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LETTER TO THE EDITOR

Male Health

GATA4 mutations are uncommon in patients with 46,XY disorders of sex development without heart anomaly

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Dear Editor,

Disorders of sex development (DSDs) are a group of conditions in which chromosomal, gonadal, or anatomical sex is atypical.[1] DSD in genetic males (46,XY DSD) primarily results from impaired androgen production or action or perturbed genital morphogenesis.[1] The current understanding of the genetic basis of 46,XY DSD remains fragmentary. For example, although more than 15 genes have been implicated in the development of nonsyndromic hypospadias,[2] one of the most common forms of 46,XY DSD,[1] mutations in these genes account for <20% of the cases.[3]

GATA-binding protein 4 (GATA4) is a transcription factor involved in cardiac and sexual development.[4–7] GATA4 harbors two zinc finger domains which mediate the interaction with various proteins such as friend of GATA2/zinc finger protein, multitype 2 (FOG2/ZFPM2) and nuclear receptor subfamily 5, group A, member 1 (NR5A1).[4,5] Known target genes of GATA4 include sex-determining region Y (*SRY*), SRY box-9 (*SOX9*), and anti-Mullerian hormone (*AMH*) involved in testicular or genital development.[4,5] *GATA4* was initially reported as a causative gene for congenital heart anomalies.[6] To date, heterozygous *GATA4* mutations have been identified in more than 140 patients with ventricular septal defect, tetralogy of Fallot, or other cardiac malformations.[7] In 2011, Lourenço *et al*.[8] identified a heterozygous *GATA4* missense mutation (p.G221R) in three 46,XY DSD patients from one family. Two of the three patients manifested ambiguous external genitalia with hypospadias and intra-abdominal gonads, and the remaining one patient exhibited male-type genitalia with micropenis and intra-abdominal gonads. The proband had no heart anomaly, whereas the other affected males and two female family members showed various cardiac abnormalities including tetralogy of Fallot. *In vitro* assays confirmed that the p.G221R mutant protein failed to bind to FOG2 and did not transactivate the *Amh* promoter. Subsequently, Eggers *et al*.[9] identified two likely pathogenic *GATA4* variants (p.P407Q and p.W228C), together with two variants of uncertain significance, in six patients with 46,XY DSD without heart anomalies. These findings suggest that *GATA4* mutations can underlie 46,XY DSD without heart malformations. However, there has been no further report of *GATA4* sequencing analyses for large 46,XY DSD cohorts, and therefore, the clinical significance of *GATA4* mutations as the cause of 46,XY DSD remains uncertain.

Here, we performed *GATA4* mutation screening for 119 patients with 46,XY DSD without heart anomalies. Patients with additional congenital anomalies, cytogenetically detectable chromosomal abnormalities, or pathogenic mutations in the major known causative genes for 46,XY DSD, androgen receptor (*AR*), chromobox 2 (*CBX2*), desert hedgehog (*DHH*), mitogen-activated protein kinase kinase kinase 1 (*MAP3K1*), *NR5A1, SOX9, SRY,* steroid 5 alpha-reductase 2 (*SRD5A2*), Wilms tumor 1 (*WT1*), and *ZFPM2*,[3] were excluded from the study group. Detailed methods are described in **Supplementary Information**. This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development, Tokyo, Japan, and performed after obtaining written informed consent. Of the 119 patients, 111 manifested male-type external genitalia with micropenis, cryptorchidism, and/or hypospadias, while the remaining eight presented with ambiguous genitalia. All patients were of Japanese origin. The coding region and splice sites of *GATA4* were analyzed using next-generation sequencers. We selected variants whose allele frequencies in Exome Aggregation Consortium (ExAC) Browser (http://exac.broadinstitute.org/; last accessed on 25 September 2017) and the 1000 Genomes Browser (http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/; last accessed on 25 September 2017) were both <1.0%. All variants of interest were confirmed by Sanger sequencing and subjected to *in silico* functional prediction. Furthermore, the transactivating activity of each variant protein was examined by *in vitro* reporter assays using the *Amh* promoter. In these assays, the activities of the variants were compared to that of p.G221R, a mutation previously identified in three patients with 46,XY DSD.[8] To clarify the frequency of each variant in the Japanese general population, we sequenced DNA samples from 100 unaffected Japanese males. We also analyzed familial samples of variant-positive patients, when possible.

