

**Methylation-Sensitive High-Resolution Melting-Curve Analysis of the *SNRPN* Gene as a Diagnostic Screen for Prader-Willi and Angelman Syndromes,** *Helen E. White,\* Victoria J. Hall, and Nicholas C.P. Cross* (National Genetics Reference Laboratory (Wessex), Salisbury District Hospital, Odstock, Salisbury, Wiltshire, United Kingdom; \* Address correspondence to this author at: National Genetics Reference Laboratory (Wessex), Salisbury District Hospital, Salisbury, Wiltshire, SP2 8BJ, United Kingdom; fax (44) 1722 338095, e-mail H.E.White@soton.ac.uk)

**Background:** Angelman syndrome (AS) and Prader-Willi syndrome (PWS) are 2 distinct neurodevelopmental disorders caused primarily by deficiency of specific parental contributions at an imprinted domain within the chromosomal region 15q11.2–13. Lack of paternal contribution results in PWS either by paternal deletion (approximately 70%) or maternal uniparental disomy (UPD) (approximately 25%). Most cases of AS result from the lack of a maternal contribution from this same region, by maternal deletion (70%) or paternal UPD (approximately 5%). Analysis of allelic methylation differences at the small nuclear ribonucleoprotein polypeptide N (*SNRPN*) locus differentiates the maternally and paternally inherited chromosome 15 and can be used as a diagnostic test for AS and PWS.

**Methods:** Methylation-sensitive high-resolution melting-curve analysis (MS-HRM) using the DNA binding dye EvaGreen was used to analyze methylation differences at the *SNRPN* locus in anonymized DNA samples from individuals with PWS (n = 39) or AS (n = 31) and from healthy control individuals (n = 95). Results from the MS-HRM assay were compared to those obtained by use of a methylation-specific PCR (MSP) protocol that is used commonly in diagnostic practice.

**Results:** With the MS-HRM assay 97.6% of samples were unambiguously assigned to the 3 diagnostic categories (AS, PWS, normal) by use of automated calling with an 80% confidence percentage threshold, and the failure rate was 0.6%. One PWS sample showed a discordant result for the MS-HRM assay compared to MSP data.

**Conclusions:** MS-HRM is a simple, rapid, and robust method for screening methylation differences at the *SNRPN* locus and could be used as a diagnostic screen for PWS and AS.

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Angelman syndrome (AS) and Prader-Willi syndrome (PWS) are distinct neurodevelopmental disorders caused by loss of function of imprinted genes localized in a 2-Mb domain in the chromosomal region 15q11–13. Loss of imprinted genes from the paternally inherited chromosome 15q11–13 region results in PWS either as a result of interstitial deletion on the paternally derived chromosome 15 (approximately 70% of cases) or from uniparental disomy (UPD), in which both chromosomes 15 are mater-

nal in origin (approximately 25% of cases) (1–3). In AS, the lack of maternal contribution from 15q11–13 can occur as a result of maternal deletions or paternal UPD (approximately 70% and 5% of cases, respectively). In approximately 25% of AS cases there is biparental inheritance of chromosome 15 and a normal pattern of allelic methylation at 15q11–q13. In this group, AS has been shown to be caused by mutations in the ubiquitin protein ligase E3A (human papilloma virus E6-associated protein, Angelman syndrome) (*UBE3A*) gene (4, 5). In a small number of cases (1% in PWS and 2%–4% in AS), aberrant imprinting and gene silencing are thought to be responsible for disease, and in approximately 27% of AS cases mosaic methylation is detected (6).

In newborns and young children PWS and AS are often difficult to diagnose on the basis of clinical examination alone, and molecular and/or cytogenetic analysis is required for definitive diagnosis. Rapid, simple, and accurate molecular tests that enable diagnosis of PWS and AS by detecting allelic methylation differences are therefore of clinical importance. Paternal and maternal copies at this imprinted region can be identified by examination of DNA methylation at a molecular level. Methylation analyses of D15S63 (PW71) and the promoter region of the small nuclear ribonucleoprotein polypeptide N (*SNRPN*) gene regions have been recognized as clinically and scientifically valid diagnostic tests for PWS/AS by the American Society of Human Genetics/American College of Medical Genetics Test and Technology Transfer Committee (7). The most studied site of the *SNRPN* gene is the CpG island at the 5' end, which appears to be completely methylated on the maternal chromosome, whereas the paternal chromosome remains unmethylated (8). Several molecular strategies have been used to study allelic methylation differences at the *SNRPN* locus in PWS/AS: Southern blotting (9, 10), methylation-specific PCR (MSP) (11–14), PCR after restriction digestion of bisulfite-treated DNA (15), methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) (16), methylation-specific melting analysis (17, 18), and pyrosequencing (19).

