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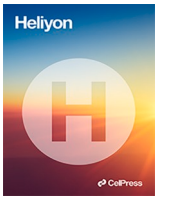

Research article

# DNA methylation changes in the genome of patients with hypogonadotropic hypogonadism

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ABSTRACT

Although some Mendelian neurodevelopmental disorders have been shown to entail specific DNA methylation changes designated as epi-signatures, it remains unknown whether epi-signatures are consistent features of other genetic disorders. Here, we analyzed DNA methylation profiles of patients with hypogonadotropic hypogonadism (HH), a rare neuroendocrine disorder typically caused by monogenic or oligogenic mutations. First, we performed microarray-based genome-wide methylation analyses of nine patients with HH due to *ANOS1*, *SOX2*, or *SOX10* variants and 12 control individuals. The results showed that 1118 probes were differentially methylated in one or more patients. The differentially methylated probes were highly variable among patients. No significant methylation changes were observed in genes functionally associated with *ANOS1*, *SOX2*, or *SOX10*. Then, we performed pyrosequencing of six selected CpG sites in the nine patients and 35 additional HH patients. The results of the patients were compared with those of 48 fertile men. There were no common methylation changes among these patients, with the exception of hypermethylation of two CpG sites in the *ZNF245* promoter of three patients. Hypermethylation of the promoter has previously been reported as a very rare epigenetic polymorphism in the general population. These results indicate that genomes of HH patients have considerable DNA methylation changes; however, these changes are more likely to be physiological epigenetic variations than disease-specific epi-signatures. Our data suggest a possible association between hypermethylation of the *ZNF254* promoter and HH, which needs to be examined in future studies.

*Abbreviations:* HH, hypogonadotropic hypogonadism; KS, Kallmann syndrome.

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## 1. Introduction

Recent studies have shown that some Mendelian neurodevelopmental disorders entail specific DNA methylation changes designated as epi-signatures [1,2]. For example, Butcher et al. demonstrated that CHARGE syndrome caused by *CHD7* mutations and Kabuki syndrome caused by *KMT2D* mutations have disease-specific epi-signatures [3]. These epi-signatures distinguished the patients with these syndromes from unaffected individuals and from each other. Furthermore, there were some common methylation changes between CHARGE and Kabuki syndromes, which may partly explain the phenotypic similarities between these syndromes. These results suggested that individuals with similar phenotypes may share common epi-signatures despite having different genetic backgrounds. Subsequently, Aref-Eshghi et al. analyzed DNA methylation profiles of patients with 42 Mendelian neurodevelopmental disorders and identified 34 robust disease-specific epi-signatures [4]. Accumulating evidence suggests that epi-signatures can be used as diagnostic tools for these disorders [1,2,4]. However, it remains unknown whether epi-signatures are consistent features of other genetic disorders.

Hypogonadotropic hypogonadism (HH) is a rare neuroendocrine disorder characterized by hypomasculinization in male neonates and delayed sexual maturation in adolescents of both sexes [5]. HH occurs either as an isolated endocrinopathy or as a component of congenital syndromes such as CHARGE and Kallmann syndromes [6]. HH typically arises from monogenic or oligogenic mutations; more than 60 causative genes have been reported to date [6]. Of these, *ANOS1* (alias, *KAL1*), *SOX2*, and *SOX10* are the major causative genes for both isolated and syndromic HH [6].

The present study aimed to clarify whether HH is associated with specific epi-signatures. To this end, we conducted microarray-based DNA methylation analysis for the entire genome and pyrosequencing of selected CpG sites.

## 2. Materials and methods

### 2.1. Patients

A total of 44 patients diagnosed with isolated HH or Kallmann syndrome participated in this study. These patients were recruited from Japanese hospitals between 2003 and 2019. Five patients have been described in our previous reports [7–10]. We focused on male patients, to exclude the effects of sex-biased DNA methylation. The 44 patients had no chromosomal abnormalities or chronic disorders that may have affected gonadotropin secretion. Prior to this study, all patients underwent mutation screening of 11 major causative genes for HH (*ANOS1, CHD7, FGF8, FGFR1, GNRH1, GNRHR, KISS1R, PROKR2, TACR3, SOX2*, and *SOX10*), and 13 patients, including one sibling-pair, were found to carry pathogenic variants of these genes. Clinical and molecular findings of the 13 patients are summarized in Table 1. Of the 13 patients, nine (patients 1–6 with *ANOS1* variants, patient 7 with a *SOX2* variant, and patients 8–9 with *SOX10* variants) were subjected to both microarray-based DNA methylation analysis and pyrosequencing. The remaining four patients (patients 10–11 with *ANOS1* variants, patient 12 with a *SOX2* variant, and patient 13 with a *SOX10* variant) and 31 additional HH patients without monogenic variants (patients 14–44) underwent only pyrosequencing.

