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Original articles

Molecular screening for proximal 15q abnormalities in a mentally retarded population

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Abstract

Paternal or maternal deletions in the 15q11.2-q13 region are known to result in Prader-Willi syndrome (PWS) or Angelman syndrome (AS), respectively. Maternal duplications in 15q11.2-q13 have been found in patients with autism. A population of adults with moderate to profound mental retardation was studied to examine the usefulness of PCR based molecular methods in screening for proximal chromosome 15 abnormalities. Two hundred and eighty-five subjects were initially screened at five microsatellite markers with average heterozygosity values of 0.74 (range 0.54-0.82). Of these subjects, four had a single allele at all five loci, suggestive of a deletion or uniparental isodisomy. The four samples were further screened with additional markers located within 15q11.2-q13 as well as markers telomeric to this region. One subject had uniparental disomy (UPD) and three subjects had a deletion. To determine the parental origin of the 15q11-q13 region containing the single haplotype, samples were analysed with a newly developed methylation specific PCR technique at the SNRPN locus. Each of the four subjects showed presence of the paternal allele and absence of the maternal allele. All cases had a phenotype consistent with Angelman syndrome as expected for the level of mental retardation, but the subject with UPD was distinct from the other subjects with an absence of a history of seizures and presence of bilateral undescended testes and Parkinsonism. Although Angelman syndrome has an estimated population prevalence of 0.008 %, at least 1.4 % of the moderately to profoundly mentally retarded subjects screened were found to have Angelman syndrome.

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Prader-Willi syndrome (PWS) and Angelman syndrome (AS) represent a class of disorders which involve genomic imprinting. Deletions in the 15q11.2-q13 region are known to result in Prader-Willi syndrome (PWS) or Angelman syndrome (AS) when the deletion is on the paternal or maternal chromosome, respectively. While both PWS and AS include mental deficiency, the syndromes are clinically and genetically distinct.

Infants with PWS are characterised by decreased fetal activity, muscular hypotonia, short stature, obesity, hypothalamic hypogonadism, and small hands and feet. Mental retardation (MR) is usually mild.[1] In contrast, AS tends to be difficult to diagnose until after the age of 2 years and is characterised by seizures, hypotonia, hyperreflexia, hypopigmentation, excessive laughter/smiling, a jerky gait, and moderate to profound MR.[2–9]

While both PWS and AS are characteristically associated with deletions in the 15q11.2-q13 region, there have been case reports of duplications in this region giving rise to abnormal phenotypes. An association between large inverted duplications of chromosome 15 (pseudodicentric 15), containing the PWS/AS critical region, and an abnormal phenotype which includes developmental delay has been reported.[10 11] In several cases, a small pseudodicentric 15 was associated with PWS or AS owing to UPD.[12] Cases of autism with a maternally inherited duplication of the PWS/AS region have been reported.[13–17] Cook *et al*[18] have reported a family in which the mother is phenotypically normal with a de novo paternally inherited duplication of proximal 15q. Of the three children in this family, two have autism or atypical autism and have maternal inheritance of a 15q11-q13 duplication. A third child who did not inherit this duplication is unaffected. In this family, a paternally inherited duplication is phenotypically silent while a maternally inherited duplication results in autism, suggesting a parent of origin effect for these duplications.

As duplications and deletions in 15q11.2-q13 are shown to be significant in PWS and AS and, more recently, autism, molecular detection of such abnormalities becomes more important. A population of adults with moderate to profound MR was studied to examine the prevalence of proximal chromosome 15 abnormalities detectable by automated microsatellite analysis.

Table 1 Comparison of study subjects with entire resident population

	Study	*Entire population*
No	285	1056
Male (%)	59	59
Age (SD)	37.5 (11.4)	36.3 (13.3)
Profound MR (%)	86	83
Severe MR (%)	11	13
Moderate MR (%)	2	3

Methods

SUBJECTS

As part of a Human Subjects Protection Committee approved study among subjects with mental retardation, letters were sent to the parents or guardians of the entire resident population of a large developmental centre. Consent was obtained for 40% of the population, and 76% of these subjects were tested using methods described below.

As indicated in table 1, the study population did not differ from the population as a whole with regard to age, severity of mental retardation, or gender. Sixty-nine percent of the study population was on at least one psychotropic medication, predominantly carbamazepine and phenobarbital. Of this sample, approximately 40% required skilled nursing care and were non-ambulatory. Of this 40%, two-thirds were on anticonvulsants.

