Original articles

The elusive Angelman syndrome critical region

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Abstract

DNA mapping studies in two families provide further information on the Angelman syndrome critical region, which has recently been defined by the gene UBE3A. The first family has probable familial Angelman syndrome with a maternally imprinted inheritance pattern. A 5 year old girl with this disorder has a 14 year old brother and an 11 year old male cousin who have less typical clinical features. DNA microsatellite analysis has shown that the three share a common segment of the same grandpaternal chromosome 15q11-q13 that overlaps with UBE3A. The child with typical Angelman syndrome has an additional maternal recombination 5' to UBE3A. The second family is a mother and son both of whom have mental retardation but no other features of Angelman syndrome despite an extensive DNA deletion on the telomeric side of UBE3A. Together, the two families identify a region between loci D15S210 and D15S986 which forms part of the Angelman syndrome critical region. A new microsatellite (D15S1234) is described which can be used in place of the LS6-1 marker at locus D15S113.

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an abnormality in the imprinting process; (4) the remainder are considered to have a mutation affecting a single gene, which is likely to be UBE3A. Familial recurrence has been reported in groups (3) and (4).¹

Positional cloning to find the AS gene has progressed slowly until recently when UBE3A was shown to be mutated in cases which were non-deleted, non-disomic, and normal in respect to imprinting mutations.^{2,3} Although not apparently imprinted in adult life, UBE3A is involved in ubiquitin dependent protein proteolysis, which would play a critical role during brain development.^{2,4} Four mutations have been described which lead to truncated proteins via premature stop codons or frameshifts in this gene.^{2,3} However, its role in the AS phenotype remains to be determined. To define further the ANCR (Angelman critical region, the minimal chromosomal region associated with AS) we have studied a family of Turkish-Cypriot origin in which the proband has features of AS but two relatives (a brother and a male cousin) are less severely affected. DNA microsatellite analysis shows that the three children share a common haplotype which overlaps UBE3A. A DNA recombination just telomeric to UBE3A in the proband and further refinement of the centromeric extent of a chromosome 15q11-q13 deletion in a second family, which does not have AS, enables the ANCR to be extended beyond UBE3A.

Materials and methods

CLINICAL REPORT

Family ML is of Turkish-Cypriot origin and came to the attention of the Genetics Clinic because of delay in speech development affecting five children who are the offspring of three sisters (fig 1). The proband (III.3) was aged 5 when recently assessed. She has features of AS which include unsteady gait, typical facies and EEG findings, delayed psychomotor development, a happy disposition, prognathism, a large mouth with widely spaced teeth, and a small head (3rd centile). Hypopigmentation was not present. Her speech defect was atypical of AS since she can use about 50 words. A cousin and brother were also assessed (III.1, III.2) and considered to be less typical of AS although review by two independent clinical geneticists confirmed that the three shared common

Angelman syndrome (AS), first described in 1965 by Harry Angelman, is a multisystem disorder which can be difficult to diagnose, particularly in the first few years of life. Clinical features include severe mental retardation with lack of speech, delayed motor milestones, characteristic facies, inappropriate laughter, good nature, hypopigmentation, and brachycephaly. Seizures associated with a typical spike and slow wave activity on EEG are common. The incidence of AS is estimated to be 1 in 20 000. It is predominantly a sporadic disorder although familial cases have been described. At the molecular level, AS can be subdivided into: (1) ~70% of cases have a deletion involving the maternally derived chromosome 15q11-q13; (2) ~3-5% of cases result from paternal uniparental disomy of chromosome 15; (3) ~8% have

Figure 1 Pedigree of family ML. Three sisters have five affected offspring. Not shown on the pedigree are three other sisters with five normal children and one brother with a normal child. The three children examined are III.1, III.2, and III.3 who are aged 11, 14, and 5 years, respectively.

features. The remaining two affected children, both female cousins, are said to have retarded development with little speech but were not available for study. The second family (BS/DH) has been published and includes a mother and son who have mild to moderate mental retardation with no other features of AS.⁵ Both have a deletion involving chromosome 15q11-q13.

MOLECULAR STUDIES

DNA was prepared from family members and analysed for microsatellite polymorphisms at the following loci: D15S122, D15S10,

D15S210, D15S986, D15S113, D15S1234, and GABRB3.^{6,7} All except D15S1234 have previously been reported. D15S1234 is defined by (CTTT)_n, a microsatellite which we isolated from a λ phage contig prepared from YAC 229A2 (GDB accession number 698716). Methylation analysis of DNA from III.3 was undertaken with the SNRPN probe.⁸ DNA from BS and DH was analysed with microsatellite markers D15S122, D15S210, and D15S986.

