

ORIGINAL INVESTIGATION

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## Screening for *UBE3A* gene mutations in a group of Angelman syndrome patients selected according to non-stringent clinical criteria

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**Abstract** The Angelman syndrome (AS) is caused by genetic abnormalities affecting the maternal copy of chromosome region 15q12. Until recently, the molecular diagnosis of AS relied on the detection of either a deletion at 15q11–13, a paternal uniparental disomy (UPD) for chromosome 15 or imprinting mutations. A fourth class of genetic defects underlying AS was recently described and consists of mutations of the *UBE3A* gene. The vast majority of mutations reported so far are predicted to cause major disruptions at the protein level. It is unclear whether mutations with less drastic consequences for the gene product could lead to milder forms of AS. We report on our results obtained by screening 101 clinically diagnosed AS patients for mutations in the *UBE3A* gene. Non-stringent clinical criteria were purposely applied for inclusion of AS patients in this study. The mutation search was carried out by single-strand conformation polymorphism (SSCP), and SSCP/restriction fragment length polymorphism (RFLP) analyses and revealed five novel *UBE3A* gene mutations as well as three different polymorphisms. All five mutations were detected in patients with typical features of AS and are predicted to cause frameshifts in four cases and the substitution of a highly conserved residue in the fifth. The results we obtained add to the as yet limited number of reports concerning *UBE3A* gene mutations. Important aspects that emerge from the data available to date is that the four classes of genetic defects known to underlie AS do not appear to cover all cases. The genetic defect underlying approximately 10% of AS cases, including some familial cases, remains unknown.

### Introduction

The Angelman syndrome (AS) is a neurological disorder characterised by severe mental retardation, absence of speech, paroxysms of laughter, epilepsy and abnormal encephalographic activity (EEG; MIM 105830). The syndrome is caused by genetic abnormalities of an imprinted region on chromosome 15q11.5. Before the role of the *UBE3A* gene in AS was known, the molecular diagnosis of the syndrome relied on the detection of approx. 80% of cases, which fell into the following three categories: a “common” 15q11–q13 deletion of about 4 Mb is detected in approx. 65–70% of AS patients; a paternal uniparental disomy (UPD) for chromosome 15 in 2–3% of cases; and the occurrence of an imprinting defect is reported in about 3–5% of patients. The discovery of the role played by *UBE3A* in the pathogenesis of the syndrome led initially to the postulation that mutations in this gene could be responsible for the remaining approx. 20% of cases (Kishino et al. 1997; Matsuura et al. 1997; Rougeulle et al. 1997; Vu and Hoffmann 1997). The initial optimism was, however, soon dampened by the detection of a lower than expected frequency of mutations (Fang et al. 1999; Malzac et al. 1998; Moncla et al. 1999a).

The *UBE3A* gene, also known as E6-AP, is an approx. 120-kb gene located in the AS critical region (Nakao et al. 1994). The *UBE3A* gene product is a ubiquitin-protein ligase and catalyses the aggregation of ubiquitin to substrates in E6-dependent pathways as well as E6-independent pathways. The resulting protein complexes are a target for proteasome degradation (Huibregtse et al. 1993a; Nawaz et al. 1999). Although the gene is expressed from both alleles in a wide range of tissues, selective imprinting was recently reported in brain (Rougeulle et al. 1997; Vu and Hoffman 1997), whereby expression occurs only from the maternal copy. Patients with mutations in the *UBE3A* gene present with typical clinical AS features (Jiang et al. 1999; Moncla et al. 1999a), such that the gene is probably responsible for the major phenotypic manifestations of the syndrome. Phenotypic differences are known

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to occur amongst AS patients (Bottani et al. 1994; Bürger et al. 1996; Moncla et al. 1999a, 1999b). The most severe forms of AS are usually found in patients with deletions, followed by patients with *UBE3A* mutations; milder forms of AS are described for patients with UPD15 and imprinting mutations.

