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ORIGINAL

Growth hormone deficiency in monozygotic twins with autosomal dominant pseudohypoparathyroidism type Ib

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Abstract. Pseudohypoparathyroidism (PHP) is associated with compromised signal transductions via PTH receptor (PTH-R) and other G-protein-coupled receptors including GHRH-R. To date, while GH deficiency (GHD) has been reported in multiple patients with PHP-Ia caused by mutations on the maternally expressed *GNAS* coding regions and in two patients with sporadic form of PHP-Ib accompanied by broad methylation defects of maternally derived *GNAS* differentially methylated regions (DMRs), it has not been identified in a patient with an autosomal dominant form of PHP-Ib (AD-PHP-Ib) accompanied by an *STX16* microdeletion and an isolated loss of methylation (LOM) at exon A/B-DMR. We studied 5 4/12-year-old monozygotic twins with short stature (both –3.4 SD) and GHD (peak GH values, <6.0 μg/L after arginine and clonidine stimulations). Molecular studies revealed maternally derived *STX16* microdeletions and isolated LOMs at exon A/B-DMR in the twins, confirming the diagnosis of AD-PHP-Ib. *GNAS* mutation was not identified, and neither mutation nor copy number variation was detected in *GH1*, *POU1F1*, *PROP1*, *GHRHR*, *LHX3*, *LHX4*, and *HESX1* in the twins. The results, in conjunction with the previous finding that *GNAS* shows maternal expression in the pituitary, suggest that GHD of the twins is primarily ascribed to compromised GHRH-R signaling caused by AD-PTH-Ib. Thus, resistance to multiple hormones including GHRH should be considered in AD-PHP-Ib.

Key words: Growth hormone deficiency, Pseudohypoparathyroidism, Autosomal dominant, *STX16* microdeletion

PSEUDOHYPOPARATHYROIDISM (PHP) is a rare endocrine disorder caused by PTH resistance due to loss of maternal expression of the *GNAS* gene that encodes the subunit of the stimulatory G protein (Gsα) mediating the signal transductions of PTH receptor (PTH-R) [1]. This condition is divided into two types, PHP-Ia with Albright's hereditary osteodystrophy (AHO) and PHP-Ib without AHO. PHP-Ia is usually caused by mutations on the coding exons of the maternally inherited *GNAS*, whereas PHP-Ib results from methylation defects of maternally derived *GNAS* differentially methylated regions (DMRs) [2]. Furthermore, PHP-Ib is divided into two forms: a sporadic form that is caused by broad methylation defects with gain of methylation (GOM) at NESP55-DMR and loss of methylation (LOM) at AS-DMR, XLas-DMR, and exon A/B-DMR; and an autosomal dominant form (AD-PHP-Ib) that is associated with an isolated LOM at exon A/B-DMR and a microdeletion of *STX16* at the position ~220 kb upstream of *GNAS* (a ~3 kb microdeletion involving *STX16* exons 4–6 is commonly observed) [2–4].

Gsα also plays an important role in signal transductions of several G-protein-coupled receptors other than PTH-R, such as TSH-R and LH-R/FSH-R. Indeed, elevated TSH and LH/FSH have often been observed in patients with PHP [2]. Furthermore, since Gsα is also involved in the signal transduction of GHRH-R, it is predicted that GHRH-R signaling is also compromised in patients with PHP. Consistent with this, while GHRH itself has not been measured, GH deficiency (GHD) has been described in multiple patients with PHP-Ia and in two cases with sporadic PHP-Ib with broad methylation defects [5–8]. To our knowledge,

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however, there has been no report describing GHD in a patient with AD-PHP-Ib. Here, we report GHD in monozygotic twins with AD-PHP-Ib.

Case Reports

The index patients were monochorionic-diamniotic male twins of triplets (the two males and a female sib with a different placenta) born to a 25-year-old mother at 34 weeks of gestation with small birth sizes (Fig. 1A and Table 1). The mother was afflicted with irregular menses, and failed to conceive for several years of normal conjugal life. Thus, artificial insemination by husband was performed after ovulation induction therapy, producing the triplets. Blood TSH values were below the cutoff level of the neonatal mass screening in the triplets.

