Molecular Diagnostics and Genetics

Quantitative Analysis of *SRNPN* Gene Methylation by Pyrosequencing as a Diagnostic Test for Prader–Willi Syndrome and Angelman Syndrome

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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. In most cases, lack of paternal contribution leads to PWS either by paternal deletion (~70%) or maternal uniparental disomy (UPD; ~30%). Most cases of AS result from the lack of a maternal contribution from this same region by maternal deletion (~70%) or by paternal UPD (~5%). Analysis of allelic methylation differences at the small nuclear ribonucleoprotein polypeptide N (*SNRPN*) locus can differentiate the maternally and paternally inherited chromosome 15 and can be used as a diagnostic test for AS and PWS.

Methods: Sodium bisulfite–treated genomic DNA was PCR-amplified for the *SNRPN* gene. We used pyrosequencing to individually quantify the resulting artificial C/T sequence variation at CpG sites. Anonymized DNA samples from PWS patients (n = 40), AS patients (n = 31), and controls (n = 81) were analyzed in a blinded fashion with 2 PCR and 3 pyrosequencing reactions. We compared results from the pyrosequencing assays with those obtained with a commonly used methylation-specific PCR (MS-PCR) diagnostic protocol.

Results: The pyrosequencing assays had a sensitivity and specificity of 100% and provided quantification of methylation at 12 CpG sites within the *SNRPN* locus. The resulting diagnoses were 100% concordant with those obtained from the MS-PCR protocol.

Conclusions: Pyrosequencing is a rapid and robust method for quantitative methylation analysis of the *SNRPN* locus and can be used as a diagnostic test for PWS and AS.

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Angelman syndrome (AS)[1] and Prader–Willi syndrome (PWS) are clinically distinct neurodevelopmental disorders caused by loss of function of imprinted genes localized in a 2-Mb domain in the chromosomal region 15q11-13. PWS results from loss of imprinted genes from the paternally inherited chromosome as a result of either interstitial deletion (~70% of cases) or maternal uniparental disomy (UPD; ~30% of cases) *(1–4)*. In AS, the lack of maternal contribution at 15q11-13 can occur as a result of maternal deletions or paternal UPD (~70% and 5% of cases, respectively). In ~25% of AS cases, there is biparental inheritance of chromosome 15 and a normal pattern of allelic methylation at 15q11-q13. In this group, sequence variations in the ubiquitin protein ligase E3A (*UBE3A*)[2] gene have been shown to be a cause of AS *(5, 6)*. 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 mosaic methylation is detected in ~27% of these AS cases *(7)*.

At the molecular level, paternal and maternal copies of this imprinted region can be distinguished by examining DNA methylation. Differentially methylated sites have been identified in 15q11-q13, and the 2 regions most commonly investigated for the diagnosis of PWS/AS are D15S63 (PW71) and the promoter region of the small nuclear ribonucleoprotein polypeptide N (*SNRPN*) gene. Methylation analysis of these regions has been recognized

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[1] Nonstandard abbreviations: AS, Angelman syndrome; PWS, Prader–Willi syndrome; UPD, uniparental disomy; MS-PCR, methylation-specific PCR; and AQ, Allele Quantification (software).

[2] Human genes: *UBE3A*, ubiquitin protein ligase E3A (human papilloma virus E6-associated protein, Angelman syndrome); *SNRPN*, small nuclear ribonucleoprotein polypeptide N.

by the American Society of Human Genetics/American College of Medical Genetics (ASHG/ACMG) Test and Transfer Committee as a clinically and scientifically valid diagnostic test for PWS/AS (8). The most studied site is the CpG island at the 5′ end of the *SNRPN* gene, which appears to be completely methylated on the maternal chromosome, whereas the paternal chromosome remains unmethylated (9). Southern blot analysis of DNA cleaved with methylation-sensitive restriction enzymes has been used as a diagnostic test for PWS/AS but has intrinsic disadvantages, such as problems with partial cleavage. Many European diagnostic laboratories test PWS and AS referrals by methylation-specific PCR (MS-PCR). MS-PCR is performed with DNA treated with sodium bisulfite, which converts cytosine, but not 5-methylcytosine, to uracil; hence, allelic methylation differences are detectable as sequence differences. Amplification with primers specific for methylated and unmethylated DNA allows differentiation between paternal and maternal alleles. Two MS-PCR protocols are widely used in Europe (10, 11), but a recent German external quality assessment scheme (2001/2002) showed that, because it is semiquantitative, the method published by Zeschnigk et al. (11) is more reliable at detecting mosaic DNA methylation in AS (12).

Several studies have reported the use of Pyrosequencing® technology for quantitative methylation analysis of multiple CpG sites (13–16). Pyrosequencing is a real-time sequencing method for the analysis of short- to medium-length DNA sequences (17). Incorporation of a nucleotide into the template strand leads to the release of pyrophosphate, which is quantified with a luciferase reaction. The signal produced is proportional to the amount of pyrophosphate released; thus, methylation at CpG sites can be detected and quantified by analyzing the chemically induced C/T sequence differences with Allele Quantification (AQ) software (Biotage AB). Here we describe a new diagnostic test for PWS/AS that uses 3 pyrosequencing assays to analyze and quantify 12 CpG sites within the 5′ end of the *SNRPN* gene. We tested a group of patients referred to the Wessex Regional Genetics Laboratory for PWS/AS testing (n = 71) and a cohort of healthy controls (n = 81) in whom the *SNRPN* methylation status had been determined previously with MS-PCR (11).

