Table 2 Non-synonymous *CFTR* variants detected in this study

Exon	Non-synonymous variant	Amino acid change	dbSNP135	Genotype	SIFT (score)	PolyPhen-2 (score)	Alcoholic CP (%)	Idiopathic CP (%)	Hereditary/familial CP (%)
2	c.91C>T	p.R31C	rs1800073	CT	D (0.012)	PD (0.989)	0/46 (0)	3/121 (2.5)	0/26 (0)
2	c.92G>A	p.R31H	rs149353983	GA	T (0.183)	B (0.003)	0/46 (0)	1/121 (0.8)	0/26 (0)
4	c.374T>C	p.I125T	rs141723617	TC	D (0.005)	B (0.17)	0/46 (0)	2/121 (1.6)	1/26 (3.8)
10	c.1231A>G	p.K411E	–	AG	D (0.015)	B (0.233)	0/46 (0)	1/121 (0.8)	0/26 (0)
11	c.1408G>A	p.V470M	rs213950	GA	T (1)	B (0)	21/46 (45.7)	65/121 (53.7)	11/26 (42.3)
				AA			5/46 (10.9)	19/121 (15.7)	1/26 (3.8)
12	c.1666A>G	p.I556V	rs75789129	AG	T (0.536)	B (0.334)	2/46 (4.3)	8/121 (6.6)	0/26 (0)
				GG			0/46 (0)	0/121 (0)	0/26 (0)
13	c.1753G>T	p.E585X	–	GT	–	–	1/46 (2.2)	0/121 (0)	0/26 (0)
17	c.2869delC	p.L957fs	–		–	–	0/46 (0)	1/121 (0.8)	0/26 (0)
21	c.3468G>T	p.L1156F	rs139729994	GT	T (0.163)	PD (0.994)	2/46 (4.3)	10/121 (8.3)	2/26 (7.7)
				TT			1/46 (2.2)	0/121 (0)	0/26 (0)
25	c.4045G>A	p.G1349S	rs201686600	GA	D (0)	PD (1)	1/46 (2.2)	0/121 (0)	0/26 (0)
25	c.4056G>C	p.Q1352H	rs113857788	GC	D (0)	PD (1)	5/46 (10.9)	11/121 (9.1)	4/26 (15.4)
				CC			0/46 (0)	0/121 (0)	0/26 (0)
27	c.4357C>T	p.R1453W	rs4148725	CT	D (0)	PD (0.999)	3/46 (6.5)	6/121 (5.0)	1/26 (3.8)

B benign, *CP* chronic pancreatitis, *D* damaging, *PD* probably damaging, *T* tolerated, *SIFT* Sorting Intolerant From Tolerant

Table 3 Comparison of the non-synonymous variant frequencies between the patients with CP and controls

Amino acid change	Genotype	All CP (%)	HGVD (%)	*P* value (vs. HGVD)				
				All CP	Alcoholic CP	Nonalcoholic CP	Idiopathic CP	Hereditary/ familial CP
p.R31C	CT	3/193 (1.6)	12/1102 (1.1)	0.48	>0.99	0.41	0.18	>0.99
p.R31H	GA	1/193 (0.5)	0	–	–	–	–	–
p.I125T	TC	3/193 (1.6)	5/1102 (0.5)	0.11	>0.99	0.057	0.15	0.13
p.K411E	AG	1/193 (0.5)	0	–	–	–	–	–
p.V470M	GA	97/193 (50.3)	573/1199 (47.8)	0.66	0.57	0.68	0.38	0.12
	AA	25/193 (13.0)	185/1199 (15.4)					
p.I556V	AG	10/193 (5.2)	78/1150 (6.8)	0.70	0.79	0.81	>0.99	0.45
	GG	0/193 (0)	3/1150 (0.3)					
p.E585X	GT	1/193 (0.5)	0	–	–	–	–	–
p.L957fs		1/193 (0.5)	0	–	–	–	–	–
p.L1156F	GT	14/193 (7.3)	45/1136 (4.0)	0.04	0.06	0.07	0.11	0.30
	TT	1/193 (0.5)	1/1136 (0.1)					
p.G1349S	GA	1/193 (0.5)	4/1094 (0.4)	0.56	0.19	>0.99	>0.99	>0.99
p.Q1352H	GC	20/193 (10.4)	57/1153 (4.9)	0.009	0.12	0.037	0.17	0.062
	CC	0/193 (0)	1/1153 (0.1)					
p.R1453W	CT	10/193 (5.2)	42/1144 (3.7)	0.32	0.25	0.49	0.45	>0.99

