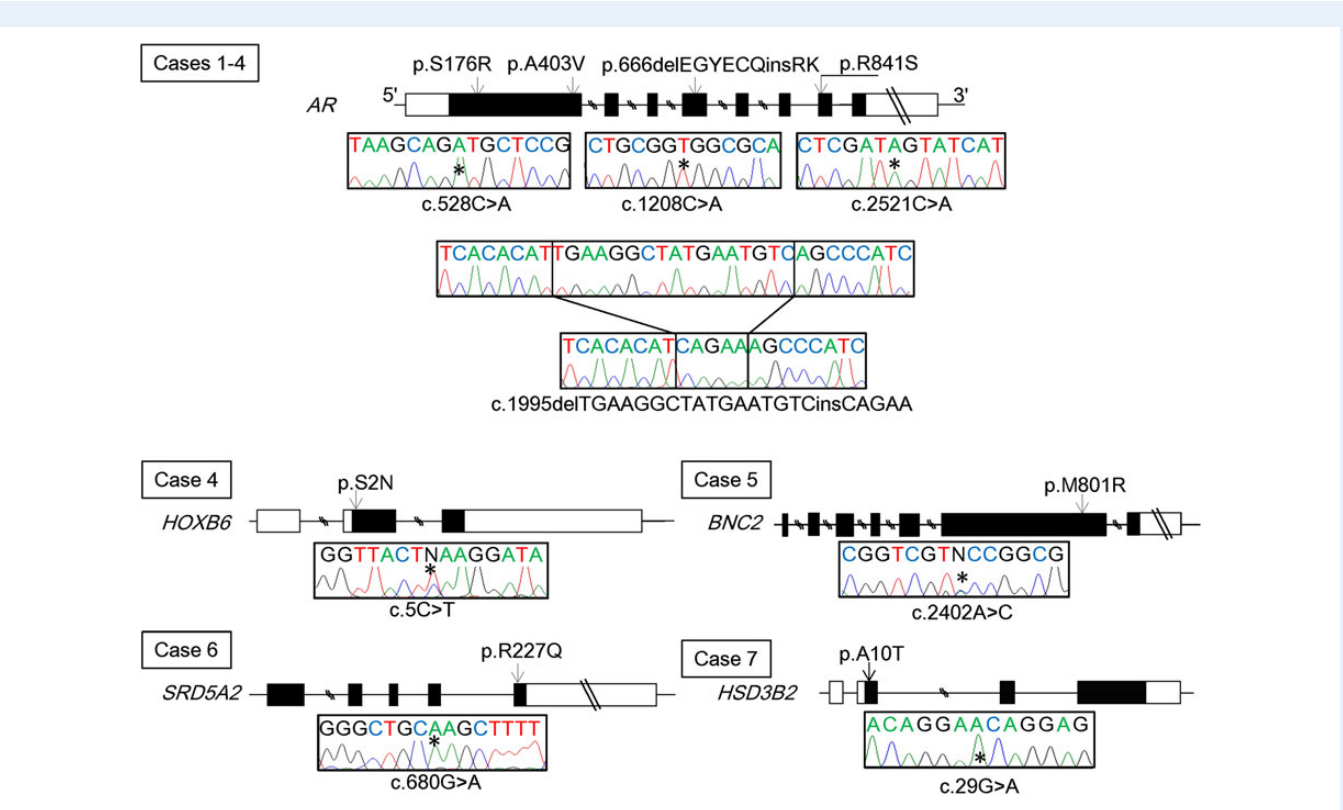

Figure 1 Putative pathogenic mutations identified in the present study. Genomic positions and chromatograms of the nucleotide substitutions are shown. Asterisks indicate the mutated nucleotides.

general population at a similar frequency, together with a SNP in *BMP4* whose frequency in the general population is unknown.

Probable benign changes were found in 13 patients (Table I and Supplementary Table SI). These substitutions included a rare SNP in *EGF* which was identified in the patient group and in the general population at similar frequency. We also detected SNPs in *ESR2* and *BNC2* that had unknown frequencies in the general population, together with a novel substitution in intron 2 of *NR5A1* (g.IVS2-5G>A) that was predicted to not affect splicing.

Copy-number analyses

One of the 62 patients (case 8) carried CNVs on the Y chromosome (Fig. 2A). These alterations consisted of copy-number gain of a ~23 Mb region from Ypter to Yq11.223 and copy-number loss of the remaining Y chromosomal region. The log2 signal ratios of most probes corresponding to the amplified and deleted regions were lower than +1.0 and higher than −2.0 respectively, indicating mosaicism of these CNVs. FISH analysis using a *SRY*-containing probe showed that case 8 had mosaic dicentric Y (Fig. 2B). CGH analysis for case 6 with an apparently homozygous *SRD5A2* mutation and case 7 with an apparently homozygous *HSD3B2* mutation excluded compound heterozygosity for a mutation and deletion (data not shown).