### 2.2. Control individuals

DNA samples obtained from healthy Japanese men were used as the controls. For microarray-based genome-wide DNA methylation analysis, we used samples from eight boys and four adults. For pyrosequencing, we analyzed samples from 48 adult men who had fathered one or more children. These individuals were recruited in our previous study conducted during 2016–2019 [11].

**Table 1**
Clinical and molecular data of 13 patients with monogenic variants.

| Patient | Clinical diagnosis | Causative variant | | | | Reference |
|---|---|---|---|---|---|---|
| | | Gene | cDNA | Protein | Zygosity | |
| **1**[a] | KS | *ANOS1* | c.318+2T>C | g.IVS3+2T>C | Hemizygous | [7] |
| **2**[a] | KS | | c.318+2T>C | g.IVS3+2T>C | Hemizygous | |
| **3** | KS | | c.811delA | p.Thr271Leufs*39 | Hemizygous | |
| **4** | KS | | c.814C>T | p.Arg272* | Hemizygous | |
| **5** | HH | | c.1933delC | p.Ala645Profs*44 | Hemizygous | |
| **6** | KS | | c.1955_1961delCGCCGGA | p.Thr652Serfs*35 | Hemizygous | |
| **7** | HH | *SOX2* | c.813_834delGGACATGATCAGCATGTATCTC | p.Gly268Alafs*96 | Heterozygous | |
| **8** | KS | *SOX10* | c.434T>C | p.Leu145Pro | Heterozygous | [9] |
| **9** | KS | | c.475C>T | p.Arg159Trp | Heterozygous | [10] |
| 10 | KS | *ANOS1* | c.196C>T | p.Gln66* | Hemizygous | |
| 11 | KS | | c.721-1G>A | g.IVS5-1G > A | Hemizygous | |
| 12 | HH | *SOX2* | c.103A>T | p.Lys35* | Heterozygous | [8] |
| 13 | KS | *SOX10* | c.1225G>T | p.Gly409* | Heterozygous | [10] |

Note: Patients subjected to genome-wide methylation analysis are boldfaced.
KS: Kallmann syndrome; HH: hypogonadotropic hypogonadism.
[a] Patients 1 and 2 are siblings.

### 2.3. Microarray-based genome-wide DNA methylation analysis

Genomic DNA samples of the patients and control individuals were extracted from the peripheral blood. Microarray-based genome-wide DNA methylation analysis was performed for patients 1–9 and 12 control individuals. Genomic DNA was treated with sodium bisulfite using the EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA, USA). Samples were hybridized to an Infinium MethylationEPIC BeadChip and analyzed using the iScan system (Illumina Inc., San Diego, CA, USA). Raw data were processed using R (version 3.6.3; https://www.r-project.org/) with the default settings of the Chip Analysis Methylation Pipeline (https://www.bioconductor.org/packages/release/bioc/html/ChAMP.html) [12]. The DNA methylation status of each probe was expressed as a β value that ranged from 0 (no methylation) to 1 (100 % methylation). We excluded probes with low signal intensity or detection *p*-values of >0.01, as well as probes on a non-CpG site or on multiple sites [13]. Probes known to show aging-related or sex-biased DNA methylation changes were also excluded [14–16].

The data were analyzed using a previously reported method [17]. First, all probes were subjected to unsupervised hierarchical clustering using the gplots R package (https://cran.r-project.org/web/packages/gplots/index.html). Then, we searched for probes differentially methylated in one or more of the nine patients. To this end, we calculated Δβ, that is, the difference between the β value of a patient and the average of 12 control individuals. We performed the Crawford–Howell *t*-test to evaluate the methylation levels in each patient [18] and calculated false discovery rate (FDR)-corrected *p*-values [19]. Probes of the patients were assessed as differentially methylated when the FDR-corrected *p*-values were <0.05. Heatmaps of the differentially methylated probes were generated using the gplots R package.

Second, we analyzed the DNA methylation profiles of 100 genes functionally associated with *ANOS1*, *SOX2*, or *SOX10*. The 100 genes were selected using GeneMANIA (https://genemania.org/), a tool used to determine various functional relationships, such as protein and genetic interactions, relevant signaling pathways, co-expression, co-localization, and protein domain similarity [20]. Heatmap generation and unsupervised hierarchical clustering were performed using the gplots R package.