CP chronic pancreatitis, *HGVB* Human Genetic Variation Database

P values were determined versus HGVD by the Fisher's exact test

Table 4 Synonymous variants in the exons of the *CFTR* gene detected in this study

Exon	Synonymous variant	Amino acid change	dbSNP135	Genotype	Alcoholic CP (%)	Idiopathic CP (%)	Hereditary/ familial CP (%)
4	c.372C>T	p.G124=	–	CT	0/46 (0)	1/121 (0.8)	0/26 (0)
13	c.1731C>T	p.Y577=	rs55928397	CT	0/46 (0)	1/121 (0.8)	0/26 (0)
15	c.2562T>G	p.T854=	rs1042077	TG	20/46 (43.5)	69/121 (57.0)	12/26 (46.2)
				GG	6/46 (13.0)	18/121 (14.9)	0/26 (0)
23	c.3723C>A	p.G1241=	rs185065886	CA	1/46 (2.2)	0/121 (0)	0/26 (0)
25	c.3975A>G	p.R1325=	–	AG	0/46 (0)	1/121 (0.8)	0/26 (0)
27	c.4254G>A	p.E1418=	–	GA	0/46 (0)	1/121 (0.8)	0/26 (0)
27	c.4389G>A	p.Q1463=	rs1800136	GA	1/46 (2.2)	3/121 (2.5)	0/26 (0)

CP chronic pancreatitis

heterozygous form (Table 6). The nonsense variant c.1753G>T (p.E585X) was found in a patient with alcoholic CP. He was diagnosed as having alcoholic CP at 28 years old. The c.1231A>G (p.411E) variant was found in a 19-year-old male with idiopathic CP. He had suffered from pancreatitis attacks since 12 years old. ERCP showed multiple stones in the main pancreatic duct. He underwent extracorporeal shock wave lithotripsy for the treatment of pancreatic stones. The patient also had the c.3468G>T (p.L1156F) variant in a heterozygous form. None of these three patients had known pancreatitis susceptibility mutations in the *PRSS1*, *SPINK1*, *CTRC*, or *CPA1* genes (Table 6). All of the patients carrying the novel synonymous variants were idiopathic CP (Table 4).

The frequency of the c.4056G>C (p.Q1352H) variant was higher in all patients with CP than that in controls ($P = 0.009$; Table 3). Stratification based on the etiologies showed that the association was significant in patients with nonalcoholic CP (combination of cases with idiopathic, hereditary, and familial CP) ($P = 0.037$). The frequency of the c.3468G>T (p.L1156F) variant was also higher in patients with CP than that in controls ($P = 0.04$). There were no significant difference for any other non-synonymous or synonymous variants detected in the exons

Table 5 Comparison of the synonymous variant frequencies between the patients with CP and controls

Synonymous variant	Genotype	All CP (%)	HGVD (%)	P value (vs. HGVD)				
				All CP	Alcoholic CP	Nonalcoholic CP	Idiopathic CP	Hereditary/familial CP
c.C372T	CT	1/193 (0.5)	0	–	–	–	–	–
c.1731C>T	CT	1/193 (0.5)	0	–	–	–	–	–
c.2562T>G	TG	101/193 (52.3)	528/1154 (45.8)	0.22	0.81	0.11	0.045	0.033
	GG	24/193 (12.4)	181/1154 (15.7)					
c.3723C>A	CA	1/193 (0.5)	3/671 (4.5)	>0.99	0.23	>0.99	>0.99	>0.99
c.3975A>G	AG	1/193 (0.5)	0	–	–	–	–	–
c.4254G>A	GA	1/193 (0.5)	0	–	–	–	–	–
c.4389G>A	GA	4/193 (2.1)	40/1112 (3.6)	0.48	>0.99	0.53	0.81	>0.99
	AA	0/193 (0)	1/1112 (0.1)					

CP chronic pancreatitis, *HGVD* Human Genetic Variation Database

P values were determined against HGVD by the Fisher's exact test

Table 6 Total *CFTR* sequencing results of patients carrying rare non-synonymous *CFTR* variants