Materials and Methods

BISULFITE TREATMENT OF DNA SAMPLES

We treated 2 μg of DNA from samples from 152 individuals [81 controls, 40 PWS patients (maternal UPD, n = 14; paternal deletion, n = 25; abnormal methylation but not maternal UPD or paternal deletion, n = 1), and 31 AS patients (paternal UPD, n = 2; maternal deletion, n = 24; abnormal methylation but not paternal UPD or maternal deletion, n = 5)] on the same day with the EZ DNA Methylation Kit (Zymo Research) in accordance with the manufacturer's instructions. Bisulfite-treated DNA was eluted in 10 μL of elution buffer and diluted 1:10 before

use. We used our current diagnostic MS-PCR (11) and 3 pyrosequencing assays to analyze the DNA samples for methylation differences at the *SRNPN* locus. The sequences of the primers used are listed in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue6.

MS-PCR AND GENESCAN ANALYSIS

Amplicons were generated in a 25-μL reaction volume containing 0.8 pmol of common primer, 0.8 pmol of maternal primer, 0.24 pmol of paternal primer, 0.18 mM deoxynucleoside triphosphates (Promega), 1× Hotstar Buffer with MgCl$_2$ (Qiagen), 1 U of Hotstar Taq (Qiagen), and 1 μL (~20 ng) of bisulfite-treated DNA. PCR was performed in a PTC-0225 DNA Engine Tetrad (MJ Research) with the following conditions: 94 °C for 15 min, followed by 29 cycles of 94 °C for 20 s, 60 °C for 35 s, and 72 °C for 35 s; 1 cycle at 72 °C for 7 min; and a final hold at 4 °C. All samples were analyzed in triplicate.

Fluorescently labeled amplicons were analyzed with an 3100 Genetic Analyzer (Applied Biosystems), and the percentage of methylation was calculated by dividing the peak area of the maternal-specific peak by the combined peak areas of the maternal- and paternal-specific peaks (Fig. 1A).

PCR AMPLIFICATION FOR PYROSEQUENCING REACTIONS

Amplicons were generated in a 50-μL reaction volume containing 10 pmol each of the forward and reverse PCR primers, 0.2 mM deoxynucleoside triphosphates (Promega), 2.5 mM MgCl$_2$, 1× Buffer II (Applied Biosystems), 1 U of AmpliTaq Gold (Applied Biosystems), and 1 μL (~20ng) of bisulfite-treated DNA. PCR was performed in a PTC-0225 DNA Engine Tetrad with the following conditions for all reactions; 94 °C for 7 min, followed by 45 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; 1 cycle at 72 °C for 7 min; and a final hold at 15 °C. Two PCR amplifications were performed for each sample to allow analysis of 12 CpG sites (9). Primers A1031F and A1032RB were used for pyrosequencing assay 1033 (analysis of CpG sites G to J), and primers A1035FB and A1036R were used for pyrosequencing assays 1037 (analysis of CpG sites R to U; Fig. 1B) and 1038 (analysis of CpG sites C to F). All samples were analyzed in triplicate.

Single-stranded biotinylated PCR products were prepared for sequencing by use of the Pyrosequencing Vacuum Prep Tool according to the manufacturer's instructions. For assays 1033 and 1038, we added 0.3 μL of single-stranded binding protein (SSB; Promega; 2.2 μg/ μL) to eliminate secondary structure in the template DNA.

