Sporadic Pseudohypoparathyroidism Type 1B in Monozygotic Twins: Insights into the Pathogenesis of Methylation Defects

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Financial Support

Disclosures

The authors have no relevant financial relationships to disclose.

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Abstract

Context

Sporadic pseudohypoparathyroidism type 1B (sporPHP1B) is an imprinting disease without a defined genetic cause, characterized by broad methylation changes in differentially methylated regions (DMRs) of the GNAS gene.

Objective

This work aims to provide insights into the causative event leading to the GNAS methylation defects through comprehensive molecular genetic analyses of a pair of female monozygotic twins concordant for sporPHP1B who were conceived naturally i.e., without assisted reproductive techniques.

Methods

Using the leukocyte genome of the twins and family members, we performed targeted bisulfite sequencing, methylation-sensitive restriction enzyme (MSRE)-qPCR, whole-genome sequencing (WGS), high-density SNP array, and Sanger sequencing.

Results

Methylation analyses by targeted bisulfite sequencing and MSRE-qPCR revealed almost complete loss of methylation at the GNAS AS, XL, and A/B DMRs and gain of methylation at the NESP55 DMR in the twins, but not in other family members. Except for the GNAS locus, we did not find apparent methylation defects at other imprinted genome loci of the twins. WGS, SNP array, and Sanger sequencing did not detect the previously described genetic defects associated with familial PHP1B. Sanger sequencing also ruled out any novel genetic alterations in the entire NESP55/AS
region. However, the analysis of 28 consecutive SNPs could not exclude the possibility of paternal heterodisomy in a span of 22 kb comprising exon NESP55 and AS exon 5.

Conclusion

Our comprehensive analysis of a pair of monozygotic twins with sporPHP1B ruled out all previously described genetic causes. Twin concordance indicates that the causative event was an imprinting error earlier than the timing of monozygotic twinning.

Keywords

Sporadic pseudohypoparathyroidism 1B, imprinting disorder, monozygotic twins, GNAS, heterodisomy, hypocalcemia
Pseudohypoparathyroidism (PHP) is the first described example of end-organ resistance to a hormone in mammals, which is primarily characterized by proximal renal tubular resistance to parathyroid hormone (PTH) (1). The molecular defects in the GNAS gene on chromosome 20, encoding Gαs subunit of the heterotrimeric G protein, play a causal role in PHP (2, 3). The GNAS locus is a highly complex, imprinted locus. In addition to the mRNA encoding Gαs, the locus gives rise to several transcripts using alternative promoters and first exons: NESP55, XLαs, and A/B. There is also a non-coding antisense transcript called GNAS-AS1 (also known as NESPAS). The promoters of these four transcripts lie within differentially methylated regions (DMRs) and are active on the non-methylated allele, leading to parent-of-origin specific expression. The AS, XLαs, and A/B DMRs are maternally methylated, while the NESP55 DMR is paternally methylated. Although the promoter of Gαs per se does not have a DMR, its expression from the paternal allele is silenced through an unknown mechanism in specific tissues, such as the proximal renal tubule, thyroid, and pituitary.

Coding pathogenic variants of the maternal Gαs result in PHP1A, in which patients have Albright’s hereditary osteodystrophy (AHO), as well as multihormone resistance. Such Gαs coding variants are not typically found in PHP1B patients, who generally lack AHO features and display end-organ resistance only to PTH and, in some cases, TSH. Instead, these patients exhibit methylation defects at the GNAS DMRs.