The triplets were examined for short stature at 5 4/12 years of age. Clinical and endocrine data of the triplets are shown in Table 1, together with those of the mother. Physical examinations of the triplets revealed no AHO phenotype except for short stature, and hand roentgenograms indicated no brachydactyly. Endocrine studies revealed GHD in the twins (peak GH values, <6.0 μg/L after arginine and clonidine stimulations), but not in the sister, although serum IGF-1 values remained normal in the twins. Serum TSH and FT_4 were normal in the triplets. Head magnetic resonance imaging delineated apparently normal pituitary glands in the twins. Thus, the twins were placed on GH therapy (0.175 mg/kg/week). Furthermore, since the mother was diagnosed as having PHP-Ib because of hypocalcemia and increased serum PTH value when she had afebrile convulsion at 26 years of age, serum calcium and PTH were examined in the triplets, showing hypocalcemia and increased PTH values in the twins, but not in the sister. Thus, the twins were also diagnosed as having PHP-Ib and were treated with 1α-(OH)-vitamin D, as their mother. On the last examinations at 8 6/12 years of age, the height was 112.5 cm (–2.8 SD) in the twin 1 and 113.0 cm (–2.7 SD) in the twin 2.

The mother exhibited no AHO phenotype. The father and the grandparents were clinically normal, and the grandmother had a normal serum calcium value (2.3 mmol/L). The height was 150 cm (–1.5 SD) in the mother, 166 cm (–1.5 SD) in the father, and 147 cm (–1.7 SD) in the grandmother (SD was evaluated by age-matched reference data reported in http://www.mhlw.go.jp/bunya/kenkou/eiyou/dl/h24-houkoku-05.pdf).

Molecular Studies

This study was approved by the Institutional Review Board Committee at National Center for Child Health and Development, and performed after obtaining written informed consent, using leukocyte genomic DNA samples and the primers shown in Supplemental Table 1.

We investigated the *STX16 – GNAS* region (Fig. 1B). Methylation-specific multiple-ligation-dependent probe amplification was performed using SALSA MLPA kit ME031A GNAS (MRC-Holland, Amsterdam) before and after digestion with a methylation-sensitive restriction enzyme *Hha*I, revealing *STX16* intragenic microdeletions in the twins, the mother, and the grandmother (Fig. 1C), and LOMs at exon A/B-DMR in the twins and the mother, but not in the grandmother (Fig. 1D). The microdeletions were shown to be ~3 kb in size (the common microdeletion) and absent from the sister, the father, and the grandfather by PCR analysis using primers flanking the microdeletions (Fig. 1E). The *GNAS* coding sequence was normal. In addition, direct sequencing and MLPA analysis using MLPA kit P216-A2 GHD were performed for *GH1*, *POU1F1*, *PROP1*, *GHRHR*, *LHX3*, *LHX4*, or *HESX1* that are known to cause GHD [9], showing no abnormal finding in the twins. The results of microsatellite analysis were consistent with monozygosity of the twins (Supplemental Table 2).

Discussion

The present study indicates that the monozygotic twins and the mother had AD-PHP-Ib. Indeed, they exhibited clinical findings compatible with PHP-Ib, and had LOM at exon A/B-DMR and a common ~3 kb microdeletion involving *STX16* exons 4–6.

The twins with AD-PHP-Ib had GHD. Since *GNAS* shows maternal expression in the pituitary as well as in the renal proximal tubules and thyroid [10, 11], compromised maternal *GNAS* expression is predicted to affect GHRH-R signaling. Furthermore, while GHD is a heterogeneous condition, no abnormal finding was detected in the seven GHD-related genes. Thus, GHD of the twins would primarily be ascribed to AD-PTH-Ib.