Results

CLINICAL

The phenotypes for III.3 and her affected brother and cousin are compared to published consensus diagnostic criteria (table 1). In this family, key features of AS, including developmental delay, speech impairment, a movement disorder, and behavioural patterns such as frequent smiling, are present but to a variable degree. The proband has the facial features of AS (fig 2) and the typical EEG pattern reported in this disorder. Facial features of her brother and cousin are provided as a contrast (fig 2). Cytogenetic studies for the proband and her brother were normal.

MOLECULAR

A difficulty with mapping in this region has been the relative positions of microsatellite markers. The location of D15S210 was defined on the basis of a λ phage contig prepared from YAC 273A2 (data not shown). It is >50 kb

Table 1 Angelman syndrome consensus criteria and comparison with family ML (from reference 9)

Clinical features		III.1	III.2	III.3 (proband)
(A)	Consistent (100%)			
	Developmental delay, functionally severe	Yes	Yes	Yes
	Speech impairment, none or minimal use of words; receptive and non-verbal communication skills higher than verbal ones	Yes	Yes	Yes
	Movement or balance disorder, usually ataxia of gait/tremulous movement of limbs	No	Yes	Yes
	Behavioural uniqueness: any combination of frequent laughter/smiling, apparent happy demeanor, easily excitable personality, often with hand flapping movements; hypermotoric behaviour, short attention span	Yes	Yes	Yes
(B)	Frequent (more than 80%)			
	Delayed, disproportionate growth in head circumference, usually resulting in microcephaly (absolute or relative) by age 2	No	No	Yes
	Seizures, onset usually <3 years of age	No	No	Yes
	Abnormal EEG, characteristic pattern with large amplitude slow-spike waves (usually 2–3/s), facilitated by eye closure	No	Slow, abnormal waves	Yes
	Associated (20–80%)			
(C)	Flat occiput	No	No	No
	Occipital groove	No	No	No
	Protruding tongue	No	No	No
	Tongue thrusting, sucking/swallowing disorders	No	No	Yes
	Feeding problems during infancy	Yes	No	No
	Prognathism	No	Yes	Yes
	Wide mouth, widely spaced teeth	No	Yes	Yes
	Frequent drooling	No	Yes	Yes
	Excessive chewing/mouthing behaviours	No	No	No
	Strabismus	No	Yes	No
	Hypopigmented skin, light hair and eye colour (compared to family), seen only in deletion cases	No	No	No
	Hyperactive lower limb deep tendon reflexes	Yes	Yes	Yes
	Uplifted, flexed arm position especially during ambulation	No	Yes	Yes
	Increased sensitivity to heat	Yes	No	No
	Sleep disturbance	Yes	No	Yes
	Attraction to/fascination with water	No*	Yes	Yes

*No, but loves swimming.

Figure 2 Physical appearance of the proband (A), her brother (B), and cousin (C). (Photographs reproduced with permission.)

telomeric to D15S10. We previously reported that D15S122 lies centromeric to D15S10⁶ and from our phage contig it is approximately 20 kb from that locus. Similarly, based on overlapping phage clones, D15S1234 is located about 20 kb telomeric to D15S113 and is highly polymorphic (heterozygosity ~0.85). It is considerably easier to use and read than the previously described D15S113 (LS6-1) (table 2). Microsatellite D15S986 lies telomeric to D15S210 in YAC 142A2 but not 378A12.⁷ Since the latter is positive for locus D15S113,¹⁰ the relative positions of these two markers is established. The order of microsatellites described is: centromeric - D15S122, D15S10, D15S210, D15S986, D15S113, D15S1234 - telomeric.