DNA EXTRACTION

Blood was collected by venepuncture into ethylenediaminetetraacetic acid (EDTA) containing vacutainer tubes. DNA was isolated by Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN). Final DNA concentration was measured on a Lambda 2 spectrophotometer (Perkin-Elmer, Norwalk, CT) and diluted to a concentration of 50 ng/µl.

MULTIPLEX PCR

Five markers, D15S97,[19] GABRB3 155CA-2,[20] D15S122, D15S128, and D15S156,[21] were selected based on location within the PWS/AS region.[22] D15S122 was selected for its location within the UBE3A gene.[8] A high degree of heterozygosity was selected for in order to increase the probability that a subject found to be homozygous at the five selected alleles would have a deletion in this region rather than being a true homozygote for all five alleles. The published heterozygosity for the markers was as follows: D15S122 (0.79), D15S128 (0.79), D15S156 (0.54), S15S97 (0.75), GABRB3 155CA-2 (0.82).

PCR amplification of markers (PCR 1) was carried out in a final volume of 10 µl consisting of 50 ng genomic DNA, 10 mmol/l Tris HCl, 50 mmol/l KCl, 0.001% gelatin, 2.5 mmol/l $MgCl_2$, 0.3 units *Taq* Gold polymerase, 200 µmol/l deoxyribonucleotides, and sense and antisense primers of the following concentrations: 100 nmol/l D15S128, 400 nmol/l D15S122, 230 nmol/l D15S97, and 76 nmol/l D15S156 (Research Genetics, Huntsville, AL). Samples were processed in a Gene Amp PCR system 9600 (Perkin-Elmer, Norwalk, CT). Initial activation step for heat activated DNA polymerase was carried out at 94°C for 12 minutes, followed by 38 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Final extension was at 72°C for 30 minutes. Markers were optimised starting with equal concentrations of all markers and were adjusted with the goal of individual peak heights at approximately one-third maximum fluorescence detection limit.[23] A second PCR (PCR 2) was run for GABRB3 155CA-2. The final volume of 10 µl consisted of 50 ng genomic DNA, 10 mmol/l Tris HCl, 50 mmol/l KCl, 0.001% gelatin, 1.5 mmol/l $MgCl_2$, 0.6 units *Taq* polymerase, 200 µmol/l dATP, 200 µmol/l dCTP, 200 µmol/l dTTP, 100 µmol/l 7-deaza-dGTP, 100 µmol/l dGTP, 5% by volume DMSO, and sense and antisense primers of the following concentrations: 325 nmol/l GABRB3 155CA-2 (Applied Biosystems, Foster City, CA). Samples were amplified in a Perkin Elmer Gene Amp PCR system 9600. Initial activation for DNA polymerase was carried out at 95°C for two minutes, followed by 41 cycles at 95°C for 30 seconds, 61°C for 30 seconds, and 72°C for 60 seconds. Final extension was at 72°C for 10 minutes.

Four additional markers were run for homozygous subjects to confirm homozygosity within the PWS/AS region: D15S217,[24] D15S10,[25] D15S113,[26] and D15S1506.[27] The following markers were run to determine homozygosity outside the PWS/AS deletion region: D15S117, D15S120, D15S126, D15S127, D15S131, D15S153, D15S165, and D15S205.[28]

SIZING OF ALLELES

PCR products were combined for analysis in the ABI PRISM 310 Genetic Analyser (Applied Biosystems, Foster City, CA) in a final volume consisting of 12 µl deionised formamide, 0.5 µl TAMRA 500 size standard, 0.4 µl PCR 1, and 0.2 µl PCR 2. Products were separated using POP4 polymer and sized with Genescan and Genotyper software (Applied Biosystems, Foster City, CA).

METHYLATION SPECIFIC PCR ASSAY (M-PCR)

To determine if there was an absence of maternal or paternal alleles in the PWS/AS region, M-PCR at SNRPN was performed with the four subjects who had complete homozygosity in the PWS/AS region as previously described.[29]

Results

Two hundred and eighty-five patients were screened at five microsatellite markers in the PWS/AS region. Of these, four (1.4 %) were homozygous at all five loci (fig 1). These four samples were then tested with additional markers. D15S1506, D15S217, D15S10, D15S165, and D15S113 were chosen to confirm homozygosity within 15q11.2-q13. In addition, eight markers were used to test for homozygosity outside 15q11.2-q13. D15S165 is a marker that is outside the common deletion region but within the common duplication region.

