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A Single-Tube PCR Test for the Diagnosis of Angelman and Prader-Willi Syndrome Based on Allelic Methylation Differences at the SNRPN Locus

Key Words

Prader-Willi syndrome
Angelman syndrome
DNA methylation
PCR

Abstract

The analysis of allelic methylation differences in 15q11-q13 has been established as a valid test for the Angelman and Prader-Willi syndromes. Current tests use methylation-sensitive restriction enzymes and Southern blot analysis. Here we describe a single-tube PCR test. It is based on sodium bisulfite treatment of DNA, which converts unmethylated, but not methylated cytosine residues to uracil, and PCR primers specific for the maternal and the paternal allele. The method was validated in a blinded retrospective study on 87 DNA samples from normal controls and patients. Prospective studies by independent laboratories will be needed before this assay can replace Southern blot analysis in routine diagnostic procedures.

Introduction

The Angelman syndrome (AS) and the Prader-Willi syndrome (PWS) are distinct neurogenetic disorders which are caused by a deficiency of maternal (AS) or paternal (PWS) contributions for chromosome 15. The affected genes are located in an imprinted chromosomal domain of 2 Mb within 15q11-q13. At the molecular level, the paternal and maternal copies of this region can be distinguished by DNA methylation, DNA replication timing, and gene expression. Each of these differences can be employed for a diagnostic test [1-3]. Based on extensive data obtained by methylation analysis of PWS and AS patients with PW71B and SNRPN probes [1, 4-9], the ASHG/ACMG Test and Technology Transfer Committee has established this analysis as a scientifically and clinically valid test [10].

Current methylation tests for AS or PWS are based on Southern blot analysis of DNA cleaved with methylation-sensitive restriction enzymes such as *HpaII* or *CfoI* for the PW71B locus or *NotI* for the SNRPN locus [4, 7, 8]. The tests are reliable, but Southern blot analysis has inherent disadvantages. It works best with radioactive probes, and rare restriction fragment length variants and partial cleavage may complicate interpretation. Partial cleavage is especially a problem with *NotI*, which is used for SNRPN, and can lead to false-positive PWS and false-negative AS results.

A more sensitive method to detect DNA methylation combines the use of methylation-sensitive restriction enzymes and PCR [11]. After digestion of the DNA with the enzyme, uncut DNA molecules are amplified with primers flanking the enzyme recognition site. This approach is not useful for analyzing allelic methylation differences, because only the methylated allele can be studied directly.

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Furthermore, it is very sensitive to partial digests, because any uncleaved DNA will be amplified and yield a false-positive result. These problems can be circumvented by using the methylation-specific PCR assay described by Herman et al. [12]. This assay entails initial modification of DNA by sodium bisulfite, which converts unmethylated, but not methylated cytosine residues to uracil, and subsequent amplification with primers specific for methylated versus unmethylated DNA.

By using the bisulfite protocol of genomic sequencing [13, 14], we have recently studied allelic methylation differences around SNRPN exon 1 and PW71 [15]. After bisulfite treatment, the paternal and maternal copies of these regions differ by DNA sequence and can be amplified specifically. Based on this strategy, we have developed a single-tube PCR assay for AS and PWS. We have chosen the SNRPN exon 1 region, because the density of differentially methylated CpG dinucleotides in this region is higher compared to D15S63 [15], and the determination of differential SNRPN methylation can be applied to blood, lymphoblastoid cell lines, cultured amniotic fluid cells, and chorionic villus samples [16].

Materials and Methods

Genomic DNA Samples

Genomic DNA from patients and normal controls was prepared as described by Kunkel et al. [17]. DNA samples were typed by Southern blot methylation analysis (PW71B) and by chromosome 15 restriction fragment length polymorphisms or microsatellites [1, 4, 18, 19, and unpubl. data].