GHD remains rare in patients with PHP-Ib, while it has often been identified in patients with PHP-Ia [5–7]. Indeed, GHD has been found only in two patients with a sporadic form of PTH-Ib accompanied by broad methylation defects [8], and was identified for the first time

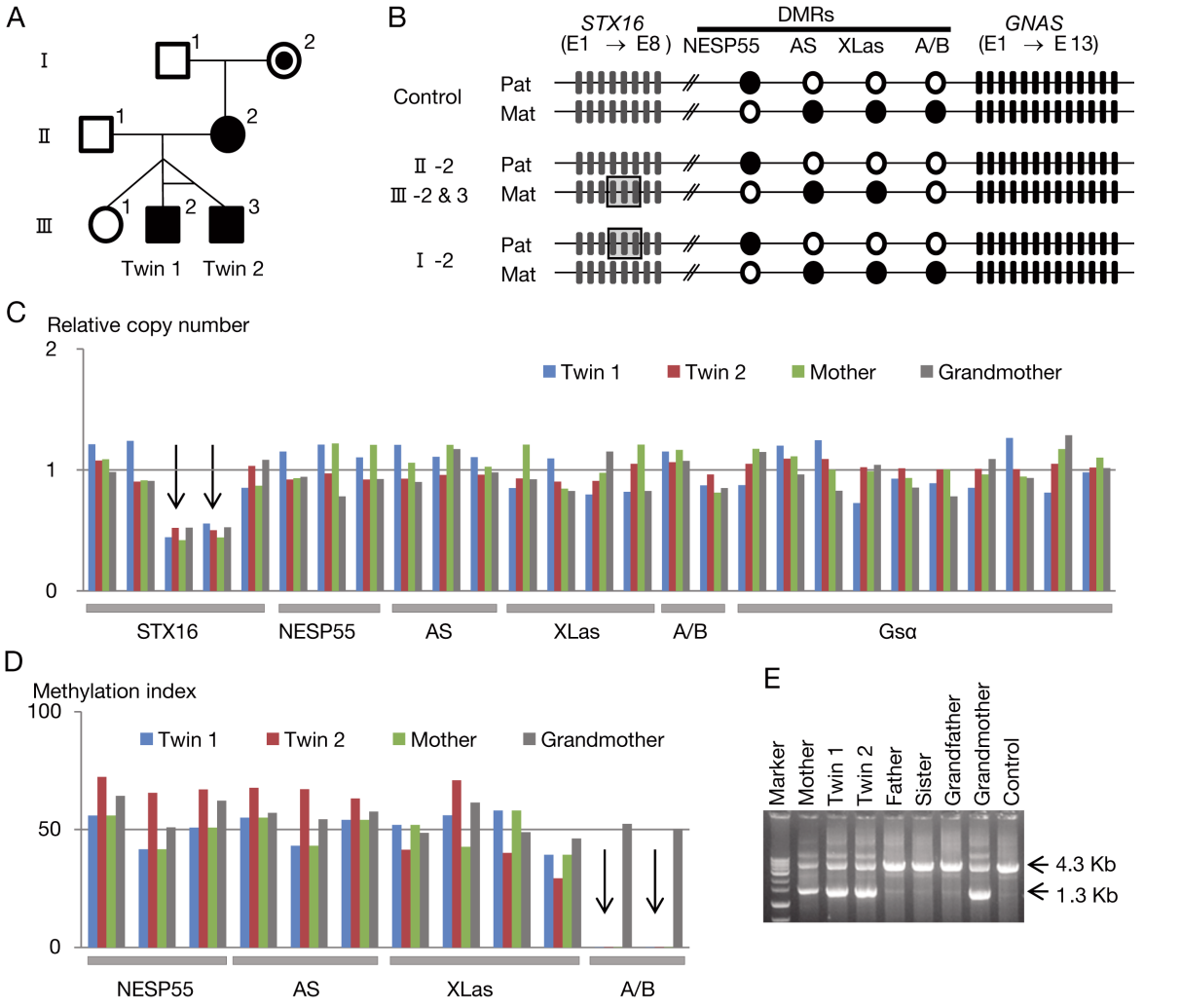

Fig. 1 The family examined in this study and the molecular findings.

A. The pedigree of the family. The twins (III-2 and III-3) and the mother (II-2) have clinical data (low serum calcium and high serum PTH) and molecular findings (*STX16* microdeletion and isolated LOM at exon A/B-DMR) compatible with AD-PHP-Ib. While the grandmother (I-2) has the same *STX16* microdeletion, she is free from the isolated LOM at exon A/B-DMR and AD-PHP-Ib phenotype.