PYROSEQUENCING REACTIONS AND DATA ANALYSIS

Pyrosequencing reactions were performed according to the manufacturer's instructions, using the PSQ 96 SNP Reagent Kit (Biotage AB). The degree of methylation at
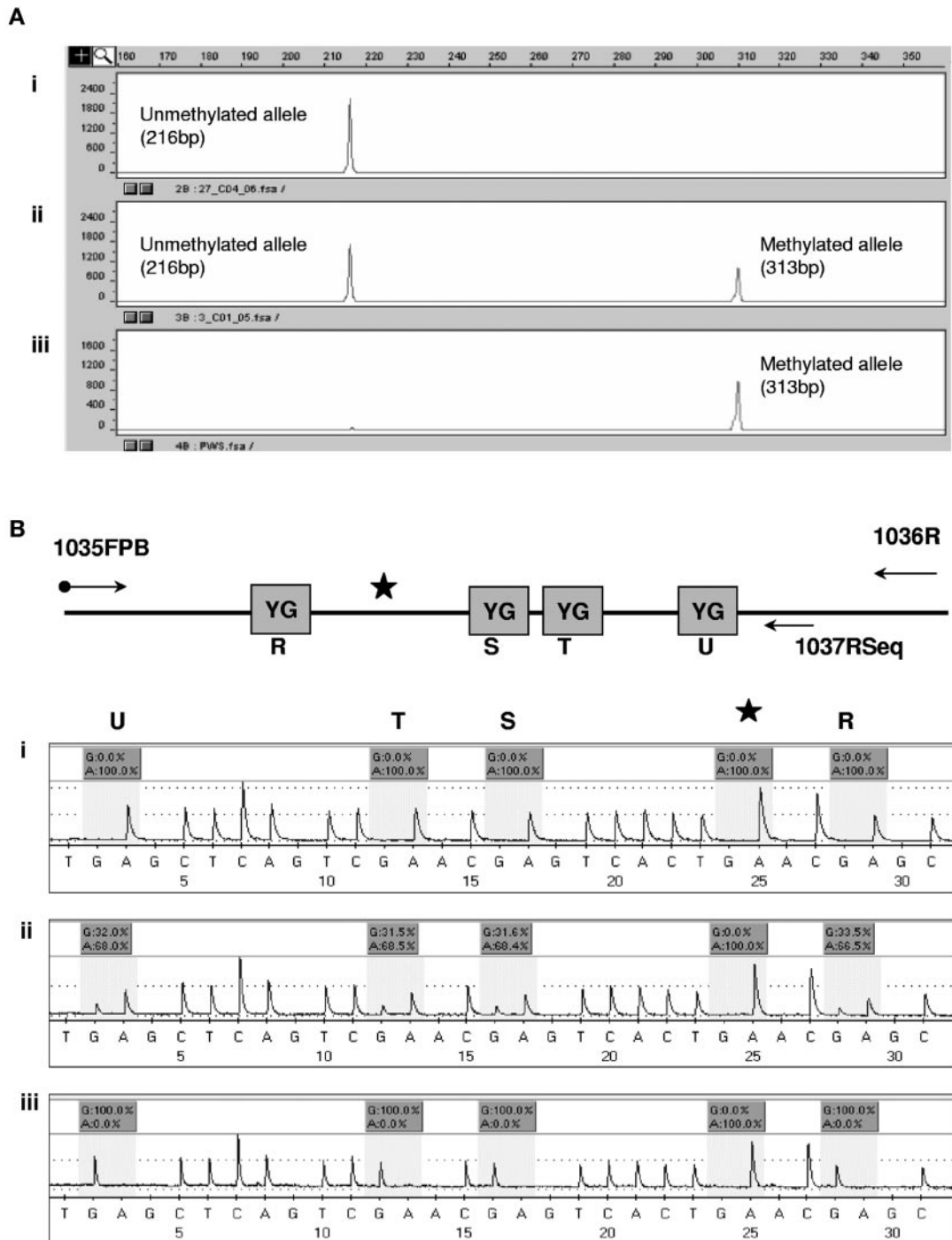
Fig. 1. MS-PCR (*A*) and pyrosequencing (*B*) results.

(*A*), representative GeneScan traces from the MS-PCR assay for an AS patient (*i*), a healthy control (*ii*), and a PWS patient (*iii*). (*i*), the presence of the paternal (unmethylated allele) PCR product at 216 bp and the absence of the maternal (methylated allele) PCR product at 313 bp indicates complete loss of maternal methylation, which is consistent with a diagnosis of AS. (*ii*), the presence of the paternal PCR product at 216 bp and maternal (methylated allele) PCR product a 313 bp indicates a percentage of methylation that is consistent with a normal methylation profile. (*iii*), the presence of the maternal (methylated allele) PCR product at 313 bp and the absence of the paternal (unmethylated allele) PCR product at 216 bp indicates complete loss of the paternal allele, which is consistent with a diagnosis of PWS. (*B*), schematic diagram of pyrosequencing assay 1037 showing the region amplified by primers 1035FPB (biotinylated) and 1036R. Using sequencing primer 1037, CpG sites R, S, T, and U can be analyzed by AQ software *(9)*. A bisulfite treatment control in which conversion of an unmethylated cytosine residue to uracil is also analyzed (★). Representative programs are shown for an AS patient (*i*), a healthy control (*ii*), and a PWS patient (*iii*). (*i*), AQ values (*shaded boxes*) for the methylated (G) allele at all CpG sites are 0%, which indicates the absence of the maternal (methylated) allele. This is consistent with a diagnosis of AS. (*ii*), AQ values (*shaded boxes*) for the methylated (G) allele range from 31.5% to 33.5% which, after correction for PCR amplification bias, equates to 50% methylation. This is consistent with a normal methylation profile in which both the methylated (maternal) and unmethylated (paternal) alleles are present. (*iii*), AQ values (*shaded boxes*) for the methylated (G) allele at all CpG sites are 100% which indicates the absence of the paternal (unmethylated) allele. This is consistent with a diagnosis of PWS. In all cases, the bisulfite control (★) shows complete conversion of the unmethylated cytosine residue because the AQ value is 100% for the unmethylated (A) allele.

each CpG site was determined by AQ Software (Fig. 1B). Nucleotide dispensation orders are listed in the online Data Supplement.

