UBE3A/E6-AP mutations cause Angelman syndrome

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Angelman syndrome (AS), characterized by mental retardation, seizures, frequent smiling and laughter, and abnormal gait, is one of the best examples of human disease in which genetic imprinting plays a role[1]. In about 70% of cases, AS is caused by *de novo* maternal deletions at 15q11–q13 (ref. 2). Approximately 2% of AS cases are caused by paternal uniparental disomy (UPD) of chromosome 15 (ref. 3) and 2–3% are caused by 'imprinting mutations'[4]. In the remaining 25% of AS cases, no deletion, uniparental disomy (UPD), or methylation abnormality is detectable, and these cases, unlike deletions or UPD, can be familial[5–7]. These cases are likely to result from mutations in a gene that is expressed either exclusively or preferentially from the maternal chromosome 15. We have found that a 15q inversion inherited by an AS child from her normal mother disrupts the 5' end of the *UBE3A* (E6-AP) gene, the product of which functions in protein ubiquitination[16]. We have looked for novel *UBE3A* mutations in nondeletion/non-UPD/non-imprinting mutation (NDUI) AS patients and have found one patient who is heterozygous for a 5-bp *de novo* tandem duplication. We have also found in two brothers a heterozygous mutation, an A to G transition that creates a new 3' splice junction 7 bp upstream from the normal splice junction. Both mutations are predicted to cause a frameshift and premature termination of translation. Our results demonstrate that *UBE3A* mutations are one cause of AS and indicate a possible abnormality in ubiquitin-mediated protein degradation during brain development in this disease.

No AS candidate gene has been described previously that either has a mutation in nondeletion/non-UPD/non-imprinting mutation (NDUI) AS or that is expressed solely from the maternal chromosome 15. By contrast, five genes have been identified that are active exclusively on paternal 15q11–q13 and may play a role in the pathogenesis of Prader-Willi syndrome[8–11]. Previous mapping of the postulated AS gene by analysis of unbalanced chromosome rearrangements and by analysis of linkage in familial AS cases has established that it lies proximal to pTD3-21 (*D15S10*)[12] and that in one pair of NDUI AS siblings the mutation responsible for AS lies distal to locus *D15S122* (ref. 13).

The P1 clone 198 spans the 15q11–q13 breakpoint of a balanced paracentric inversion that has been transmitted from a normal mother to her AS daughter (V. Greger *et al.* manuscript submitted). It contains locus *D15S10*, recently reported to be the telomeric boundary of the AS critical region[12]. We reasoned that this inversion may inactivate a maternally active gene at the breakpoint, and that lack of a functional maternal allele may lead to AS. We isolated 4 exons by exon trapping of P1 clone 198, ranging in size from 64 to 128 bp and located centromeric to the inversion breakpoint. None of these showed homology to sequences in the GenBank database. One 120-bp exon, designated exon 4 (Fig. 1), did show 97% identity with a mouse expressed sequence tag (EST), W14234. A GenBank search with this mouse EST revealed that the region of homology to exon 4 was flanked by 43 bp that showed 90% identity to the human *UBE3A* gene and by 81 bp that showed 70% identity to the human genomic sequence OP2 (Genbank Accession No. L23501)[14], which contains a CpG island. Our mapping of these sequence elements (exon 4, *UBE3A*, and OP2) indicates that they are located centromeric to pTD3-21 and that OP2 lies telomeric to *D15S122*, within the previously defined critical region (Fig. 1). The position of *UBE3A*, proximal to pTD3-21, is consistent with STS analysis of YACs from the region[15]. Exon 4 and *UBE3A* are located centromeric to the inversion breakpoint and OP2 is located telomeric to the breakpoint, indicating that the human homologue of the gene from which the mouse EST is derived should be disrupted by the inversion.