PHP1B manifests as autosomal dominant (AD-PHP1B) or sporadic (sporPHP1B) forms. AD-PHP1B patients have deletions in STX16 or NESP55 loci on the maternal allele and generally show loss-of-methylation restricted to the A/B DMR (4-9). SporPHP1B patients have broad methylation changes at the GNAS DMRs with unknown causes, except in 8-10% of cases with paternal uniparental disomy involving the long arm of chromosome 20 (2, 3). A previous study suggested a post-zygotic event caused somatic mosaicism in sporPHP1B patients with partial GNAS methylation defects (10). However, in sporPHP1B patients with complete GNAS methylation defects, the mechanistic basis of methylation defects, including the timing of the causative event, is still unclear.
In imprinting diseases with unknown etiology, like sporPHP1B, the findings from monozygotic twin cases provide critical information. In particular, to estimate the timing of the causative imprinting errors, monozygotic twin discordance for imprinting diseases is essential: twins are clinically discordant if one sibling is (completely) affected and the other is unaffected (or partially affected); and twins are epigenetically discordant if they show differences in methylation levels. Previous studies have demonstrated that most monozygotic twin cases with imprinting diseases, such as Beckwith-Wiedemann syndrome and Silver-Russel syndrome, show both clinical and epigenetic discordance (11-16). In patients with Beckwith-Wiedemann syndrome, based on the discordance between monozygotic twins, it is speculated that the causative methylation defects can trigger monozygotic twinning (17). By contrast, only one set of monozygotic twins with sporPHP1B have been described to date (18).

In this study, we report a pair of monozygotic twins with sporPHP1B. The twins were concordant for clinical phenotype and the GNAS methylation defects. Our comprehensive molecular genetic analyses have ruled out the previously described genetic defects underlying PHP1B. The current findings provide insights into the causative event leading to the GNAS methylation defects in sporPHP1B.

Patients and Methods

Twin patients and their family

The probands were a pair of 26-year-old Japanese female monozygotic twins. Twin 1 was referred to our department for further evaluation of incidentally found hypocalcemia at a local clinic. Twin 2 had noticed occasional hand numbness and facial spasm for two years, and she visited our department due to the diagnosis of hypocalcemia in twin 1. The parents were nonconsanguineous (Fig. 1A). The twins were conceived without assisted reproductive techniques and were born of an uncomplicated pregnancy and term delivery. We could not obtain information on amnionicity and chorionicity of the twins. Both twins had normal intellectual and physical development. On physical examination, each showed a positive Chvostek’s sign but no evidence of AHO. In both twins, the laboratory tests
revealed hypocalcemia, hyperphosphatemia, hypocalciuria, and elevated serum intact PTH levels. The Ellsworth-Howard tests showed blunted urinary cAMP excretion and phosphaturia in response to teriparatide. The twins were treated with alfacalcidol (with calcium lactate for twin 2) (Fig. S1) (19). The calcium, phosphate, and intact PTH levels of other family members were within the normal range, except for the father’s slightly elevated PTH level, probably due to vitamin D deficiency. There was no other family history of endocrine or bone disease (Table 1). Genomic DNA of all family members was extracted from peripheral blood leukocytes using QIAamp DNA Mini Kit (Qiagen). Analyses were performed with informed consent from each family member using forms approved by the Ethics Committee of Tazuke Kofukai Medical Research Institute, Kitano Hospital.

Targeted bisulfite sequencing

Targeted bisulfite sequencing was performed on all family members using SureSelect Human Methyl-Seq (Agilent). Target epigenetic regions on fragmented DNA were hybridized with biotinylated RNA probes and captured on beads. The selected fragments were bisulfite-treated, PCR-amplified, and sequenced on the HiSeq platform. After sequencing, BSMAP 2.90 package was used to align reads and calculate the methylation ratio of every single cytosine location.