Clinical findings of patients with putative pathogenic defects

Putative pathogenic defects were associated with both anterior and posterior hypospadias (Table II). Endocrine evaluation of cases 1–8 remained fragmentary; blood hormone levels in cases 3 and 7 were within the normal range (Table II).

Discussion

Systematic mutation screening identified putative pathogenic mutations in 7 of 62 patients with non-syndromic hypospadias. These results, in conjunction with previous studies showing that ~30% of cases with severe hypospadias are ascribable to specific defects such as mutations in *AR* or *SRD5A2* (Albers *et al.*, 1997; Boehmer *et al.*, 2001), demonstrate the significant role of mutations in known causative genes in the etiology of non-syndromic hypospadias. Furthermore, our results support the previously proposed notion that genetic defects in *AR* account for a substantial percentage of cases with various types of 46,XY DSD (Albers *et al.*, 1997; Boehmer *et al.*, 2001; Audi *et al.*, 2010) and that mutations in *HSD3B2* can lead to non-syndromic hypospadias as a sole clinical manifestation, although *HSD3B2* plays an essential role in adrenal function (Boehmer *et al.*, 2001; Codner *et al.*, 2004; Audi *et al.*, 2010). Case 3 carried the p.R841S mutation in *AR*, which have been identified in patients with ambiguous genitalia (Melo *et al.*, 2003), suggesting the phenotypic diversity of missense mutations in *AR*. Notably, two of our patients had putative pathogenic mutations in multiple genes. Case 4 carried a hemizygous in-frame deletion/insertion in *AR* and a heterozygous missense substitution in *HOXB6*. Likewise, case 7 with a homozygous missense mutation in *HSD3B2* had an additional heterozygous missense mutation in *SRD5A2* that retains 3% of enzymatic activity (Makridakis *et al.*, 2000; Sasaki *et al.*, 2003). These data imply for the

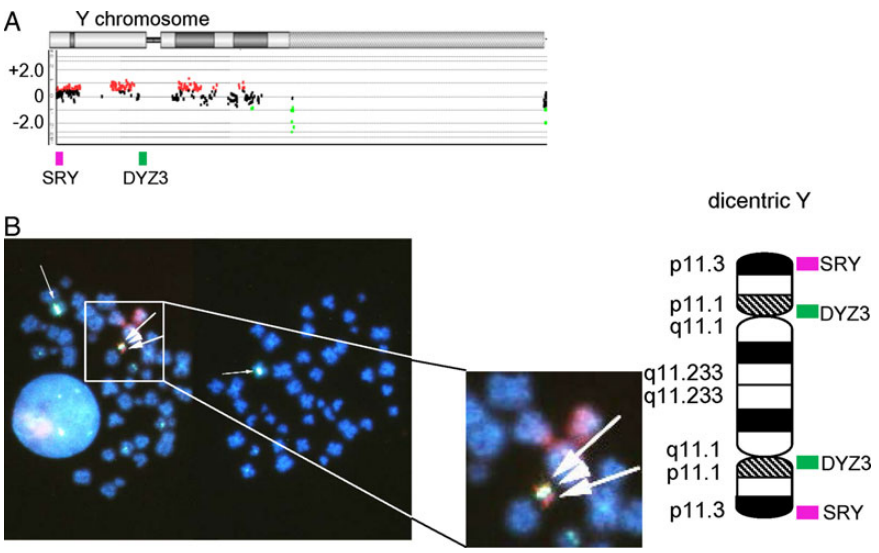

Figure 2 Copy-number alterations identified in case 8. Results of array-based comparative genomic hybridization (CGH) (**A**) and fluorescence *in situ* hybridization (FISH) analysis and schematic representation of the dicentric Y chromosome (**B**) are shown. The black, red and green dots in CGH denote signals indicative of the normal, increased (>+0.4) and decreased (<−0.8) copy-numbers, respectively. The arrowhead and thick arrows in FISH indicate a signal of DYZ3 (Y centromeric probe) and signals of *SRY*-containing probe (Yp11.3), respectively. The thin arrows in the left panel indicate signals of X centromeric probe.

Table II Molecular and clinical findings of patients with putative pathogenic abnormalities.

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
Affected gene/region	*AR*	*AR*	*AR*	*AR/HOXB6*	*BNC2*	*SRD5A2*	*HSD3B2*	Y chromosome[a]
Ethnic origin	Japanese	Japanese	Japanese	Japanese	Japanese	Vietnamese	Vietnamese	Japanese
Family history of DSD	No	No data	No	No data	No	No	No	No
Clinical features								
Hypospadias[b]	Anterior	No data	Posterior	No data	Posterior	Posterior	Posterior	Posterior
Cryptorchidism	No	No data	No	No data	No	No	Yes (right)	No
Micropenis	No	No data	Yes	No data	No	Yes	Yes	No
Other features	No	No data	No	No data	No	No	No	Borderline MR
Endocrine findings								
Age at examination	No data	No data	15 months	No data	No data	No data	3.5 years	No data
LH (IU/l)[c]	No data	No data	<0.2 (<0.2–0.3)	No data	No data	No data	No data	No data
FSH (IU/l)[c]	No data	No data	<1.0 (<1.0–1.5)	No data	No data	No data	No data	No data
Testosterone (nmol/l)[c]	No data	No data	0.17 (0.10–0.45)	No data	No data	No data	0.16 (0.10–0.45)	No data

