

A rapid, PCR based test for differential molecular diagnosis of Prader-Willi and Angelman syndromes

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Abstract

Approximately 98% of Prader-Willi syndrome (PWS) and 80% of Angelman syndrome (AS) cases have deletions at a common region in chromosome 15q11-13, uniparental disomy for chromosomes 15 (UPD15), or mutations affecting gene expression in this region. The resulting clinical phenotype (PWS or AS) in each class of mutation depends upon the parent of origin. Both disorders are characterised at the molecular level by abnormal methylation of imprinted genes at 15q11-q13 including the small nuclear ribonucleoprotein N gene (SNRPN). Current diagnostic strategies include high resolution cytogenetics, fluorescence in situ hybridisation (FISH), Southern blot hybridisation, or microsatellite typing.

We have developed a novel and rapid diagnostic test for PWS and AS based on differential digestion of expressed (paternally imprinted) SNRPN sequences by the methylation sensitive endonuclease *NotI* or repressed (maternally imprinted) SNRPN sequences by the methylation requiring nuclease *McrBC*, followed by PCR amplification of the SNRPN promoter. We have evaluated this test by blinded analysis of 60 characterised DNA samples (20 PWS, 20 AS, and 20 unaffected controls). SNRPN sequences could not be amplified from PWS patient DNA which had been digested with *McrBC*, nor from AS patient DNA which had been digested with *NotI*. We were able to make a correct diagnosis of PWS, AS, or unaffected in all 60 samples tested. This novel test is rapid and has a high specificity and sensitivity for deletion and UPD15 cases. These features make this new test suitable as the initial step in a molecular diagnostic strategy for PWS/AS.

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Prader-Willi syndrome (PWS) and Angelman syndrome (AS) are complex and distinct mental retardation syndromes caused by genetic lesions in a common chromosomal region, 15q11-q13.^{1,2} The characteristic PWS phenotype includes short stature, sexual infantilism, hypotonia, mild to moderate mental retardation and behavioural problems, characteristic facies, hyperphagia leading to obesity, and a

tendency to diabetes in adolescence.^{2,3} AS presents with a distinct phenotype including severe mental retardation, characteristic facial appearance with protruding tongue and inappropriate laughter, ataxic gait, microcephaly, seizures, little or absent speech, and jerky movements.^{1,4} Both syndromes can be difficult to diagnose clinically.

Approximately 70% of PWS and AS have deletions in a common region of 15q11-13 either on the paternal chromosome in PWS or the maternal chromosome in AS.^{1,2,5} Approximately 28% of PWS and 3-5% of AS cases are caused by uniparental disomy at chromosome 15 (UPD15, inheriting two normal copies of chromosome 15 from only one parent).⁶ PWS patients are disomic for maternal chromosomes 15 and AS patients disomic for paternal chromosomes 15.^{4,7} A small proportion (approximately 1% of PWS and 4% of AS) have neither deletions nor disomy, but abnormal patterns of methylation at loci in 15q11-q13 ("imprinting" mutations).⁸⁻¹¹ Rare PWS and AS patients have cytogenetically visible chromosome rearrangements involving 15q11-q13.¹² At least some of the 20% or so non-deletion, non-UPD15, normally methylated, and cytogenetically normal AS patients have mutations in the E6-AP ubiquitin-protein ligase gene (UBE3A).^{13,14} It is important to be able to distinguish the molecular pathology in PWS/AS for counselling purposes since a significant recurrence risk is associated with imprinting mutations, chromosomal translocations, and UBE3A mutations, but not with deletions and UPD.

Genes at 15q11-q13 show parent of origin specific expression patterns ("imprints"). PWS/AS caused by deletion, UPD15, or abnormal methylation is characterised by abnormal expression of imprinted genes. The gene for the small nuclear ribonucleoprotein polypeptide N (SNRPN) is the best characterised imprinted gene from this region^{9,10,15} and is a useful diagnostic marker for PWS and AS.¹⁶ PWS patients have only a maternal SNRPN imprint (methylated and inactivated) and AS patients have only a paternal SNRPN imprint (unmethylated and expressed).

