Molecular analysis of the GATA3 gene in five Japanese patients with HDR syndrome

Akie Nakamura1), Fumie Fujiwara1), Yukihiro Hasegawa2), Katsura Ishizu1), Akiyo Mabe3), Hiroyasu Nakagawa4), Keisuke Nagasaki5), Wakako Jo1) and Toshihiro Tajima1)

1)Department of Pediatrics, Hokkaido University School of Medicine, Sapporo 060-8635, Japan
2)Endocrinology and Metabolism Unit, Tokyo Metropolitan Children’s Hospital, Fuchu 183-8561, Japan
3)Department of Child Development, Kumamoto University Graduate School, Kumamoto 860-0811, Japan
4)Department of Pediatrics, Fukuiken Saiseikai Hospital, Fukui 918-8503, Japan
5)Division of Pediatrics, Department of Homeostatic Regulation and Development, Niigata University Graduate School of Medicine and Dental Sciences, Niigata 951-8510, Japan

Abstract. GATA3 is a member of the GATA family of transcription factors. Heterozygous GATA3 abnormalities are associated with hypoparathyroidism, sensorineural deafness, and renal abnormality (HDR syndrome). However, this triad of symptoms does not occur in all HDR patients and other clinical features may be present in some cases. We report the clinical phenotypes and the molecular analysis of GATA3 in five Japanese HDR patients, including two familial cases. All five patients had hypoparathyroidism and sensorineural deafness, however renal abnormalities were absent in four patients. In addition, two patients with different mutations of GATA3 had female genital tract abnormalities. Sequence analysis of GATA3 demonstrated three novel (R262G, c1063delC and C318) and two reported mutations (c.432insG and c.1051-1G>T). Transient transfection assay using the GATA3 activating reporter system revealed that the transactivating activity of the R262G, c.1063delC, C318S and c.432insG mutants were markedly decreased, indicating that all four mutations are loss-of-function. In conclusion, this study reiterates the clinical variability in HDR syndrome and identifies three novel mutations of GATA3.

Key words: GATA3, HDR syndrome, Mutations, Genital tract anomaly

THE COMBINATION of hypoparathyroidism, sensorineural deafness and renal dysplasia is a rare congenital disease first described in 1992 by Bilous et al. [1] and named HDR syndrome by Hasegawa et al. [2]. The hypoparathyroidism is characterized by symptomatic or asymptomatic hypocalcemia with undetectable or low serum levels of parathyroid hormone (PTH) [3]. The sensorineural deafness is usually bilateral, although the degree of hearing loss varies. Renal anomalies are also heterogeneous with variable penetrance, including renal dysplasia, hypoplasia, or aplasia, and vesico-ureteric reflux, but renal anomalies are absent in a few cases [3-5].

GATA 3 is one of six members of the GATA family of transcription factors that bind the consensus response element 5’-(A/T)GATA(A/G)-3’[3]. Human and mouse GATA3 expression are observed in the developing parathyroid glands, inner ear and kidney, together with the thymus and central nervous system (CNS) [6, 7]. GATA3 encodes a 444-amino-acid protein that contains two transactivating domains (TA1 and TA2) and two zinc finger domains (ZnF1 and ZnF2) [3, 8, 9]. ZnF2 is highly conserved and essential for binding of its consensus response element, whereas ZnF1 is thought to stabilize this binding and to interact physically with the multi-type zinc finger Friends of GATA (FOG) proteins, which were cloned as GATA-specific factors [9, 10].

Van Esch et al. [11] first identified one nonsense mutation and two intragenic deletions of GATA3 in HDR syndrome. To date, gross deletions, missense
mutations, nonsense mutations and small insertions or deletions (resulting in frameshifts) of GATA3 have been reported in human HDR syndrome [2, 4, 5, 11-16], and thus haploinsufficiency of GATA3 is the mechanism of HDR syndrome [11].

In this study, we report three novel and two previously reported mutations in five Japanese patients with HDR syndrome. Functional studies demonstrated that these mutants were loss-of-function.

Patients

Clinical characteristics, biochemical findings and mutations of GATA3 are summarized in Table 1. Additional phenotypes and carriers of mutations in familial cases are summarized in Fig. 1.

