Detection and Discrimination between Deletional and Non-Deletional Prader-Willi and Angelman Syndromes by Methylation-Specific PCR and Quantitative Melting Curve Analysis

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Prader-Willi syndrome (PWS) and Angelman syndrome (AS) are clinically distinct neurological disorders caused by a lack of expression of oppositely imprinted genes in chromosomal region 15q11-13. The loss of expression can be due to parent-specific segmental deletions or can arise from non-deletional mechanisms, such as uniparental disomy of chromosome 15 or defects in imprinting. Most current diagnostic methods to distinguish PWS from AS require separate amplification and detection steps, and some methods cannot differentiate between deletional and non-deletional forms of these syndromes. We have developed a single-step, methylation-specific PCR, and quantitative melting curve analysis assay to identify methylation differences and copy number changes in PWS and AS. In this strategy, duplex amplification followed by melting curve analysis was performed to detect the maternally and paternally imprinted *SNRPN* alleles and *LIS1* reference gene. To discriminate between deletional and non-deletional PWS and AS, relative peak height ratios of maternal or paternal *SNRPN:LIS1* were determined, respectively. To validate the diagnostic accuracy of the analysis, methylation-specific multiplex ligation-dependent probe amplification was performed on all PWS and AS samples. Complete concordance between the melting curve analysis and methylation-specific multiplex ligation-dependent probe amplification results was observed for all PWS and AS samples. Methylation-specific PCR and quantitative melting curve analysis represents a simple, rapid, and robust alternative to methylation-specific multiplex ligation-dependent probe amplification for the detection of and discrimination between deletional and non-deletional

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Prader-Willi syndrome (PWS) and Angelman syndrome (AS) are two distinct neurological disorders caused by lack of expression of oppositely imprinted genes in chromosomal region 15q11-q13.^{1,2} Absence of a paternally contributed allele leads to PWS whereas absence of a maternally imprinted allele results in AS. Parent-specific segmental deletions, uniparental disomy of chromosome 15 (UPD15), and imprinting defects together constitute the most common causal mechanisms in both PWS and AS. Approximately 70% to 75% of PWS results from a deletion of the paternal chromosome 15q11-q13 region, while another 25% to 29% is caused by maternal UPD15. Imprinting defects and balanced translocations involving breaks in the 15q11-q13 region each account for less than 1% of PWS. Therefore, greater than 99% of PWS is associated with a detectable methylation abnormality in the 15q11-q13 imprinted region (<http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=gene&part=pws>; accessed March 24, 2009). In contrast, ~68% of AS results from a deletion of the maternal chromosome 15q11-q13 region, while paternal UPD15 and imprinting defects account for another ~7% and ~3%, respectively. As such, ~78% of AS is associated with a detectable methylation abnormality in the 15q11-q13 imprinted region (<http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=gene&part=angelman>; accessed March 24, 2009). Of the remaining ~22% of AS patients, whose methylation profiles are normal, approximately half carry a mutation in the *UBE3A* ubiquitin-ligase gene, while the molecular basis for the remaining patients is undetermined.

Small nuclear ribonucleoprotein polypeptide N (*SNRPN*) is one of several paternally expressed genes in this

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region, and its parent-of-origin specific methylation has been recognized as a diagnostic indicator of PWS and AS.^{3,4} Apart from widely used cytogenetic methods involving high-resolution chromosome studies and fluorescence *in situ* hybridization for detection of deletional PWS/AS, several molecular diagnostic assays have been developed based on the study of allelic methylation differences at the *SNRPN* locus, for instance, methylation-specific PCR (MS-PCR),⁵⁻⁷ methylation-dependent digestion followed by PCR,⁸ methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)⁹⁻¹¹ and pyrosequencing.¹² Some of these strategies can discriminate between deletional and non-deletional PWS/AS, but involve separate gene amplification, genotyping and detection steps. Recently, simple and rapid single-step strategies integrating amplification of bisulfite modified DNA and fluorescence melting curve analysis (MCA) have been reported, but they cannot discriminate between deletional and non-deletional PWS/AS.¹³⁻¹⁵ We have developed an improved single-step MS-PCR and MCA assay capable of differentiating between deletional and non-deletional PWS and AS.

Materials and Methods

A total of 143 DNA samples were analyzed in this study, including samples from PWS patients, AS patients and normal controls (Table 1). Genomic DNAs were extracted from peripheral whole blood or cultured lymphoblasts. Each 1 μ g of genomic DNA was bisulfite-treated and eluted in 20 μ l of elution buffer using the EZ DNA Methylation Kit (Zymo Research). Sodium bisulfite treatment converts unmethylated cytosines to uracil, while methylated cytosines are protected from conversion. Therefore, methylated and unmethylated alleles of a gene fragment can become significantly different in sequence after bisulfite-mediated modification, with resulting differences in amplicon thermal stabilities.