Bisulfite Treatment

Genomic DNA (4 µg in 70 µl) was denatured for 15 min at 37 °C by adding 8 µl freshly prepared 3 M NaOH. For complete denaturation, the samples were incubated at 95 °C for 3 min and immediately cooled on ice. The bisulfite solution was prepared by dissolving 8.1 g sodium bisulfite (Sigma) in 15 ml degassed water, adding 1 ml of 40 mM hydroquinone and adjusting the pH to 5.0 by adding 600 µl of 10 M NaOH. The denatured DNA solution was mixed with 1 ml bisulfite solution, overlaid with mineral oil, and incubated at 55 °C for 16 h in a water bath in the dark. The DNA was recovered by using 5 µl glassmilk (GeneClean II Kit, Bio 101 Inc.) and eluted in 100 µl H₂O. Subsequently, 11 µl of 3 M NaOH was added, and the sample was incubated for 15 min at 37 °C. The solution was then neutralized by adding 110 µl of 6 M NH₄OAc, pH 7.0. The DNA was precipitated with ethanol, washed in 70% ethanol, dried and resuspended in 20 µl H₂O. The concentration of bisulfite treated DNA was estimated with the help of DNA DipSticks™ (Invitrogen, San Diego, Calif., USA).

Polymerase Chain Reaction

PCR was performed in a reaction volume of 25 µl in a Perkin-Elmer GeneAmp PCR System 9600 using the following conditions:

50–100 ng of bisulfite-treated DNA, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 1 µM each of primers MAT and COMMON, 0.25 µM of primer PAT, 225 µM of each dNTP and 0.5 units AmpliTaq (Perkin Elmer). After initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s and extension at 72 °C for 30 s were performed, followed by a final extension at 72 °C for 5 min. PCR products were analyzed on 2% agarose gels. The primer sequences were as follows:

MAT

5'-TATTGCGGTAAATAAGTACGTTTGCGCGGTC-3'

PAT

5'-GTGAGTTTGGTGTAGAGTGGAGTGGTTGTTG-3'

COMMON

5'-CTCCAAAACAAAAACTTTAAAACCCAAATTCC-3'

Results

Rationale

Sodium bisulfite converts unmethylated, but not methylated cytosine residues to uracil. After modification, opposite DNA strands are no longer complementary, and a PCR reaction on this DNA is strand specific. We have developed primers that amplify the sense strand of the SNRPN exon 1 region. PCR is initiated with a primer annealing to a site downstream of exon 1, which is identical in both parental alleles (fig. 1). As the sense strands of the paternal and the maternal alleles differ at CpG dinucleotides showing parent-of-origin specific methylation, a paternal and a maternal specific DNA strand is synthesized. In the second PCR cycle, DNA is synthesized with a primer binding specifically to the paternal strand and a primer binding specifically to the maternal strand. In the following cycles, the maternal and the paternal alleles are specifically amplified in a duplex PCR reaction sharing the common primer. To distinguish between the two parental alleles, the specific primers are chosen so that the maternal product is 313 bp and the paternal product is 221 bp in length. In this way, the agarose gel pattern resembles a Southern pattern in which the methylated allelic fragment is uncut by the restriction enzyme and therefore longer than the paternal allelic fragment. Unreacted DNA is not amplified, because the primers do not anneal to unmodified DNA.

Methylation-Specific PCR

PCR conditions were optimized using DNA samples from normal controls, PWS patients and AS patients. By using 50–100 ng of DNA and 1 µM of each primer, the paternal product was more abundant than the maternal product, and PWS samples exhibited a faint paternal band (data not shown). Therefore, the concentration of