Methylation sensitive restriction enzyme-quantitative PCR (MSRE-qPCR)

Genomic DNA was digested with methylation-sensitive restriction enzyme HpaII or methylation-insensitive restriction enzyme MspI. qPCR reactions were performed using KOD SYBR qPCR mix (TOYOBO) and primers flanking each CpG island. In every qPCR experiment, we confirmed a single melt curve peak in each sample. The calibration curve was generated using Ct values of serially diluted undigested genome samples. The relative methylated ratios were calculated based on the Ct values of the HpaII-digested samples. Primer sequences are available upon request.
Whole-genome sequencing (WGS) analysis

WGS was performed on the twins using TruSeq DNA PCR-Free Kit (Illumina) and the Illumina Novaseq6000 (Illumina) in a 2 x150 bp paired-end mode. The average coverage depths of twin 1 and twin 2 were 34.95 x and 29.59 x, respectively. Structural variants were detected using Manta structural variant caller.

High-density single nucleotide polymorphism array

The genome-wide SNP typing was performed on Twin 1, the parents, and the brother, using the InfiniumOmn15-4v1-2_A1 (Illumina). Genomic DNA was amplified, fragmented, and hybridized on BeadChip. Array data were extracted, processed, and analyzed using Illumina GenomeStudio v.2 with copy number variation (cnvPartition v3.2.0).

Sanger sequencing and fragment analysis

The genomic region from upstream of AS exon 5 to the XL DMR of the twins was PCR-amplified with 46 amplicons and Sanger-sequenced. PCR amplicons were designed to overlap each other. Especially, the region from upstream of AS exon 5 to exon NESP55 was completely Sanger-sequenced in all family members. In other regions, all amplicons containing the twins’ informative heterozygous SNPs were sequenced in other family members. PCR reactions were performed using KOD ONE (TOYOBO), a high-fidelity DNA polymerase. Sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific), and the samples were analyzed by a 3730xl DNA Analyzer (Thermo Fisher Scientific). For fragment analysis, PCR reactions were performed using 6FAM-labelled primers, and the products were analyzed by a 3730xl DNA Analyzer (Thermo Fisher Scientific) using GeneMapper Software v5 (Thermo Fisher Scientific).
Results

Both monozygotic twin patients showed concordant clinical phenotypes consistent with PHP1B: hypocalcemia, hyperphosphatemia, and resistance to PTH without AHO. To investigate the cause of PHP1B in the twins, we performed comprehensive molecular genetic analyses: targeted bisulfite sequencing, MSRE-qPCR, WGS, high-density SNP array, and Sanger sequencing (Fig. 1B).

Methylation analysis with targeted bisulfite sequencing of all family members revealed almost complete loss of methylation at the AS, XL, and A/B DMRs and gain of methylation at the NESP55 DMR in the twins but not in other family members (Fig. 2A, B). We further confirmed the GNAS methylation defects by MSRE-qPCR (Fig. S2) (19). Consistent with the clinical presentation, the results indicated that the twins had concordant, broad, and almost complete methylation defects at the GNAS locus. In targeted bisulfite sequencing, except for the GNAS locus, we did not find apparent methylation defects at multiple other representative imprinted genome loci of the twins (Fig. 2C). Therefore, it was unlikely that the twins had multi-locus imprinting disturbances (MLID), which has been reported in some patients with PHP1B (20).

To identify the causative genetic defect, we performed WGS of the twins. There were no coding variants at the GNAS locus. We found no deletions, insertions, or inversions at the GNAS and STX16 loci, which are known to be associated with AD-PHP1B (4-9). A high-density SNP array also did not detect any copy number alteration on chromosome 20 of the twin 1 (Fig. 2D) and family members (Fig. S3) (19). Additionally, Sanger sequencing of the region between exon NESP55 and AS exon 3 in the twins ruled out microdeletions that have been reported in AD-PHP1B patients with loss of methylation at all three maternal DMRs (7). We found at least one heterozygous SNP in every two consecutive amplicons of this region, which excluded the possibility of monoallelic PCR amplification. We further screened coding variants in candidate genes involved in imprinting control, including the known causative genes of MLID (Table S1) (19, 21), but no coding variants were found. From WGS and SNP array, we found no regions of suspected uniparental isodisomy on chromosome 20 of the twins (Fig. 2E). Taken together, these results ruled out the previously described genetic defects in AD- or sporPHP1B patients.
Based on the results of SNP array and Sanger sequencing of the NESP55/AS region of all family members, we could not exclude the possibility of paternal heterodisomy around exon NESP55. We further Sanger-sequenced an approximately 30-kb region and identified a 22-kb region, including 28 consecutive informative heterozygous SNPs, in which segmental paternal heterodisomy could not be excluded (Fig 2F). The region extended from upstream of AS exon 5 to exon NESP55 and partially included the NESP55 DMR. The unaffected brother also had SNP genotypes similar to the twins in this region, except for one SNP located within the NESP55 DMR. No informative microsatellite markers were found within this region.