Then, using the data from the genome-wide analysis, we selected target CpG sites for further analyses. These sites were either differentially methylated in multiple patients or clustered in a small region of the genome and were located within the promoter regions of genes expressed in the hypothalamus and/or pituitary gland. In this study, a cluster of differentially methylated probes was defined as a <2 kb region containing three or more consecutive probes with $|\Delta\beta|$ values of >0.1. We referred to the GTEx portal (https://www.gtexportal.org/home/datasets) to examine the expression profile of each gene.

### 2.4. Pyrosequencing of selected CpG sites

The methylation status of the target CpG sites was analyzed by pyrosequencing, which is known as an accurate and convenient method [21,22]. Genomic regions containing the CpG sites were PCR-amplified using bisulfite-treated DNA samples and analyzed on PyroMark Q24 (QIAGEN, Valencia, CA, USA). The primer sequences are shown in Table S1. DNA methylation indices were calculated using the PyroMark Q24 software. The reference range for the methylation level of each CpG site was determined using the minimum and maximum values of the 48 control individuals. When the methylation changes in patients 1–9 were confirmed by pyrosequencing,

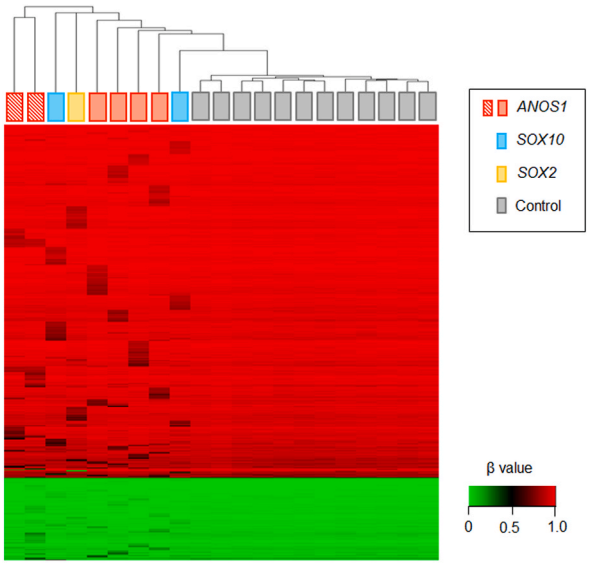


**Fig. 1.** Heatmap of differentially methylated probes in patients 1–9 and control individuals. The methylation status of 1118 differentially methylated probes is shown. Patients with variants in *ANOS1*, *SOX2*, and *SOX10* are depicted as red, yellow, and blue boxes, respectively. The red striped boxes indicate sibling cases (patients 1 and 2). Control individuals are shown as gray boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

we analyzed additional samples obtained from four patients with monogenic variants (patients 10–13) and 31 patients without monogenic variants (patients 14–44). We conducted two independent pyrosequencing experiments for all samples, except for some samples from patients 10–44 that were insufficient for the second analysis.

## 3. Results

### 3.1. Microarray-based genome-wide DNA methylation analysis

A total of 761,198 probes in the microarray passed the quality control. Unsupervised hierarchical clustering of these probes did not distinguish nine patients from 12 control individuals (Fig. S1). We identified 1118 probes that were differentially methylated in one or more of the nine patients (Table S2). Hierarchical clustering of the 1118 probes discriminated patients 1–9 from control individuals but did not classify the patients with *ANOS1*, *SOX2*, and *SOX10* variants (Fig. 1).

Next, we examined the methylation status of 12,325 probes in 100 genes functionally associated with *ANOS1*, *SOX2*, or *SOX10*. Most probes were normally methylated in patients 1–9 (Table S3). Clusters of differentially methylated probes were not detected. Hierarchical clustering did not distinguish the patients from the controls (Fig. S2).

None of the 1118 differentially methylated probes were shared by multiple patients, except for one probe in the *PLEKHA1* promoter (cg10576280) that was invariably hypermethylated in all patients and two probes in the ZNF254 promoter that was hypermethylated in patients 2 and 4 (Table S4). The sibling pair (patients 1 and 2) shared 50 common differentially methylated probes, whereas the other seven patients had few overlapping probes (Table 2). Twenty-four of 1118 probes were clustered on three chromosomes (Table S4). Of these, nine were located in the promoter regions of the genes expressed in the hypothalamus and/or pituitary gland (*GRIK2*, *TRIM68*, and *ZNF254*). In addition, *PLEKHA1* is known to be expressed in various tissues including the hypothalamus and pituitary gland. Thus, we selected the nine probes, together with one probe in the *PLEKHA1* promoter, for pyrosequencing (Table 3).