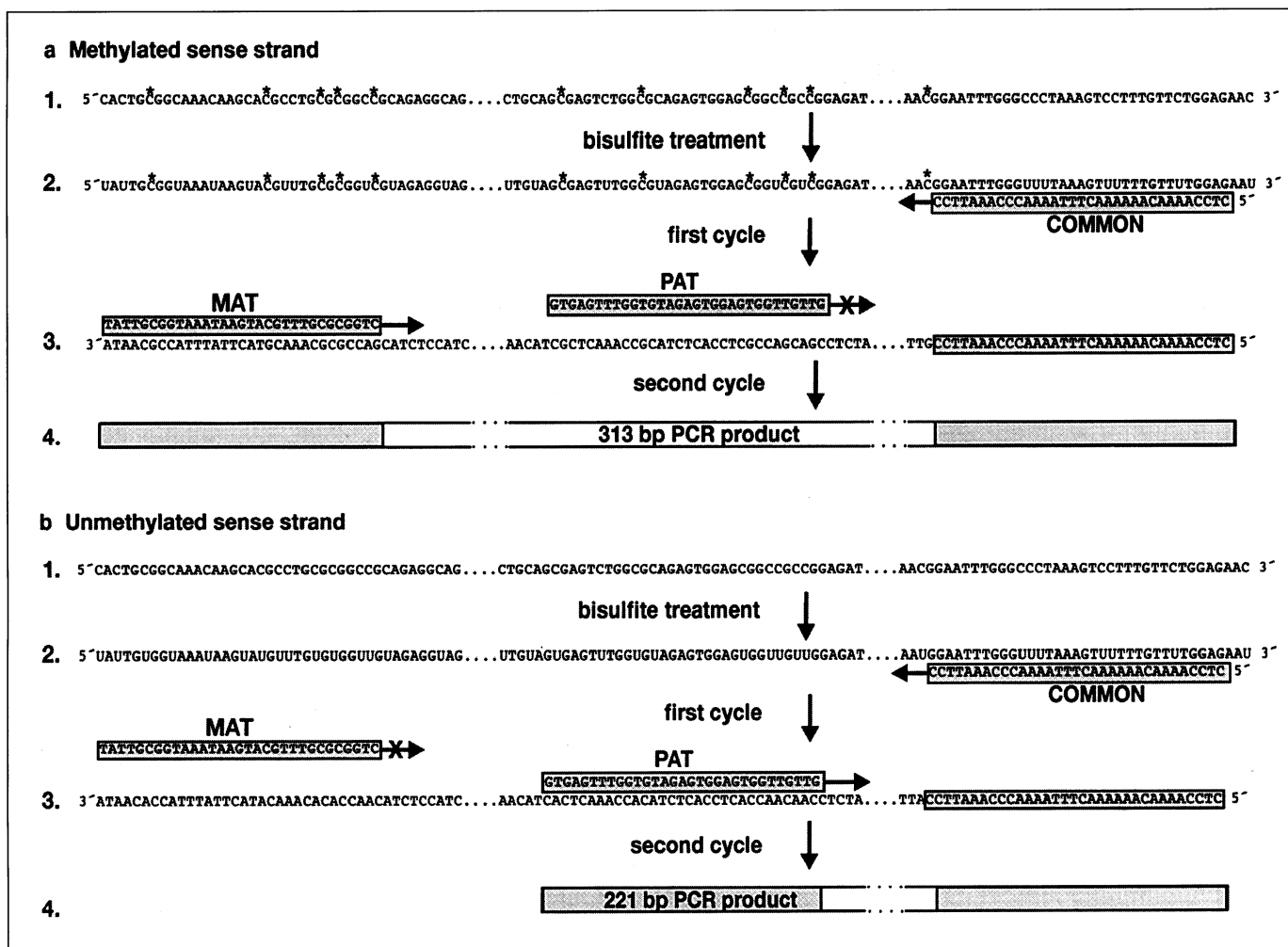


Fig. 1. Schematic overview of the bisulfite treatment and amplification of methylated (a) and unmethylated (b) DNA. The relevant parts of the sense strand sequence of the SNRPN exon 1 region before and after bisulfite treatment [15] are presented in the first two lanes each. Sequence interruptions are indicated by dots, and methylated cytosine residues are marked by an asterisk. PCR primers are

boxed. In the first cycle, the COMMON primer anneals to a site identical in both DNA molecules, but directs the synthesis of two different antisense strands (lanes 3). In the second cycle, the MAT and PAT primers bind specifically to the two antisense strands and direct the synthesis of a 313-bp and a 221-bp DNA fragment, respectively (lanes 4).

the paternal primer was lowered to 0.25 μ M. When using these conditions, we obtained a maternal and a paternal PCR product of similar intensity in normal controls. PWS had a visible maternal band only, whereas AS patients had a visible paternal band only (fig. 2). No PCR products were observed with untreated DNA. The addition of untreated DNA to bisulfite-treated DNA did not affect the PCR results (data not shown).

To validate the test, we performed a blinded retrospective study on 87 DNA samples (86 blood samples and one chorionic villus sample) previously studied by Southern blot methylation analysis and microsatellite typing.