Case#	Etiology	Age at onset	Rare variant	Additional non-synonymous variants	c.1210-34TG(9_13) c.1210-12T(5_9)	Mutation in other pancreatitis susceptibility genes[a]
A1	Idiopathic	34	p.R31C/-	p.R1453W/-	TG11/TG11, 7T/7T	–
A2	Idiopathic	8	p.R31C/-	–	TG11/TG12, 7T/7T	–
A3	Idiopathic	16	p.R31C/-	–	TG11/TG12, 7T/7T	–
A4	Idiopathic	10	p.R31H/-	–	TG11/TG12, 7T/7T	–
A5	Idiopathic	16	p.I125T/-	p.L1156F/-	TG11/TG12, 7T/7T	*CTRC* p.R29Q/-
A6	Idiopathic	2	p.I125T/-	–	TG11/TG12, 7T/7T	–
A7	Hereditary	28	p.I125T/-	p.R1453W/-	TG11/TG12, 7T/7T	–
A8	Idiopathic	19	p.K411E/-	p/L1156F/-	TG11/TG12, 7T/7T	–
A9	Alcoholic	28	p.E585X/-	p.I556V/-	TG11/TG11, 7T/7T	–
A10	Idiopathic	21	p.L957fs/-	p.Q1352H/-	TG11/TG12, 7T/7T	–
A11	Alcoholic	40	p.G1349S/-	–	TG11/TG11, 7T/7T	–

[a] Pancreatitis-associated mutations in the *PRSS1*, *SPINK1*, *CTRC*, and *CPA1* genes

between all patients with CP and controls (Tables 3, 5). The frequency of the c.2562T>G variant was different between the controls and the patients with idiopathic or hereditary/familial CP.

The 5T and, more rarely, 3T splicing variants of the intron 9 acceptor splice site [c.1210-12T(5_9)] are considered to be variants associated with CFTR-RD [16]. The 5T or 3T allele is a polymorphic variant with variable penetrance, causing less efficient exon 10 splicing and a lower *CFTR* transcript level [28]. The splicing efficiency of exon 10 is further affected by the length of the adjacent TG repeat [c.1210-34TG(9_13)]. The distribution of the c.1210-34TG(9_13) and c.1210-12T(5_9) variants is shown in Table 7. In our cohort, nine patients with CP had the 5T allele, all in a heterozygous form. Four patients (two alcoholic, one idiopathic, one hereditary) had the 5T-TG13. No patient had the haplotype TG10-7T-M470, which was reported to increase the risk of idiopathic CP [28].

It has been increasingly recognized that compound and trans-heterozygosity in the pancreatitis susceptibility genes are an overt risk factor for idiopathic CP [29–32]. Among the 193 patients with CP enrolled in this study, 29 patients had pancreatitis-associated mutations in the *PRSS1*, *SPINK1*, *CTRC*, and *CPA1* genes. Among these, nine patients had the non-synonymous *CFTR* variants, which are probably damaging based on the SIFT and/or the PolyPhen-2 prediction (Table 8).

Discussion

In this study, we performed comprehensive analysis of the variants in the *CFTR* gene by targeted NGS. To our knowledge, this is the first study to analyze pancreatitis susceptibility genes by targeted NGS. Comprehensive analysis by targeted NGS enabled us to identify novel and

Table 7 Distribution of the c.1210-34TG(9_13) and c.1210-12T(5_9) variants in patients with CP

c.1210-34TG(9_13), c.1210-12T(5_9)	All CP (%)	Alcoholic CP (%)	Idiopathic CP (%)	Hereditary/ familial CP (%)
TG10/TG11, 7T/9T	1/193 (0.5)	0/46 (0)	1/121 (0.8)	0/26 (0)
TG11/TG11, 7T/7T	46/193 (23.8)	15/46 (32.6)	23/121 (19.0)	8/26 (30.8)
TG11/TG11, 7T/9T	4/193 (2.1)	1/46 (2.2)	3/121 (2.5)	0/26 (0)
TG11/TG12, 5T/7T	5/193 (2.6)	0/46 (0)	4/121 (3.3)	1/26 (3.8)
TG11/TG12, 6T/7T	1/193 (0.5)	0/46 (0)	1/121 (0.8)	0/26 (0)
TG11/TG12, 7T/7T	124/193 (64.2)	27/46 (58.7)	81/121 (66.9)	16/26 (61.5)
TG11/TG13, 6T/7T	1/193 (0.5)	1/46 (2.2)	0/121 (0)	0/26 (0)
TG11/TG13, 7T/7T	1/193 (0.5)	0/46 (0)	1/121 (0.8)	0/26 (0)
TG12/TG12, 7T/7T	6/193 (3.1)	0/46 (0)	6/121 (5.0)	0/26 (0)
TG12/TG13, 5T/7T	4/193 (2.1)	2/46 (4.3)	1/121 (0.8)	1/26 (3.8)