High-resolution melting-curve analysis (HRM) is a simple and cost-effective post-PCR technique that can be used for methylation profiling (20). The technique requires the use of standard PCR reagents and double-stranded DNA-binding dyes that can be used at saturating concentrations without inhibiting PCR amplification. This closed-tube screening method has advantages over current techniques because it requires no post-PCR handling (minimizing the risk of PCR contamination) and no separation step, characteristics that improve analysis time. We have developed a methylation-sensitive HRM (MS-HRM) assay that uses the Rotor-Gene 6000 (Corbett Life Sciences) to analyze differential methylation at the *SNRPN* locus.

Using the EZ DNA Methylation Kit (Zymo Research) in accordance with the manufacturer's instructions, we bisulfite-treated 2  $\mu\text{g}$  of genomic DNA ( $n = 165$ ) from anonymized samples from healthy controls ( $n = 95$ ), PWS patients ( $n = 39$ ), and AS patients ( $n = 31$ ). The bisulfite-treated DNA was eluted in 10  $\mu\text{L}$  of elution buffer. To ensure that all of the DNA samples contained the same salt and buffer concentration after bisulfite treatment, we diluted 5  $\mu\text{L}$  of bisulfite-treated DNA in 95  $\mu\text{L}$  of molecular biology grade water and further purified the diluted DNA with Montage™ SEQ<sub>96</sub> plates (Millipore) and then resuspended the samples in 50  $\mu\text{L}$  of elution buffer from the EZ DNA Methylation Kit. We used both our current diagnostic MSP (12) and the MS-HRM assay to analyze samples for methylation differences at the *SNRPN* locus. MSP and GeneScan analyses were carried out as described previously (19); 2.5  $\mu\text{L}$  (approximately 50 ng) of bisulfite-treated DNA was amplified using 2 pmol of forward primer 5'-AGGGAGTTGGGATTTTGTATT-3' (GenBank accession no. NG\_002690; 173957–173979) and reverse primer 5'-CCCCAACTATCTCTTAAAAAAAAC-3' (NG\_002690; 174170–174195). PCR was performed by use of a 2 $\times$  custom designed EvaGreen HRM PCR master mix (Quantace). PCR and MS-HRM were performed by use of a Rotor-Gene 6000, and the PCR conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s and 1 cycle at 72 °C for 7 min. The same control samples for normal ( $n = 3$ ), AS ( $n = 3$ ), and PWS ( $n = 3$ ) were amplified in each run. PCRs were monitored in real time by acquiring data with the Green channel (470/510 nm). The 238-bp *SNRPN* amplicons containing 21 CpG dinucleotides were melted immediately after PCR. Samples were heated to 95 °C for 5 s, cooled to 50 °C for 5 min, and melted from 70 °C to 95 °C, with the temperature increasing by 0.2 °C increments with a 1-s hold at each step. HRM data were acquired by use of the HRM channel (470/510 nm) with a read rate of 1000 reads/°C.

HRM data were analyzed by use of the dedicated HRM software (Rotor-Gene 6000 Series Software 1.7, build 34). Normalization regions for the leading/trailing ranges were set at 73–79 °C/86–93 °C. Data generated from the amplification plots were used to determine reaction quality by use of the comparative amplification function of the software. Samples were considered to have failed if amplification had begun after 30 cycles or if the relative fluorescence values fell <10 units on the raw melting-curve plot. The failure rate for the MS-HRM assay was 0.6% (1 sample).