A variety of complementary cytogenetic and molecular techniques are used to investigate PWS and AS, including high resolution cytogenetic analysis, fluorescent in situ hybridisation (FISH), methylation analysis by Southern blot hybridisation, and deletion/UPD analysis by microsatellite typing. Each technique has its own advantages and limitations.⁶

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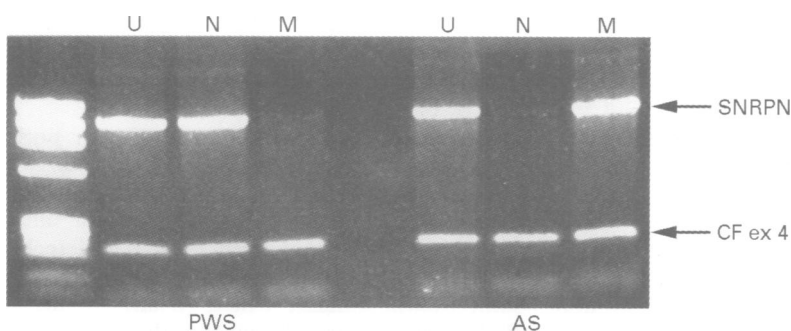


Figure 1 Molecular diagnosis of PWS and AS. Ethidium bromide stained agarose gel showing PCR products from PWS or AS DNA either undigested (U) or following *NotI* (N) or *McrBC* (M) restriction digestion. The 1088 bp SNRPN product is absent following *McrBC* digestion (track 4) or *NotI* digestion (track 6) confirming diagnosis of PWS and AS respectively. The CFTR exon 4 amplification control is present in all tracks as expected. Track 1 contains molecular weight markers.

Diagnostic method(s) of choice will depend upon local resources and expertise but many molecular genetics laboratories use a strategy in which the first step is to screen for deletions, disomy, or imprinting mutations by restriction digestion with methylation sensitive endonucleases followed by Southern blot hybridisation to probes such as PW71b (D15S63) or SNRPN.^{16 17}

Novel PCR based diagnostic tests for PWS and AS have recently been reported. Wevrck and Francke¹⁸ reported an assay for PWS only based on RT-PCR analysis to determine expression levels of SNRPN and two other groups independently reported assays based on modification of unmethylated DNA by sodium bisulphite before allele specific amplification of SNRPN sequences.^{19 20}

We present another novel test for SNRPN methylation status using differential restriction endonuclease digestion of genomic DNA and PCR. This strategy uses either *NotI* to digest unmethylated (paternally imprinted) DNA or *McrBC* to digest methylated (maternally imprinted) DNA followed by PCR amplification of the promoter and first exon of SNRPN to diagnose AS or PWS respectively.

Methods

PATIENTS

Twenty subjects, mostly from North Thames, were referred with a strong or suspected clinical diagnosis of PWS. Mean age at referral was 10 years (range 9 months to 49 years). Twenty patients from the same region were referred with suspected AS. Mean age at referral was 10 years (range 3 months to 39 years). In each case, molecular genetic analysis was requested to confirm the clinical diagnosis. Normal control DNA samples were from healthy members of the department and anonymised.

The diagnosis in each affected patient had been confirmed previously by Southern blot methylation analysis using probes at either D15S63 (PW71b) or SNRPN. UPD15 had been confirmed in 3/20 PWS and 2/20 AS patients by microsatellite analysis of the affected child and both parents.

PCR BASED METHYLATION ANALYSIS

Genomic DNA (approximately 500 ng) was digested with either *NotI* (20 units in a 20 μ l

reaction volume) or *McrBC* (10 units in a 10 μ l reaction volume) according to the manufacturer's instructions (New England Biolabs), but with additional GTP (to 2 mmol/l) and BSA (to 100 μ g/ml) in the *McrBC* reaction buffer. DNA was digested at 37°C overnight, followed by inactivation at 65°C for 20 minutes before PCR amplification.

PCR amplification was performed on 100 ng DNA either undigested (amplification control) or digested with *NotI* or *McrBC*. PCR included both "diagnostic" primers S1 and S2 (to amplify a 1088 bp fragment containing the SNRPN promoter and exon 1), and "amplification control" primers C1 and C2 (to amplify a 300 bp fragment of exon 4 of the CFTR gene). Primer sequences were: S1 (sense) 5'-AGGTCATTCCGGTGAGGGAGG-3'; S2 (antisense) 5'-CCCCTCCTCTAGACAGCAATGAT-3'; C1 (sense) 5'-CTATGACCCGGA TAACAAGGAGGAGC-3'; C2 (antisense) 5'-AGAATATATGTGCC ATGGGCCTGTG-3'. Each 50 μ l reaction contained 0.1 μ mol/l of each primer in 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 1.0 mmol/l MgCl₂, 200 μ mol/l dNTPs (Pharmacia), and 1 U *Taq* polymerase ("Amplitaq", Perkin Elmer). "Hot start" PCR amplification was used with a temperature profile of five minutes at 94°C, followed by 30 cycles of 94°C for one minute, 60°C for one minute, 72°C for one minute, followed by a final extension at 72°C for 10 minutes. PCR products (typically 12 μ l) were separated on 0.8% agarose minigels and visualised by ethidium bromide staining and UV illumination.