To confirm this, we isolated *UBE3A* cDNAs from a human fetal brain library. The two longest cDNAs, c7-3 and c10, contain the entire published *UBE3A* sequence[16] as well as exons 3, 4 and 5 in the 5' untranslated region (Fig. 2). c7-3 and c10 contain 52 bp and 60 bp, respectively, from OP2, confirming that the human *UBE3A* gene contains sequences from OP2 and therefore spans the inversion breakpoint. The genomic organization of *UBE3A*, including the novel 5' exons, is shown in Fig. 1.

Northern blot analysis using probes from *UBE3A* gave results consistent with previous reports, indicating that an mRNA species of approximately 5 kb[15,16] is widely expressed. Fetal brain showed high levels of expression, with mRNA sizes of approximately 6 and 8 kb (data not shown).

In order to determine whether any NDUI AS patients might have sequence alterations in *UBE3A* that could lead to the AS phenotype, we performed SSCP on genomic DNA from 22 NDUI AS patients. Among these, 13 were from nonfamilial cases and 9 were from 5 AS families. SSCP analysis with primers flanking exon 16

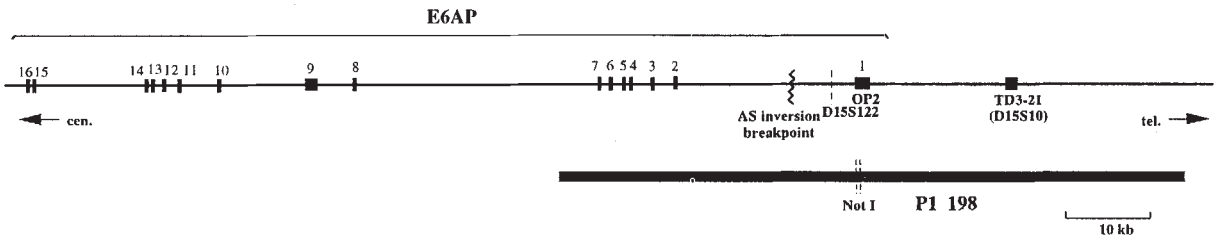

Fig. 1 Map of *UBE3A* exons and the inversion breakpoint. The map is based on restriction maps of phage subclones from P1 clone 198 and a second partially overlapping P1 clone, as well as clones from a human genomic phage library. P1 clone 198 was oriented with respect to the centromere by FISH with a *D15S10* probe, which showed that the inversion breakpoint is between the centromere and *D15S10* (V. Greger *et al.*, manuscript submitted). OP2 is a genomic clone[14] that contains the 5' end of *UBE3A* cDNA clones c7-3 and c10, referred to here as exon 1. Exons 2,3, 4 and 5 were isolated by exon trapping. Exon 6 was identified by cDNA sequencing. Exon 7 contains the 5' end of the published *UBE3A* cDNA sequence [16]. The size of the filled rectangles is not proportional to exon size.

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showed that one patient, WB141, was heterozygous for an abnormally large band that was absent in both parents. Sequence analysis revealed the mutation to be a *de novo* 5-bp tandem duplication (Fig. 3), predicting premature termination of the UBE3A protein and disruption of the 'hect domain' that is conserved in a variety of putative ubiquitin protein ligases from yeast to human[17]. This mutation was not observed in 100 control individuals. It occurs 50 bp proximal to the imperfect pentanucleotide repeat $TAAAA(CAAAA)_3$-TAAAACAAAA in the 3' untranslated region of the *UBE3A* gene. Slipped mispairing of this repeat during replication is a possible explanation for this type of mutation[18].