Differences in sequence content between the methylated and unmethylated alleles after bisulfite treatment can influence the melting and annealing properties of the PCR amplification. We constructed calibration curves for the forward and reverse pyrosequencing PCRs to determine the extent of PCR amplification bias *(15)*. DNA from a PWS and an AS patient was quantified, and 2 μg was treated with bisulfite. The DNA samples were then mixed before PCR to generate samples with 0%–100% methylation (in 10% increments). The samples were PCR-amplified and subjected to pyrosequencing. To ensure that the pyrosequencing assays were providing unbiased quantification, we mixed PCR products generated from a PWS patient and an AS patient before pyrosequencing to generate samples with 0%–100% methylation (in 20% increments).

EFFECT OF BISULFITE TREATMENT

To determine whether variations in quantification at CpG sites occurred between batches of bisulfite treatment, we treated DNA samples from the healthy controls (n = 16) on different days, using different batches of the EZ DNA Methylation Kit (Zymo Research).

EFFECT OF DNA CONCENTRATION

To determine whether the amount of template DNA added to the PCR reaction affected the reproducibility of quantification of methylation, we treated 2 μg of DNA from samples from healthy controls (n = 4) with bisulfite. We assumed 100% recovery and performed assay 1037 in triplicate, using 50, 20, 10, 5 and 2.5ng of template DNA.

Results

PYROSEQUENCING PCR AMPLIFICATION BIAS

The calibration curves for pyrosequencing assay 1037 are shown in Fig. 2, with the curve generated with DNA from a PWS and an AS patient mixed before PCR shown in panel A, and the curve generated with PCR products amplified from an PWS and AS sample mixed after PCR shown in panel B. The curve generated from the mixed-template DNA samples (Fig. 2A) shows a second-order polynomial fit with a correlation coefficient (R^2) of 0.992, demonstrating that there has been preferential PCR amplification of the unmethylated allele. This must be taken into account when absolute quantification of methylation for a PWS/AS sample is required. However, the highly linear relationship ($R^2 = 0.994$) observed for the pyrose-
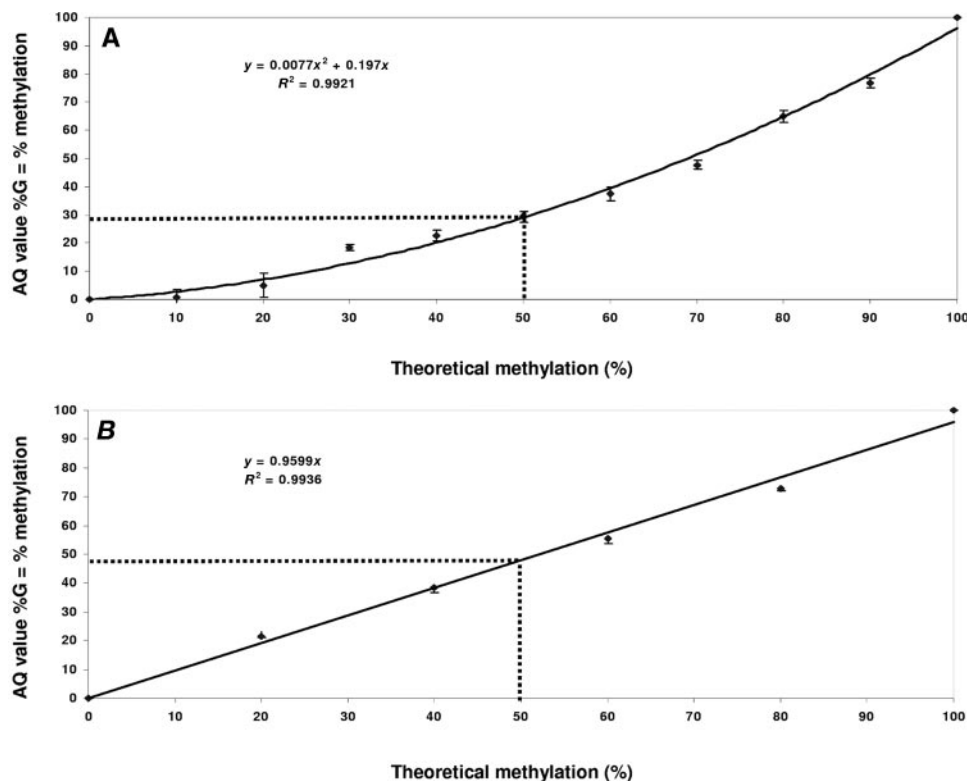

Fig. 2. Calibration curves for pyrosequencing assay 1037.

Curves were constructed by mixing equal concentration of PWS and AS patient DNA samples before PCR to generate samples with theoretical methylation from 0% to 100% in 10% increments (*A*), and mixing PCR products obtained from a PWS and an AS patient after amplification to generate samples with theoretical methylation from 0% to 100% in 20% increments (*B*).

quencing assay run using mixed PCR products obtained from a PWS and an AS patient indicated that the pyrosequencing reaction was absolutely quantitative (Fig. 2B). Calibration curves for the forward pyrosequencing assay 1033 produced similar results (data not shown).