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rare variants in the *CFTR* gene. The c.1753G>T (p.E585X) variant is a nonsense variant, and the c.2869delC (p.L957fs) variant leads to a stop codon afterward at amino acid 967. These variants result in a heavily truncated protein missing nearly two-thirds (p.E585X) or more than one-third (p.L957fs) of its amino acids. Because we did not perform functional assays, we do not have direct evidence that these two variants cause loss of the CFTR expression and/or function. However, a general acknowledgment has been agreed that mutations of this type, called class I mutations, are associated with complete loss or near complete loss of the CFTR function (<3 % of wild-type CFTR function) [33, 34]. The pathogenic potential of another novel variant, c.1231A>G (p.K411E), is currently unknown, but the in silico analyses suggest that this variant is deleterious. Importantly, the clinical phenotype of this patient might be complicated by the presence of another variant, p.L1156F. Noone et al. [30] reported that pancreatitis risk was increased approximately 40-fold by having two *CFTR* mutations. This is also the case with the c.374T>C (p.I125T) variant. Two of the three patients carrying this variant had other non-synonymous variants (p.I556V and p.R1453W). This p.I125T variant was originally reported in Chinese patients with idiopathic bronchiectasis and considered to be associated with CFTR-RD [35].

There are considerable regional and ethnic variations in the spectrum of the *CFTR* mutations [15]. Approximately 70 % of individuals with CF in the Caucasian population are homozygous for the F508del mutation, and almost 90 % of the patients have at least one F508del allele [36]. This mutation is extremely rare in the Japanese population, accounting for the rare presentation of classical CF in this region (approximately 1/350,000 live births) [37]. It is not surprising that the CF-causing mutations are frequently found in Caucasians, but very rarely in East Asia. Audrézet et al. [29] reported from France that at least 20 % of the patients with idiopathic CP carried one of the most common *CFTR* mutations. Fujiki et al. [38] reported from Japan that none of the 20 common CF-causing mutations was found in 65 Japanese patients with CP (51 alcoholic and 14 idiopathic). Wang et al. [39] reported comprehensive screening of pancreatitis susceptibility genes including *CFTR* in 75 pediatric patients with idiopathic CP from China. They identified a novel 8-bp deletion in exon 4, but not the common CF-causing mutations. In this study, we found no common severe CF-causing mutations, in agreement with these previous studies from East Asia.

We found a significant association between the p.Q1352H variant and CP. This finding confirms the previous reports from Japan and Korea showing that this variant was over-presented in patients with CP compared to controls [38, 40]. Fujiki et al. [38] from Japan reported that the frequency of this variant was higher in patients with CP (8/65, 12.3 %) than in controls (6/162, 3.7 %). Lee et al. [40] reported from Korea that 14.3 % (4/28) of the patients with CP had this variant, whereas only 0.9 % (1/117) of the controls did. Glutamine at 1,352 is located in the second nucleotide-binding fold of CFTR, and its change to histidine (p.Q1352H) causes reductions in both the protein expression and channel activity of CFTR [40]. Similarly, we found that the p.L1156F variant was overexpressed in patients with CP. A functional study reported reduced Cl^-/HCO_3^- permeability in the presence of the p.L1156F variant [41].

Gene–gene interactions of known pancreatitis susceptibility genes, especially between the *CFTR* and *SPINK1* genes, have been increasingly recognized. Indeed, seven out of 25 patients carrying the *SPINK1* variant(s) had the *CFTR* p.Q1352H and/or p.L1156F variants. One patient was trans-heterozygous for the *CTRC* p.R29Q and *CFTR* p.I125T/p.L1156F variants. Noone et al. [30] reported that pancreatitis risk was increased approximately 40-fold by having two *CFTR* mutations, 20-fold by having the *SPINK1* p.N34S variant, and 900-fold by having both. Trans-heterozygosity of the *SPINK1* p.N34S with the

Table 8 Total *CFTR* sequencing results of patients with *SPINK1*, *PRSS1*, *CTRC*, or *CPA1* mutations

