

Diagnosis of Prader-Willi syndrome and Angelman syndrome by targeted nanopore long-read sequencing

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ABSTRACT

The CpG island flanking the promoter region of *SNRPN* on chromosome 15q11.2 contains CpG sites that are completely methylated in the maternally derived allele and unmethylated in the paternally derived allele. Both unmethylated and methylated alleles are observed in normal individuals. Only the methylated allele is observed in patients with Prader-Willi syndrome, whereas only the unmethylated allele is observed in those with Angelman syndrome. Hence, detection of aberrant methylation at the differentially methylated region is fundamental to the molecular diagnosis of Prader-Willi syndrome and Angelman syndromes. Traditionally, bisulfite treatment and methylation-sensitive restriction enzyme treatment or methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) have been used. We here developed a long-read sequencing assay that can distinguish methylated and unmethylated CpG sites at 15q11.2 by the difference in current intensity generated from nanopore reads. We successfully diagnosed 4 Prader-Willi syndrome patients and 3 Angelman syndrome patients by targeting differentially methylated regions. Concurrent copy number analysis, homozygosity analysis, and structural variant analysis also allowed us to precisely delineate the underlying pathogenic mechanisms, including gross deletion, uniparental heterodisomy, uniparental isodisomy, or imprinting defect. Furthermore, we showed allele-specific methylation in imprinting-related differentially methylated regions on chromosomes 6, 7, 11, 14, and 20 in a normal individual together with 4 Prader-Willi patients and 3 Angelman syndrome patients. Hence, presently reported method is likely to be applicable to the diagnosis of imprinting disorders other than Prader-Willi syndrome and Angelman syndrome as well.

1. Introduction

Prader-Willi syndrome (PWS) is characterized by poor feeding and hypotonia in infancy, and excessive eating and obesity after early childhood, whereas Angelman syndrome (AS) is characterized by epilepsy, severe intellectual disability with severely limited speech, and truncal ataxia. These two prototypic imprinting disorders are caused by aberrant genomic imprinting on chromosome 15q11.2. The underlying mechanisms include gross deletion, uniparental disomy, and defect of the genomic region called the imprinting center which is differentially methylated.

The promoter region of the *SNRPN* gene contains a CpG island that is

completely methylated in the maternally derived allele and unmethylated in the paternally derived allele. Both unmethylated and methylated alleles are observed in normal individuals (Glenn et al., 1996). Only the methylated allele is observed in patients with PWS (Nicholls et al., 1989a, 1989b), whereas only the unmethylated allele is observed in those with AS (Knoll et al., 1989; Malcolm et al., 1991).

Detection of abnormal methylation in the promoter of *SNRPN* is the basis of molecular diagnosis of PWS and AS, and three methods, including the bisulfite treatment and methylation-sensitive restriction enzymatic treatment and methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) have been used (Moelans et al., 2018). In 1997, we and others developed a polymerase chain

Abbreviations: PWS, Prader-Willi syndrome; AS, Angelman syndrome.

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reaction (PCR)-based assay to evaluate the methylation status of the CpG island of the *SNRPN* gene (Kosaki et al., 1997). The assay was based on the observation that methylated cytosines in the CpG dinucleotide are resistant to chemical modification by sodium bisulfite, whereas bisulfite treatment converts all unmethylated cytosines to uracil (Clark et al., 1994; Nygren et al., 2005; Zeschnigk et al., 1997). These assays require bisulfite or enzymatic treatment prior to DNA sequencing and complex follow-up tests, including dosage analysis and microsatellite analysis to uncover the mechanism of abnormal imprinting.

Nanopore sequencing (Oxford Nanopore Technologies) is a long-read sequencing technology that can detect CpG methylation through differences in electrical current intensities produced from nanopore reads of unmethylated and methylated bases (Laszlo et al., 2013; Schreiber and Karplus, 2015). Several articles have described the performance of nanopore technology for the detection of CpG methylation, but clinical applications are still awaited (Yuen et al., 2021). The paucity of clinical applications for the detection of CpG methylation using nanopore sequencing can be attributed to the high cost of nanopore sequencing. A potential solution could be the use of adaptive sampling (Loose et al., 2016; Payne et al., 2021), which accepts DNA molecules that are inside the pre-set target sequences but rejects molecules that are outside the target sequences. Here, we applied nanopore adaptive sampling technology to the targeted analysis of the methylation statuses of imprinted regions of the human genome, enabling the successful diagnosis of patients with PWS and AS.