All samples were first analyzed by methylation-specific PCR to identify methylation differences at the *SNRPN* locus.⁷ PWS/AS samples were further analyzed by MS-

Table 1. Differentiating between Deletional and Non-Deletional PWS/AS by Analysis of *SNRPN:LIS1* Peak Height Ratios

Genotype	No.	<i>SNRPN:LIS1</i> peak height ratio $\pm$ SD	
		Paternal	Maternal
Deletional PWS	16	—	0.67 ± 0.20
Non-deletional PWS	8	—	1.60 ± 0.53
Deletional AS	13	1.66 ± 0.34	—
Non-deletional AS	2	4.24 ± 0.86	—
Paternal duplication (PWS)	1	8.31	0.62
Normal	103	2.38 ± 0.83	0.87 ± 0.53

Figure 1. MS-PCR and quantitative MCA analysis of the *LIS1* and *SNRPN* loci. The highlighted columns indicate the *LIS1*, paternal *SNRPN*, and maternal *SNRPN* peaks. Three peaks are observed in a normal sample (A), namely *LIS1* ($T_m = \sim 76.0^\circ\text{C}$), paternal *SNRPN* ($T_m = \sim 83.3^\circ\text{C}$), and maternal *SNRPN* ($T_m = \sim 87.7^\circ\text{C}$). Only the *LIS1* and paternal *SNRPN* peaks are observed in PWS affected patients (B, C). The maternal *SNRPN* peak is lower than the *LIS1* peak in deletional PWS (B), but higher than *LIS1* in non-deletional PWS (C). Only the *LIS1* and paternal *SNRPN* peaks are observed in AS affected patients (D, E). The paternal *SNRPN* peak is higher than the *LIS1* peak in both deletional (D) and non-deletional (E) AS, but the peak height ratio differs between the two mutation groups without overlap. In a sample with paternal *SNRPN* duplication (F), the *LIS1*, paternal *SNRPN*, and maternal *SNRPN* peaks are observed. However, the paternal *SNRPN* peak is much higher than the *LIS1* peak, as compared with a normal sample. In both (A) and (F), the arrow indicates an additional minor peak lying between the *LIS1* and paternal *SNRPN* peaks, which may have been derived from melting of a heteroduplex of paternal and maternal *SNRPN* products.

MLPA (P028 PWS/AS, MRC-Holland) to differentiate between deletional and non-deletional causes.¹⁴

In parallel, all samples were analyzed by a single-step MS-PCR and quantitative MCA assay. A 238 bp fragment of the *SNRPN* promoter was amplified using 0.5 μ mol/L each of previously described primers,¹³ together with a 107 bp fragment of exon 11 of the *LIS1* gene using 0.1 μ mol/L each of primers 5'-GTTTTTGGTGTGTTTATAGG-GATGTGTAATGTG-3' (GenBank U72342; 1328→1359) and 5'-CAAACACTATATTCCTCCCACTCCTTTC-3' (GenBank U72342; 1434→1406). The *LIS1* gene (OMIM # 601545), also known as *PAFAH1B1*, is located on chromosome 17p13.3. Haploinsufficiency of *LIS1* is rare and causes a severe brain malformation and profound mental retardation disorder.¹⁶ Conversely, *LIS1* duplications are even rarer, and are associated with a clinical phenotype distinct from PWS and AS.¹⁷ Since loss or gain of *LIS1* copy number is phenotypically recognizable from the normal two-copy state, and given the extreme improbability of concurrent *LIS1* duplication/deletion and PWS/AS, *LIS1* serves as an ideal two-copy reference gene for the PWS/AS quantitative MCA assay.

Each duplex amplification reaction was performed in a 20 μ l volume containing the pairs of primers, 0.2 mmol/L of each deoxynucleotide triphosphate (Roche Applied Science), 0.5 U HotStarTaq DNA polymerase (Qiagen), 0.5 mmol/L $MgCl_2$, 1 $\times$ EvaGreen (Biotium), 1 $\times$ supplied PCR buffer (containing 1.5 mmol/L $MgCl_2$), and 4 μ l of the bisulfite-modified DNA. PCR amplification was performed in a LightCycler 480 instrument (Roche Applied Science) under the following conditions: an enzyme activation step at 95°C for 15 minutes followed by 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. The MCA was performed automatically after PCR amplification under the following conditions: fragment denaturation at 95°C for 5 seconds, cooling to 65°C for 1 minute, and slow temperature ramping from 65°C to 95°C, with fluorescence data acquisition rate of 10 readings per degree using the 483–533 nm filter channel.

Melting curve fluorescence data were analyzed using the melting temperature (T_m) calling function in the LightCycler 480 software. The T_m calling function converts the melting-curve data into melting-peak plots, determines the T_m for each PCR product as the highest point of the corresponding melting peak, and calculates the peak height for each melting peak. The starting fluorescence signal strength in the raw melting curve plot indicates the reaction quality. A low starting fluorescence intensity in the melting curve plot usually yields a melting peak with high background, leading to inaccuracy in peak height determination. Therefore, we considered the assay to have failed if the starting fluorescence intensity of a melting curve plot falls below the 10 unit threshold.¹³ To discriminate between deletional and non-deletional PWS or AS, the peak height data were exported into a Microsoft Excel worksheet to calculate the ratio between the maternal or paternal *SNRPN* and *LIS1* peak heights.