B. Schematic representation of the *GNAS*– *STX16* region. Pat: paternally derived chromosome; and Mat: maternally derived chromosome. Filled and open circles indicate methylated and unmethylated state of *GNAS*-DMRs, respectively. In a control subject, NESP55-DMR is methylated and unmethylated after paternal and maternal transmissions, respectively, and AS-DMR, XLas-DMR, and A/B-DMR are methylated and unmethylated after maternal and paternal transmissions, respectively. The stippled rectangles represent microdeletions involving *STX16* exons 4–6. In the mother (II-2) and the twins (III-2 and III-3), the microdeletion resides on the maternally inherited chromosome, and is associated with isolated hypomethylation of A/B-DMR. In the grandmother (I-2), the microdeletion resides on the paternally inherited chromosome, and is accompanied by normal methylation patterns of the DMRs.

C. Relative copy number analysis by MS-MLPA using non-treated genomic DNA samples (before *Hha*I digestion).The value of 1.0 denotes two copies of the examined regions. Thus, the decreased (~0.5) values for the *SXT16* sequence (exon 5 and exon 6) denote heterozygous deletion of the corresponding regions in the twins, the mother, and the grandmother (arrows).

D. Methylation indices (MIs) of *GNAS*-DMRs by MS-MLPA using genomic DNA samples digested with methylation-sensitive *Hha*I that recognizes unmethylated, but not methylated, GCGC sequence. The MIs of 50% indicate the normal patterns of *GNAS*-DMRs, with hypomethylated NESP55-DMR and hypermethylated exon A/B-DMR, XLas-DMR, and AS-DMR on the maternally derived allele, and hypermethylated NESP55-DMR and hypomethylated exon A/B-DMR, XLas-DMR, and AS-DMR on the paternally inherited allele. Thus, the extremely low MIs for exon A/B-DMR (arrows) indicate LOM of this DMR on the maternally derived allele with the *STX16* microdeletion in the twins and the mother, whereas the normal MIs for this DMR in the grandmother represent the presence of the *STX16* microdeletion on the paternally inherited allele.

E. PCR analysis using primers hybridizing to intron 3 and intron 6 of *STX16*, showing the presence of the ~3 kb common microdeletions involving *STX16* exons 4–6 in the twins, the mother, and the maternal grandmother.

Table 1 Clinical findings of the twins, the sister, and the mother

	Twin 1	Twin 2	Sister		Mother	
Sex	Male	Male	Female		Female	
Examined age (year:month)	5:4	5:4	5:4		26:0	
<Pre & postnatal growth>						
Birth length (cm) (SDS)[a]	41.0 (– 1.9)	37.0 (– 3.7)	42.5 (–1.2)		Unknown	
Birth weight (g) (SDS)[a]	1,324 (– 3.4)	1,072 (– 4.4)	1,616 (– 2.2)		Unknown	
Height (cm) (SDS)[b]	93.0 (– 3.4)	93.3 (– 3.4)	98.1 (– 2.3)		150.0 (– 1.5)	
Weight (kg) (SDS)[b]	12.7 (– 2.0)	12.9 (– 2.0)	13.6 (– 1.9)		50.0 (– 0.8)	
Bone age (year:month)[c]	3:9	3:9	4:6		…	
AHO phenotype	No (SS only)	No (SS only)	No (SS only)		No	
<Blood laboratory data>						
Basal & Peak GH (μg/L)						
Arginine stimulation test[d]	0.04 → 1.26	0.98 → 3.75	0.47 → 7.97	(> 6) (peak)		
Clonidine stimulation test[e]	0.07 → 1.90	2.50 → 1.59	0.54 → 7.13	(> 6) (peak)		
IGF-1 (μg/L) (male)	86.1	78.2	…	(44 – 193)		
IGF-1 (μg/L) (female)	…	…	138.5	(56 – 252)		
TSH (mIU/L)	2.1	4.0	3.2	(0.7 – 6.4)	2.7*	(0.5 – 5.0)
Free T4 (pmol/L)	16.6	14.3	16.3	(10 – 28)	13.0*	(10 – 30)
Calcium (mmol/L)	1.8	1.6	2.4	(2.2 – 2.7)	1.4*	(2.1 – 2.5)
Inorganic phosphate (mmol/L)	**1.9**	**1.9**	1.5	(1.2 – 1.8)	1.4*	(0.8 – 1.4)
Intact PTH (pmol/L)	**31.0**	**21.1**	3.4	(0.95 – 6.8)	**19.7***	(0.95 – 6.8)

The values above the age- and sex-matched Japanese reference ranges (shown in parentheses) are boldfaced, and those below the reference ranges are underlined.