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As a result, we identified p.R265C (c.793C>T) in one patient (Case 1) and p.P407Q (c.1220C>A) in four patients (Case 2–5) (**Figure 1a**). Case 1–5 manifested male-type genitalia and hypospadias with and without micropenis and cryptorchidism. The variants were present in a heterozygous state. The p.R265C and p.P407Q variants were extremely rare polymorphisms accounting for 2 of 120 802 and 68 of 121 396 alleles with ExAC Browser, respectively (**Supplementary Table 1**). *In silico* analyses scored both variants as deleterious (**Supplementary Table 1**). The p.R265C variant was predicted to alter the protein structure (**Figure 1b**). The structural prediction of the p.P407Q variant was unobtainable, because of the lack of information of the C-terminal crystal structure of wild-type GATA4. *In vitro* assays revealed that p.R265C retained normal transactivation activity, while p.P407Q had markedly decreased activity similar to that of p.G221R (**Figure 1c**). None of our 100 control individuals carried p.R265C, whereas two had p.P407Q. We also analyzed DNA samples obtained from parents of Case 1–3 and siblings of Case 1 and 2. The variants of Case 1 and 2 were shared by their unaffected fathers, while the variant of Case 3 was identified in the mother. In addition, a younger brother of Case 1 also carried p.R265C. None of the variant-positive relatives of Case 1–3 had heart anomalies.

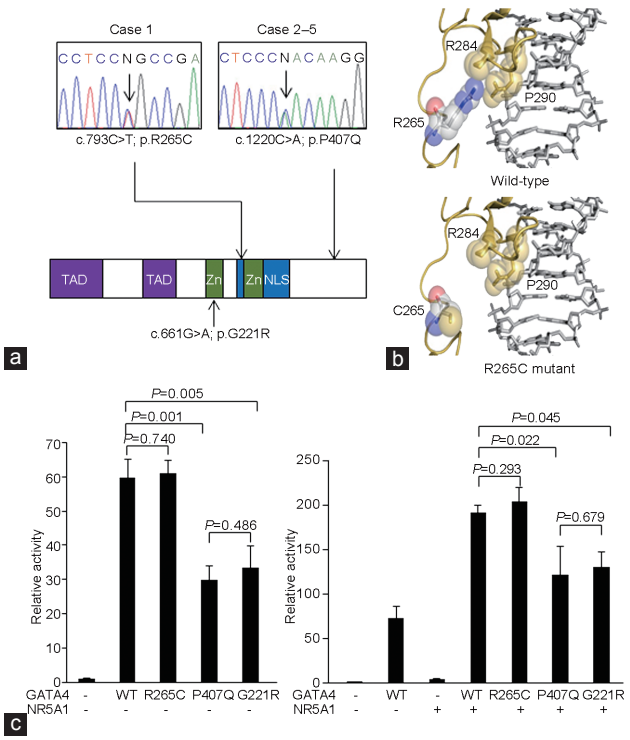

Figure 1: *GATA4* variants in Case 1–5. (**a**) Upper panel: chromatograms of the p.R265C and p.P407Q variants. Arrows indicate mutated nucleotides. Lower panel: protein structure of GATA4. The p.G221R is a previously reported mutation associated with 46,XY DSD. (**b**) Three-dimensional structure of GATA4 proteins. The structures of wild-type and the p.R265C variant are shown. GATA4 and its target DNA are shown in gold and silver, respectively. Amino acids at the 265th codon are illustrated as rainbow spheroidal shapes. Amino acids at the 284th and 290th positions in C-terminal zinc finger domain are illustrated as gold spheroidal shapes. The wild-type likely interacts with amino acids in the C-terminal zinc finger domain. Color code: red, oxygen; blue, nitrogen; yellow, sulfur; gray, others. (**c**) Representative results of luciferase assays using the murine *Amh* promoter. The GATA4 expression vector, with and without the NR5A1 expression vector, was transfected into COS-1 cells. The transactivating activities of the wild-type GATA4 (WT), the p.R265C mutant (R265C), the p.P407Q mutant (P407Q), and the previously reported p.G221R mutant (G211R) were compared with that of the empty expression vector (-). The results are expressed as mean ± standard deviation. NLS: nuclear location sequence; TAD: transactivation domain; Zn: zinc finger domain.