### 3.2. Pyrosequencing for selected CpG sites

Pyrosequencing of patients 1–9 did not recapitulate the hypermethylation of the four CpG sites in the *GRIK2* promoter but confirmed differential methylation of the six CpG sites in the promoters of *PLEKHA1*, *TRIM68,* and *ZNF254* (Table 3). Thus, we examined the methylation statuses of the six CpG sites in 35 additional patients. Two pyrosequencing experiments of these CpG sites yielded consistent results in all tested samples. The results showed that three CpG sites in the *TRIM68* promoter were normally methylated in the 35 patients, whereas cg09060057 and cg04571847 in the *ZNF254* promoter were hypermethylated in both patients 27 and 41 and only in patient 41, respectively (Table 3, Fig. 2). The CpG site in the *PLEKHA1* promoter was either normally methylated or slightly hypomethylated in the 35 patients.

## 4. Discussion

Microarray-based genome-wide DNA methylation analysis did not distinguish nine patients with monogenic HH from 12 control individuals. Moreover, although we identified 1118 probes (CpG sites) that were differentially methylated in the patients, each of these probes showed methylation changes in only one or two. Furthermore, patients 1–9 showed no significant DNA methylation changes in genes functionally associated with *ANOS1*, *SOX2*, or *SOX10*. These data indicate that the methylation changes in patients 1–9 are not closely associated with the HH phenotype. Since previous studies have documented considerable inter-individual variations in DNA methylation profiles [23], the differential methylation observed in patients 1–9 is likely to be physiological epigenetic variations. In this regard, the relatively similar methylation profiles of patients 1 and 2 may reflect some common genetic or environmental factors in this family.

Next, patients 1–9 were subjected to pyrosequencing for ten CpG sites that were differentially methylated in multiple patients or clustered within a small region of the genome. The results confirmed hypermethylation of the *PLEKHA1* promoter in patient 1 and that of the promoters of *TRIM68* and *ZNF254* in patients 2 and 4, respectively. Further pyrosequencing of 35 patients detected hypermethylation of the *ZNF254* promoter in patients 27 and 41, while methylation changes in the *PLEKHA1* and *TRIM68* promoters were not observed. Importantly, patients 4 and 41 had hypermethylation of both CpG sites in the *ZNF254* promoter. *ZNF254* is a ubiquitously expressed gene of unknown function (UCSC Genome Browser, https://genome.ucsc.edu/). Previous studies have suggested that the *ZNF254* promoter is differently methylated in a very small percentage of the general population; hypermethylation of more than

**Table 2**
The number of common differentially methylated probes between two patients.

| | Patient 2[a] | Patient 3 | Patient 4 | Patient 5 | Patient 6 |
|---|---|---|---|---|---|
| Patient 1[a] | 50 | 1 | 1 | 4 | 1 |
| Patient 2[a] | | 3 | 6 | 5 | 1 |
| Patient 3 | | | 0 | 3 | 1 |
| Patient 4 | | | | 2 | 6 |
| Patient 5 | | | | | 2 |

[a] Patients 1 and 2 are siblings.

**Table 3**
Probes selected for pyrosequencing.

| Gene name | Target CpG site | Chromosome | Location (hg19) | Array-based DNA methylation analysis | | Pyrosequencing |
|---|---|---|---|---|---|---|
| | | | | Patients with differential methylation (patients 1–9) | Average Δβ of the probes | Patients with differential methylation (patients 1–44) |
| *PLEKHA1* | cg10576280 | 10 | 124,133,823 | Patients 1–9 | 0.275 (0.262–0.301)[a] | Patient 1 |
| *GRIK2* | cg22541254 | 6 | 101,846,780 | Patient 2 | 0.165 | None of patients 1–9 (Patients 10–44 were not examined) |
| | cg05942459 | | 101,846,806 | | | |
| | cg18193094 | | 101,846,906 | | | |
| | cg10591607 | | 101,846,917 | | | |
| *TRIM68* | cg00364778 | 11 | 4,629,411 | Patient 2 | 0.113 | Patient 2 |
| | cg26847010 | | 4,629,417 | | | |
| | cg16469099 | | 4,629,433 | | | |
| *ZNF254* | cg09060057 | 19 | 24,269,920 | Patients 2 and 4 | 0.119 (Patient 2), 0.177 (Patient 4) | Patients 4, 27, and 41 |
| | cg04571847 | | 24,270,008 | | | Patients 4 and 41 |

[a] The median value (minimum-maximum) of nine patients.