Discussion

We describe a pair of monozygotic twins with sporPHP1B, who were concordant for the clinical phenotype: hypocalcemia, hyperphosphatemia, elevated PTH levels, and no AHO features. No other family members showed these clinical features. In addition, the twins were concordant for methylation defects at the GNAS locus: almost complete loss of methylation at the AS, XL, and A/B DMRs and gain of methylation at the NESP55 DMR. These methylation defects were not observed in other family members. From the above, the twins were considered to be sporadic or de novo AD-PHP1B.

Comprehensive genetic analyses with WGS, SNP array, and Sanger sequencing ruled out previously described genetic causes of PHP1B, including deletion of the STX16, deletion of the NESP55/AS region, or large paternal uniparental disomy (2, 3). In addition, targeted bisulfite sequencing excluded MLID, and WGS confirmed the absence of pathogenic variants in the known causative genes for MLID and other methylation regulatory genes. Collectively, our analyses excluded all known causes of PHP1B, suggesting that the cause is a previously uncharacterized event that leads to an epimutation limited to the GNAS locus.

Monozygotic twin cases affected by imprinting disorders have important implications for estimating the timing of the causative event of methylation defects (11). Traditionally, monozygotic twinning has been explained by the splitting theory, which assumes postzygotic splitting events before
13 days postfertilization (22, 23). Another recent hypothesis is that monozygotic twinning occurs earlier, at the first division of the zygote, although this hypothesis is controversial (23-25). In any case, monozygotic twinning must precede the onset of germ layer formation 14 days postfertilization (22). Notably, several lines of evidence support monozygotic twin discordance in imprinting disorders. In Beckwith-Wiedemann syndrome, the frequency of monozygotic twins is much higher than the general population, and most twin pairs are discordant for the clinical phenotype (11-14). Those twins also show discordant methylation defects at an imprinted region of 11p15.5 in certain tissues, suggesting mosaicism (11-14). These observations support the hypothesis that the monozygotic twinning is triggered, at least partly, by the failure of methylation maintenance close to the timing of twinning and the resultant growth asymmetry (17). Similar predominant clinical and epigenetic discordances are also reported for monozygotic twins with Silver-Russel syndrome or transient neonatal diabetes (11, 15, 16). By contrast, our twins with sporPHP1B were concordant for the clinical phenotype and GNAS methylation defects. To date, only one more pair of monozygotic twins was included in a previous study of 22 cases with sporPHP1B, and they were also concordant (18). These findings suggest that the timing of the causative event of sporPHP1B is earlier than those of other imprinting diseases mentioned above and must be before monozygotic twinning (Fig. 2G). This argument is apparently in line with a previous study speculating that an epimutation before the formation of the germ layer causes sporPHP1B (10). The current findings can further narrow the window for the causative event because monozygotic twinning is earlier than triple germ layer formation (22).