EFFECT OF BISULFITE TREATMENT ON ABSOLUTE QUANTIFICATION OF METHYLATION

As shown in Fig. 3A, DNA samples treated with bisulfite on different days gave slightly different quantitative results in the 1037 pyrosequencing assay. The mean difference in quantification of methylation at CpG site U was 4.5% (range, 0.9%–8%); however, this variation was less pronounced with pyrosequencing assays 1033 and 1038 (data not shown).

EFFECT OF DNA CONCENTRATION ON REPRODUCIBILITY

The amount of DNA added to the pyrosequencing PCR can affect the reproducibility of methylation quantification. Low amounts of DNA template may introduce bias into the PCR reaction because preferential amplification of either allele during the first few rounds of amplification can occur (16). The effect of DNA concentration in assay 1037 is shown in Fig. 3B. The average standard deviations at CpG site U for 50, 20, 10, 5, and 2.5 ng of template DNA were 2.8, 3.0, 4.1, 7.2 and 6.7, respectively. These data indicate that a minimum of 10–20 ng of template DNA should be added to the PCR if reproducible results are to be obtained.
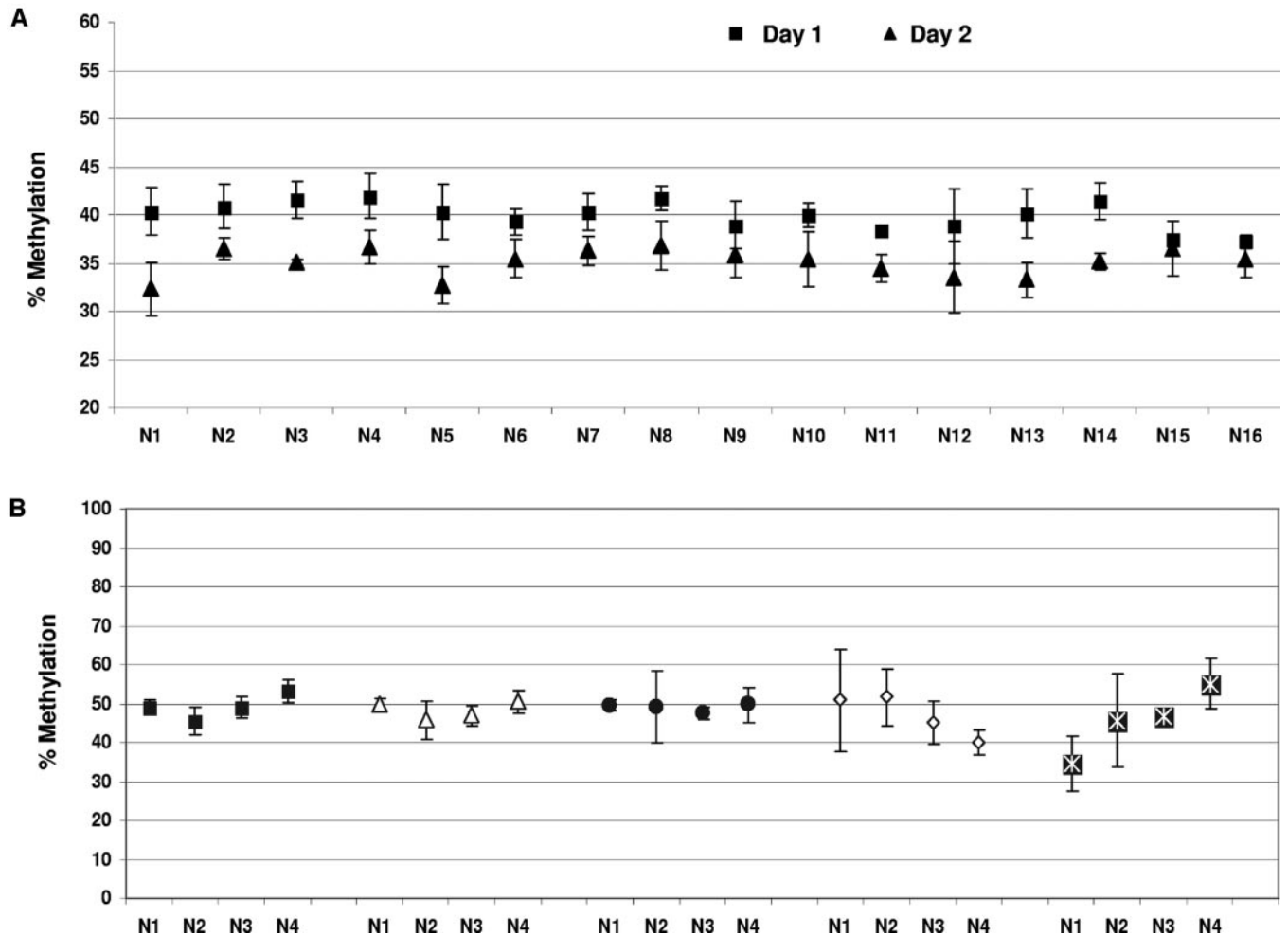

Fig. 3. Effects of bisulfite treatment and DNA concentration on absolute quantification of CpG methylation at CpG site U, as determined by pyrosequencing assay 1037.