2. Materials and methods

The present research protocol was approved by an institutional board. Informed consent was obtained from the patients or the patients' parents. The patients' diagnoses of PWS or AS had been suspected clinically and had been confirmed by fluorescent in-situ hybridization (FISH) studies, microsatellite marker analyses of the patient-parents trio, and methylation-specific polymerase chain reactions. Among the four PWS patients, two had deletions of chromosome 15q11.2, one had maternal uniparental heterodisomy of chromosome 15, and one had an imprinting defect. Among the three AS patients, one had a deletion of chromosome 15q11.2, one had paternal uniparental heterodisomy of chromosome 15, and one had paternal uniparental isodisomy of chromosome 15.

Genomic DNA samples from these seven patients were subjected to targeted sequencing using the nanopore adaptive sampling technology. Libraries were prepared for sequencing according to the protocol described in the Ligation Sequencing Kit SQK-LSK110 (Oxford Nanopore Technologies [ONT], Oxford, UK). Each library was loaded onto an R9.4.1 flow cell FLO-MIN106 for sequencing on a GridION instrument (ONT, Oxford, UK) running MinKNOW control software, v4.3.11.

Genomic regions subject to medically relevant parent-of-origin methylation (Court et al., 2014; Eggermann et al., 2015; Monk et al., 2018), including the CpG dinucleotides of the *SNRPN* promoter region known as SNURF:TSS-DMR (Kosaki et al., 1997; Monk et al., 2018) were set as the target regions in the BED format file. More specifically, the regions listed in Table 1 of the report by Court et al. and their 10-kb flanking sequences were selected as the targets for adaptive sampling and subsequent methylation analysis (Court et al., 2014).

In addition, regions covering the entire coding sequences (i.e., from the start codon to the stop codon) of 3601 known human disease-causative genes and their 10-kb flanking sequences were also selected as targets for adaptive sampling. These genes were selected with reference to ClinGen (<https://clinicalgenome.org>). In addition, disease-causing genes defined in the OrphaData database (<http://www.orphadata.org>) and the Clinical Genomic Database (<https://research.nhgri.nih.gov/CGD>) were included if the gene was designated as disease-causative by both databases. The total size of the target regions was 259,114,649 bps. Information on target regions will be available upon request. Among these 3601 genes were *UBE3A* and *MAGEL2*, pathogenic

variants of which cause the AS (Matsuura et al., 1997) and PWS-related disorder Schaaf-Yang syndrome (Schaaf et al., 2013), and other genes related to imprinting disorders such as *ZFP57*, *KCNK9*, *CDKN1C*, *IGF2*, *DLK1*, *MKRN3*, and *GNAS* (Akbari et al., 2021).

Base-calling was performed using Guppy software from ONT (v6.0.6) with a super accuracy model (dna_r9.4.1_450bps_sup.cfg). The base-called reads were then aligned to the human reference genome (GRCh37) using Minimap2 (v2.24) (Li, 2018).

Haplotype-aware small variant calling was accomplished using PEPPER-Margin-DeepVariant (v0.8) (Shafin et al., 2021), which uses nanopore long reads to link adjacent single nucleotide variants and then phases the mapped reads to infer the haplotypes. The structural variations were called using Sniffles (version 2.0.3) (Sedlazeck et al., 2018). The Megalodon pipeline (v2.3.4) (Liu et al., 2020) was used to call CpG methylation from the raw nanopore data. In the pipeline, raw reads were base called with 5 mC modifications on the CpG motif using Guppy v.5.0.11 software and the dna_r9.4.1_450bps_modbases_5mc_hac model. The obtained reads were then aligned onto the reference genome sequences of GRCh37, and the results were generated as a BAM file format where reference-anchored per-read modified base calls were recorded with Mm and Ml tags. These tags were used to visualize the methylation status using haplotype information inferred by PEPPER-Margin-DeepVariant in the Integrative Genomics Viewer (v2.11.2) (Robinson et al., 2011).

From the aligned bam data generated using Minimap2, copy number aberrations were detected using the CNVpytor software, which utilizes the read depth and allele imbalance (Suvakov et al., 2021). From the variant call data, the region of homozygosity was detected by the H3M2 software (Magi et al., 2014).