Haplotypes for family ML are shown in fig 3. A recombination event (indicated by X) has occurred between markers D15S210 and D15S113 in two females, I.2 and II.3. The AS phenotype cosegregates with the haplotype ebd/ which includes exon 1 of the UBE3A gene and its immediate 5' flanking region.² Unfortunately, microsatellite D15S986, which is placed

Table 2 Allele sizes and frequencies for microsatellite D15S1234*

Allele	Size	Frequency
A1	308	0.02
A2	304	0.08
A3	300	0.08
A4	296	0.02
A5	292	0.08
A6	288	0.08
A7	284	0.02
A8	280	0.25
A9	276	0.15
A10	272	0.13
A11	268	0.02
A12	264	0.05
A13	256	0.02

*The allele frequency was determined from 74 unrelated subjects. Primers: FP1: 5'-GCGGGGAGCCACTATAA GACTTC-3'; RP2: 5'-GCCTGGCAACAGAGTGAGACA CTC-3'. PCR was carried out in a 25 µl volume with 25 ng genomic DNA, 20 pmol each primer, 200 µmol/l each dNTP, 50 mmol/l KCl, 10 mmol/l Tris-HCl pH 8.3, 10 mmol/l MgCl₂, 0.001% gelatin, and 0.5 U *Taq* polymerase. Initial denaturation was for four minutes at 95°C followed by 24 cycles of denaturation (94°C, one minute), annealing (55°C, two minutes), and extension (72°C, two minutes) with the final cycle extending for seven minutes. PCR products were separated on 8% acrylamide gels and visualised by silver staining.

between D15S210 and D15S113, was uninformative in this family. The methylation imprint in III.3 was normal, that is, both maternal and paternal specific bands were detected.

Microsatellite analysis in BS/DH (the family without AS) showed that DNA from the mother and son was biallelic at markers D15S122 and D15S210 and both were deleted at D15S986. This is consistent with previous data showing an intact D15S10 and a deleted D15S113.⁵ The D15S986 result extends the centromeric extent of the deletion in BS/DH from D15S113 to D15S986.

Discussion

The long awaited breakthrough in the positional cloning for the AS gene has now come with the finding of mutations in the UBE3A gene. In terms of function, the gene, as a member of the ubiquitin protein ligases, can play a number of critical roles during development, for example, regulation of protein turnover, phosphorylation, and endocytosis.²⁻⁴ Further complexity associated with UBE3A is the finding of alternative transcripts with the potential to encode three protein isoforms which differ at their N-termini, a genomic region extending over 60 kb, and a 5' untranslated region which remains to be characterised but is presumed to be long, based on sizing of mRNA and a relatively short 3' non-coding region.¹¹ Thus, definitive characterisation of UBE3A and its role in AS will take some time as extensive mutation analyses enable genotype/phenotype correlations to be completed. Already there is evidence that the 5' untranslated region requires further study since an AS family with two affected sibs has placed the mutation locus distal to D15S122, that is, either the first exon of UBE3A or further upstream.^{2, 12}

Before the identification of UBE3A, approximately 15-20% of AS cases had no demonstrable molecular abnormality, that is, deletion, uniparental disomy, or methylation

Figure 3 Microsatellite DNA haplotypes for family ML. The position of two crossover events is indicated by an X. The 5' end (exon 1) of the UBE3A gene is shown by → and the gene then extends beyond D15S122.

Figure 4 Mapping data in families ML and BS/DH (not drawn to scale). (a) The relation of UBE3A to D15S122 and D15S10; (b) the segment of chromosome 15q11-q13 shared by the proband and her two relatives and the segment which has recombined; (c) the normal (intact) and deleted segments in family BS/DH. Dashed line indicates uncertainty in terms of size or extent of a region.

difference.¹ It is into this group that affected subjects in family ML fit. The different phenotypes in family ML are puzzling. There is little doubt that the proband (III.3) has the syndrome and her two male relatives have a milder but related disorder. The difference could be explained by the three sharing a mutation involving UBE3A and the more

severe phenotype then resulting from the recombination event which has occurred in the proband. The mutation in UBE3A would need to have minimal effect on the protein's function, for example, a splicing defect which did not lead to a frameshift or a missense change. There are precedents for different mutations in the same gene leading to mild phenocopies, for example, cystic fibrosis and β thalassaemia. In this respect, it is noteworthy that a missense change (Cys21Tyr) in UBE3A was present in a patient with a less classical phenotype.³ An additional recombination event affecting a 5' regulatory or controlling region, such as an enhancer, could further interfere with the protein's expression or function. In the case of family ML, the recombination has occurred between D15S210 and D15S113 (fig 4). Clinically, the family again emphasises the importance of a thorough history to show the familial nature of the disorder in this case. Subject III.3, who is critical to the diagnosis, might not have been identified if more distant family members had presented initially and had subsequently been investigated superficially. This is particularly relevant for family ML which is at high risk for recurrence of AS.

