Investigation of *UBE3A* and *MECP2* in Angelman Syndrome (AS) and Patients With Features of AS

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Angelman syndrome (AS) is an imprinted neurobehavioral disorder characterized by mental retardation, absent speech, excessive laughter, seizures, ataxia, and a characteristic EEG pattern. Classical lesions, including deletion, paternal disomy, or epigenetic mutation, are confirmatory of AS diagnoses in 80% of cases. Loss-of-function mutations of the *UBE3A* gene have been identified in ∼8% of AS cases, failing to account for the remaining patient population, and there appears to be a higher prevalence of mutations in familial than sporadic cases. We screened *UBE3A* in 45 index cases of AS without obvious 15q11-13 abnormalities. Pathological mutations were identified in 3/6 (50%) familial and 4/39 (>10%) sporadic cases. By combining our data with those of the literature, we demonstrate statistically that the frequency of *UBE3A* mutations is significantly higher in the familial than sporadic subsets of AS. This indicates that an independent molecular mechanism or 'phenocopy' exists for the sporadic group. Rett syndrome (RS), caused by mutations of the *MECP2* gene, and patients with deletions of 22q13.3 → qter, have overlapping clinical features with AS. We screened 24 of the sporadic AS cases without detectable *UBE3A* mutations for mutations of *MECP2*, but found none. A separate cohort of 43 atypical patients with features common to AS and RS, in whom 15q11-13 lesions and 22q13.3 → qter deletion had been ruled out, were also screened for *MECP2* mutations. One male patient was mosaic for a frameshift mutation of this gene (previously reported). While *MECP2* mutations can cause a phenotype reminiscent of AS in rare cases, they fail to account for the excess of sporadic patients with a definitive clinical diagnosis of AS. © 2003 Wiley-Liss, Inc.

KEY WORDS: Angelman syndrome; *UBE3A*; Rett syndrome; *MECP2*

INTRODUCTION

Angelman syndrome (AS) is a well characterized neurobehavioral disorder with strict clinical criteria for diagnosis. The main clinical features of AS are mental retardation, seizures, ataxia, lack of speech, paroxysms of laughter, movement stereotypes including hand-flapping, acquired microcephaly, facial anomalies, and a characteristic EEG pattern [Williams et al., 1995; Laan et al., 1997]. In 80% of patients, the clinical diagnosis is supported by molecular detection of one of the typical 15q11-13 abnormalities including chromosomal deletion, paternal uniparental disomy 15 (pUPD15), or an imprinting center mutation (reviewed by Nicholls et al., 1998]. Heterozygous loss-of-function mutations of the ubiquitin protein ligase 3A (E6-AP) gene (*UBE3A*) have been identified in ∼8% of cases [Malzac et al., 1998; Fang et al., 1999; Webber and Wagstaff, 1999; Hennies et al., 1999]. Although the pathogenesis of AS remains to be clearly defined, there is unequivocal genetic evidence that mutations of *UBE3A* cause AS: *UBE3A* is imprinted, with maternal expression in the brain in both human and mouse, consistent with the neurobehavioral manifestations of this disorder [Albrecht et al., 1992; Rougeulle et al., 1997; Vu and Hoffman, 1997]. *UBE3A* mutations are only pathogenic when maternally transmitted, as witnessed in a number of familial cases [Fang et al., 1999; Moncla et al., 1999]. Despite this, the yield of *UBE3A* mutations in AS cases with no classical

abnormality of 15q11-13 is low. Furthermore, there appears to be a disparity in frequency of mutations in familial and sporadically occurring cases, with a higher rate of *UBE3A* mutations in familial cases in which linkage to 15q12 is demonstrable [Malzac et al., 1998; Fang et al., 1999]. The lack of mutations in sporadic patients poses the greatest concern with respect to genetic counseling, as an accurate risk of recurrence cannot be given [Stalker and Williams, 1998].