Patient 1

Patient 1 is now a 8 year-old Japanese girl, who was born after normal vaginal delivery at full term without asphyxia. During the neonatal period, she had convulsion due to hypoglycemia and hypocalcemia, however, no further medical attention was paid to the condition. At age 1 month, an endocrine consultation at another

### Table 1 Phenotypes, genotypes, and hormonal data of the five patients

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at manifestation</td>
<td>1 month</td>
<td>11 months</td>
<td>2 years</td>
<td>1 month</td>
<td>13 years</td>
</tr>
<tr>
<td>Symptoms</td>
<td>poor body weight gain</td>
<td>seizure</td>
<td>pain on lower limb</td>
<td>seizure</td>
<td>muscle cramp</td>
</tr>
<tr>
<td>Ca$^a$ (mg/dL)</td>
<td>4.8</td>
<td>4.4</td>
<td>unknown</td>
<td>6.0</td>
<td>5.6</td>
</tr>
<tr>
<td>IP$^a$ (mg/dL)</td>
<td>unknown</td>
<td>9.0</td>
<td>unknown</td>
<td>11.5</td>
<td>6.8</td>
</tr>
<tr>
<td>Intact-PTH$^b$ (pg/mL)</td>
<td>7-10</td>
<td>5.9</td>
<td>20</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>Sensorineural deafness</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Right/Left (dB)</td>
<td>60/60</td>
<td>60/45</td>
<td>60/80-100</td>
<td>80/80</td>
<td>40-50/40-50</td>
</tr>
<tr>
<td>Renal anomaly</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
<td>left renal dysplasia</td>
<td>(-)</td>
</tr>
<tr>
<td>Other phenotypes</td>
<td>epilepsy, mental retardation</td>
<td>epilepsy</td>
<td>(-)</td>
<td>uterus duplex</td>
<td>heterotropia</td>
</tr>
</tbody>
</table>

### Family history

- **Maternal aunt:** mild mental retardation, deafness, tetanic episode
- **Mother:** hypoparathyroidism, sensorineural deafness
- **Family history**
  - Father: hypoparathyroidism, sensorineural deafness, hydronephrosis
  - Sister: hypoparathyroidism, sensorineural deafness, right renal dysplasia, vaginal atresia

### Mutations of GATA3

- Intron 5/exon 6 boundary: c.1051-1G>T, p.1351fsX18
- Exon 5: c.942T>A, p.C318S

IP; inorganic phosphorus  
$^a$ Normal reference ranges are as follows: Ca, 8.7~10.3 mg/dL; IP, 2.5~4.7 mg/dL; intact-PTH, 10-60 pg/mL

![Fig. 1 Pedigree of patient 3 and 5](#)

(A) Family tree of patient 3. (B) Family tree of patient 5. The presence of symptoms of HDR syndrome and other features is indicated by blackening of each quartered area. The asterices indicate mutations of GATA3.
hospital was carried out because of poor weight gain. At this time, laboratory data showed that her serum calcium level was decreased (4.8 mg/dL) with low serum intact PTH level (7 pg/mL, normal range; 10-60 pg/mL), and she was diagnosed as having idiopathic hypoparathyroidism. Treatment with 1α-hydroxyvitamin D3 and calcium lactate was started. Renal ultrasonography revealed no renal anomaly.

She had delayed developmental milestones (head control at age 5 months, walking without support at age 2 years) and had a limited vocabulary. At age 8 months, she had convulsions with normal serum calcium level and was diagnosed as epilepsy due to abnormal spikes in the electroencephalogram, although a brain CT was normal. At age 2.8 years, an audiogram examination demonstrated bilateral sensorineural deafness (60 dB bilaterally).

From the interview of patient’s mother, her maternal aunt had several tetanic episodes, moderate mental retardation and deafness, however a detailed clinical examination was not done. In this family, DNA analysis of only patient 1 was performed.

