Counselling dilemmas associated with the molecular characterisation of two Angelman syndrome families

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Abstract

We report the molecular characterisation of two families with Angelman syndrome referred for prenatal diagnosis, in which atypical molecular findings resulted in counselling dilemmas. The first is a familial case of Angelman syndrome in which the two affected children have mutations which affect the imprinting mechanism, as shown by the presence of paternal DNA methylation patterns at D15S63 and SNRPN and biparental inheritance of 15q11-q13 markers. DNA prepared from a 21 week fetal blood sample detected a fetus with normal maternal and paternal DNA methylation patterns at D15S63, but inheritance of the same maternal chromosome 15q11-q13 as the two affected sibs. This is probably a result of germline mosaicism in the mother. The second is a case of Angelman syndrome with an atypical deletion of 15q11-q13, which involves both unusual proximal and distal breakpoints. The deletion was characterised in order to assess the risk of Angelman syndrome in a second pregnancy in the mother of this child.

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Angelman syndrome is a neurogenetic disorder characterised by severe mental retardation, ataxia, absent speech, seizures, inappropriate laughter, and hypopigmentation.¹ Angelman syndrome has an estimated incidence of 1 in 20 000² and is associated with the absence of a maternal genetic contribution from chromosome 15q11-q13, a region associated with genomic imprinting. Absence of a paternal genetic contribution from this region results in Prader-Willi syndrome, a clinically distinct neurogenetic disorder characterised by global developmental delay, hypogonadism, infantile hypotonia, short stature, small hands and feet, and obesity.³

Approximately 70% of Angelman and Prader-Willi syndrome cases arise from a large de novo deletion of the 15q11-q13 region.⁴⁻⁸ These deletions commonly span approximately 4 Mb and are associated with constant proximal and distal breakpoints.⁹⁻¹¹ The proximal breakpoint generally lies between D15S541 and D15S9, with an alternative

breakpoint lying proximal to D15S541. The distal breakpoint lies distal to D15S12. Almost all the remaining cases of Prader-Willi syndrome result from uniparental maternal disomy¹²⁻¹³ and, in a small number of cases, Angelman syndrome results from uniparental paternal disomy.¹⁴⁻¹⁵ These mechanisms are associated with a low recurrence risk. Approximately 20-25% of Angelman syndrome subjects and a small number of Prader-Willi syndrome subjects, including almost all familial cases, have neither of these mechanisms. These people are thought to have a mutation affecting the imprinting process¹⁶⁻¹⁹ or, in the case of Angelman syndrome, a mutation in a single maternally expressed gene.²⁰⁻²² It is in the families of these subjects that there is a recurrence risk of up to 50%.

Parent of origin specific DNA methylation patterns have been observed at the D15S9, D15S63, and SNRPN loci.¹⁹⁻²³⁻²⁶ Familial Angelman and Prader-Willi syndrome patients who have a putative mutation affecting the imprinting process (imprintor mutation) show aberrant 15q11-q13 DNA methylation imprints with biparental inheritance of 15q11-q13 markers.¹⁶⁻¹⁹ Inherited microdeletions have been identified in some of these patients, affecting a region around and upstream of SNRPN (exon α).²⁷⁻²⁸ These deletions appear to block the paternal to maternal imprint switch in the female germline, resulting in offspring with Angelman syndrome, whereas in the male germline, the maternal to paternal imprint switch is blocked, resulting in offspring with Prader-Willi syndrome.²⁹

Two probes have been used diagnostically to detect DNA methylation imprints. PW71B, which lies close to the transcriptional site of a set of proposed upstream exons of SNRPN, detects a 6.6 kb maternal and a 4.7 kb paternal *HindIII/HpaII* fragment.²⁵ SNRPN (exon α), which contains the first 5' exon of SNRPN, detects 4.2 kb maternal and 3.0 kb, 0.9 kb, 0.15 kb, and 0.1 kb paternal *XbaI/NotI* fragments.¹⁹ Normal subjects have both the maternal and paternal DNA methylation imprints, whereas Angelman syndrome deletion, uniparental paternal disomy, and imprintor mutation cases have a paternal methylation imprint only. The converse is observed in Prader-Willi syndrome, where a maternal methylation imprint only is observed. As Angelman and Prader-Willi syndrome cases resulting from deletions and uniparental dis-