The majority of *UBE3A* mutations reported so far represent frameshift mutations or other insertions and deletions that would lead to drastic consequences for the gene product (Fang et al. 1999; Fung et al. 1998; Kishino et al. 1997; Malzac et al. 1998; Matsuura et al. 1997; Moncla et al. 1999a; Tsai et al. 1998). It is still unclear whether DNA mutations with less dramatic predicted changes at the amino acid level might lead to milder forms of the syndrome. The work presented here aimed at providing a screening for *UBE3A* mutations in a large number of clinically assessed AS patients, which were selected according to non-stringent criteria.

## Subjects and methods

### Subjects

Patients diagnosed by geneticists in our Institute or referred to us over the past decade for molecular investigations of AS were included in the study after exclusion of a 15q11–q13 deletion, paternal UPD15 and an imprinting defect. A total number of 101 cases have been analysed so far. In three patients the syndrome was familial, with two or more affected siblings.

The diagnostic criteria compiled by Williams et al. (1995) were applied, wherever possible, in order to differentiate between patients with strong clinical AS features and those with mild to uncertain phenotypes. Table 1 summarises the clinical features observed in our group of AS patients. Precise clinical descriptions were unfortunately not available for all patients referred to us, and the very young age of some probands did not allow an assessment of their psychomotor development.

### Methods

The screening procedure for mutations in the *UBE3A* gene was at first carried out by SSCP and SSCP/RFLP analysis. Further inves-

**Table 1** Summary of clinical features of a group of Angelman syndrome (AS) patients. Groups 1–4, typical AS features; groups 5–13, mild AS features; group 14, unknown clinical features

Group	Clinical findings	Patients (n)	Mutations/polymorphisms
1	Developmental delay, speech impairment, typical ataxic gait, characteristic laughter, seizures, abnormal EEG, typical associated features (e.g. protruding tongue, wide mouth, hyperactivity of lower limbs)	13 Including two familial cases	4 Mutations: AS298 (familial), AS311, AS454, AS542 1 Polymorphism: AS328
2	As group 1; however: Abnormal EEG, no seizures Normal EEG, seizures Normal EEG, no seizures	3 2 5	None None None
3	No clinical details provided; however, patients seen by experienced geneticists and defined as "typical"	23	1 mutation: AS476
4	Patients for whom only a few characteristics were provided Developmental delay, speech impairment, associated features (as in group 1) Developmental delay and ataxic gait Developmental delay, ataxia, seizures, abnormal EEG Developmental delay, ataxia, seizures Developmental delay, ataxia, characteristic laughter, unclear EEG Developmental delay, microcephaly	1 1 1 1 1 1	None None None None None None
5	Patients with mild ataxia Otherwise as group 1, without seizures With developmental delay, characteristic laughter, mild to normal speech, with or without seizures With developmental delay and characteristic laughter Otherwise as group 1, with mild associated features With developmental delay, speech impairment, abnormal EEG, no seizures	2 4 1 1 1	None None 1 Polymorphism: AS520 None None
6	Patients with no characteristic laughter Otherwise as group 1, with abnormal EEG and seizures Otherwise as group 1, with normal EEG and no seizures With seizures and abnormal EEG, mild ataxia, mild speech problem	2 3 1	1 Polymorphism: AS446 None None
7	Patients with slightly impaired to normal speech With developmental delay and characteristic laughter, with or without seizures With ataxia, characteristic laughter, normal EEG, no seizures	3 1	None None
8	Abnormal EEG with or without seizures	3	None
9	Developmental delay, ataxia with or without associated features and seizures	4	None
10	Differential diagnosis: AS/RET syndrome	5	None
11	Differential diagnosis: AS/Prader-Willi syndrome	3	None
12	Differential diagnosis: mosaic trisomy 8 (negative)/AS	1	None
13	Differential diagnosis: fragile X syndrome (negative)/AS	2	1 Polymorphism: AS500
14	Referred to us, no clinical data provided	12	1 Polymorphism: AS259

tigations included DNA sequencing of the larger exons and long-range PCR amplifications of genomic segments encompassing intron sequences.