[a] Assessed by the gestational age- and sex-matched Japanese reference data for single newborns. (http://www.e-stat.go.jp/SG1/estat/List.do?lid=000001085635); there are no reference data for birth size of triplets.

[b] Assessed by the age- and sex-matched Japanese reference data (http://jspe.umin.jp/medical/taikaku.html).

[c] Assessed by the TW-2 method standardized for Japanese (17).

[d] Arginine (0.5 g/kg) i.v. over a 30 minute time period; blood sampling at 0, 30, 60, 90, and 120 minutes.

[e] Clonidine (0.15 mg/m^2) p.o.; blood sampling at 0, 30, 60, 90, and 120 minutes.

SDS: standard deviation score; AHO: Albright's hereditary osteodystrophy; and SS: short stature.

*Examined at the time of afebrile convulsion at 26 years of age.

in the twins with AD-PTH-Ib. This would primarily be explained by assuming that *GNAS* expression is more severely affected by mutations than by methylation defects. In support of this, it has been reported that *GNAS* expression and Gsα activity in erythrocytes are more severely compromised in patients with *GNAS* mutations than in those with methylation defects [12]. Furthermore, this notion would explain why TSH resistance is less frequent in patients with PHP-Ib (40-80%) than in those with PHP-Ia (~80%), and why resistance to several hormones other than PTH and TSH is barely present in PHP-Ib but often observed in PHP-Ia [8, 13, 14].

Several points should be made for the GHD in the twins. First, the twins had apparent intrauterine growth retardation that is often associated with relatively poor GH secretion [15]. Second, GH secretary function was studied only by arginine and clonidine stimulation tests (although we actually attempted to perform more potent GHRH and/or GHRP-2 stimulation tests after diagnosis of AD-PTH-Ib, this was refused by the parents because the twins had already received GH therapy). Third, serum IGF-1 values remained within the normal range in the twins. Fourth, the twins lacked TSH resistance that is much more prevalent than GHD in AD-PHP-Ib as well as in sporadic PHP-Ib [2,8], although this could be explained by assuming that the twins had some genetic and environmental factor(s) that raises the susceptibility to the development of GHD, but not to that of TSH resistance. Lastly, the mother with AD-PHP-Ib lacked short stature, although such intra-familial phenotypic variation has been reported in various disorders [16]. Taken together, it would remain tenable that GHD of the twins is a co-incidental finding independent of AD-PTH-Ib.

Two findings are also noteworthy in this study. First, the mother had irregular menses, and failed to conceive for several years of normal conjugal life. This might

be due to LH-R/FSH-R signaling defects, although there is no report describing increased LH/FSH values in patients with AD-PHP-Ib [2, 8, 14]. Second, the grandmother had normal phenotype and normal methylation patterns of four examined DMRs including exon A/B-DMR, in the presence of the *STX16* microdeletion. In this regard, since LOM at exon A/B-DMR can occur only when the *STX16* microdeletion is present on the maternally inherited chromosome 20 [3, 4], it is likely that the microdeletion of the grandmother resided on the paternally inherited chromosome 20. Furthermore, since the grandmother and the previously reported individuals with the *STX16* microdeletion on the paternally derived chromosome 20 have normal phenotype with neither AHO phenotype nor hormone resistance, it is assumed that LOM at exon A/B-DMR rather than *STX16* microdeletion is relevant to the development of AD-PHP-Ib.

In summary, we observed GHD in monozygotic twins with AD-PHP-Ib. Although it remains to be determined whether the GHD is causally related to AD-PHP-Ib, the results suggest that AD-PHP-Ib may lead to resistance to several hormones other than PTH and TSH. Further studies will permit to clarify the phenotypic spectrum of AD-PHP-Ib.

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Disclosure Summary

All authors have nothing to disclose.