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Figure 1 A map of the AS/PWS region within 15q11-q13 showing the position of the microsatellite markers and DNA methylation probes used in this study.

omy also show aberrant DNA methylation imprints, imprintor mutation cases can only be distinguished through the observation of biparental inheritance of markers from 15q11-q13. Unexpected problems were encountered in two Angelman syndrome families requesting prenatal diagnosis. One case involved a family with an imprintor mutation and the second a child with an atypical deletion.

Materials and methods

DNA METHYLATION STUDIES

Probes for the D15S63 and SNRPN loci were used to investigate the methylation imprints in the patients and their families. *HindIII/HpaII* blots were prepared for PW71B analysis and *XbaI/NotI* digests were used for SNRPN (exon α). DNA methylation studies were carried out according to the published methods.¹⁹⁻²⁵

MICROSATELLITE ANALYSIS

DNA from the patients and all available family members was analysed with a combination of (CA)_n repeats from the 15q11-q13 region (fig 1): D15S541,¹¹ D15S11,³⁰ D15S128 and D15S210,³¹ D15S10,³² D15S113,³³ GABRB3,³⁴ and D15S97.³⁵ D15S11, D15S10, D15S113, GABRB3, and D15S97 were amplified and visualised using conditions previously described.⁷ For D15S541, D15S128, and D15S210, 25-100 ng DNA was added to a reaction mix containing 160 mmol/l (NH₄)₂SO₄, 670 mmol/l Tris-HCl (pH 8.8 at 25°C), 25 pmol of each primer, 0.2 mmol/l dATP, dGTP, and dTTP, and 0.1 mmol/l dCTP, 1 μ Ci of ³²P-dCTP, 1.5-2.5 U of *Taq* polymerase, and magnesium ions (the concentrations of which were determined by titration). The reactions were "hot started" by adding the *Taq* polymerase after the reaction mixture had been denatured at 95°C for five minutes. Twenty-five cycles were performed, involving one minute denaturation at 95°C, one minute annealing at 56°C for D15S541, 58°C for D15S128, and 57°C for D15S210, one minute extension at 72°C. A final extension step at 72°C was added after the last cycle. The radiolabelled PCR products were resolved on a 6% denaturing polyacrylamide gel.

RFLP ANALYSIS

42P1.1-5 is a 1.1 kb *PstI* fragment subcloned from a cosmid that contains the D15S113 locus.³⁶ It is located within 20 kb of D15S113 and detects polymorphic *HindIII* fragments of 8.0 and 7.6 kb. These alleles both have frequencies of 0.5 in the white population. The polymorphism can be detected after double digestion with *HindIII/HpaII*, allowing blots already prepared for PW71B analysis to be used for this probe.

FISH ANALYSIS

FISH analysis was performed as previously described³⁶ with the following changes: 2 μ g of cosmid DNA was labelled and coprecipitated with human Cot-1 DNA (20 μ g) and herring sperm DNA (20 μ g) and resuspended in 100 μ l of hybridisation buffer; 200-400 ng probe was used in the hybridisation and following denaturation at 70°C for 10 minutes, preannealing was carried out for 10 minutes at 37°C. After hybridisation, the slides were washed in 50% formamide/2 $\times$ SSC at 45°C, followed by 0.1 $\times$ SSC at 60°C. Signals were detected with avidin-FITC (Vector laboratories) and amplified with biotinylated antiavidin (Vector laboratories) and a second round of avidin-FITC.

Results

FAMILY 1

Family 1 contains two affected sibs with Angelman syndrome and was referred to the Molecular Genetics Unit at the Institute of Child Health when the mother was 17 weeks pregnant. An imprintor mutation was identified in the two affected sibs, based on paternal DNA methylation patterns at D15S63 (fig 2) and SNRPN, together with biparental inheritance of markers at D15S541, D15S128, and D15S113. Both parents had normal DNA methylation patterns at D15S63 and SNRPN. A 21 week fetal blood sample was taken for prenatal diagnosis of the mutation in the fetus.