The individual exons of the coding region of *UBE3A* (exons 7–16) as well as exons 4 and 5 were amplified from genomic DNA by polymerase chain reaction (PCR) using standard methods. The PCRs consisted of 35 cycles of: a denaturing time of 30 s (3 min in the first cycle) at 94°C, primer annealing at 50–56°C for 45 s, and primer extension at 72°C for 1 min. The nucleotide sequences of the primers used were as follows: exon 4, forward TGT ATT TCA TT TAC AAT GAC<sup>a</sup>, reverse TAA AGT GTT CTA ACC AAA GG<sup>a</sup>; exon 5, forward TTT CTC TAG AAG TTT TTA TAA C<sup>a</sup>, reverse ATC GCA GAA AAT ATG ATC AC<sup>a</sup>; exon 7, forward ATG GAA TAT TTT GCT AAC TG, reverse TAA GAA CCA CAG TCT CAA CC; exon 8, forward GCT TAT TGT TTG AAT GTT TGG, reverse GGT TTT CAG GCA ACA ATT CTC; exon 9, forward (a) GCA ACA GAG TAA ACA TAC ATA TT, forward (b) TGC AGA CCA GAT TCG GAG, reverse (a) AAT GAA TTC ACT GAA CTG TAT CAT G, reverse (b) TCT CCG AAT CTG GTC TGC; exon 10, forward GCA ATC ATC TTC TTT TCA TGT T<sup>b</sup>, reverse CGA CAC CAT AAT CAC ATT AC<sup>b</sup>; exon 11, forward TTA AAA ATC ATT TCT TAT AGG, reverse GAA TTA AAA AAA TGA CAA AG; exon 12, forward GCC TCA ATT TAC CAT TTC TG, reverse AAT GAA GAG ACA AAA TGT GAC; exon 13, forward (a) GAA GTT CTT GTG ATT AAT GT<sup>a</sup>, forward (b) AAA ACA GTT CAA GGC TTT TC<sup>a</sup>, reverse (a) CTT TAA GGG AGA TTC ATT GG<sup>a</sup>, reverse (b) CGA TAC ATG ACT TTT TGC AG<sup>a</sup>; exon 14, forward AGT AGT ATA GCA GAT AAC TAA GAC<sup>a</sup>, reverse CCC TTT GGT GAA TCA AAT CTT CC<sup>a</sup>; exon 15, forward GAA TGC CAA ACT GAA ACC AG, reverse GTC ACA AGT TAA TAA TTA CC; exon 16, forward ACC ATG ACT TAC AGT TTT CCT<sup>b</sup>, reverse TGG GAC ACT ATC ACC ACC AA<sup>b</sup>.

Primers indicated by *superscript a* were synthesised according to the sequences reported by Malzac et al. (1998), and those by *superscript b*, according to Kishino et al. (1997). The other primers were designed on the basis of the genomic sequences published by Kishino and Wagstaff (1998). The oligonucleotide primers were used in the unlabelled form for PCR and SSCP reactions, and labelled at the 5' end with Cy5 for sequencing reactions.

The SSCP and SSCP/RFLP analysis were carried out using standard methods. Aliquots of the PCR products representing exons 8 and 9 were digested with different restriction enzymes prior to SSCP, as follows: exon 8 PCR products were digested separately with *XhoI* and *NdeII*; exon 9 PCR products were digested separately with *TaqI*, *HinfI*, *MspI* + *AvaII* + *HaeIII* + *Sau3AI*, *Tru9I*, *HinfI* + *XbaI* + *Sau3AI* and *DdeI* + *Sau3AI*. The SSCP analysis was carried out using a Genephor electrophoresis unit (Pharmacia). DNA Sequencing reactions were performed using the Pharmacia Cycle Sequencing Kit and were analysed using an ALF automatic sequencer (Pharmacia). Long-range PCR amplifications

were carried out essentially as described above. The extension time was increased to 2 min and the primers were chosen to cover intron sequences.