For each run, the genotyping function in the software was used to assign the control samples for normal ( $n = 3$ ), AS ( $n = 3$ ), and PWS ( $n = 3$ ) as normal, AS, or PWS. With automated calling with an 80% confidence percentage threshold, 97.6% of the 165 test samples were correctly assigned to 1 of the 3 diagnostic categories (AS, PWS, normal). Two normal control samples, an AS sample, and

a PWS sample could not be assigned to a disease category at a confidence threshold above 80%, i.e., they were classified as variant. The 2 normal control samples showed a biphasic melting curve consistent with normal samples (Fig. 1) and could be assigned as normal at confidence thresholds of 76.2% and 71.9%, respectively. Samples such as these may reflect true population methylation variation at the *SNRPN* locus, as has been observed in other studies (8, 19). The AS sample classified as variant was known to harbor a point mutation and, therefore, the curve shape did show variation from the AS control samples. The curve shape, however, was consistent with loss of methylation and hence a diagnosis of AS. Examination of the melting curve shape for the PWS sample classified as variant suggested that this sample either harbored a mutation or was possibly a PWS mosaic. Mosaicism was not found by pyrosequencing or MSP, however, and sequencing of the genomic DNA sample revealed no mutations. The melting curve profile was reproducible for this sample when tested by HRM by use of other double-stranded DNA binding dyes (data not shown), and the cause for this discrepant result is unknown.

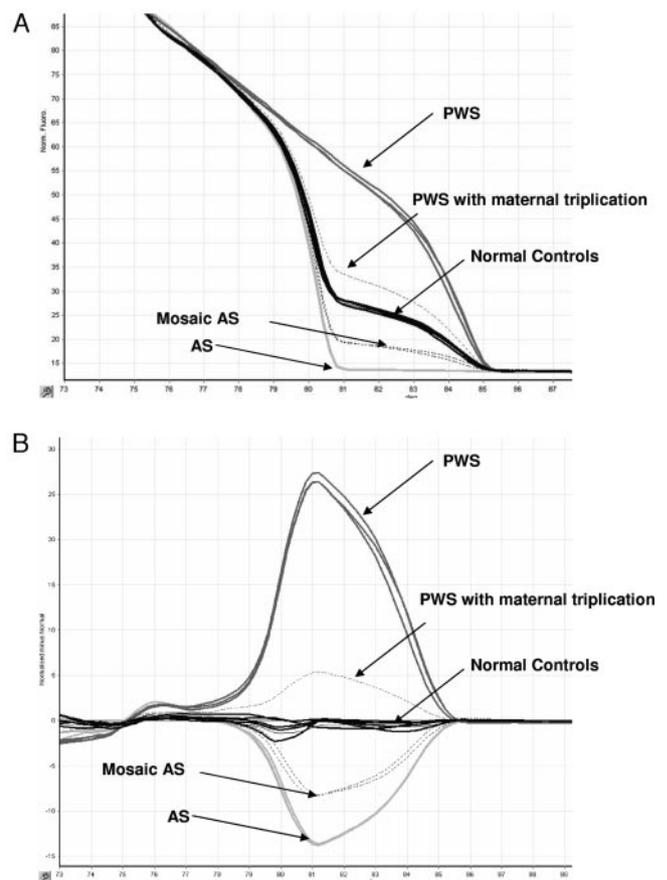


Fig. 1. Representative (A) high-resolution melting curves and (B) difference plots for PWS, AS, and normal samples.

The mosaic AS cases and PWS with a putative maternal triplication of the *SNRPN* locus can be clearly distinguished from nonmosaic AS/PWS cases and normal controls.

To determine the capability of HRM to detect mosaicism, the assay was also performed using bisulfite-treated DNA from 2 AS patient samples known to be mosaic and a PWS patient sample with a putative triplication of the *SNRPN* promoter region on the maternally derived chromosome 15 (as determined by semiquantitative MSP, fluorescence in situ hybridization, and microsatellite analysis). The 2 mosaic AS cases were classified as variant at a confidence percentage threshold of 80%, and the melting curve shapes could be clearly distinguished from the AS and normal control groups (Fig. 1). The PWS sample with a putative triplication at the *SNRPN* locus on the maternally methylated chromosome was also classified as variant at a confidence percentage threshold of 80% and showed a melting curve shape that clearly differed from the normal control and PWS groups (Fig. 1).

We conclude that MS-HRM is a simple, rapid, and robust method for screening methylation differences at the *SNRPN* locus and could be used as a diagnostic screen for PWS and AS. Samples that cannot be unequivocally assigned to a diagnostic category at a confidence percentage threshold of 80% should be investigated further using another technique. MS-HRM has the potential to be developed as a diagnostic tool for other methylation/imprinting disorders, such as Beckwith–Wiedemann syndrome, for which mosaicism is more commonly observed.

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