Of the four samples tested, one subject was found to be homozygous for all 17 markers tested, suggestive of isodisomy of chromosome 15. Three subjects had a single allele for mark-

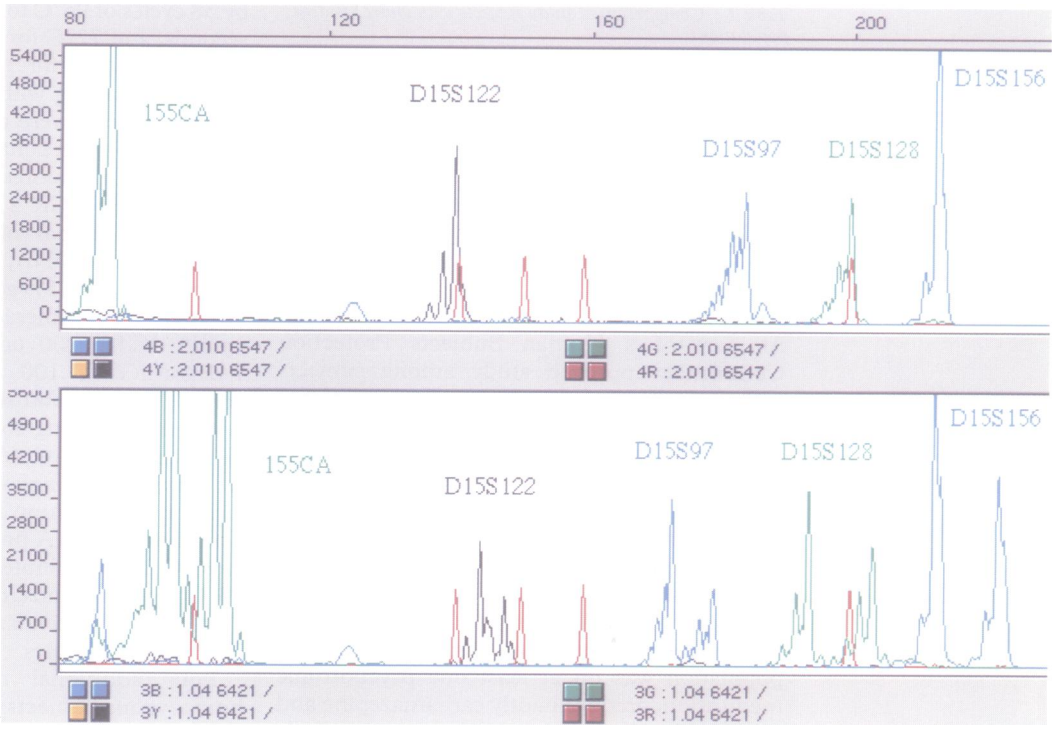

Figure 1 Microsatellite analysis using the ABI 310 genetic analyser. Electrophoretograms of multiplex PCR products from the screening set of five microsatellite markers in the PWS/AS region are presented for two patients. The top panel is an example of a subject with a single allele at all five markers and identified as a suspected case of either PWS/AS region deletion or UPD. The bottom panel is an example of a subject that was heterozygous at each marker and not found to have either deletion of the entire PWS/AS region or UPD. Size standard peaks are red.

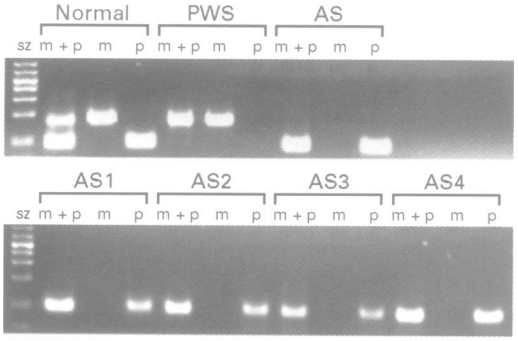

Figure 2 Methylation specific PCR at SNRPN. Bisulphite treated genomic DNA was amplified by PCR using three reactions: (1) duplex reaction with maternal specific (174 bp) and paternal specific (100 bp) primers (m+p); (2) maternal specific primers only (m); and (3) paternal specific primers only (p). In the upper row, one normal control was analysed to confirm positive reactions for both sets of primers followed by one known PWS patient, showing amplification of only the maternal specific primers, and one known AS patient, showing amplification of only the paternal specific primers. The second row shows the results of the four patients from this study indicating amplification of only the paternal specific primers consistent with a diagnosis of AS. sz=size marker.

ers within the PWS/AS critical region, but were heterozygous at the markers that extended outside the region, consistent with deletion. No cases of possible duplications represented by three or more alleles at a locus or threefold increase in amplitude of one allele relative to another were found.