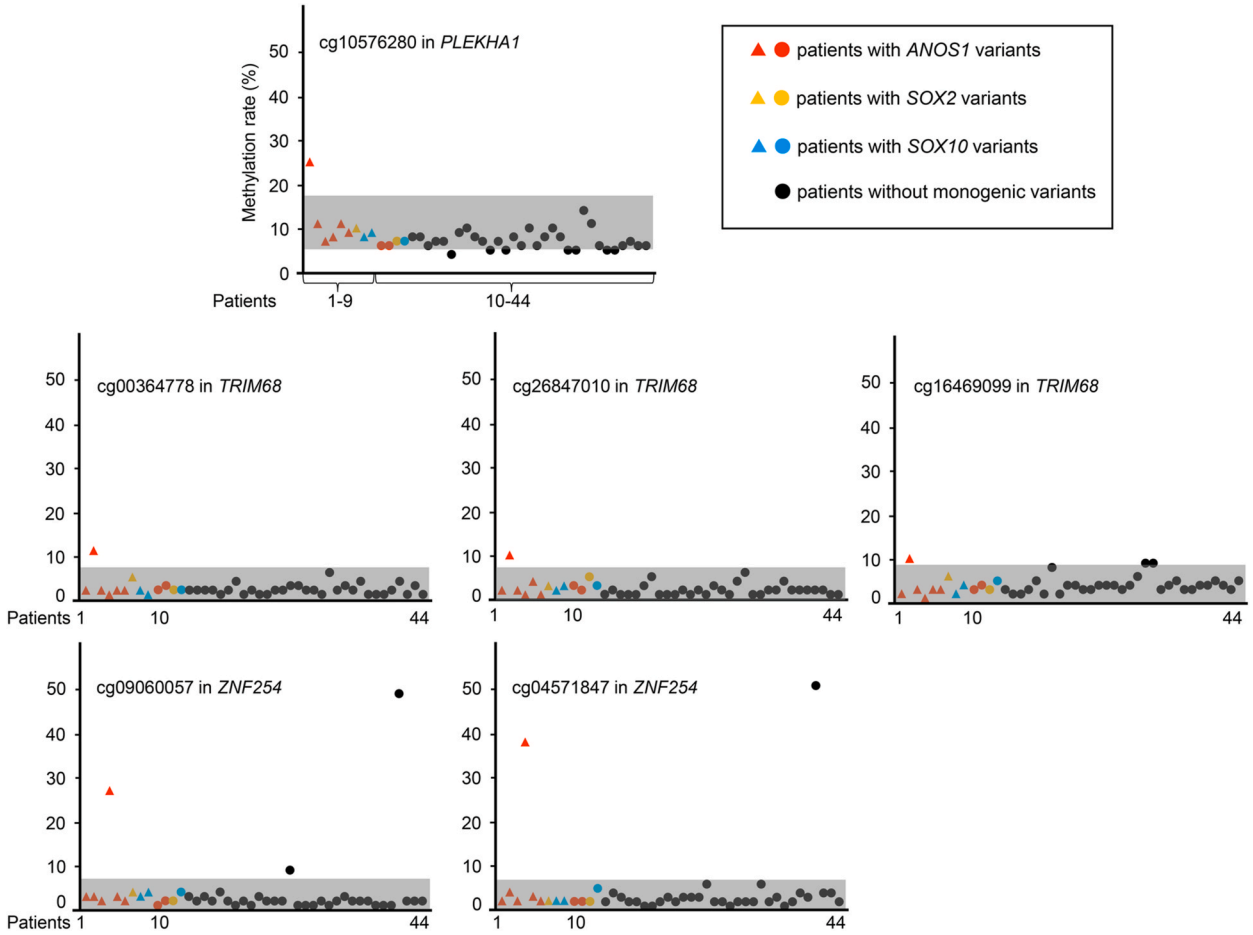


**Fig. 2.** Representative results of pyrosequencing. The results of six CpG sites are shown. The triangles and circles indicate the initially analyzed patients (patients 1–9) and additional patients (patients 10–44), respectively. Patients with variants in *ANOS1*, *SOX2*, and *SOX10* are shown in red, yellow, and blue, respectively. The gray-shaded areas depict the reference ranges determined by the maximum and minimum values of 48 control individuals. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

three consecutive probes in this region was observed in 21 of 23,116 individuals [23]. Since the frequency of hypermethylation of CpG sites in the *ZNF254* promoter was higher in our patients than in the general population (3/44 vs. 21/23,116), this epigenetic change may be associated with the HH phenotype. However, this notion awaits further validation because the present and previous studies used different methods for DNA methylation analyses [23]. In addition, while 7 of 35 patients showed 1–2% hypomethylation of the *PLEKHA1* promoter, this slight hypomethylation is unlikely to exert significant effects on phenotypes.