Case#	Etiology	*CFTR* variants[a]	c.1210-34TG(9_13) c.1210-12T(5_9)	*SPINK1*	*PRSS1*	*CTRC*	*CPA1*
B1	Familial	p.Q1352H/-	TG11/TG12, 7T/7T	p.N34S/p.N34S			
B2	Idiopathic	–	TG12/TG12, 7T/7T	p.N34S/p.N34S			
B3	Idiopathic	–	TG11/TG12, 7T/7T	p.N34S/p.N34S			
B4	Idiopathic	p.L1156F/-, p.Q1352H/-	TG11/TG11, 7T/7T	p.N34S/-			
B5	Idiopathic	p.Q1352H/-	TG11/TG12, 7T/7T	p.N34S/-			
B6	Idiopathic	p.Q1352H/-	TG11/TG12, 7T/7T	p.N34S/-			
B7	Idiopathic	–	TG11/TG12, 7T/7T	p.N34S/-			
B8	Idiopathic	–	TG11/TG12, 7T/7T	p.N34S/-			
B9	Idiopathic	–	TG11/TG12, 7T/7T	p.N34S/-			
B10	Idiopathic	–	TG11/TG12, 7T/7T	p.N34S/-			
B11	Idiopathic	–	TG11/TG12, 7T/7T	p.N34S/-			
B12	Idiopathic	–	TG11/TG12, 7T/7T	p.N34S/-			
B13	Idiopathic	–	TG11/TG12, 7T/7T	p.N34S/-			
B14	Alcoholic	–	TG12/TG13, 5T/7T	p.N34S/-			
B15	Idiopathic	–	TG11/TG12, 7T/7T	p.N34S/IVS3+2T>C			
B16	Idiopathic	p.R1453W/-	TG11/TG11, 7T/7T	p.N34S/IVS3+2T>C			
B17	Idiopathic	–	TG11/TG12, 7T/7T	IVS3+2T>C/IVS3+2T>C			
B18	Idiopathic	–	TG11/TG12, 7T/7T	IVS3+2T>C/IVS3+2T>C			
B19	Hereditary	p.I125T/-, p.L1156F/-	TG11/TG12, 5T/7T	IVS3+2T>C/-			
B20	Familial	p.L1156F/-	TG11/TG12, 7T/7T	IVS3+2T>C/-			
B21	Idiopathic	–	TG11/TG12, 7T/7T	IVS3+2T>C/-			
B22	Alcoholic	p.Q1352H/-	TG11/TG12, 7T/7T	IVS3+2T>C/-			
B23	Alcoholic	–	TG11/TG12, 7T/7T	IVS3+2T>C/-			
B24	Idiopathic	–	TG11/TG12, 7T/7T	p.P45S/-			
B25	Idiopathic	–	TG12/TG12, 7T/7T	IVS3+2T>C/-	p.R122H/-		
B26	Hereditary		TG11/TG12, 7T/7T		p.R122H/-		
B27	Idiopathic	p.I556V/-	TG11/TG12, 7T/7T		p.N29I/-		
B28	Idiopathic	p.I125T/-, p.L1156F/-	TG11/TG12, 7T/7T			p.R29Q/-	
B29	Idiopathic	–	TG11/TG12, 7T/7T				p.T368_Y369ins20/-

Nine patients had the non-synonymous *CFTR* variants, which are probably damaging based on the SIFT or the PolyPhen-2 prediction

The p.I556V variant appeared to be benign based on the SIFT or the PolyPhen-2 prediction

Case B28 is the same as A5 in Table 6

[a] We excluded the p.V470M variant from the list because of its similar frequencies in patients and controls

CFTR p.R75Q was reported to increase CP risk [31]. 6.5 % of the patients with idiopathic or hereditary CP carried variants in at least two pancreatitis susceptibility genes [32]. Whether the coinheritance of variants/mutations in pancreatitis susceptibility gene is a *bona fide* example of digenic inheritance or interaction between a disease-causing gene and a genetic modifier is unclear in most cases [42].

We used targeted sequence capture and high-throughput NGS to detect variants in the *CFTR* gene. Due to the large size (27 exons, 1,480 amino acids), traditional technologies, such as PCR and capillary sequencing, are time- and cost-consuming. A major advantage of the HaloPlex-targeted enrichment system is the convenient workflow, integrating both capture and library preparation. The protocol allows one person to prepare a set of finished libraries within two working days and requires no larger specialized instruments. Sequence capture eliminates the necessity of setting up hundreds of PCR, instead allowing for parallel

enrichment of target regions in a single experiment. A weakness of this method is that the detection of larger copy number variations would require different methods. We have designed the HaloPlex platform for more than 70 genes, including the known pancreatitis susceptibility genes such as *CFTR*, *PRSS1*, *SPINK1*, *CTRC,* and *CPA1*. This system has allowed us to perform rapid screening of the known susceptibility genes simultaneously and gives an overview of potentially pathogenic variants in patients with pancreatitis. In addition, our HaloPlex platform includes candidates of novel pancreatitis susceptibility genes such as pancreatic digestive enzymes, those highly expressed in the pancreas and those related to autophagy and endoplasmic reticulum stress. This system might contribute to the identification of novel pancreatitis susceptibility genes in the future.

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Conflict of interest None.

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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.

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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 1). 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 1 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