There are several disorders with a number of clinical features in common with AS including the phenotype associated with a deletion of 22q13.3 → qter and Rett syndrome (RS). It is possible that sporadic patients with either disorder have been misdiagnosed as AS due to phenotypic overlap, especially in young children prior to the emergence of differential diagnostic features [Williams et al., 2001]. Patients with a terminal deletion of 22q13.3 have mental retardation and severe speech delay, with seizures and a happy affect also reported in some cases [Precht et al., 1998; Knight et al., 1999; De Vries et al., 2000]. RS is characterized by mental retardation, seizures, muscular hypotonia, awkward gait, hand-movement stereotypes, and acquired microcephaly, in common with AS [Clarke, 1996]. RS occurs almost exclusively in females and is sporadic in >95% of cases. Heterozygous mutations of the X-linked methyl CpG binding protein 2 gene (*MECP2*) have been identified in girls with RS [Amir et al., 1999; Bienvenu et al., 2000]. Clinical heterogeneity exists within RS, which may be attributable to the type of mutation, somatic mosaicism [Wan et al., 1999; Bourdon et al., 2001], and non-random X-inactivation within families [Amir et al., 2000]. Furthermore, hemizygous *MECP2* mutations have been reported in a small number of males with severe mental retardation and movement disorders [Orrico et al., 2000; Meloni et al., 2000; Imessaoudene et al., 2001]. Recently, two reports have described *MECP2* mutations in 11 patients with AS features [Watson et al., 2001; Imessaoudene et al., 2001], including an "Angelman-like" male mosaic for a frameshift mutation of this gene [Watson et al., 2001].

In this study, we investigated our cohort of 45 cases with a clear diagnosis of AS, without 15q11-13 abnormalities, for mutations of *UBE3A*. To investigate RS as a 'phenocopy' for AS, we also screened a proportion of the sporadic AS patients in whom no *UBE3A* mutations were identified, for mutations of *MECP2*. In addition, we analyzed a second group of 43 patients with a broader phenotype, who had clinical features common to both AS and RS, for mutations of *MECP2*.

MATERIALS AND METHODS

Patients

Patients were referred to the Great Ormond Street Hospital for Sick Children (GOSH) and examined by experienced clinical geneticists for differential diagnosis of AS. Blood samples for DNA analysis and generation of lymphoblastoid cell lines were taken with the informed consent of the patient or guardians, and ethical approval for this study was provided by the GOSH Ethical Committee (application 642).

A group of 45 index cases meeting the diagnostic criteria for AS [Williams et al., 1995] was screened for *UBE3A* mutations. Deletion of 15q11-13, pUPD15 [Chan et al., 1993], and imprinting mutations had previously been ruled out. This group included six familial cases in which the affected siblings were previously shown to share the same maternal 15q12 haplotypes (of which five cases were described by Clayton-Smith et al., 1992), and 39 sporadically occurring cases. A subset of 24 sporadic patients, for whom further DNA was available, was additionally screened for *MECP2* mutations.

A second group of 43 atypical patients did not meet the clinical diagnostic criteria for AS, but had been referred for methylation analysis by a Clinical Geneticist due to clinical suspicion of AS. The clinical characteristics of these patients have previously been described [de Vries et al., 2002]. In summary, all patients had mental retardation, the majority had developmental delay, speech impairment, and a movement or balance disorder, but less than half had a happy demeanor, seizures, microcephaly, and as AS-like EEG pattern. Typical abnormalities of 15q11-13 and monosomy 22q13.3 → qter had been previously been ruled out [de Vries et al., 2002]. This group, comprising 14 males and 29 females, were screened for mutations of *MECP2* only.