Supplemental Table 1 Primers utilized in this study

	Forward (5'→3')	Reverse (5'→3')
<*STX16* microdeletion analysis>		
Exons 4–6	TTGGCAGATAACTGCTGTGG	CGAGGCATGGGAAGAGAATA
<*GNAS* mutation analysis>		
Exon 1	CCTCCCGGCCCGCGTGA	CTGCGGGGCGCCCTTCGA
Exon 2	AAAATGCCTCCTTCATAACCTGA	GCCCACCTATACTTCCTAAAG
Exon 3	ATGGTTGAGGAATGTAGAGAGACTG	CAGTATGATCTTCATGTTTGTTTG
Exons 4–5	ATGAAAGCAGTACTCCTAACTGA	TGGATGCTCCTGCCCATGTG
Exon 6	ATTAGTTCAAGCTCTTGCCTTTC	TTGTCTGTTTTATGTGCTGATGG
Exons 7–9	TGCTGCATAACTGTGGGACG	TTAACCAAAGAGAGCAAAGC
Exons 10–11	TGTTAGGGATCAGGGTCGCTG	AGAACCACCGCAATGAACAGCC
Exon 12	AGACTTCAGGAGCTACAGAGA	AGAGGAGGAACAAGAGAGGAA
Exon 13	ATAGGGTGGTTCCTGGCGAGG	TCACTCGGGGGAAGGGAAAGG
<*GH1* mutation analysis>		
Exon 1	ACAGGTGGGGTCAACAGTGG	CCAGGGACCAGGAGCTTTCT
Exon 2	CGGCTCCCTCTGTTGCCCTCT	CCCCTTCCTGCCACCCCTGAT
Exon 3	AATGGGAGCTGGTCTCCAGCG	GGGGCTCTGACTACAGGTCTC
Exon 4	GTGGATGCCTTCTCCCCAGGC	GGGGCTCCAGGATTGGGGAC
Exon 5	GAATGAGAAAGGGAGGGAACAGTA	CTGGAGTGGCAACTTCCAGGG
<*POU1F1* mutation analysis>		
Exon 1	TGGGGTAAAAAAGGAAGACGG	GGAGGGGTAAAATGAAAGATG
Exon 2	CGTCAGAGAACTTACCCAAAA	ATGGTCTCTGTGGGTGTCACA
Exon 3	AGGCTAGAGCAGAATAATGGG	GCAATAAAGATTTGCAAACCA
Exon 4	TGGAAAGTTGGAGCTGATGGT	TTTTAGGTTAAAACACAGCAC
Exon 5	TTGAAATCCAAATCAGATCG	AAAATACCAGTTTTGTCCTC
Exon 6	AATTTCACCCCCTATGTCCA	GAAAGGAATGAAACGGGAGA
<*PROP1* mutation analysis>		
Exon 1	CAGGTGTGGAGAGGAGCTGC	ATGCTTTCAGCCTCACACC
Exon 2	AGACTGGAGCAGCACAGGAC	AGCACCAAAGAAATCTGCATT
Exon 3a	CTTGTCATTGGAGTAGGGTGTC	AGTCCTCAGACTGGTGTGAC
Exon 3b	TACAGCCATGCCCTCCCTTC	TTCTAGTCGCTGAGCTGAC