DNA methylation analysis

DNA methylation studies with PW71B showed that both normal maternal and paternal DNA methylation imprints were present in the fetus (fig 2). Before analysing the fetal blood sample from family 1, a normal 21 week fetal blood sample was probed with PW71B to ensure that normal maternal and paternal DNA methylation patterns were obtained, as these patterns are known to be abnormal in cultured chorionic villus samples (fig 2).³⁷ A recent publication has shown that the methylation status at SNRPN is consistent in all cell types tested including amniotic fluid and cultured chorionic villus samples.³⁸ At the time of the prenatal diagnosis, the SNRPN (exon α) probe was not available. No DNA from the fetal blood sample remained to reassess the methylation status after SNRPN (exon α) became available.

Microsatellite analysis

Segregation analysis of markers in the fetus showed that the two affected sibs and the fetus shared the same maternal allele for D15S128 (fig 3).

P1.1-5 analysis

Reprobing the *HindIII/HpaII* blot used for the DNA methylation studies with 42P1.1-5 (D15S113) showed that at least one of the two affected sibs had inherited the same maternal allele as the fetus (as only one sib was informative) at this locus (fig 3).

The linkage and methylation data gave contradictory results. After counselling, the parents decided to terminate the pregnancy. Sub-

Figure 2 Southern blot results of *HindIII/HpaII* digested genomic DNA hybridised with the probe PW71B showing (1) a normal control, (2) an AS subject, (3) a PWS subject, (4) a normal 21 week fetus and followed by the subjects tested from family 1. All samples were extracted from peripheral blood apart from the fetal sample and the control in track 4 which were 21 week fetal blood samples.

sequently, a deletion of the imprinting control locus was identified.²⁸ Two probes (L48.3I and L48.6I) detected an 11.0 kb *EcoRI* junction fragment in the two affected sibs and their mother. The DNA from the fetal blood sampling did not carry this altered fragment.

FAMILY 2

In family 2, the proband is the only daughter of normal parents and presented to the clinic at 7 years of age, where a clinical diagnosis of Angelman syndrome was made. Characterisation of the molecular defect resulting in Angelman syndrome in this child was necessary to ascertain the recurrence risk of Angelman syndrome in this family, as the mother was 7 weeks pregnant. The father of the affected child was unavailable for study.

DNA methylation analysis

PW71B detected both the 6.6 kb maternal and 4.7 kb paternal bands in the proband and her mother (fig 4A), thus excluding the possibility of a deletion of the D15S63 locus or uniparental paternal disomy and placing the pregnancy in the high risk category. SNRPN (exon α) detected both the 4.3 kb maternal and 3.0 kb paternal bands in the mother but only the 3.0 kb paternal band in the proband (fig 4B).

Figure 3 Pedigree of family 1. The haplotypes suggested by the chromosome 15q11-q13 markers are also depicted in the pedigree.

Microsatellite analysis

The proband was heterozygous for the microsatellite markers at GABR β 3 and D15S97. The mother was homozygous for the markers at D15S113 and D15S210, while the proband inherited a single different band to the mother at these two loci, implying a deletion. These results indicate that the distal deletion breakpoint in the proband is atypical in that it does not extend as far distal as D15S12. The markers at D15S10 and D15S128 were uninformative. The results of the DNA methylation analysis with PW71B indicate that the proximal deletion breakpoint is also atypical in that it lies distal of D15S63.

FISH analysis

FISH analysis with a cosmid from the D15S113 locus (E24) produced signal on one chromosome 15 homologue only in the proband. Signal was observed on both chromosome 15 homologues in the mother, confirming that the proband has a *de novo* deletion at the D15S113 locus and the mother does not carry any translocation detectable at this level (data not shown). This placed the pregnancy in the low risk category and the parents were given a probable risk of 1%. The parents decided against prenatal diagnosis. A normal boy was born, who was examined at the Child Development Clinic when a few months old. He was found to be developing normally.