3. Results

Nanopore sequencing showed that in all of the four patients with PWS, virtually all of the CpGs within the differentially methylated region of the promoter region of the *SNRPN* locus were methylated (Fig. 1, PWS-A, PWS-B, PWS-C, and PWS-D), whereas virtually none of the CpGs were methylated in all the three patients with AS (Fig. 1, AS-A, AS-B, and AS-C). In a normal control, approximately half of the reads were methylated, and the remaining half were unmethylated (Fig. 1, bottom). Hence, nanopore adaptive sampling technology was successfully applied to the diagnosis of patients with PWS and AS.

Furthermore, the nanopore adaptive sampling technology was able to correctly delineate the underlying pathogenesis (i.e., deletion, uniparental isodisomy, uniparental heterodisomy, and imprinting defect). In the PWS and AS patients with FISH-positive deletions, a copy number analysis showed a deletion in a region corresponding to 15q11.2 (Fig. 2a, b, and 2d). In the PWS and AS patients with known uniparental isodisomy and uniparental heterodisomy, the copy number was neutral for all of chromosome 15 (Fig. 2c, e, and 2f). In one patient with PWS and one patient with AS who had previously been diagnosed as showing uniparental heterodisomy based on a patient-parents trio microsatellite marker analysis, evaluation of variant homozygosity (Magi et al., 2014) of the entire chromosome 15 showed heterozygosity of multiple single nucleotide variants (Fig. 3c and e), whereas in one patient with AS who had previously been diagnosed as having uniparental isodisomy, such evaluation showed runs of homozygous variants (Fig. 3f). In one PWS patient (PWS-D) with presumed imprinting defect (i.e., no gross deletion or uniparental disomy, but aberrant methylation compatible with PWS), a 9-kb deletion, GRCh37/hg19 15q11.2(25,197,764-25,207,033)x1 (ClinVar SCV002549144), involving SNURF:TSS-DMR, a critical imprinting center, was detected (Fig. 4).

Differentially methylated regions other than the *SNRPN* promoter region SNURF:TSS-DMR were evaluated to explore the applicability of nanopore long read sequencing-based methylation analysis to other imprinting disorders. In a normal individual and the 4 PWS and 3 AS patients, one allele was methylated and the other allele was