This study protocol was conducted under Institutional Review Board approval #EC200711152.

Figure 2. Peak height ratios for deletional and non-deletional PWS/AS samples from replicate PCR experiments. Variations in the peak height ratios were observed between runs. However, the ratios in the deletion and non-deletion groups within each run did not overlap.

Results

The *SNRPN* amplicon derived from a bisulfite-modified maternal (normally methylated) allele melts at ~87.7°C, compared with the paternal (normally unmethylated) allele, which melts at ~83.3°C due to the higher GC content of the former amplicon (Figure 1, A–F). The *SNRPN* fragment was amplified together with a fragment from *LIS1*, which functions as the reference two-copy gene for the quantitative MCA analysis. Therefore, DNA samples from normal individuals should produce three melting peaks, including the *LIS1* peak with a T_m of ~76.0°C, and both the paternal and maternal *SNRPN* peaks. In contrast, the presence of the *LIS1* peak and only the maternal or the paternal *SNRPN* peak is diagnostic of PWS and AS, respectively (Figure 1, A–F). Discrimination between deletional and non-deletional PWS and AS was achieved by determining the ratio between the maternal or paternal *SNRPN* peak height and the *LIS1* peak height. The *SNRPN* to *LIS1* peak height ratio in a deletional PWS/AS patient is expected to be less compared with the *SNRPN*:*LIS1* peak height ratio in a non-deletional PWS/AS patient (Figure 1, Table 1). This is because patients with deletional PWS/AS contain only one copy of *SNRPN*, while patients with non-deletional PWS/AS, due either to UPD15 or imprinting center defects, contain two copies of *SNRPN* that are identically imprinted. Comparison of the peak height ratios from deletional and non-deletional PWS and AS patients confirms a higher *SNRPN*:*LIS1* ratio in non-deletional patients compared with patients with deletions (Figure 1, Table 1).

In replicate assay runs, we observed variations in the peak height ratio between samples within each genotype group; however, these variations did not result in an overlap in peak height ratios between the deletional and non-deletion groups within each run, thus allowing their unambiguous discrimination (Figure 2). Nevertheless, positive control samples are recommended for each assay run to mitigate the effects of variations between assay runs.

Given its quantitative nature, this assay is also capable of detecting rare duplications within the PWS/AS critical region. This is illustrated by the DNA sample from a Coriell Repository cell line GM12135, which was previously shown to carry a paternal segmental duplication of chromosome 15 both cytogenetically and by MS-MLPA.¹⁴ In this sample, we observed three peaks corresponding to *LIS1*, paternal *SNRPN*, and maternal *SNRPN*, a normal maternal *SNRPN:LIS1* peak height ratio of 0.62 (range in normal samples 0.87 ± 0.53), but a much higher paternal *SNRPN:LIS1* peak height ratio of 8.31 (range in normal samples 2.38 ± 0.83) (Figure 1F, Table 1).

A total of 25 PWS and 15 AS patient samples were analyzed by MS-PCR and quantitative MCA in parallel with MS-MLPA and concordant results were observed for all of the patient samples.

Discussion

Using essentially standard procedures and reagents, we have modified the sodium bisulfite mediated MS-PCR assay for PWS/AS into a quantitative method to enable discrimination between the more common deletional and the rarer non-deletional forms. After sodium bisulfite treatment, PCR and quantitative MCA screening can be performed continuously without further hands-on manipulation and the reaction can be completed within 2 hours. Metaphase fluorescence *in situ* hybridization has been widely used for the detection of deletional PWS/AS, as well as the rarer translocations, but cannot detect PWS/AS due to UPD15 or imprinting center defects. Furthermore, the procedure requires cell culture, is comparatively more labor-intensive, and requires the use of fluorescent hybridization probes.

Recently, MS-MLPA has been developed for the molecular diagnosis of PWS/AS.^{9–11} It avoids the need for sodium bisulfite treatment of the DNA sample but instead subjects the DNA to digestion with a methylation sensitive restriction enzyme *HhaI*. Using multiple specially designed probes specific for gene sequences in and around the PWS/AS critical region as well as reference genes outside this region, MS-MLPA can simultaneously detect methylation pattern differences and copy number changes in patients. However, this method is also comparatively more labor-intensive, makes use of fluorescently labeled PCR primers, and requires post-PCR analysis on a fluorescent fragment separation and analysis instrument.

Our MS-PCR and quantitative MCA represents a simple, rapid and robust alternative to MS-MLPA for the detection of, and discrimination between, deletional and non-deletional forms of PWS and AS. As is the case with MS-MLPA, one limitation of quantitative MCA is its inability to differentiate between UPD and imprinting defects, for which multiplex microsatellite marker analysis, single nucleotide polymorphism haplotype analysis, and/or direct PCR and sequencing of the imprinting center is/are required.

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