The present study provided no evidence that pathogenic variants of *ANOS1*, *SOX2*, or *SOX10* entail epi-signatures. These results are inconsistent with those of the studies by Butcher et al. and Aref-Eshghi et al. in which several Mendelian neurodevelopmental disorders including HH-associated CHARGE syndrome were shown to have specific epi-signatures [3,4]. The difference between the present and previous results may reflect the differences in the functions of the mutated genes. Indeed, many causative genes of neurodevelopmental disorders such as *CHD7* are known to be involved in epigenetic regulation [4,24]. Furthermore, the negative results of the present study may be due to the small number of subjects or the limited power of the methods. Mutations in *ANOS1*, *SOX2*, and *SOX10* may be associated with different epi-signatures. In addition, mild or tissue-specific methylation changes may have been overlooked in this study. Further studies are necessary to clarify the clinical significance of epi-signatures in various disorders and monogenic mutations.

## 5. Conclusion

This study demonstrated considerable DNA methylation changes in the genomes of patients with HH. However, most of these changes are likely to be physiological epigenetic polymorphisms, rather than HH-associated epi-signatures. The possible association between hypermethylation of the CpG sites in the *ZNF254* promoter and HH needs to be examined in future studies.

## Data availability statement

The data associated with this study has not been deposited into a publicly available repository. Data will be made available on request.

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## Ethics approval

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development (Project code #512). Written informed consent was obtained from all participants.

## CRediT authorship contribution statement

**Erina Suzuki:** Writing – original draft, Methodology, Investigation, Formal analysis. **Kazuhiko Nakabayashi:** Writing – review & editing, Validation, Software, Resources, Methodology. **Saki Aoto:** Writing – review & editing, Validation, Software, Methodology. **Tsutomu Ogata:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation. **Yoko Kuroki:** Writing – review & editing, Supervision, Investigation. **Mami Miyado:** Writing – review & editing, Validation, Supervision, Investigation. **Maki Fukami:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization. **Keiko Matsubara:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Formal analysis, Data curation.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37648.

## References

[1] B. Sadikovic, M.A. Levy, E. Aref-Eshghi, Functional annotation of genomic variation: DNA methylation episignatures in neurodevelopmental Mendelian disorders, Hum. Mol. Genet. 29 (R1) (2020) R27–R32, https://doi.org/10.1093/hmg/ddaa144.

[2] E. Aref-Eshghi, E.G. Bend, S. Colaiacovo, M. Caudle, R. Chakrabarti, M. Napier, L. Brick, L. Brady, D.A. Carere, M.A. Levy, J. Kerkhof, A. Stuart, M. Saleh, A. L. Beaudet, C. Li, M. Kozenko, N. Karp, C. Prasad, V.M. Siu, M.A. Tarnopolsky, P.J. Ainsworth, H. Lin, D.I. Rodenhiser, I.D. Krantz, M.A. Deardorff, C.

E. Schwartz, B. Sadikovic, Diagnostic utility of genome-wide DNA methylation testing in genetically unsolved individuals with suspected hereditary conditions, Am. J. Hum. Genet. 104 (4) (2019) 685–700, https://doi.org/10.1016/j.ajhg.2019.03.008.

[3] D.T. Butcher, C. Cytrynbaum, A.L. Turinsky, M.T. Siu, M. Inbar-Feigenberg, R. Mendoza-Londono, D. Chitayat, S. Walker, J. Machado, O. Caluseriu, L. Dupuis, D. Grafodatskaya, W. Reardon, B. Gilbert-Dussardier, A. Verloes, F. Bilan, J.M. Milunsky, R. Basran, B. Papsin, T.L. Stockley, S.W. Scherer, S. Choufani, M. Brudno, R. Weksberg, Charge and kabuki syndromes: gene-specific DNA methylation signatures identify epigenetic mechanisms linking these clinically overlapping conditions, Am. J. Hum. Genet. 100 (5) (2017) 773–788, https://doi.org/10.1016/j.ajhg.2017.04.004.