(*A*), analysis of 16 control DNA samples (N1 to N16) treated with bisulfite on day 1 (■) and day 2 (▲). The methylation values (*% Methylation*) are the AQ values for the percentage of G and have not been corrected for methylation bias. The *error bars* indicate the standard deviation for triplicate analyses. (*B*), analysis of 4 control samples (N1 to N4) in which 50 ng (■), 20 ng (△), 10 ng (●) 5 ng (◇), and 2.5 ng (⊠) of template DNA was added to the PCR. The values for percentage of methylation have been corrected for methylation bias. The *error bars* indicate the standard deviations for triplicate analyses.

PYROSEQUENCING ASSAYS

Pyrosequencing assays were performed on the products from triplicate PCRs from all DNA samples in a blinded fashion. Data were analyzed by use of AQ software, which calculated the percentage of methylated (maternal) and unmethylated (paternal) alleles as chemically induced C/T sequence differences (or G/A), respectively, at each CpG site. Each position was given a quality score by the software; passed, checked, or failed. Replicates that had failed or that had checked results for 3 or more CpG sites were excluded from the analysis. Data were corrected for PCR amplification bias by use of the appropriate calibration curve. The samples were scored as being indicative of AS, PWS, or unaffected, and the results were compared with the original diagnosis obtained by MS-PCR. Once the assays had been fully optimized, the 152 samples were correctly identified; the results are shown in Fig. 4.

Analysis of CpG sites U, T, S, and R by assay 1037 revealed some unexpected methylation patterns for 5 AS patients and 1 PWS patient. In 5 AS cases (maternal deletions), methylation was detected at CpG sites S and R, with values ranging from 13% to 36.5%. These results were confirmed by pyrosequencing of the CpG sites in the opposite orientation (data not shown). One PWS patient (PWS58, maternal UPD) showed unexpected loss of methylation at CpG site R. DNA sequencing revealed that this patient had a C-to-T sequence change at this site. Outlier samples were observed in the control population (indicated by plus signs in Fig. 4).

With the exception of CpG sites S and R in assay 1037, the 3 pyrosequencing assays were capable of providing unambiguous classification of PWS, AS, or control samples. The mean (SD) percentages of methylation for each CpG site for each group of patients are shown in Table 1. The 3 diagnostic categories are clearly distinct, and no overlapping values for percentage of methylation were observed when the range of methylation values for each category was defined as being 3 SD from the mean. Classification of the samples was 100% concordant with data obtained from MS-PCR.
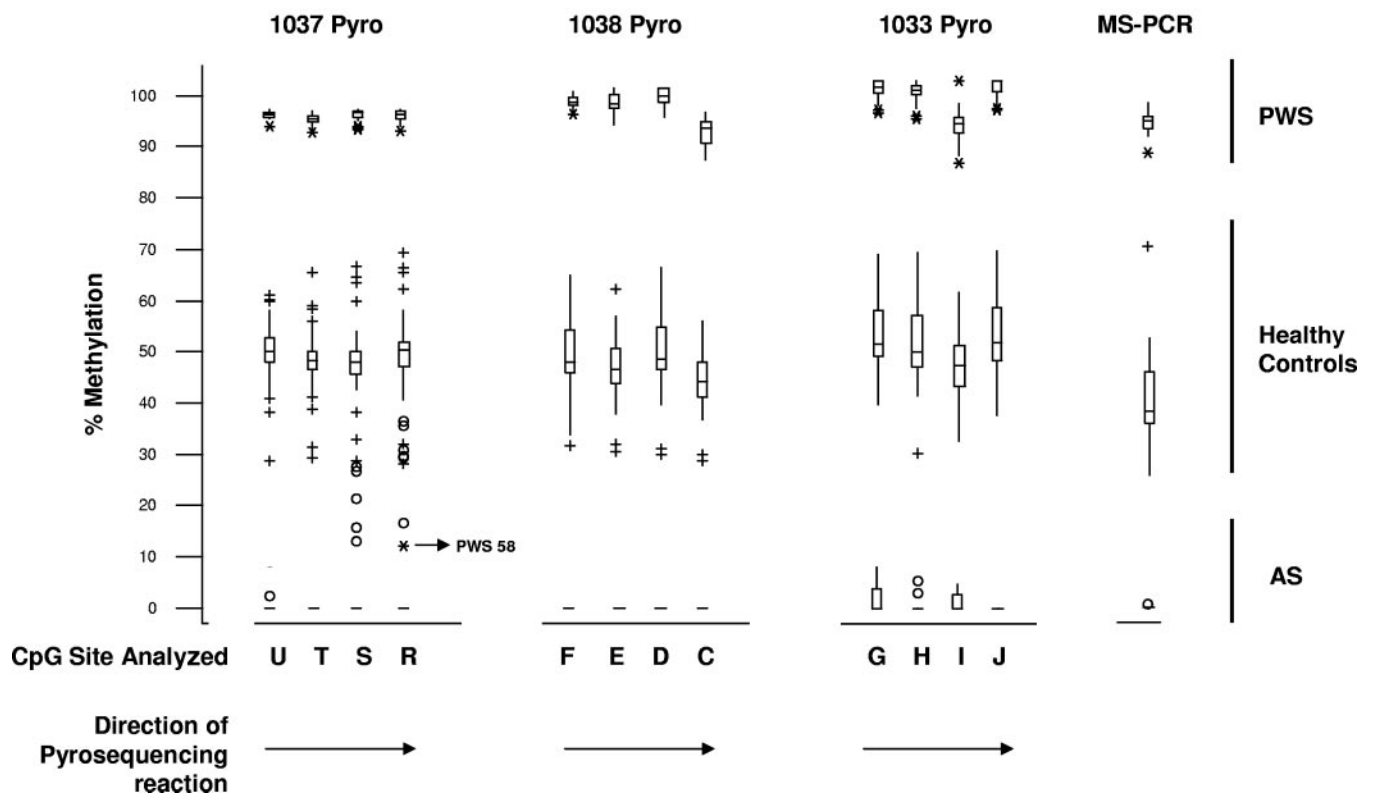