Results

This test provides differential molecular diagnosis of PWS and AS. PWS samples are expected to give SNRPN specific PCR products after only *NotI* digestion, AS after only *McrBC* digestion, and unaffected control DNA is expected to yield products after both *NotI* and *McrBC* digestion.

The test was evaluated on 60 genomic DNA samples, 20 each from patients with PWS and AS and 20 unaffected controls. The evaluation was performed with the disease status of each sample unknown to the operator. The methylation status of all 60 samples was scored correctly giving a test specificity of 100% and sensitivity (for PWS and AS caused by deletion or UPD15) also of 100%. Representative examples are shown in fig 1.

Discussion

We have developed a rapid, PCR based test for differential molecular diagnosis of Prader-Willi and Angelman syndromes. This test makes use of two restriction endonucleases with mutually exclusive methylation requirements to analyse the imprinted methylation status of the SNRPN promoter. *NotI* is methylation sensitive and *McrBC* has an absolute requirement for methylated substrate DNA (*McrBC* is the only commercially available enzyme we are aware of with such a requirement). The 1088 bp PCR product encompasses the SNRPN promoter and first exon. This region includes 23 CpG dinucleotides, more than 96% of

which are methylated (^mCpG) on maternally inherited chromosomes and effectively none of which are methylated on paternal chromosomes.²¹ Seventeen of these 23 CpG dinucleotides are immediately preceded by A or G and are therefore potential substrates for *McrBC* (recognition site: Pu^mC[N₄₀₋₂₀₀₀]/[N₄₀₋₂₀₀₀]Pu^mC). Four imprinted CpGs lie within *NotI* sites (recognition sequence GC/GGCCGC). These observations suggest that this novel test should be highly specific and sensitive for PWS and AS resulting from deletion, UPD15, or imprinting mutations. Our results support this since we were able correctly to diagnose the disease status of all 60 PWS, AS, or control samples.

Two groups have recently published PCR based methods of differential molecular diagnosis of PWS and AS.^{19,20} In both methods, patient genomic DNA was treated with sodium bisulphite (which converts unmethylated cytosine to uracil but does not modify methylated DNA) followed by PCR with primers specific to either the maternal or paternal alleles. In both reports, these methods correctly identified PWS, AS, or normal DNA in blinded analysis of samples of previously determined genotype.

The bisulphite based methods and our own method reported here appear to be completely effective at differentially diagnosing PWS and AS; however, each approach has potential advantages and disadvantages. Our method, based on differential restriction digestion, involves fewer technical steps than the reported bisulphite based tests and will be a familiar technique in molecular genetics laboratories. In addition, the published bisulphite based methods require PCR with either three¹⁹ or four²⁰ allele specific primers, increasing start up costs compared with our method.

There is the potential for incorrect typing with either approach. In the case of the bisulphite based methods there is the potential for preferential PCR amplification of smaller (paternal) alleles producing a false diagnosis of Angelman syndrome. The most important potential problem associated with our method is that of false negatives resulting from incomplete restriction digestion. In particular, some laboratories are known to observe partial restriction digestion with *NotI* in Southern blot hybridisation studies (although we have not encountered this problem). Partial *NotI* digestion is more likely to be a problem with Southern analysis where typically 5–10 µg of genomic DNA is digested than with the PCR protocol reported here where only 500 ng or less of genomic DNA is digested. However, a false PCR signal could potentially be seen if only a very small proportion of the *NotI* treated target molecules remain intact (although in such cases we might expect to observe band intensity differences between the normal control and partially *NotI* digested AS patient DNA). Our protocol aims to minimise the likelihood of partial digestion by treating 500 ng genomic DNA with 20 units of *NotI* overnight (approximately 16 hours). This should represent a significant overdigestion. In addition,

alongside each batch of patient samples, PWS, AS, and normal control DNA are analysed which should allow detection of partial digestion by either enzyme.

Despite these potential sources of error, both the bisulphite methods and our *NotI/McrBC* method present substantial advantages over previous techniques. Only a formal and blinded evaluation of both PCR based techniques on a common reference set of patient samples will indicate which (if any) of these PCR based assays will become the method of choice.

The test reported here will miss AS patients with *UBE3A* mutations and the very small proportion of PWS or AS patients with chromosomal abnormalities involving 15q11-q13. In addition, the test will not distinguish deletions from UPD15 or imprinting mutations. However, it will confirm diagnosis in the great majority of PWS and AS cases and is rapid, non-isotopic, requires a minimal blood sample from the affected child, and does not require parental samples. These features represent substantial advantages over existing approaches (Southern blot, microsatellite analysis, or FISH) and will make this test suitable as the initial screening step in a general diagnostic strategy to confirm or exclude a clinical diagnosis of Prader-Willi or Angelman syndromes.

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