[4] E. Aref-Eshghi, J. Kerkhof, V.P. Pedro, D.I France Groupe, M. Barat-Houari, N. Ruiz-Pallares, J.C. Andrau, D. Lacombe, J. Van-Gils, P. Fergelot, C. Dubourg, V. Cormier-Daire, S. Rondeau, F. Lecoquierre, P. Saugier-Veber, G. Nicolas, G. Lesca, N. Chatron, D. Sanlaville, A. Vitobello, L. Faivre, C. Thauvin-Robinet, F. Laumonnier, M. Raynaud, M. Alders, M. Mannens, P. Henneman, R.C. Hennekam, G. Velasco, C. Francastel, D. Ulveling, A. Ciolfi, S. Pizzi, M. Tartaglia, S. Heide, D. Héron, C. Mignot, B. Keren, S. Whalen, A. Afenjar, T. Bienvenu, P.M. Campeau, J. Rousseau, M.A. Levy, L. Brick, M. Kozenko, T.B. Balci, V.M. Siu, A. Stuart, M. Kadour, J. Masters, K. Takano, T. Kleefstra, N. de Leeuw, M. Field, M. Shaw, J. Gecz, P.J. Ainsworth, H. Lin, D.I. Rodenhiser, M.J. Friez, M. Tedder, J.A. Lee, B.R. DuPont, R.E. Stevenson, S.A. Skinner, C.E. Schwartz, D. Genevieve, B. Sadikovic, Evaluation of DNA methylation episignatures for diagnosis and phenotype correlations in 42 Mendelian neurodevelopmental disorders, Am. J. Hum. Genet. 106 (3) (2020) 356–370, https://doi.org/10.1016/j.ajhg.2020.01.019.

[5] D.M. Styne, Physiology and disorders of puberty, in: S. Melmed (Ed.), Williams Textbook of Endocrinology Philadelphia PA, 2019, pp. 1085–1117, 14th.

[6] R.P. Grinspon, Genetics of congenital central hypogonadism, Best Pract. Res. Clin. Endocrinol. Metabol. 36 (1) (2022) 101599, https://doi.org/10.1016/j.beem.2021.101599.

[7] Y. Izumi, E. Suzuki, S. Kanzaki, S. Yatsuga, S. Kinjo, M. Igarashi, T. Maruyama, S. Sano, R. Horikawa, N. Sato, K. Nakabayashi, K. Hata, A. Umezawa, T. Ogata, Y. Yoshimura, M. Fukami, Genome-wide copy number analysis and systematic mutation screening in 58 patients with hypogonadotropic hypogonadism, Fertil. Steril. 102 (4) (2014) 1130–1136, https://doi.org/10.1016/j.fertnstert.2014.06.017.

[8] H. Shima, A. Ishii, Y. Wada, J. Kizawa, T. Yokoi, N. Azuma, Y. Matsubara, E. Suzuki, A. Nakamura, S. Narumi, M. Fukami, SOX2 nonsense mutation in a patient clinically diagnosed with non-syndromic hypogonadotropic hypogonadism, Endocr. J. 64 (8) (2017) 813–817, https://doi.org/10.1507/endocrj.EJ17-0078.

[9] E. Suzuki, Y. Izumi, Y. Chiba, R. Horikawa, Y. Matsubara, M. Tanaka, T. Ogata, M. Fukami, Y. Naiki, Loss-of-function SOX10 mutation in a patient with Kallmann syndrome, hearing loss, and iris hypopigmentation, Horm. Res. Paediatr. 84 (3) (2015) 212–216, https://doi.org/10.1159/000436965.

[10] H. Shima, E. Tokuhiro, S. Okamoto, M. Nagamori, T. Ogata, S. Narumi, A. Nakamura, Y. Izumi, T. Jinno, E. Suzuki, M. Fukami, SOX10 mutation screening for 117 patients with Kallmann syndrome, J Endocr Soc 5 (7) (2021) bvab056, https://doi.org/10.1210/jendso/bvab056.

[11] K. Matsubara, M. Itoh, K. Shimizu, S. Saito, K. Enomoto, K. Nakabayashi, K. Hata, K. Kurosawa, T. Ogata, M. Fukami, M. Kagami, Exploring the unique function of imprinting control centers in the PWS/AS-responsible region: finding from array-based methylation analysis in cases with variously sized microdeletions, Clin. Epigenet. 11 (1) (2019) 36, https://doi.org/10.1186/s13148-019-0633-1.