**Patient 2**

Patient 2 is now a 27 year-old Japanese female. At age 11 months, she manifested several episodes of febrile generalized seizures with frequent spiky discharges as observed by an electroencephalogram, although a brain CT was normal. A diagnosis of epilepsy was made and treatment of phenobarbital was begun. At age 1 year, she was referred to our hospital for further follow-up. Her developmental milestones were normal. She was found to have hypocalcemia (4.4 mg/dL) and hyperphosphatemia (9.0 mg/dL) by regular blood examination at age 1.6 years. Serum intact PTH level was also low (5-9 pg/mL). Based on these findings, she was diagnosed as having idiopathic hypoparathyroidism. Administration of 1α-hydroxyvitamin D3 and calcium lactate was initiated. At this time abdominal CT demonstrated right renal calcification, but no renal anomaly. At age 8 year, audiogram demonstrated bilateral sensorineural deafness (right; 60 dB, left; 45 dB). During follow-up, at age 9 year, she demonstrated absence seizure despite normal serum calcium levels. An electroencephalogram showed abnormal spikes at this time, and a diagnosis of epilepsy was accordingly made again.

**Patient 3**

Patient 3 is now a 31-year-old female. She was born via normal vaginal delivery. Based on an interview with her mother, she had several tetanic episodes at age 2 years, and was evaluated at the local hospital. At this time, she had hypocalcemia and hyperphosphatemia. She was diagnosed as having hypoparathyroidism and treatment of 1α-hydroxyvitamin D3 was started. She had normal psychomotor development. At age 11 years, an audiogram demonstrated bilateral sensorineural deafness (right; 60 dB, left; 80-100 dB).

Renal ultrasonography did not show abnormality in the kidneys. Her mother was also diagnosed as having hypoparathyroidism at the local hospital due to tetanic episodes at age 27 years, but clinical data were unavailable. She stated that she was also diagnosed with sensorineural deafness during childhood, but refused further evaluation for renal function. DNA from patient 3 and her mother was subjected to analysis.

**Patient 4**

Patient 4 is now a 5-month-old female born via normal vaginal delivery. At 1 month, she was evaluated because of several episodes of apnea and tetany. Laboratory findings demonstrated hypocalcemia (6.1 mg/dL) and low serum intact PTH (9 pg/mL). She was diagnosed as having hypoparathyroidism and treated by 1α-hydroxyvitamin D3. Renal ultrasonography showed left kidney hypoplasia. Magnetic resonance imaging of the abdomen revealed a uterus didelphys, however she had normal female genitalia. The audiogram demonstrated bilateral sensorineural deafness (80 dB bilaterally). There was no family history of hypoparathyroidism and kidney disease.

**Patient 5**

Patient 5 is now a 13-year-old male and was born via normal vaginal delivery. When he was in elementary school, he was diagnosed as having bilateral sensorineural deafness (40-50 dB bilaterally). At age 12 years, he had episodes of muscle cramp during exercise. By laboratory examination, he was found to have hypocalcemia (5.6 mg/dL) and low intact PTH (13 pg/mL). He was diagnosed as having hypoparathyroidism and treated by 1α-hydroxyvitamin D3. Renal ultrasonography showed no renal abnormality. His father had bilateral sensorineural deafness (50 dB bilaterally), hydronephrosis and mild hypocalcemia (6.7 mg/dL). His sister had bilateral sensorineural deafness (40-50 dB bilaterally) and a right multicystic kidney with ectopic ureter. Serum calcium (8.0 mg/dL) and intact PTH levels (19 pg/mL) of his sister were at the normal range.
lower limits of the normal range. She also had vaginal atresia. DNA analysis was performed in patient 5, his father and his younger sister.

Methods

DNA amplification and sequence analysis

The ethical committee of Hokkaido University School of Medicine admitted this study. Written informed consent to participate in the study was obtained from the patients and their parents. Genomic DNA was extracted from peripheral blood leukocytes. Each exon of GATA3 was amplified by PCR and sequenced directly with the primers reported by Van Esch et al. [11].