<*GHRHR* mutation analysis>		
Exon 1	AACGGCTGTGTCAGGGGACAG	CAGAGGCCAGAGGGTCTCAG
Exons 2–3	GACACCCAAATGGCTTGGCTCAT	GCCACTTCCAGATGAAAGCACCTC
Exon 4	GTGTCCCTGGGCAGGTTAGAC	GTGGGTCACCCTTCAGTAACTTA
Exon 5	TGTCAAGCCTTTTTTTCTTCCAAA	AAAGTTAGACATGGGTATGGCG
Exon 6	TGATTCGATTCACCTCATGCC	TTAGGGGGTGACATGGGAAAG
Exon 7	CTGCAGGTCCCAGCCTAACAT	TAGCGAGGGAGTCTGCATCCT
Exon 8	AGATCTCAGAGTCAAGGATGCAGA	TCCACTCCACACCCCATGTAG
Exon 9	GAAACGTTTGTCCATCTAGGTG	TCTGCAACCAGCACATTAGGC
Exon 10	CATTCACCTGCACATTCTCACG	GAAGGAAGGGGCCAGGTCTATT
Exon 11	GGAGGTGGTAGGAAGTGAGAGGA	ATGAACTCAGCCAAGGCCC
Exon 12	AGGCCAAAGGGTTCTGATGGG	TTAGGTCTGGTGGGAGGGGGA
Exon 13	GACCTTCCTAAACGTCCTCTTC	CAGCTGGGGTGGGGATGTGGC
<*LHX3* mutation analysis>		
Exon 1a	ACGCTGACCTAGGAGGAGCG	TGCCGCCCTTGCCGTTTCTG
Exon 1b	AAAGTTCGGGACTGGAGAGTG	ATCTCCTGGTCTCCCCGGTGCAG
Exon 2	TTCACTGCCTCCTGGTCTACGAGG	GCTCCCACACTGAGGTGAGG
Exon 3	GAAATGAGCCTCGCGCTTCCG	AGACCAGGAAAGGTGGGAGCG
Exons 4–5	CTGCCGCGCCTCACCGCTCG	CCGAGCTCCGCGATCCCTCCG
Exon 6	ACTCAGGGGTGAGGGCAGTG	GGGCCTCAGCAAGGTGACAT
<*LHX4* mutation analysis>		
Exon 1	TTCTGAATCGAGCTAGAGCG	TGAGGCTGCTAGCTGTCTTG
Exon 2	TGGCCTGGTTAGCAGGGCTG	AACTTCCCCCTCACTGCTTG
Exon 3	AAGTTGGGGGAAGCCAGATC	CATGAGAAGGGCACCTCAGG
Exon 4	CTTAGGTGCAGAAAGGATGG	GCTGATGTCACTCAGGATAC
Exon 5	TCCATTCAGGCTTCAGTCTG	GTGCCAGGGATTACAGATTC
Exon 6	TCCTGGCAGCTGACAATAAA	TTGGCGTACTTTCGATCCTT
<*HESX1* mutation analysis>		
Exon 1	CAGGAAGATCCCAGCCCTAT	GGGCAAATTAAACACTGTAAATGA
Exon 2	TGGTTTTTGGTTAACGGGGT	TGGCAATTAATCTGGATGCCT
Exon 3	GCATCCAGATTAATTGCCAAG	AACATTTCAACATCATGAATAACAA
Exon 4	TTGTTATTCATGATGTTGAAATGTT	CTGATTCTTCATGCTCTGCAA

Supplemental Table 2 Microsatellite analysis of the triplet and their parents.

Locus	Chr. location	Father	Mother	Sister	Twin 1	Twin 2
D20S100	20q13.2	201/203	203/205	201/205	201/203	201/203
D20S120	20q13.2	211/215	211	211	211	211
D20S149	20q13.31	280/290	288/300	280/300	280/288	280/288
D20S173	20q13.33	182/188	180/182	182/188	180/182	180/182
D20S192	20q12.3	286/288	286/292	286/292	286/288	286/288
D20S200	20q11.21	249/251	253/255	249/255	249/255	249/255
D20S917	20q12.3	156/158	158/160	156/160	156/158	156/158
D7S484	7p14.2	102/150	102/106	106/150	102/106	102/106
D7S531	7p22.2	216/240	216/244	216/240	216/240	216/240
D7S550	7q36.3	187/189	189/195	187/189	187/189	187/189
D7S684	7q34	170/180	176/180	170/176	170/180	170/180
D7S669	7q21.11	118/126	118/128	118	118/128	118/128
D7S672	7q1.22	132/142	152	132/152	132/152	132/152
D7S1824	7q34	174/186	174/186	174/186	186	186
D7S2846	7p14.1	178/182	174/178	174/182	178/182	178/182

The Arabic numbers indicate the PCR product sizes (bp).
Genomic DNA samples were PCR-amplified with fluorescently labeled forward primers and unlabeled reverse primers. The PCR product sizes were determined on an ABI PRISM 3130 autosequencer using GENEMAPPER software version 4.1 (Life Technologies).
The primer sequences have been described in the Human Genome Database (http://www.gdb.org/).

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