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  1 GGCGAGGGCG ACTACGACCA GTGAGGCGGC CGCCGCAGCC CAGGCGCGGG
 51 GGCGACGACA GGTTAAAAAT CTGTAAGAGC CTGATTTTAG AATTCACCAG
101 CTCCTCAGAA GTTTGGCGAA ATATGAGTTA TTAAGCCTAC GCTCAGATCA
151 AGGTAGCAGC TAGACTGGTG TGACAACCTG TTTTTAATCA GTGACTCAAA
201 GCTGTGATCA CCCTGATGTC ACCGAATGGC CACAGCTTGT AAAAGAGAGT
251 TACAGTGGAG GTAAAAGGAG TGGCTTGCAG GATGGAGAAG CTGCACCAGT
301 GTTATTGGAA GTGAGCCACC ATTTGAATTT GCTAGCTCAT GCTGCAGTAT
351 TCAGATTATG CCAGCAGGTT TATTTTTTGT TTTGCAAGCC AGCTCTGCCT
401 CCTTACAGTA TGACATCTGA TGCTGGAGGG TCGCACTTTC AAAAATGAGT
451 CAGCTGGTAC ATGGGGTTAT CATCAATTTT TAGCTCTTCT GTCTGGGAGA
501 TACAAGTTTG GAAGCAATCT TGGGGTACTT ACCCACAAGG CTGGTGGAGA
551 CCAGATCAGG AGAACCTCAG TCTGACGACA TTGAAGCTAG CCGAATGAAG
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Fig. 2 Sequence of the 5' ends of *UBE3A* cDNAs c7-3 and c10. The 5' ends of these cDNA fragments are identical with two exceptions: c10 contains 60 bp from OP2 whereas c7-3 contains only 53 bp from OP2, and c10 contains only 65 bp from exon 5 as a result of a splice site within this exon. Nts 1–61 (red): exon 1 (OP2 sequence nucleotides 2340–2399); nts 62–125: exon 3; nts 126–245: exon 4; nts 246–358: exon 5; nts 359–554: exon 6; nts 555–596: exon 7; nts 597–: exon 8. Sequence corresponding to the 5' end of the published *UBE3A* cDNA[16] is shown in blue. The OP2 sequence, Genbank Accession No. L23501, lacks a G between positions 2384 and 2385 that would be predicted from the sequence of both cDNA clones. The presence of this G has been confirmed by sequencing genomic clones of this region.

SSCP analysis of products derived from two NDUI AS brothers (WJK14 and WJK15; refs. 19,20) with primers flanking exon 10 showed an abnormal band that was also present in their normal mother but not in their father. Sequence analysis showed that the brothers are heterozygous for an A to G transition at position –8 of the 3' splice junction. The mutation creates a new consensus splice site and generates a *Tsp45*I restriction site (Fig. 4). Utilization of the newly created splice junction, 7 bp upstream from the normal junction, would lead to a frameshift and to premature termination of translation. This mutation was not present in 100 normal control individuals. The normal phenotype of the mother is presumably a consequence of paternal inheritance of the mutation.

Our identification of *UBE3A* mutations that presumably result in premature termination of translation in one sporadic and one familial AS case, together with mapping of an AS balanced inversion breakpoint within the 5' region of the *UBE3A* gene, indicates that *UBE3A* mutations are one cause of AS. Mapping of the AS mutation distal to *D15S122* (ref. 13) in two siblings would suggest that this mutation is in exon 1, the promoter or the 5' regulatory sequences of *UBE3A*.

The AS gene is predicted to be expressed either exclusively or preferentially from the maternal allele, because paternal transmission of inferred AS gene mutations has no phenotypic effect in the offspring[5,7]. Nakao *et al.*[15] showed that *UBE3A* is expressed in lymphoblasts and fibroblasts in patients with PWS and AS deletions. Therefore, its expression is clearly not exclusively from the maternal allele. However, there are a large and growing number of examples of genes for which imprinted expression is either tissue-specific[21,22], specific to developmental stage[23,24], species-specific[25-27], promoter-specific[28], or partial[29]. Detailed characterization of the imprinting pattern of *UBE3A* in specific tissues at different developmental stages will require identification of nucleotide sequence polymorphisms to allow analysis of allele-specific expression.