Wild-type and mutant GATA3 cDNA construction

Human GATA3 cDNA was prepared by polymerase chain reaction using human embryonic brain cDNA (Stratagen, La Jolla, CA). After verification of the DNA sequence, the GATA3 cDNA was cloned into the PCR 2.1 TA cloning vector (Invitrogen, Carlsbad, CA). The GATA3 cDNA was subsequently cloned into the eukaryotic expression vectors, pCDNA3 containing the cytomegalovirus promoter (Invitrogen, Carlsbad, CA). Mutant GATA3-cDNA (MT-GATA3-cDNA) corresponding to the observed genomic mutations were created using the QuickChange kit (Stratagen, La Jolla, CA).

A 6X GATA response element-containing luciferase vector (GATA-response) was used for the functional analysis as described previously [17].

Cell culture

COS-7 cells were obtained from American Type Cell Culture (Manassas, VA) and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum.

Transient gene expression

To assay promoter activity, COS-7 cells were plated in 6-well plates to 70% confluency, and transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with either (1) the empty expression vector (0.5 µg), (2) WT-GATA3 vector (0.5 µg), or (3) each MT-GATA3 vector (0.5 µg), together with the GATA-response vector (0.5 µg).

Cell extracts were prepared 48 hours after transfection, and luciferase assays were performed. Luciferase measurements were divided by the respective β-galactosidase activity to control for transfection efficiency. All transfections were performed in triplicate and the experiment was repeated 3 times. The mean of each triplicate reaction was expressed as a percentage of the WT-GATA3 activity for that study to allow comparison of data from different experiments. The results represent the mean ± SEM from 3 independent experiments.

Fluorescence analysis and microscopy

WT-GATA3 and MT-GATA3 constructs were subcloned into the mammalian expression vector pEGFP-C1 (BD Biosciences Clontech, Mountain View, CA) as previously described [10]. COS-7 cells were transfected with either WT-GATA3-GFP or MT-GATA3-GFP, using Lipofectamine 2000, and the cells were placed onto glass coverslips and fixed in 4% (vol/vol) formaldehyde/PBS at 48hr after transfection. Cells were permeabilized in 0.1% Triton/PBS and examined on a Nikon TE2000E inverted microscope (Tokyo, Japan). Nuclear was stained by DAPI (Invitrogen, Carlsbad, CA).

Results

PCR-direct sequencing of GATA3

Sequence analysis of GATA3 identified three novel mutations (c.1063delC, R262G and C318S) and two previously reported mutation (c.432insG and c.1051-1G>T) (Figs. 2, 3). These mutations were not identified in DNA obtained from 50 normal Japanese individuals.

In patient 1, a single base deletion (c.1063delC) was observed in exon 6 (Fig. 2A) generating a premature stop codon at codon 355, and resulting in the loss of 90 amino acids at the C-terminal region. A single base duplication (c.432insG) in exon3 (Fig. 2B), which has been reported previously [16], was identified in patient 2. This mutation shifts the reading frame, generating a premature stop codon at codon 303 in exon 4. Patients 3 and her mother had a heterozygous novel missense mutation (R262G) in exon 4 (Figs. 1A, 2C). This mutation was located adjacent to the first zinc finger domain of GATA3. Patient 4 had a previously reported acceptor splice site mutation, G>T transversion at the boundary of intron 5/exon 6 (Fig. 2D), generating a new open reading frame encoding a missense peptide with a premature termination at codon 367 [10]. Finally, a second missense mutation in the second zinc finger region
**Fig. 2** Results of GATA3 analysis
The wild-type sequence is also shown. Arrow indicates mutation sites. (A) The sequence analysis of the patient 1 showed a base deletion (c.1063delC) in exon 6. (B) The sequence analysis of the patient 2 demonstrated a duplication of G nucleotide (c.432insG). Note the double bands present after the mutation site. (C) In the patient 3, R262G (CTG to CCG) was identified in exon 4. (D) The patient 4 had a one base change (c.1051-1G>T) in an intron 5/exon 6 boundary. (E) In the patient 5, C318S (CAG to CTG) was identified in exon 5. The chromatogram of (B) shows the sense strand. The chromatogram of (A), (C), (D) and (E) show the antisense strand.

**Fig. 3** Schematic representation of GATA3 mutations
The ZnFs indicate zinc finger domains. The TAs represent transactivation domain. Previously reported mutations are shown by white boxes. Mutations reported in this study are shown by dark boxes in upper portion.
(C318S) was identified in patient 5 and his affected family members (Figs. 1B, 2E).