Paternal iso- or hetero-disomies have been identified in a few patients with sporPHP1B (26). Intriguingly, based on the result of SNP genotyping, we could not exclude the possibility of paternal heterodisomy in the upstream region of the GNAS locus, maximum 22 kb in length, partially including the NESP55 DMR. It is unclear whether such a small region of paternal heterodisomy in the current twins could play a causal role in the observed GNAS methylation defects. Note that very small uniparental heterodisomies have been identified in other diseases and suggested to play a causative role (27-33). Based on human (9) and mouse (34, 35) studies, NESP55 transcription and/or the
genomic region comprising exon NESP55 is crucial for the control of GNAS imprinting. While a study suggested a role for NESP55 transcription during oogenesis (34), two other studies indicated that this region may also be required for methylation maintenance at downstream GNAS DMRs in other cells after fertilization (6, 36). It is thus tempting to speculate that the paternal heterodisomy led to the suppression of NESP55 transcription on both alleles very early in the zygote, leading to broad methylation defects at the GNAS locus. However, except for one in the NESP55 DMR, the brother shared SNP genotypes with the twins in the same region. Therefore, it is difficult to draw a definitive conclusion on the causal role of this region of possible paternal heterodisomy.

In this study, we performed a comprehensive analysis on a pair of monozygotic twins with sporPHP1B, ruling out all previously described genetic causes. Twin concordance indicates that the causative event was an imprinting error earlier than the timing of monozygotic twinning.
Acknowledgments

We thank the patients and other family members for their participation. We are grateful to Dr. Harald Jüppner for helpful suggestions. We also thank the managing staff of the medical research institute, Kitano Hospital, for their support of our research.

Data Availability

Restrictions apply to the availability of some or all data generated or analyzed during this study to preserve patient confidentiality or because they were used under license. The corresponding author will on request detail the restrictions and any conditions under which access to some data may be provided.
Reference

1. Albright F, Burnett CH, Smith PH, Parson W. Pseudohypoparathyroidism - an example of “Seabright-Bantam syndrome.” Endocrinology. 1942;30:922-32.

2. Jüppner H. Molecular Definition of Pseudohypoparathyroidism Variants. J Clin Endocrinol Metab. 2021;106(6):1541-52.

3. Mantovani G, Bastepe M, Monk D, et al. Diagnosis and management of pseudohypoparathyroidism and related disorders: first international Consensus Statement. Nat Rev Endocrinol. 2018;14(8):476-500.

4. Bastepe M, Fröhlich LF, Hendy GN, et al. Autosomal dominant pseudohypoparathyroidism type Ib is associated with a heterozygous microdeletion that likely disrupts a putative imprinting control element of GNAS. J Clin Invest. 2003;112:1255–63.

5. Bastepe M, Fröhlich LF, Linglart A, et al. Deletion of the NESP55 differentially methylated region causes loss of maternal GNAS imprints and pseudohypoparathyroidism type Ib. Nat Genet. 2005;37(1):25–27.

6. Chillambhi S, Turan S, Hwang DY, Chen HC, Jüppner H, Bastepe M. Deletion of the noncoding GNAS antisense transcript causes pseudohypoparathyroidism type Ib and biparental defects of GNAS methylation in cis. J Clin Endocrinol Metab. 2010;95(8):3993–4002.

7. Rezwan FI, Poole RL, Prescott T, Walker JM, Temple IK, Mackay DJG. Very small deletions within the NESP55 gene in pseudohypoparathyroidism type 1b. Eur J Hum
8. Takatani R, Molinaro A, Grigelioniene G, et al. Analysis of multiple families with single individuals affected by pseudohypoparathyroidism type Ib (PHP1B) reveals only one novel maternally inherited GNAS deletion. J Bone Miner Res. 2016;31(4):796–805.

9. Richard N, Abeguîle G, Coudray N, et al. A new deletion ablating NESP55 causes loss of maternal imprint of A/B GNAS and autosomal dominant pseudohypoparathyroidism type Ib. J Clin Endocrinol Metab. 2012;97(5):E863-7.

10. Elli FM, Bordogna P, Arosio M, Spada A, Mantovani G. Mosaicism for GNAS methylation defects associated with pseudohypoparathyroidism type 1B arose in early post-zygotic phases. Clin Epigenetics. 2018;10:16.