The product of the *UBE3A* gene was initially identified as a protein that forms a complex with the E6 protein of certain human papillomaviruses (HPVs); hence its original name is the E6-associated protein (E6-AP)[16]. This E6-AP/E6 complex inactivates the p53 tumor-suppressor protein. E6-AP/UBE3A is a member of a

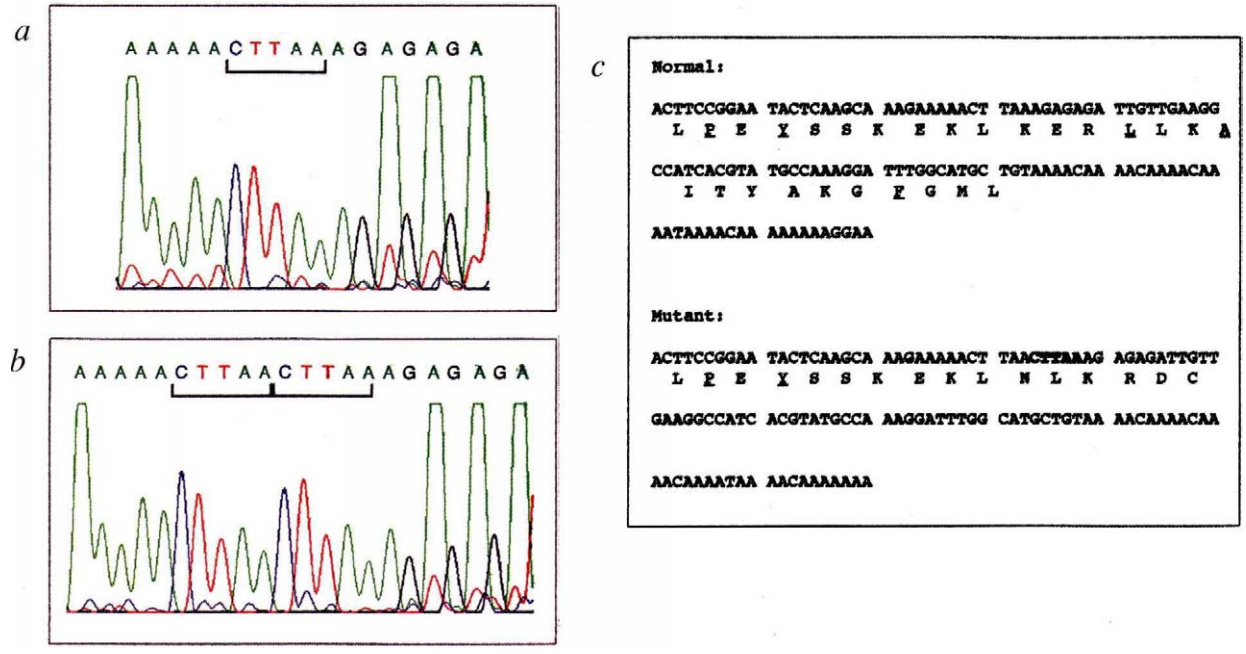

Fig. 3 Sequence of normal *UBE3A* exon 16 and a mutant exon 16 with a 5-bp tandem duplication. ***a***, Chromatogram of sequence from clone of normal allele. ***b***, Chromatogram of sequence from clone of mutant allele. ***c***, Sequence of normal and mutant alleles. Sequence of normal allele is the same as that beginning with nucleotide 2514 in a previous study[16]. Amino acid residues identical in at least 10 of 11 hect domain proteins are underlined. The 5-bp tandem duplication of CTTAA is shown in boldface. The duplication is predicted to lead to premature termination of the UBE3A protein.