Fig. 4. *Box plots* showing distribution of AQ data corrected for PCR amplification bias for each diagnostic category for pyrosequencing assays 1037, 1038, and 1033 and the distribution of values for percentage of methylation obtained with the fluorescent MS-PCR protocol.

The data are shown for each CpG site analyzed in each assay. The *boxes* indicate the distribution of data between the 1st and 3rd quartiles (Q1 and Q3). The *line inside* each *box* indicates the median value, and the *whiskers* extend to 1.5 times the interquartile range of Q1 and Q3. For AS, in some cases, only the median value (0%) is shown (indicated by a *horizontal line*) because no variation was observed. Outlier samples are indicated as ○ (AS), + (healthy controls) and ∗ (PWS). The 5 AS samples with apparent methylation at CpG sites S and R (assay 1037) can been seen as ○, and PWS sample 58 with a homozygous C-to-T sequence change at CpG site R is also identified. Outlier samples observed at individual CpG sites (except CpG sites R and S) for the AS and PWS population were attributable exclusively to the presence of a checked result at a single CpG position, which caused the mean result of the triplicate analysis to deviate from the expected 100% methylation for PWS patients and 0% methylation for the AS patients. None of these samples were classified as outliers for more than a single CpG site within an individual assay. Outliers in the control population may be an artifact of bisulfite treatment or variable DNA quality, which is often found with diagnostic samples or may reflect typical population variation.

Table 1. Means and standard deviations of the percentage of methylation detected at each CpG site for samples from patients with PWS or AS, and healthy controls.[a]

| | | Methylation, % | | | | | | | | |
| | | AS (n = 31) | | | Healthy controls (n = 81) | | | PWS (n = 40) | | |
Assay	CpG site[b]	Mean	SD	Mean ± 3 SD	Mean	SD	Mean ± 3 SD	Mean	SD	Mean ± 3 SD
1038	C	0	0	0	44.5	5.3	28.6–60.4	92.9	2.4	85.7–100
	D	0	0	0	49.8	5.9	32.1–67.5	99.9	1.4	95.7–100
	E	0	0	0	47.2	5.2	31.6–62.8	98.7	1.8	93.3–100
	F	0	0	0	49.7	5.9	32.0–67.4	98.9	1.1	95.6–100
1033	G	1.6	2.2	0–8.2	53.2	6.0	35.2–71.2	100	1.6	95.2–100
	H	0	0	0	51.8	6.8	31.4–72.2	100	1.9	94.3–100
	I	0.9	1.4	0–5.1	47.3	6.2	28.7–65.9	94.2	3.2	84.6–100
	J	0	0	0	53.2	5.5	36.7–69.7	100	1.8	94.6–100
1037	R[c]	0	0	0	50.1	5.7	33–67.1	96.2	0.9	93.7–98.9
	S[c]	0	0	0	48.1	5.3	32.1–64	96.4	1.0	93.4–99.4
	T	0	0	0	48.3	4.8	33.9–59.4	95.5	0.9	92.8–98.2
	U	0.1	0.5	0–1.6	50.2	4.6	36.4–64	96.3	0.8	93.9–98.7
MS-PCR	B-F, L-P	0	0	0	40.2	7.3	18.3–62.1	94.8	1.9	89.1–100

[a] Each category can be clearly differentiated because the ranges of percentage of methylation for each group (presented as the mean ± 3 SD) show no overlap.

[b] As assigned by Zeschnigk et al. (9).

[c] Data for the 5 unusual AS syndrome cases and the PWS patient (PWS58) with a homozygous C-to-T sequence change at CpG R have been excluded from this analysis.

MS-PCR

Triplicate analysis of DNA samples by MS-PCR (11) was performed in a blinded fashion. The box-plots for the MS-PCR data are shown in Fig. 4, and the mean (SD) percentage of methylation for each diagnostic category is shown in Table 1. The 3 diagnostic groups were clearly distinct when the range of methylation for each category was defined as being 3 SD from the mean. An outlier sample was observed in the control population that had higher than normal percentages of methylation.