11. Ollikainen M, Craig JM. Epigenetic discordance at imprinting control regions in twins. Epigenomics. 2011;3(3):295-306.

12. Blick J, Alders M, Maas SM, et al. Lessons from BWS twins: Complex maternal and paternal hypomethylation and a common source of haematopoietic stem cells. Eur J Hum Genet. 2009;17(12):1625-34.

13. Cohen JL, Duffy KA, Sajorda BJ, et al. Diagnosis and management of the phenotypic spectrum of twins with Beckwith-Wiedemann syndrome. Am J Med Genet A. 2019;179(7): 1139-47.

14. Weksberg R, Shuman C, Caluseriu O, et al. Discordant KCNQ1OT1 imprinting in sets of monozygotic twins discordant for Beckwith–Wiedemann syndrome. Hum Mol Genet. 2002;11(11):1317-25.

15. Riess A, Binder G, Ziegler J, Begemann M, Soellner L, Eggermann T. First report on concordant monozygotic twins with Silver-Russell syndrome and ICR1 hypomethylation. Eur J Med Genet. 2016;59(1):1-4.

16. Laborie LB, Mackay DJG, Temple IK, et al. DNA hypomethylation, transient neonatal
diabetes, and prune belly sequence in one of two identical twins. Eur J Pediatr. 2010;169(2):207-13.

17. Bestor TH. Imprinting errors and developmental asymmetry. Philos Trans R Soc Lond B Biol Sci. 2003;358:1411-15.

18. Linglart A, Bastepe M, Jüppner H. Similar clinical and laboratory findings in patients with symptomatic autosomal dominant and sporadic pseudohypoparathyroidism type Ib despite different epigenetic changes at the GNAS locus. Clinical Endocrinology. 2007;67:822–831.

19. Keidai Y, Iwasaki Y, Iwasaki K, Honjo S, Bastepe M, Hamasaki A. Supplemental material for: Sporadic Pseudohypoparathyroidism Type 1B in Monozygotic Twins: Insights into the Pathogenesis of Methylation Defects. Dryad Digital Repository 2021. Deposited 30 September 2021. https://doi.org/10.5061/dryad.5mkkwh76v

20. Rochtus A, Martin-Trujillo A, Izzi B, et al. Genome-wide DNA methylation analysis of pseudohypoparathyroidism patients with GNAS imprinting defects. Clin Epigenetics. 2016;8:10.

21. Monk D, Mackay D, Eggermann T, Maher E, Riccio A. Genomic imprinting disorders: lessons on how genome, epigenome and environment interact. Nat Rev Genet. 2019;20(4):235-248.

22. Hall J. Twinning. Lancet. 2003;362:735–43.

23. McNamara HC, Kane SC, Craig JM, Short RV, Umstad MP. A review of the mechanisms and evidence for typical and atypical twinning. Am J Obstet Gynecol. 2016;214(2):172-191.

24. Herranz G. The timing of monozygotic twinning: a criticism of the common model. Zygote. 2015;23(1):27-40.

25. Denker H-W. Comment on G. Herranz: The timing of monozygotic twinning: a criticism
of the common model. Zygote (2013). Zygote. 2015;23(2):312-314.

26. Colson C, Decamp M, Gruchy N, et al. High frequency of paternal iso or heterodisomy at chromosome 20 associated with sporadic pseudohypoparathyroidism 1B. Bone. 2019;123:145–152.

27. Eggermann T, Mergenthaler S, Eggermann K, et al. Identification of interstitial maternal uniparental disomy (UPD) (14) and complete maternal UPD(20) in a cohort of growth retarded patients. J Med Genet. 2001;38(2):86-9.

28. Rio M, Ozilou C, Cormier-Daire V, et al. Partial maternal heterodisomy of chromosome 17q25 in a case of severe mental retardation. Hum Genet. 2001;108(6):511-5.