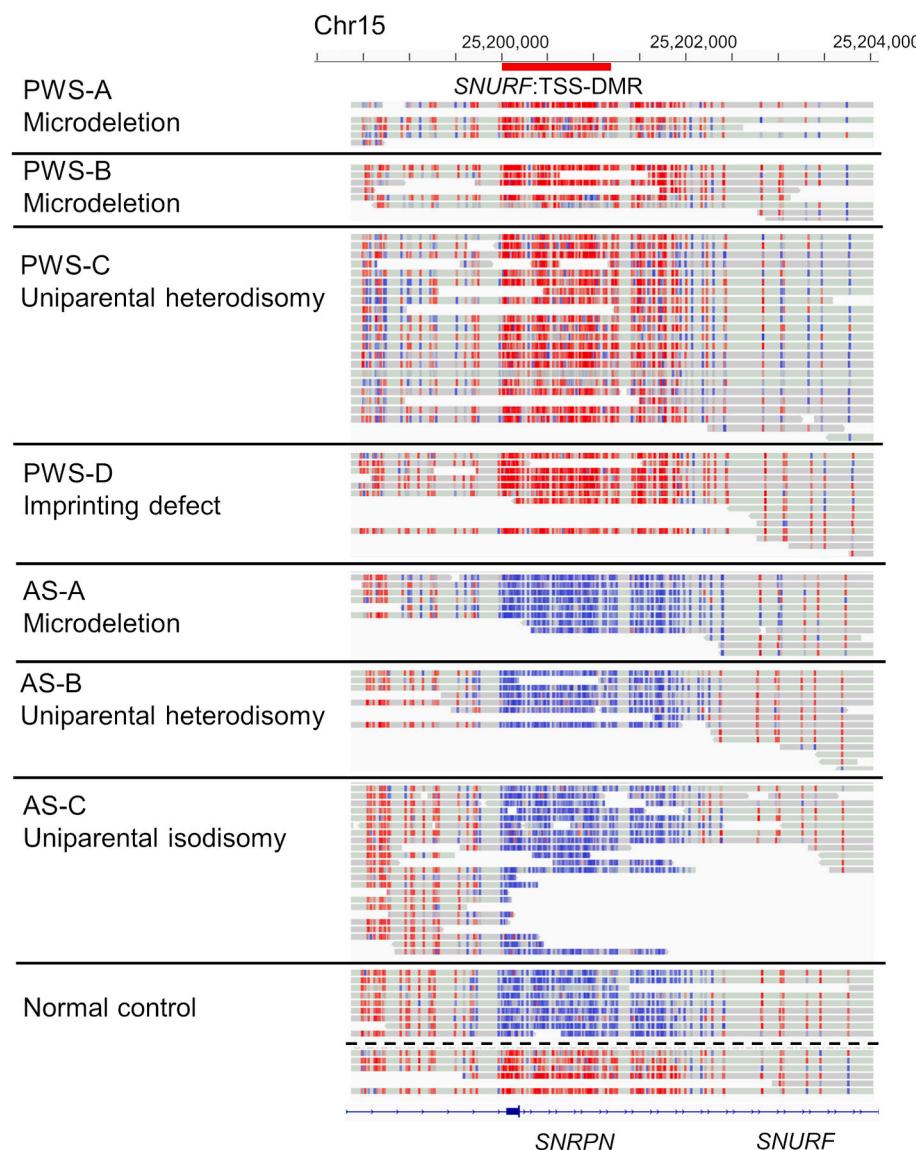


Fig. 1. Integrative Genomics Viewer (IGV) screen capture images of bam files showing the differentially methylated region of the PWS imprinting center spanning the differential methylated region at the *SNRPN* locus (SNURF: TSS-DMR). Methylated bases are colored in red and unmethylated bases are colored in blue. Images of the bam files of (top to the bottom) four Prader syndrome (PWS) patients and three Angelman syndrome (AS) patients, and a normal control. SNURF: TSS-DMR is completely methylated in PWS patients (PWS-A, PWS-B, PWS-C, and PWD-D) and completely unmethylated in AS patients (AS-A, AS-B, and AS-C). The patterns are essentially the same irrespective of underlying mechanisms (i.e., microdeletion, uniparental disomy or imprinting defect). For the normal individual, the top and bottom of the black dotted line in the center of each row represent two distinctive haplotypes, presumably from each parent. Both methylated and unmethylated alleles are observed in the normal individual. The chromosome positions are shown according to the GRCh37 assembly. PWS: Prader-Willi syndrome. AS: Angelman syndrome. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

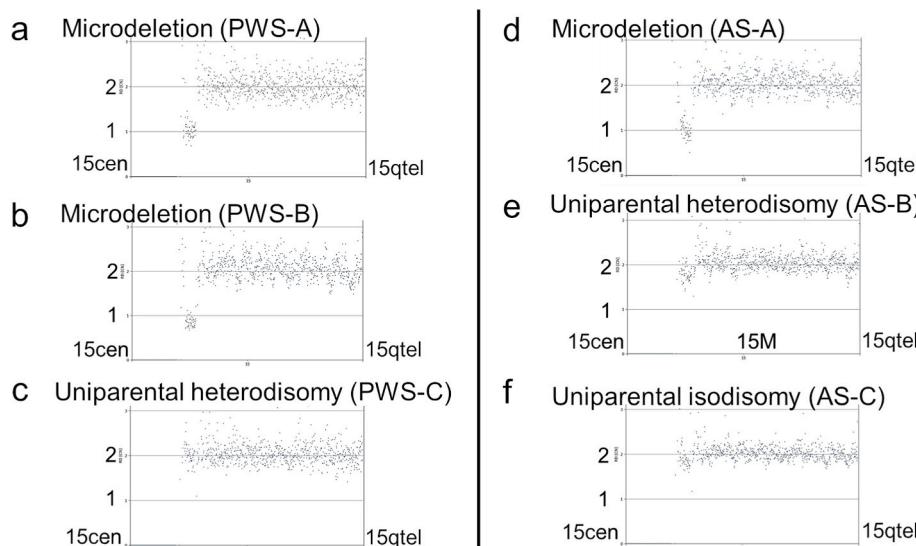


Fig. 2. Diagnosis of deletion of chromosome 15 by copy number analysis. Figures were generated by CNVpytor program. Horizontal axis denotes relative genome coordinate of chromosome 15 from the centromere (15cen) to telomere of the long arm (15qtel). Vertical axis denotes relative copy number (log₂ ratio). Note region of decreased copy number in deletion cases (2a, 2b, and 2d) but not in uniparental disomy cases (2c, 2e, and 2f). PWS: Prader-Willi syndrome. AS: Angelman syndrome.