DSD, disorders of sex development; MR, mental retardation; LH, luteinizing hormone; FSH, follicle stimulating hormone.
[a]Copy-number alterations on Y chromosome.
[b]Position of urethral opening.
[c]Hormone values in parentheses indicate the reference ranges of age- and sex-matched control individuals.

first time that non-syndromic hypospadias results from digenic mutations. On the other hand, we did not observe the accumulation of rare SNPs in the patient group. Our data suggest that previously reported susceptibility SNPs play no or only minor roles in the development of non-syndromic hypospadias in the Japanese population. However, we cannot exclude the possibility that oligogenicity of these SNPs increases the risk of the disease, because a small number of our patients carried these SNPs as biallelic or digenic substitutions. Considering the small number of participants of this study, further investigations are necessary to clarify the possible association between rare SNPs and the disease phenotype.

Genome-wide copy-number analysis identified cryptic CNVs only in one patient. Case 8 carried a copy-number gain of a ~23 Mb region

on Yp and Yq and copy-number loss of the remaining Y chromosomal region. FISH analysis revealed that case 8 had mosaic dicentric Y, which has been described in multiple patients with hypospadias (Drummond-Borg *et al.*, 1988; Kojima *et al.*, 2001). It has been proposed that dicentric Y results in hypospadias by mosaic loss of the rearranged Y chromosome or by aberrant expression of Y chromosomal genes (Drummond-Borg *et al.*, 1988; Kojima *et al.*, 2001). The lack of pathogenic CNVs in the remaining 61 cases suggests the rarity of cryptic CNVs as genetic causes of non-syndromic hypospadias.

In this study, putative pathogenic defects were identified predominantly in patients with severe (posterior) hypospadias, while an *AR* mutation was detected in case 1, who manifested mild (anterior) hypospadias without micropenis or cryptorchidism. In this regard, previous studies have shown that syndromic hypospadias often arises from known gene mutations or chromosomal rearrangements (van der Zanden *et al.*, 2012). These data imply that monogenic mutations can underlie various types of hypospadias, although they are more strongly associated with severe or syndromic hypospadias than with mild non-syndromic hypospadias. Since identification of pathogenic defects can help to predict disease outcomes and improves the accuracy of genetic counseling, genetic analyses should be considered in patients with hypospadias of various clinical severities.

It should be pointed out that the present study has some limitations. First, the patient group consisted of only Japanese and Vietnamese individuals. Since the prevalence of hypospadias varies among countries (Nassar *et al.*, 2007; Serrano *et al.*, 2013), there may be ethnicity-specific causes of hypospadias. For example, mutations in *ATF3*, which account for ~10% of cases in the USA (Kalfa *et al.*, 2008), were absent from our cohort. In contrast, the p.A10T mutation in *HSD3B2* and the p.R227Q mutation in *SRD5A2* were detected exclusively in Vietnamese patients in homozygous state. Thus, our results are not simply applicable to other ethnic groups. Second, the frequency of monogenic defects may be underestimated in this study, because we focused on protein-altering mutations in 25 genes. Mutations/variations in regulatory regions, defects in unexamined genes and epigenetic abnormalities may be hidden in our mutation-negative patients. Lastly, clinical information of our patients remained fragmentary. Although previous studies have revealed that several factors such as low birthweight, placental insufficiency and maternal hypertension are associated with the risk of hypospadias (Stoll *et al.*, 1990; Weidner *et al.*, 1999; Fredell *et al.*, 2002; Brouwers *et al.*, 2010), the contributions of such factors to the disease phenotype of our patients are yet to be studied. Moreover, since endocrine data were unavailable for most of our mutation-positive cases, further studies are needed to elucidate the hormonal characteristics of each monogenic disorder.

Conclusion

The present study indicates that mutations in known causative genes and submicroscopic CNVs account for >10% of cases with non-syndromic hypospadias. Pathogenic defects appear to underlie both severe and mild hypospadias. On the other hand, previously reported risk SNPs are unlikely to play a major role in the development of the disease; further studies are required to validate this observation. Most importantly, this is the first report documenting the possible oligogenicity of non-syndromic hypospadias.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

M.K., K.No., T.O., and M.F. designed the study. M.K., E.S., V.C.D., Y.H., T.M., K.Mu., K.U., N.I., K.Nag., Y.O., T.H., K.Y., M.I, Y.K.-F., K.Nak., K.Hay., K.Hat., Y.M., K.Mo., and T.O. contributed to the acquisition of data. M.K. and M.F. analyzed data and wrote the paper. All authors were involved in revising the paper and approved the final version of the manuscript for submission.

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Conflict of interest

The authors declare that there is no conflict of interest.

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