The second family (BS/DH), which has a DNA deletion but no features of AS apart from the non-specific finding of mental retardation, narrows the region of interest since it excludes locus D15S986. Thus, the potential regulatory or controlling region is situated between D15S210 and D15S986 (fig 4). The size of this DNA segment remains to be defined because the relative position of D15S986 to microsatellites on either side was determined on the basis of overlapping YAC clones. It is likely to contain a recombination hot spot since, in the report by Paldi *et al.*,⁷ all five recombinations in 86 informative female meioses detected in this segment of chromosome 15q11-q13 occurred between D15S210 and a more telomeric D15S97 locus. The two recombinations involving females in family ML occurred between D15S210 and D15S113. Only one of the 14 recombination events in males were localised to this region, the remainder being situated on the centromeric side of D15S122.⁷

Mutation analysis involving UBE3A is proceeding in family ML, although this will take some time as the exon-intron boundaries for the gene have not been sufficiently defined to enable all exons to be characterised from genomic DNA. The more rapid RT-PCR approach using peripheral blood lymphocytes remains to be evaluated. As with all large genes, the identification of mutations in introns will lag considerably behind those found in exons. Irrespective of the potential to look for mutations in the UBE3A gene, the initial molecular approaches to laboratory confirmation of AS will continue to rely on FISH, microsatellite analysis, and methylation studies. A limitation to microsatellite analysis has been the availability of markers in this region including the potential for null alleles, for example, the LS6-1 marker for D15S113.¹³ The microsatellite D15S1234 is technically

easier to use and will prove helpful in this respect.

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- 1 American Society of Human Genetics/American College of Medical Genetics Test and Technology Transfer Committee. Diagnostic testing for Prader-Willi and Angelman syndromes: report of the ASHG/ACMG test and technology transfer committee. *Am J Hum Genet* 1996;58:1085-8.
- 2 Kishino T, Lalonde M, Wagstaff J. *UBE3A*/E6-AP mutations cause Angelman syndrome. *Nat Genet* 1997;15:70-3.
- 3 Matsuura T, Sutcliffe JS, Fang P, et al. *De novo* truncating mutations in E6-AP ubiquitin-protein ligase gene (*UBE3A*) in Angelman syndrome. *Nat Genet* 1997;15:74-7.
- 4 Ciechanover A. The ubiquitin-proteasome proteolytic pathway. *Cell* 1994;79:13-21.
- 5 Michaelis RC, Skinner SA, Lethco BA, et al. Deletion involving D15S113 in a mother and son without Angelman syndrome: refinement of the Angelman syndrome critical deletion region. *Am J Med Genet* 1995;55:120-6.
- 6 Malcolm S, Donlon TA. Report of the second international workshop on human chromosome 15 mapping 1994. *Cytogenet Cell Genet* 1994;67:1-22.
- 7 Paldi A, Gyapay G, Jami J. Imprinted chromosomal regions of the human genome display sex-specific meiotic recombination frequencies. *Curr Biol* 1995;5:1030-5.
- 8 Glenn CC, Saitoh S, Jong MTC, et al. Gene structure, DNA methylation and imprinted expression of the human *SNRPN* gene. *Am J Hum Genet* 1996;58:335-46.
- 9 Williams CA, Angelman H, Clayton-Smith J, et al. Angelman syndrome: consensus for diagnostic criteria. *Am J Med Genet* 1995;56:237-8.
- 10 Mutirangura A, Jayakumar A, Sutcliffe JS, et al. A complete YAC contig of the Prader-Willi/Angelman chromosome region (15q11-q13) and refined localization of the *SNRPN* gene. *Genomics* 1993;18:546-52.
- 11 Yamamoto Y, Huibregtse JM, Howley PM. The human EP-AP gene (*UBE3A*) encodes three potential protein isoforms generated by differential splicing. *Genomics* 1997;41:263-6.
- 12 Greger V, Reis A, Lalonde M. The critical region for Angelman syndrome lies between D15S122 and D15S113. *Am J Med Genet* 1994;53:396-8.
- 13 Burke LW, Wiley JE, Glenn CC, et al. Familial cryptic translocation resulting in Angelman syndrome: implications for imprinting or location of the Angelman gene? *Am J Hum Genet* 1996;58:777-84.