The aforementioned data raise questions about the causal relationship between the *GATA4* variants and 46,XY DSD in our participants. Particularly, p.R265C retained normal *in vitro* transactivation activity for the *Amh* promoter and was shared by the unaffected father and brother of Case 1. Hence, although p.R265C is extremely rare in the general population and was predicted to be deleterious by multiple *in silico* analyses, this variant is unlikely to disrupt sexual development in genetic males. Similarly, p.P407Q seems to be insufficient to cause 46,XY DSD, because this variant was shared by the unaffected father of Case 2 and two of the 100 control males. However, we cannot exclude the possibility that p.P407Q exerts some deleterious effects on male sex development, because this variant showed compromised transactivation activity for the *Amh* promoter. Notably, this variant has been reported as a pathogenic mutation responsible for tetralogy of Fallot, atrial septal defect, and ventricular septal defect.[7] The lack of heart anomalies in Case 2–5 with p.P407Q may reflect incomplete penetrance of heart anomalies resulting from *GATA4* mutations,[10] although the relatively high frequency of heart anomalies in previously reported cases suggests that developing hearts are more vulnerable to *GATA4* dysfunction than developing testes.[6]

Collectively, the results of this study demonstrate that *GATA4* mutations are rare in patients with 46,XY DSD without heart anomalies. Further studies such as pull-down assays using FOG2 and NR5A1 antibodies and mutation screening for large patient cohorts are necessary to clarify whether *GATA4* variants, including p.P407Q, contribute to the genetic predisposition of 46,XY DSD.

AUTHOR CONTRIBUTIONS

MI and MF designed this study; KM, YK, YH, and TO participated in the acquisition of patients' data; MI, MK, and SN conducted the experiments; MI and MF drafted the manuscript; and all authors have reviewed and approved the final version.

COMPETING INTERESTS

All authors declared no competing interests.

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Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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Supplementary Table 1: Allele frequencies and *in silico* functional prediction of *GATA4* variants

cDNA	*Protein*	*dbSNP*	*ExAC*[a]	*1000G*[b]	*MutationTaster*[c]	*Polyphen-2*[d]		*SIFT*[e]		*CADD*[f]		*M-CAP*[g]	
					Prediction	*Score*	*Prediction*	*Score*	*Prediction*	*Score*	*Prediction*	*Score*	*Prediction*
c.793C>T	p.R265C	rs776523140	2/120 802	0/5008	Disease causing	1.000	Probably damaging	0.00	Deleterious	35.0	Deleterious	0.781	Deleterious
c.1220C>A	p.P407Q	rs115099192	68/121 396	6/5008	Disease causing	0.675	Possibly damaging	0.00	Deleterious	23.8	Deleterious	0.210	Deleterious

All analyses were performed by using the default parameters. [a]ExAC Browser (http://exac.broadinstitute.org/). Current Version: 0.3.1, GRCh37; [b]1000 Genomes Browser (http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/). Current Version: 3.7, GRCh37.[c]MutationTaster (http://www.mutationtaster.org/). Current version: MutationTaster2, GRCh37/Ensembl 69; [d]Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/). Current version: 2.2.2, GRCh37. Scores between 0.909 and 1, between 0.447 and 0.908, and below 0.446 were assessed as probably damaging, possibly damaging, and benign, respectively; [e]SIFT (http://sift.jcvi.org/). Current version: JCVI-SIFT v1.03. GRCh37/Ensembl 63. Scores <0.05 were assessed as deleterious; [f]CAAD (http://cadd.gs.washington.edu/). Current version: v1.3, GPCh37. Scores >20 were assessed as deleterious; [g]M-CAP (http://bejerano.stanford.edu/mcap/). Current version: M-CAP v1.0, GPCh37. Scores >0.025 were assessed as deleterious. ExAC: Exome Aggregation Consortium; CAAD: Combined Annotation Dependent Depletion; M-CAP: Mendelian Clinically Applicable Pathogenicity; dbSNP: The single nucleotide polymorphism database