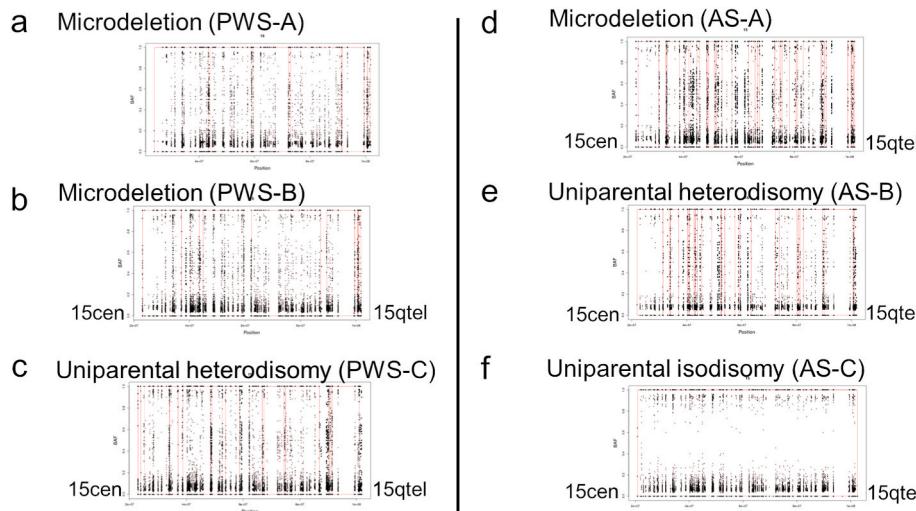


Fig. 3. Diagnosis of uniparental isodisomy of chromosome 15 by homozygosity mapping. Figures were generated by the H3M2 programs. Horizontal axis denotes relative genome coordinate of chromosome 15 from the centromere (15cen) to telomere of the long arm (15qtel). Vertical axis denotes B-allele frequency. Cloud-like dots in the middle shows heterozygous variants. Note region of homozygosity throughout chromosome 15 in uniparental isodisomy (3f). PWS: Prader-Willi syndrome. AS: Angelman syndrome.

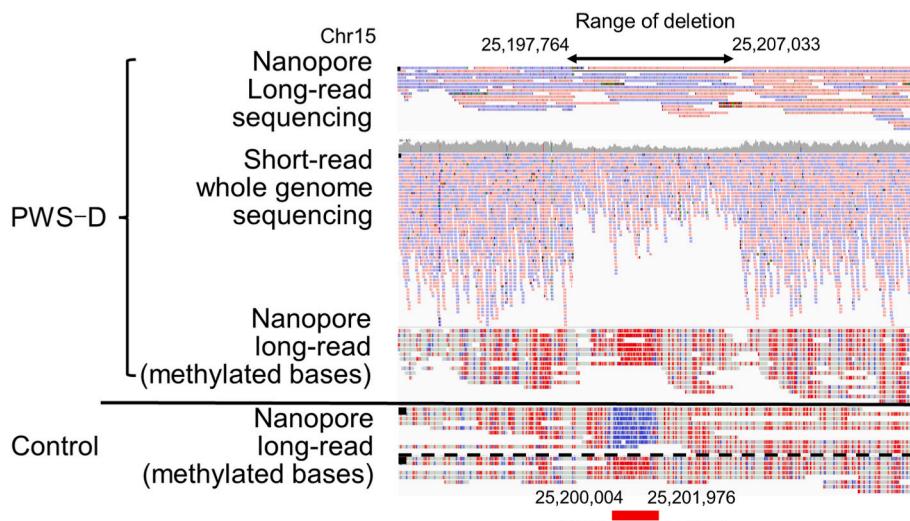


Fig. 4. Integrative Genomics Viewer screen capture images of bam files showing a 9-kb deletion involving the critical imprinting center, SNURF:TSS-DMR. Top: Image of a bam file of nanopore long-read sequencing and short-read whole genome sequencing. Note the split-mapped read spanning the 9-kb deletion in the nanopore long-read. Second row: Image of a bam file of short-read whole genome sequencing. Third row: Image of a nanopore long-read sequencing in the PWS patient with the 9-kb deletion, GRCh37/hg19 15q11.2(25,197,764-25,207,033)x1. Methylated bases are colored in red and unmethylated bases are colored in blue. Fourth row: Image of nanopore long-read sequencing of a normal individual. Reads are phased and methylated bases are colored in red. Note that only methylated alleles are observed in the patient with PWS, whereas both methylated and unmethylated alleles are observed in the normal individual in the differentially methylated region, the official name of which is SNURF:TSS-DMR. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