## Results

The screening for *UBE3A* mutations in our group of patients with suspected AS resulted in the detection and characterisation of five novel *UBE3A* mutations and three polymorphisms. The major characteristics of the eight DNA variations are summarised in Table 2. All DNA variations were detected by SSCP and SSCP/RFLP. Systematic DNA sequencing of the largest exons (exons 8, 9 and 16) in all patients did not reveal further DNA variations, nor were further mutations detected by the long-range PCR analysis aimed at detecting larger deletions.

The first mutation listed in Table 2 represents the only familial *UBE3A* mutation we have detected in our group of AS patients so far. Two affected brothers, currently aged 13 years and 10 years, inherited the same allele from their asymptomatic mother. The mutation in this family as well as those detected for patients AS 476 (a 9-year-old girl) and AS 311 (a 15-year-old girl) are all predicted to have drastic consequences at the protein level, leading in each case to a frameshift and a truncated protein. The mutation detected in the next patient (AS 454, a 9-year-old girl) causes the substitution of a highly conserved amino acid (Fig. 1). The last *UBE3A* mutation that we found, detected in an 8-year-old girl (AS 542), consisted in a short tandem duplication of 16 bp, which is predicted to cause a frameshift and an extension of the protein.

Three further DNA variations were considered as polymorphisms. The base substitution detected in patient AS 520 leads to the exchange of the alanine at codon 178 for a threonine. Although this protein region is homologous in humans and mice, it doesn't seem to be highly conserved in other species. However, the main argument in favour of a polymorphism is obviously the paternal transmission of the DNA variation. The second polymorphism listed in Table 2 (detected in the DNA sample of patient AS 259) is a T-to-C base substitution, which occurs at the

**Table 2** *UBE3A* mutations and polymorphisms detected in our group of clinically diagnosed AS patients. The nucleotide and codon positions refer to the complete *UBE3A* cDNA sequence, accession number U84404

Patient ID	Exon	DNA variation	Predicted aa change	Familial/sporadic	Comments
AS 298	8	762–763insGA	Frameshift	Familial: brother, affected; mother, carrier	AS mutation
AS 454	9	2102 C to T	Arg506Cys	Sporadic: mother, no signs of mosaicism; father, normal	AS mutation
AS 476	9	1296insT	Frameshift	Sporadic: mother, no signs of mosaicism; father, normal	AS mutation
AS 311	11	2376delG	Frameshift	Sporadic: mother, no signs of mosaicism; brother, normal	AS mutation
AS 542	16	3120ins16	Frameshift	Sporadic: mother, no signs of mosaicism; father, normal	AS mutation
AS 520	9	1118 G to A	Ala178Thr	Inherited from father	Polymorphism
AS 259	12–13 Intron	T to C	No effect?	Parental samples not available	Polymorphism
AS 446	16	3'UTR del14 <sup>a</sup>	No effect?	Inherited from mother	Polymorphism
AS 500	16	3'UTR del14 <sup>a</sup>	No effect?	Inherited from father	Polymorphism
AS 328	16	3'UTR del14 <sup>a</sup>	No effect?	Parental samples not available	Polymorphism

<sup>a</sup>Corresponds to the polymorphism previously described by Fung et al. (1998)

Patient "AS 454" a*		codon 506 C	
Homo sapiens	a*	Q G Q Q L N P Y L R L K V R R	D H I I D D A L V R L E M I A
Mus spretus	b*	Q G Q Q L N P Y L R L K V R R	D H I I D D A L V R L E M I A
Mus musculus	c*	Q G Q Q L N P Y L R L K V R R	D H I I D D A L V R L E M I A
Saccharomyces pombe	d*	V E P Y L M I R V R R	D R L L E D S L
Saccharomyces cerevisiae	e*	K G K S V D V Y F K I K V R R	D V I S H D S L
Mya arenaria	f*	G M P T M P Y L R I R V S R N R	I I D D A L V A L E M V A