COST-EFFECTIVENESS AND SPEED OF ANALYSIS

The costs for the pyrosequencing assays and fluorescent MS-PCR using 2005 list prices were comparable, with the cost per sample being £1.20 (GBP) and £1.17, respectively, excluding system costs and instrument maintenance contracts. The analysis time for pyrosequencing is 1 h for up to 96 samples. Analysis times for MS-PCR varies depending on the type of genetic analyzer used.

Discussion

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 are suitable for use in the diagnosis of PWS and AS by detecting allelic methylation differences are therefore of clinical importance. Several molecular strategies have been used to study allelic methylation differences at the *SNRPN* locus in PWS/AS: Southern blotting]for examples, see, Refs. (18, 19)], MS-PCR [for examples, see Refs. (10, 11, 20–22)], PCR after restriction digestion of bisulfite-treated DNA (23), and

methylation-specific multiplex ligation-dependent probe amplification (24). In general, these methodologies are qualitative with the exception of MS-PCR and methylation-specific multiplex ligation-dependent probe amplification, which are considered to be semiquantitative. After bisulfite treatment of DNA, the resulting C/T sequence differences can be used to more accurately quantify allelic methylation differences via a variety of techniques, including denaturing HPLC (25), matrix-assisted laser desorption/ionization mass spectrometry (26), adaptations of SnaP-shot™ (13), and Pyrosequencing (13–16).

Using pyrosequencing, we analyzed bisulfite-treated DNA samples from 81 healthy controls, 40 patients with PWS, and 31 patients with AS and compared the results with those obtained from a commonly used MS-PCR protocol (11). We have demonstrated that the assays can be used for the accurate diagnosis of PWS/AS and can also be used to simultaneously quantify the degree of methylation at multiple CpG sites in a single assay. Analysis of pyrosequencing data showed complete segregation of the 3 diagnostic categories (Table 1); therefore, these assays can be used individually or in combination to diagnose 100% of PWS cases and ~80% of AS cases with a sensitivity and specificity of 100%. The assays should also be capable of detecting rare cases of mosaicism. Construction of calibration curves for the pyrosequencing assays (Fig. 2) showed PCR bias that is indicative of preferential amplification of the unmethylated allele. This means that the lowest reliable percentage of methylation detected by these assays is ~10%. Theoretically, mosaic samples with 10% methylation would produce AQ values of 2.7%. It is important to be aware that if results from the

AQ software are to be used in a truly quantitative manner, the AQ data require correction by use of the appropriate equation from the calibration curve generated by mixing DNA from a PWS and an AS patients before PCR. This is particularly important if mosaicism is suspected.

Factors that may affect the reproducibility of absolute quantification of individual CpG sites include bisulfite treatment and DNA concentration/quality. We have shown that the DNA samples treated with bisulfite on different days show variation in degree of methylation ranging from 0.8% to 9% for CpG site U in assay 1037 (Fig. 3). Variability can be controlled for by ensuring that calibration curves are constructed from DNA treated with bisulfite at the same time as the test samples and that the amount of DNA treated is accurately quantified. Low concentrations (<10 ng) of template DNA (and presumably poor DNA quality) can also affect absolute quantification because PCR amplification bias can be introduced in the first few cycles of PCR. We suggest that samples be amplified in triplicate so that variations in amplification bias can be easily detected. Samples with variable replicates or those with more than 2 checked or any failed positions after analysis with the AQ software should be treated with caution and re-tested with higher concentrations of template DNA.

Methylation analysis by pyrosequencing has several theoretical advantages over MS-PCR because it allows quantification of methylation at multiple CpG sites and the assays have several built-in quality controls. Pyrosequencing assays are designed to include a bisulfite treatment control, in which the analysis of a cytosine that is not present at a CpG site is analyzed as a C/T sequence variation. Because this cytosine residue always remains unmethylated, it should undergo full conversion to uracil after bisulfite treatment (Fig. 1). AQ data generated by the PSQ MA system is scored for confidence as passed, checked, or failed, which alerts users to the quality of the assay. The CpG sites are presented in sequence context, and therefore sequence variants will be identified, as in the case of the PWS patient who harbored a C-to-T point change at CpG site R. Additional information can be gained about subtle methylation changes at individual CpG sites, as in the case of the 5 AS patients whose samples exhibited methylation at CpG sites R and S. Such information could be useful both clinically and in a research context to further understand the epigenetics of PWS/AS.

The pyrosequencing methylation assays used in this study were easy to design and optimize and could detect and quantify DNA methylation at the *SNRPN* gene locus, allowing the unambiguous diagnosis of PWS/AS in a diagnostic setting. The costs and time of analysis of MS-PCR and pyrosequencing are similar, but pyrosequencing has advantages in terms of quality control and the additional information gained from the assay data. Results from this study suggest that pyrosequencing could be developed as a diagnostic tool for other methyl-ation disorders, such as Beckwith–Wiedemann syndrome, for which mosaicism is more common and absolute quantitative analysis of methylation at individual CpG sites would be essential.

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