The following samples were investigated: 30 normal controls including the chorionic villus sample, 15 AS patients with a maternal deletion, 3 AS patients with an imprinting defect, 18 PWS patients with a paternal deletion, 18 PWS patients with maternal uniparental disomy, and 3 PWS patients with an imprinting defect. The samples were coded, so that the person performing the tests did not know the identity of the samples. After the PCR products were analyzed, the code was broken and the results were compared. All 87 samples had been correctly identified.

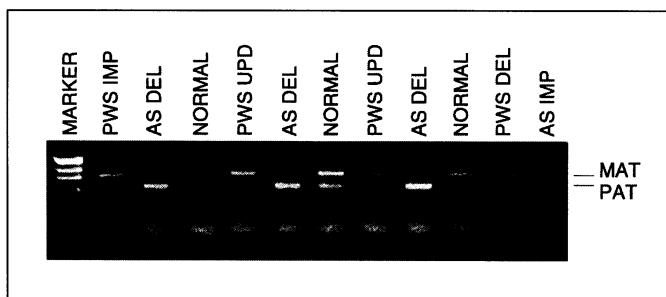


Fig. 2. PCR analysis of patients and normal controls. PWS IMP = PWS patient with an imprinting mutation; PWS UPD = PWS patient with maternal uniparental disomy; PWS DEL = PWS patient with a paternal deletion 15q11-q13; AS IMP = AS patient with an imprinting mutation; AS DEL = AS patient with a maternal deletion 15q11-q13; NORMAL = normal control; MARKER = *MspI*-digested pUC19 DNA; MAT = maternal PCR product; PAT = paternal PCR product.

Discussion

We have developed a single-tube PCR test for the diagnosis of PWS and AS based on allelic DNA methylation differences identified by the bisulfite protocol of genomic sequencing. As demonstrated by the addition of untreated DNA, the results are not affected by incomplete bisulfite treatment. However, the relative concentrations of the maternal and the paternal primers appear to be critical for obtaining maternal and paternal bands of similar intensity. Optimal results were obtained when the paternal primer was present at a four-fold lower concentration compared to the maternal primer. New batches of primers have first to be tested on control samples, and the relative primer concentrations have to be adjusted, if necessary. The presence of a faint paternal product in PCR reactions containing PWS DNA and equimolar primer concentrations may reflect a low degree of methylation mosaicism in the blood. This notion is substantiated by the finding of some hypomethylated DNA molecules in PWS patients, as determined by genomic sequencing of the SNRPN exon 1 region [15]. Despite this problem, the PWS pattern was always different from a normal pattern.

The test was validated in a blinded retrospective study and may facilitate the testing of patients suspected of having PWS or AS. This possibility is especially important, because a significant fraction of hypotonic newborns may have PWS [4]. Furthermore, the PCR test does not use radioactive reagents. Like Southern-blot-based methylation analysis, it detects deletions, uniparental disomy and imprinting defects, although it does not distinguish

between these lesions. In contrast to PWS, about 25% of AS patients have a normal methylation pattern. These patients appear to have mutations in the UBE3A gene [20, 21], and UBE3A mutation screening has to be performed in methylation-normal AS patients.

Monaghan et al. [22] have recently calculated that a diagnostic approach beginning with methylation studies becomes increasingly economical as the percentage of affected cases falls below 50%. The latter is true for most referrals to a routine diagnostic laboratory. In the past 5 years, for example, our laboratory has studied 1,050 patients suspected of having PWS and has identified 309 (29%) positive cases. The use of a methylation-specific PCR test, which is cheaper than a Southern blot test, renders a diagnostic approach beginning with methylation studies even more economical.

Hitherto, only one other PCR test for PWS has been described [3]. This test is based on the monoallelic expression of the SNRPN gene in blood cells. It entails the isolation of RNA, reverse transcription, and PCR analysis. In contrast to the methylation-specific PCR test, it cannot be used for AS. Furthermore, RNA is more labile than DNA. We believe that a DNA-based PCR assay is more robust, although larger prospective studies by independent laboratories will be needed before this test can replace Southern blot tests. Similar protocols have been developed in other laboratories [23] and these protocols need to be compared before a particular test can be recommended for routine diagnostic procedures.

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