**Fig. 1** The amino acid substitution at codon 506 in patient AS454 results in the modification of a highly conserved protein residue. The amino acid sequences reported here represent: *a\**, human ubiquitin protein ligase E3A (U84404), residues 492–521; *b\**, E6-AP ubiquitin protein ligase (AF082835), residues 489–518; *c\**, E6-AP ubiquitin protein ligase (U82122), residues 489–518; *d\**, putative ubiquitin-protein ligase (ALO32684), residues 429–447; *e\**, hypothetical 103.5-kd protein (sp/P40985/YJ06), residues 513–535; *f\**, E3 ubiquitin-protein ligase (AF154109), residues 215–243

9th base of the intron between exons 12 and 13. The parental DNA samples were unfortunately not available. The last polymorphism, a 14-bp deletion in the 3'UTR, was detected in three unrelated patients. This DNA polymorphism corresponds to that reported by Fung et al. (1998). The 14-bp deletion was present in the mother of one proband (AS 446) and in the father of a second patient (AS 500). Parental samples of the third proband (AS 328) were unfortunately not available.

## Discussion

We present the results obtained by screening clinically diagnosed AS patients for mutations in the *UBE3A* gene. The type and frequency of mutations we detected are in agreement with the results reported by other groups (Fang et al. 1999; Fung et al. 1998; Kishino et al. 1997; Malzac et al. 1998; Matsuura et al. 1997; Moncla et al. 1999a; Tsai et al. 1998). The five heteroallelic mutations reported here are all "private" mutations; they are located in four different exons and do not appear to be clustered at "hot-spot" regions, although they are all predicted to affect the hect domain (Huibregtse et al. 1995). Two frameshift mutations are located upstream of the hect domain, one within the first half of the domain and the last towards the 3' end of the domain. The fifth mutation, located at the 5' end of the hect domain, causes the substitution of a highly conserved residue (R506C).

It is still unclear whether *UBE3A* mutations, which do not directly involve the hect domain, may lead to milder forms of the disorder. Interestingly, mild AS phenotypes were observed by Matsuura et al. (1997) and Fang et al. (1999) in siblings with a mutation that is not predicted to affect the hect domain (C21Y). The missense mutation S349P, detected by Malzac et al. (1998) in three brothers

with typical AS phenotypes, is localised within a *UBE3A* region that is involved in the E6-dependent association with p53 (Huibregtse et al. 1993b).

We intentionally did not apply stringent clinical criteria for inclusion of AS patients in the study in order to investigate whether *UBE3A* mutations could also lead to milder, non-typical AS-like cases. However, amongst the 101 patients we have screened so far, *UBE3A* mutations were detected only in patients with typical features of AS (Table 1). We are aware of the fact that probably less than one-half of the patients referred to us for molecular investigations are indeed affected by AS. Based on the incidences of AS patients with deletions and UPD15, we estimated the frequency of *UBE3A* mutations in our group of AS patients at about 9–10%. The number of *UBE3A* mutations we detected in our group of patients is thus in agreement with the frequency reported by other groups. What emerges from the data available so far is that the genetic defect underlying approximately 10% of all AS cases remains unknown (Jiang et al. 1999). Amongst the unsolved cases, we have patients with typical AS features, including a couple of familial cases (Table 1).

The obvious questions are whether a large number of *UBE3A* mutations remain undetected and whether there are other "AS genes" that also lead to typical features of the syndrome. DNA mutations, in *UBE3A* itself or other genes, which would have repercussions on the transcription and expression of the *UBE3A* gene would be particularly difficult to detect and would have been missed in our screening. The major difficulties in carrying out the mutation search at the cDNA level is the biallelic expression of the gene in the majority of tissues, the low expression of the gene in blood (Kishino and Wagstaff 1998), and the presence of at least two highly homologous processed pseudogenes (Kishino and Wagstaff 1998).

It is as yet unclear how an abnormal expression in brain of the *UBE3A* gene leads to the complex phenotypes typical for AS. Major challenges in the field will be to provide a better understanding of the *UBE3A* gene in AS and animal models and to reveal the genetic defects underlying the remaining 10% or so of unsolved AS cases.

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