Functional analysis

Cotransfection of WT-GATA3 with the GATA-response vector activated the luciferase activity relative to the empty vector, however MT-GATA3 vectors containing the c.1063delC, c.432insG, R262G, and C318S mutations were inactive in this context (Fig. 4), indicating that these mutations were loss of function. The relative activities of the empty vector and the C318S mutant were not statistically different analyzed by ANOVA.

Subcellular localization of WT-GATA3 and each mutant GATA3

Proteins encoded by WT-GATA3 and MT-GATA3 of the c.1063delC, R262G and C318S mutations localized to the nucleus (Figs. 5A, 5B, 5D, 5E), whereas a protein containing the c.432insG mutation failed to local-
GATA3 mutations in HDR syndrome

In our study, we identified three novel and two previously reported mutations of GATA3 in 5 Japanese patients with HDR syndrome. Functional analysis demonstrated that four of these mutants (c.432insG, c.1063delC, R262G and C318S) failed to activate the GATA response element in transient transfection assay. Two frameshift mutations (c.432insG and c.1063delC) generated a premature stop codon in the reading frame indicating C-terminally truncated GATA3 proteins are non-functional. The R262G and C318S mutations were located adjacent to ZnF1 and ZnF2, respectively. R262 and C318 are well conserved among GATA3 across species. Results of our in vitro analysis of these two mutants further supports the importance of these conserved amino acids for normal function of GATA3. Regarding the splice acceptor site mutation, a previous study demonstrated that this mutation caused the aberrant splicing, generating a premature termination at codon 367 in exon 6 [10].

Based on the results of subcellular localization analysis of the GATA3 mutants, the c.432insG mutation did not accumulate in the nucleus, however the other mutants localized to the nucleus. It has been reported that the nuclear localization signal for GATA3 resides within residues 249-311 encompassing ZnF1[9, 19]. Since the c.432insG mutant generates a premature stop codon at codon 303 in exon 4 and lacks a portion of the nuclear localization signals and the other mutants retain these residues, our results are consistent with previous studies.

The clinical characteristics of HDR syndrome are known to be heterogeneous among individuals [4, 5, 13, 15]. Ferraris et al. [5] have summarized the clinical presentations of patients described in the literature. According to their study, 48/77 patients (62.3%) have the characteristic triad of HDR symptoms, 22 patients (28.6%) have hypoparathyroidism and deafness, and 2 patients (2.6%) have deafness and renal disease. In our study, one patient had the triad of symptoms, whereas the other four patients had only two clinical features of HDR syndrome (hypoparathyroidism and sensorineural deafness). Taking into consideration this clinical heterogeneity, screening of GATA3 mutations is worthwhile for diagnosis and genetic counseling, even when patients have only hypoparathyroidism and deafness.

Furthermore, in one familial case (the family of patient 5), the proband had hypoparathyroidism and deafness without kidney malformations. By contrast, the father and the sister with the same mutation had the triad of HDR syndrome. It has been reported that even within families with patients harboring identical GATA3 mutations, there exists variable penetrance of renal abnormalities and parathyroid disorder [5, 13]. These observations are consistent with our family case.

It is of note that patient 4 and a sister of patient 5 had a uterus didelphys and vaginal atresia, respectively. Hernandez et al. [15] have reported a familial case of HDR syndrome caused by a mutation of GATA3 with female genital tract anomalies. According to their study, the patient had a uterus didelphys with septate vagina and her mother had a septate uterus, and both patients suffered from painful menstruations. To date, the exact reason for the malformation of the vagina and uterus is unknown, however these findings suggest that the close examination of the genital tract is warranted in female patients with HDR syndrome.

In conclusion, our study has identified three novel loss-of-function mutations of GATA3 in five clinically heterogeneous HDR syndrome patients.

Acknowledgments

We thank T Yamagata (Tokyo University) for the gifts of wild type GATA3 and GATA reporter plasmid (6xGATA-tk-Luci). We are also grateful to the Nikon Imaging Center at Hokkaido University for helpful advices of confocal microscopy.

References