29. Nazarenko S, Sazhenova E, Baumer A, Schinkel A. Segmental maternal heterodisomy of the proximal part of chromosome 15 in an infant with Prader–Willi syndrome. Eur J Hum Genet. 2004;12(5):411-4.

30. Kwasnicka-Crawford DA, Roberts W, Scherer SW. Characterization of an Autism-Associated Segmental Maternal Heterodisomy of the Chromosome 15q11–13 Region. J Autism Dev Disord. 2007;37(4):694-702.

31. Lebre AS, Morinière V, Dunand O, Bensman A, Morichon-Delvallez N, Antignac C. Maternal uniparental heterodisomy of chromosome 17 in a patient with nephropathic cystinosis. Eur J Hum Genet. 2009;17(8):1019-23.

32. Eli FM, Ghirardello S, Giavoli C, et al. A new structural rearrangement associated to Wolfram syndrome in a child with a partial phenotype. Gene. 2012;509(1):168-72.

33. Kotzot D. Complex and segmental uniparental disomy (UPD): review and lessons from rare chromosomal complements. J Med Genet. 2001;38(8):497-507.

34. Chotalia M, Smallwood SA, Ruf N, et al. Transcription is required for establishment of germline methylation marks at imprinted genes. Genes Dev. 2009;23(1):105-17.

35. Fröhlich LF, Mrakovcic M, Steinborn R, Chung U, Bastepe M, Jüppner H. Targeted
deletion of the Nesp55 DMR defines another Gnas imprinting control region and provides a mouse model of autosomal dominant PHP-Ib. Proc Natl Acad Sci U S A. 2010;107(20):9275-80.

36. Mehta S, Williamson CM, Ball S, et al. Transcription driven somatic DNA methylation within the imprinted Gnas cluster. PLoS One. 2015;10(2):e0117378
Figure legend

Figure 1. Pedigree and comprehensive genetic analysis for family members

(A) Pedigree of the family. There were no consanguineous marriages in this family. Only the twins showed the biochemical features of PHP1B. (B) Methods used for genetic analysis of the family. MSRE-qPCR, Methylation sensitive restriction enzyme-quantitative polymerase chain reaction; SNP, single nucleotide polymorphism; ICR; imprinting control region.

Figure 2. Genetic analyses of the twins and family members

(A) Diagram showing the GNAS locus. The methylated allele is indicated by the purple mark at each DMR. Arrows indicate transcription start sites and the direction of transcription. (B, C) Heatmap visualization of methylation ratio at the GNAS DMRs (B) and other representative imprinted loci (C). The methylation ratio at each CpG site was measured by target bisulfite sequencing. (D, E) SNP array results of twin 1. Plots of log R ratio (D) and B allele frequency (E) in chromosome 20 are shown. (F) Sanger sequencing results of the region from AS exon 5 to the XL DMR. The blue box in the schema represents the Sanger sequenced area. Informative SNPs and fragment length results are listed. The result of fragment analysis of a nearby short tandem repeat (806CA-80085) is also shown. The double arrow indicates the 22 kb region of possible paternal uniparental heterodisomy in the twins. The base positions at both ends are shown, referred to GRCh38. In the table, the blue and the orange boxes indicate paternally and maternally derived alleles, respectively. The gray boxes indicate alleles of undetermined parental origin. (G) Schematic representation of the timing of the causative methylation error. The shaded area (orange color) represents the possible window for the error during development.
Table 1. Clinical features of all family members