A methylation specific PCR (M-PCR) assay was developed during the course of this study. Samples from the four subjects with PWS/AS deletion or chromosome 15 UPD were analysed with M-PCR to assess the presence of maternal and paternal alleles at the SNRPN locus. Each of the four subjects with UPD or full deletion showed only the presence of the paternal unmethylated SNRPN allele (fig 2).

Each of the four subjects with UPD or full deletion had clinical symptoms of Angelman syndrome in this age group.[30 31] Clinical characteristics are summarised in table 2. Each of the subjects with UPD or deletion was in the subsample requiring skilled nursing care. The frequency of detected AS in this subsample was 3.5%.

Table 2 Clinical characteristics of Angelman syndrome subjects

Subject	*Anomaly*	*Age (y)*	*Sex*	*IQ*	*Medications*	*Examination*	*Gait*	*Seizure onset (y)*	*Childhood behaviour*	*Other*
1	del	27	M	6	Phenobarbital	Spastic quad, paroxysmal choreoathetosis	Non-ambulatory	2	Rumination, pica, no speech	EEG severely abnormal, heart murmur
2	del	40	F	4	Carbamazepine, phenobarbital, imipramine	Spastic quad	Unsteady, with assistance	5	Hyperactivity, no speech	Abnormal EEG, strabismus, heart murmur
3	del	39	M	2	Ranitidine	Spastic quad, choreoathetosis	Unsteady	3	Hyperactivity, "uncontrollable giggling spells", no speech	Abnormal EEG
4	UPD	62	M	14	Bromocriptine, artane, tranxene	Spastic quad	Unsteady, wide based	None	Hyperactivity, insomnia, no speech	Abnormal EEG, strabismus, parkinsonism

Discussion

The frequency of AS caused by deletions or UPD in this cohort of institutionalised children with moderate to profound MR was approximately 1.4%. This is similar to a minimum frequency of AS deletions of 1.2% reported in a recent sample of predominantly severely or profoundly retarded adults.[32] The greater number of AS cases in the current sample relative toPWS cases is most likely a reflection of the population sampled. AS is known to cause more severe MR than PWS[1 2] and 86% of the subjects in this sample had IQs in the profoundly retarded range (below 20). One case of paternal UPD (25% of AS cases in this population compared to a frequency of 2% within the AS population) probably represents the small number of AS cases in this sample. Alternatively, this is the only study to our knowledge in which a population was screened by molecular analysis and not using clinical examination or EEG abnormalities as an initial screen. Given that the patient with UPD had a relatively normal EEG and no history of seizures, and the suggestion by some authors,[33] but not others[34] that the UPD phenotype may differ, it may be that some cases of AS associated with UPD go clinically undetected.

No duplications were detected in our population. It is possible that the sample size was not large enough to show accurately the frequency of 15q11.2-q13 duplications in this population. Some duplications may also have gone undetected by our methods which may have missed duplications in which the duplicated material is derived from the same parental chromosome. Duplications would have been missed if subjects were homozygous for three parental alleles. As another example, a duplication that was produced during meiosis II would produce a genotype that would not have three alleles at any locus, although the patient would have duplicated DNA within the region. In such a case, it would be expected that the height of half of the alleles would be doubled. Such a case could have been missed by our techniques, which relied on a visual assessment of relative peak heights. In addition, the detection of such a duplication may have been complicated by the nature of the PCR process, during which unequal amounts of product are often produced owing to allelic variation in efficiency of amplification. It is possible that the frequency of maternal deletions and paternal UPD in this population is higher than the frequency of duplications. If so, this may be because of a relatively less severe level of MR in proximal 15q duplications than in AS.

Screening by chromosomal analysis in this population will often be performed at an initial stage since many abnormalities at other sites may be identified. Routine chromosomal analysis will miss many AS deletions and all cases of AS by UPD. PCR based screening is the most efficient next step, since FISH analysis is relatively expensive and would miss cases resulting from UPD.

Conclusion

Among the institutionalised subjects in this study, 1.4% were found to have previously undiagnosed Angleman syndrome relative to a population prevalence of 1:12 000. Molecular screening for Angelman syndrome in similar populations may therefore be warranted. Initially, multiplex PCR with microsatellite polymorphisms was used to identify potential deletion and isodisomy cases of AS, but this approach will not detect AS cases resulting from meiotic non-disjunction (heterodisomy). An alternative molecular method, methylation PCR, was recently developed and will identify all cases of deletion and UPD and may therefore be the most efficient initial screening test for AS in such populations.

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