Discussion

The two cases presented in this paper illustrate the complexity of the molecular analysis in Angelman syndrome and problems which have arisen during prenatal diagnosis.

In family 1, the DNA methylation imprints observed in the two affected Angelman syndrome sibs at D15S63 and SNRPN suggested that the imprinting control locus had been disrupted. Confirmation that this was an imprintor mutation and had not arisen as a result of a deletion or uniparental paternal disomy was obtained through the analysis of microsatellite markers from 15q11-q13. The observation of normal maternal and paternal DNA methylation imprints at D15S63 in the DNA from the fetal blood sampling implied that the imprinting control locus was not disrupted and that the fetus would not have Angelman syndrome. However, analysis with the polymorphic marker 42P1.1-5 (D15S113) indicated that the fetus had inherited the same maternal allele as at least one of the affected sibs at this locus. Since the DNA methylation and 42P1.1-5 results conflicted, further 15q11-q13 markers were analysed to ascertain whether there had been a crossover between the imprinting control locus and D15S113. Segregation analysis indicated that the fetus had inherited the same maternal allele as the two affected sibs at D15S128. D15S128 maps very close to the imprinting control locus and there is virtually no chance of a double recombination between these two loci, implying that the fetus most probably inherited the same critical maternal segment as the two affected sibs and probably has the whole haplotype in common. A deletion

Figure 4 (A) The probe SNRPN (exon α) hybridised to NotI/XbaI digests from the proband and her mother from family 2, and (B) the probe PW71B hybridised to HindIII/HpaII DNA digests from the same subjects.

of the imprinting control locus was subsequently identified in family 1.²⁸ Two probes (L48.3I and L48.6I) detected an 11.0 kb *EcoRI* junction fragment in the two affected sibs and their mother. This junction fragment was not detected in the fetus. In two previously reported imprintor mutation families,²⁸ the mothers showed a reduction in intensity of the breakpoint fragments compared to that in their affected children. This suggested that they were mosaic for the deletion. Germline mosaicism would also explain the findings in family 1, suggesting that this could be a relatively frequent phenomenon in imprintor mutation cases.

In family 2, the PW71B result initially suggested that the proband was not deleted or disomic, placing her mother in the high recurrence risk class. Only the analysis of further markers and probes detected a deletion in this person. The microsatellite data showed that the deletion in the proband was atypical but failed to show whether this deletion was inherited or de novo in origin. Confirmation of a de novo deletion was ascertained through FISH analysis in the proband and her mother. The risk of Angelman syndrome to the pregnancy was therefore considered to be low and a figure of 1% was given. This allowed for a residual

possibility of germline mosaicism. In view of the findings in family 1 presented here and the two examples described by Saitoh *et al*,²⁸ germline mosaicism is a clear possibility in cases of de novo deletions of atypical size. In these established mosaic cases, the de novo deletions were within the region 5' to the coding region of SNRPN. In family 2, one of the proband's breakpoints is also apparently in this region and the possibility of germline mosaicism may be higher. The deletion in the proband from family 2 is atypical and extends over approximately 1 Mb. The distal breakpoint lies between GABR β 3 and D15S113. Since the GABR β 3 microsatellite lies approximately 50-60 kb proximal to the gene for GABR β 3, the proband is intact for the GABR β 3 gene itself. The observation of normal maternal and paternal DNA methylation imprints at D15S63 and a paternal DNA methylation imprint only at SNRPN indicates that the proximal breakpoint in this proband lies distal of D15S63. This region is highly repetitive²⁵ and the exact breakpoint has not yet been identified, although SNRPN (exon α) is most probably deleted. PW71B (D15S63) is a homologous DNA sequence that lies within 160 kb of SNRPN (exon α) and forms part of an imprinting centre transcript.²⁷ It will be interesting to determine whether the imprinting centre transcripts are affected in this subject and, consequently, to determine the mechanism by which the DNA methylation imprint at D15S63 remains unaltered.

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