[12] T.J. Morris, L.M. Butcher, A. Feber, A.E. Teschendorff, A.R. Chakravarthy, T.K. Wojdacz, S. Beck, ChAMP: 450k chip analysis methylation pipeline, Bioinformatics 30 (3) (2014) 428–430, https://doi.org/10.1093/bioinformatics/btt684.

[13] Y.A. Chen, M. Lemire, S. Choufani, D.T. Butcher, D. Grafodatskaya, B.W. Zanke, S. Gallinger, T.J. Hudson, R. Weksberg, Discovery of cross-reactive probes and polymorphic CpGs in the illumina Infinium HumanMethylation450 microarray, Epigenetics 8 (2) (2013) 203–209, https://doi.org/10.4161/epi.23470.

[14] S. Horvath, DNA methylation age of human tissues and cell types, Genome Biol. 14 (10) (2013) R115, https://doi.org/10.1186/gb-2013-14-10-r115.

[15] G. Hannum, J. Guinney, L. Zhao, L. Zhang, G. Hughes, S. Sadda, B. Klotzle, M. Bibikova, J.B. Fan, Y. Gao, R. Deconde, M. Chen, I. Rajapakse, S. Friend, T. Ideker, K. Zhang, Genome-wide methylation profiles reveal quantitative views of human aging rates, Mol Cell 49 (2) (2013) 359–367, https://doi.org/10.1016/j.molcel.2012.10.016.

[16] R.S. Alisch, B.G. Barwick, P. Chopra, L.K. Myrick, G.A. Satten, K.N. Conneely, S.T. Warren, Age-associated DNA methylation in pediatric populations, Genome Res. 22 (4) (2012) 623–632, https://doi.org/10.1101/gr.125187.111.

[17] K. Hara-Isono, K. Matsubara, T. Fuke, K. Yamazawa, K. Satou, N. Murakami, S. Saitoh, K. Nakabayashi, K. Hata, T. Ogata, M. Fukami, M. Kagami, Genome-wide methylation analysis in Silver-Russell syndrome, Temple syndrome, and Prader-Willi syndrome, Clin. Epigenet. 12 (1) (2020) 159, https://doi.org/10.1186/s13148-020-00949-8.

[18] F.I. Rezwan, L.E. Docherty, R.L. Poole, G.A. Lockett, S.H. Arshad, J.W. Holloway, I.K. Temple, D.J. Mackay, A statistical method for single sample analysis of HumanMethylation450 array data: genome-wide methylation analysis of patients with imprinting disorders, Clin. Epigenet. 7 (1) (2015) 48, https://doi.org/10.1186/s13148-015-0081-5.

[19] Y. Benjamini, Y. Hochberg, Controlling the false discovery rate: a practical and powerful approach to multiple testing, J. R. Statist. Soc. B 57 (1) (1995) 289–300, https://doi.org/10.1111/j.2517-6161.1995.tb02031.x.

[20] M. Franz, H. Rodriguez, C. Lopes, K. Zuberi, J. Montojo, G.D. Bader, Q. Morris, GeneMANIA update 2018, Nucleic Acids Res. 46 (W1) (2018) W60–W64, https://doi.org/10.1093/nar/gky311.

[21] Š. Šestáková, C. Šálek, H. Remešová, DNA methylation validation methods: a coherent review with practical comparison, Biol. Proced. Online (2019), https://doi.org/10.1186/s12575-019-0107-z.

[22] L. De Chiara, V. Leiro-Fernandez, M. Rodríguez-Girondo, D. Valverde, M.I. Botana-Rial, A. Fernández-Villar, Comparison of bisulfite pyrosequencing and methylation-specific qPCR for methylation assessment, Int. J. Mol. Sci. 21 (23) (2020) 9242, https://doi.org/10.3390/ijms21239242.

[23] P. Garg, B. Jadhav, O.L. Rodriguez, N. Patel, A. Martin-Trujillo, M. Jain, S. Metsu, H. Olsen, B. Paten, B. Ritz, R.F. Kooy, J. Gecz, A.J. Sharp, A survey of rare epigenetic variation in 23,116 human genomes identifies disease-relevant epivariations and CGG expansions, Am. J. Hum. Genet. 107 (4) (2020) 654–669, https://doi.org/10.1016/j.ajhg.2020.08.019.

[24] K.D. Wilson, E.G. Porter, B.A. Garcia, Reprogramming of the epigenome in neurodevelopmental disorders, Crit. Rev. Biochem. Mol. Biol. 57 (1) (2020) 73–112, https://doi.org/10.1080/10409238.2021.1979457.