unmethylated for other known imprinted regions on chromosomes other than chromosome 15, including regions responsible for transient neonatal diabetes mellitus type 1 (*PLAGL1*), Russell-Silver syndrome (*H19*, *MEST*), Beckwith-Wiedemann syndrome (*H19*, *KCNQ1OT1*), Temple syndrome (*MEG3*), Kagami-Ogata syndrome (*MEG3*), and pseudohypoparathyroidism (*GNAS*) (Suppl Fig. 1).

In the protocol of nanopore adaptive sequencing presented here, regions from the start codon to the stop codon of 3601 known human disease-causing genes were targeted. The mean coverage of the 3601 genes was 17.8 folds (from 11.8-fold to 24.8) among 8 samples.

4. Discussion

Here, we documented the utility of nanopore adaptive sequencing for the diagnosis of PWS and AS, two reciprocally related imprinting disorders (Fig. 1). Use of nanopore adaptive sequencing obviates the need to use the cumbersome bisulfite or methylase pre-treatment steps. Furthermore, copy number analysis (Fig. 2), homozygosity analysis (Fig. 3), and structural variant analysis (Fig. 4) also allow one to precisely delineate the underlying pathogenic mechanisms, including gross deletion, uniparental heterodisomy, uniparental isodisomy, or imprinting defect due to even a very small deletion of the imprinting

center. Hence, nanopore adaptive sequencing represents a very efficient one step assay for diagnosing PWS and AS, as well as for delineating the underlying mechanism, which is essential for precise genetic counseling.

The new technology allows simultaneous genome-wide assessments of CpG methylation. The documentation of allele-specific methylation in imprinting-related differentially methylated regions (Suppl Fig. 1) lends promising support to the concept that the presently reported method is also likely to be applicable to the diagnosis of pediatric imprinting disorders other than PWS and AS (Akbari et al., 2021). Our study findings suggest that the known imprinting disorders (i.e., epigenetic disorders) can be screened simultaneously. In contrast to PWS and AS, other imprinting disorders, such as Russel-Silver syndrome, Beckwith-Wiedemann syndrome, and GNAS-related methylation defect syndrome occur as somatic mosaic defects (Eggermann et al., 2015). The accuracy of the reported method for the measurement of mosaicism needs to be evaluated in a quantitative manner.

From a standpoint of cost, existing methods, i.e., the bisulfite treatment and methylation-sensitive restriction enzymatic treatment and MS-MLPA, are much less expensive than the method reported herein. However, the assay reported herein, which covers both epigenetic disorders (i.e., imprinting disorders) and genetic disorders in a single assay. In that genomic imprinting plays significant roles in physical growth and

neurodevelopment, screening for imprinting disorders and differentiating them from non-imprinting disorders on the basis of the medical history and physical examination alone is difficult. Simultaneous screening of more than 3000 known human disease-causing genes in parallel with methylation analysis would be promising for making a precise molecular diagnosis in children with “undiagnosed diseases” in view of the improved sensitivity and specificity for single-nucleotide substitutions of nanopore sequencing. However, the reduced sensitivity of nanopore sequencing in detecting indels remains to be addressed.

Additionally, technical rationale behind the addition of non-imprinted genes is that adaptive sequencing requires relatively large target size. Selection of only the imprinted genes as target would not work because of excessively small target size in comparison with the whole genome.

In summary, we have developed a new assay that is applicable to molecular diagnosis of PWS and AS and to definition of the underlying pathogenetic mechanisms, including gross deletion, uniparental heterodisomy, uniparental isodisomy, or imprinting defect. The method can be applied to diagnosis of other imprinting defects and can be combined with targeted gene analysis of all known human genetic disorders in a single assay.

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Author statement

MY, HO, KO, HS, FM, TT, and KK performed the genetic analysis and interpreted the results. MY wrote the original draft whilst all authors revised the final manuscript. KK supervised the work. MY, TT, and KK financed the work.

Declaration of competing interest

The authors have no conflicts of interest to disclose.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejmg.2022.104690>.

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