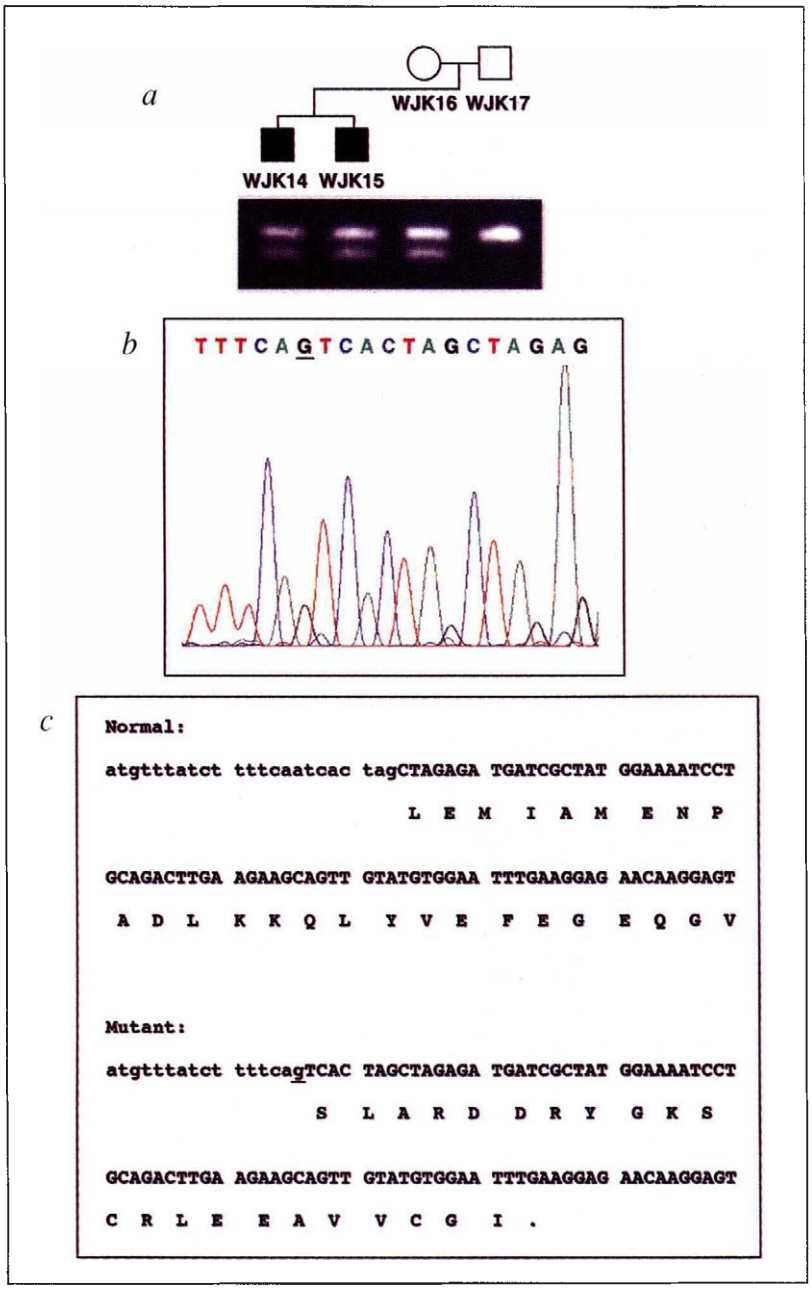

Fig. 4. *UBE3A* mutation in AS brothers WJK14 and WJK15 and their mother. ***a***, *Tsp45*I restriction enzyme analysis of exon 10 PCR products from patients and their parents. The AS brothers and their mother are heterozygous for an A to G transition that creates the new restriction enzyme site. ***b***, Chromatogram of sequence from clone of mutant allele. ***c***, Sequence of normal and mutant alleles. Intron sequence is shown in lower case, exon in upper case. Mutant nucleotide is underlined. The A to G transition creates a new 3' splice junction, which is predicted to shift the splice site 7 bp upstream, leading to frameshift and premature termination.

class of functionally related E3 ubiquitin-protein ligases defined by a 'hect (homologous to the E6-AP carboxyl terminus) domain'[17] and thought to play a role in defining substrate specificity of the ubiquitin system. However, more recent evidence has indicated that ubiquitin is transferred from E1 to a specific E2 to UBE3A, which then transfers ubiquitin directly to specific substrates[30]. Therefore, UBE3A probably plays two roles: one in catalysis and one in recognition. A UBE3A mutant protein lacking the last 6 amino acids is completely deficient in its capacity to ubiquinate p53 (ref. 17). The *de novo* tandem duplication that we have described in an AS patient results in a predicted mutant protein that lacks the last 17 amino acids, and should therefore represent a null allele.