|               | Twin1          | Twin2          | Father | Mother | Brother |
|---------------|---------------|---------------|--------|--------|---------|
| **Sex**       | Female        | Female        | Male   | Female | Male    |
| **Age at investigation (yr)** | 26            | 26            | 58     | 53     | 22      |
| **Symptoms**  | Asymptomatic  | Hand numbness, | Asymptomatic | Asymptomatic | Asymptomatic |
|               |               | Facial spasm  | c       | c      | c       |
| **Height (cm) [Percentile]** | 152.3 [10-25] | 154 [10-25]  | 168.4 [50-75] | 163.9 [95-99] | 179.5 [90-95] |
| **Body weight (kg)** | 52            | 54            | 84.3   | 78.9   | 74      |
| **Body mass index (kg/m2)** | 22.5          | 22.8          | 29.7   | 29.4   | 23      |
| **Birth weight (g)** | 2072          | 2200          |        |        | 2700    |
| **Physical and mental development** | Normal       | Normal        | Normal | Normal | Normal |
| **Physical examination** | Chvostek's sign + | Chvostek's sign + | -      | -      | -       |
| **AHO feature** | No            | No            | No     | No     | No      |
| **Past medical history** | IDA, Exotropia | IDA, AD       | Colonic polyp | No    | No      |
| **Calcium (8.8-10.1 mg/dL)** | 7.6           | 5.5           | 9.2    | 9.4    | 9.5     |
| **Phosphate (2.7-4.6 mg/dL)** | 4.8           | 6.4           | 3.8    | 4      | 4       |
| **PTH (18.5-88.0 pg/mL)**  | 509.2         | 384.9         | 96.6   | 69.2   | 42.6    |
| **25OH Vitamin D (>20 ng/mL)** | 4.5           | 12            | 12.7   | 8.9    | 19.5    |
| **1,25OH Vitamin D (20-60 pg/mL)** | 54            | 52.3          |        |        |        |
| **Urinary Ca/creatinine**  | <0.01         | <0.01         | 0.083  | 0.092  | 0.14    |
| **Bone ALP (2.6-22.6 μg/L)** | 11.6          | 18.6          |        |        |        |
| **TRACP-5b (120-420 mIU/dL)** | 302           | 487           |        |        |        |
| **TSH (0.34-3.88 μIU/mL)**  | 2.834         | 1.526         |        |        |        |
| **FT4 (0.95-1.74 ng/dL)**   | 0.942         | 0.82          |        |        |        |
Ellsworth Howard test
- increase of urinary excretion of P (>35 mg), cAMP (>1μmol)
  | P: 33.5 | P: 26.3 |
  | cAMP: 0.238 | cAMP: 0.165 |

T score of Bone density (SD);
Lumbar/Hip/Radius
  | -1.3/-0.8/-0.5 | -0.8/-1.3/-0.9 |

Abbreviations: IDA, Iron-deficiency anemia; AD, Atopic dermatitis.
Figure 1

A

Liver cirrhosis

Gallstone

Hypertension

Aortic dissection

Dementia

Heart disease

Colonic polyp

Lumbar spine surgery during high school

Twin 1

Twin 2

B

| Purpose                          | Whole-Genome Sequencing | MSRE-qPCR | Targeted Bisulfite Sequencing | High-density SNP array | SNP genotyping by Sanger Sequencing |
|---------------------------------|-------------------------|-----------|------------------------------|------------------------|-----------------------------------|
| GNAS coding variants           | ○                       | ○         | ○                            | ○                      | ○                                 |
| Copy number alterations at the GNAS and ICRs | ○                       | ○         | ○                            | ○                      | ○                                 |
| Uniparental disomy              | ○                       | ○         | ○                            | ○                      | ○                                 |
| GNAS methylation-defects        | ○                       | ○         | ○                            | ○                      | ○                                 |
| Multi-locus imprinting deficiency | ○                       | ○         | ○                            | ○                      | ○                                 |
| Copy number alterations at the GNAS and ICRs | ○                       | ○         | ○                            | ○                      | ○                                 |
| Uniparental disomy              | ○                       | ○         | ○                            | ○                      | ○                                 |
| Uniparental disomy              | ○                       | ○         | ○                            | ○                      | ○                                 |