Previous evidence of a role for ubiquitin-mediated proteolysis during CNS development has come from the identification of the *Drosophila bendless* gene product as an E2 ubiquitin-conjugating protein[31]. The phenotype of *bendless* indicates a role for this protein in the alteration of synaptic connectivity among a subset of CNS neurons and in development of the visual system. The normal role of UBE3A in human brain development may involve ubiquitin-mediated destruction of specific proteins. Alternatively, a role for ubiquitin-mediated proteolysis in the generation of active proteins from inactive precursors has been found, for example in the case of the transcription factor NF-κB [32]. The fact that almost all phenotypic abnormalities in AS can be attributed to abnormal brain development and function probably is a reflection of tissue-specific imprinting of *UBE3A*. The relevant substrates of UBE3A in developing brain are not known; UBE3A substrates other than p53 have not been identified as yet, and there is no published evidence for UBE3A-mediated degradation of p53 in non-HPV infected cells.

In summary, we have found that an inversion that causes AS when transmitted maternally disrupts the *UBE3A* gene, and we have found two mutations in NDUI AS patients that are predicted to eliminate UBE3A function. These results indicate that mutations in *UBE3A* are one cause of AS, and suggest that a process of ubiquitin-mediated proteolysis obligatory for normal brain development is disrupted in AS.

Methods

Exon trapping. Exon trapping using *BamH*I–*Bgl*II double-digested DNA from P1 clone 198 (Genome Systems, Inc. clone no. 551F) was performed using the Exon Trapping System (Gibco/BRL) with vector pSPL3 (ref. 33). Exons isolated from this clone were sequenced on an ABI automated DNA sequencer.

Mapping of exons. Phage libraries were constructed from P1 clone 198 and a partially overlapping P1 clone in vector λFIXII (Stratagene). A genomic phage contig covering the *UBE3A* gene was constructed using these libraries and a human genomic phage library. Exons and *D15S122* were mapped by PCR and by hybridization to restriction digests of these phage clones.

cDNA library screening. A normal human fetal brain cDNA library in vector λZAPII (Stratagene) was screened with a probe made by PCR amplification of human genomic DNA with the EST primers for *UBE3A* described by Nakao *et al.*[15].

SSCP analysis. PCR amplification of exon 16 of *UBE3A* was carried out with flanking primers E6Z-A (5'–ACCATGACTTACAGTTTTCCT–3') and E6Z-B (5'–TGGGACACTATCACCACCAA–3'), which generate a 277-bp product in normal human DNA. Exon 10 was amplified with intron primers E6-10A (5'–GCAATCATCTTCTTTTCATGTT–3') and E6-10B (5'–CGACACCATAATCACATTAC–3'), which generate a 217-bp product. Exon 10 contains nucleotides 1588–1732 of the published cDNA sequence[16]. PCR conditions were as follows: 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, followed by a cycle with a 5 min extension at 72 °C. PCR products were labelled by

incorporation of α-^{32}P dCTP. Exon 16 PCR products were cleaved with *Hpa*II. Products were separated on 0.5× MDE gels (A-T Biochemical) with or without 5% glycerol added and were visualized by autoradiography. PCR products from patient DNA were cloned into the pCRII TA vector (Invitrogen) and were sequenced on an ABI 373A automated DNA sequencer.

Restriction enzyme analysis. Exon 10 PCR products from WJK14, 15, 16 and 17 were digested with *Tsp45*I (New England Biolabs) according to manufacturer's recommendations and were separated on a 2% agarose gel.

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