Screen for *UBE3A* Mutations

SSCP analysis. SSCP analysis was performed using genomic DNA from AS patients and their immediate family members. Exons 1, 3–6, and 8–16 were screened for mutations using primers designed by the authors (sequences may be requested), Kishino et al. [1997], Yamamoto et al. [1997], and Malzac et al. [1998]. Exons 2 (5′-UTR) and 7 (first coding exon) were omitted, as these are <50 bp in length. Aliquots of the PCR products were denatured and electrophoresed on Mutation Detection Enhancement gels (Flowgen, Staffordshire, UK) under four different sets of conditions; at both 4°C (15 W) and room temperature (60 W), with and without 15% glycerol. The DNA was visualized by silver staining. For each amplicon, 60 normal randomized controls were analyzed alongside the AS samples.

Sequence analysis. Amplification products of *UBE3A* exons demonstrating altered SSCP migration patterns were sequenced directly to determine the nature of the change. Where necessary, the individual alleles were separated and re-sequenced by subcloning using a TA cloning kit (InVitrogen, Paisley, UK), or by excision of the shifted and normal DNA fragments from the SSCP gel, followed by re-amplification of the alleles using the original primers. Family members were also sequenced to determine the carrier status and mode of inheritance of any sequence changes identified. PCR products were purified using S-300 HR microspin columns (Pharmacia). Cloned products were sequenced from standard 'miniprep' plasmid DNA templates using vector primers. Sequencing was performed using the Big-Dye Terminator ready reaction kit (Applied Biosystems, Warrington, UK) and electrophoresed on an ABI 377 automated DNA sequencer (Applied Biosystems,

Warrington, UK). The sequences were compared to the published *UBE3A* exonic sequences by BLAST analyses and using the Sequence Navigator program. Mutant alleles were also compared to the *UBE3AP1* and *UBE3AP2* pseudogene sequences to ensure that sequence changes were not present in either pseudogene, ruling out co-amplification of these sequences [Kishino and Wagstaff, 1998]. Primers published by Kishino et al. [1997] and Malzac et al. [1998] were used to confirm the mutations by PCR and direct sequencing.

Confirmation of mutations in lymphoblastoid cDNAs. *UBE3A* mutations were confirmed in lymphoblastoid cDNA of the AS probands. Lymphoblastoid cell lines were cultured according to standard protocols. Feeding was performed within 24 hr of harvesting. Total RNA was extracted using the Trizol reagent (Invitrogen, Paisley, UK), and polyA+ RNA isolated using poly-dT beads (Dynal, Oslo, Norway). First-strand cDNAs were generated from 100 ng mRNA using poly-dT primers and MMLV reverse transcriptase (RT) (Promega, Madison, WI) according to standard protocol. PCR amplification of one-tenth of the RT reaction was performed using exonic primers crossing an intron and spanning the site of the mutation. (RT-PCR primer sequences may be requested from the authors). Amplification products were purified and sequenced.

Heteroduplex and Sequence Analyses of *MECP2*

Genomic DNA was PCR-amplified in 30 µl volumes using previously designed primer sequences and conditions [Amir et al., 1999], but with the forward primers labeled with FAM, HEX, or TET. Between 0.2 and 2 µl PCR product combined with 0.5 µl MegaBACE ET400-R size standard (Amersham Biosciences, Bucks, UK) in a final volume of 10 µl, was injected at 3 kV for 30 sec and electrophoresed at 7 kV for 75 min on a MegaBACE (Amersham). In the case of male patients, amplification products were additionally electrophoresed spiked with the equivalent product from a normal male control, to detect hemizygous mutations. Here, equal quantities of the labeled PCR products from the patient and normal control were combined, denatured by heating to 95°C for 5 min, then slowly cooled to room temperature to allow the products to reanneal, prior to injection. Where products gave an altered peak shape, the DNA was re-amplified using unlabeled primers and the PCR products purified and sequenced directly by fluorometric methods.

RESULTS

UBE3A Mutations in the GOSH Cohort of AS Patients

The *UBE3A* gene was screened for mutations in 45 index cases of AS comprising six families and 39 sporadic individuals by SSCP followed by sequence analysis of genomic DNA and lymphoblastoid cDNA. Pathogenic mutations were identified in seven index cases, as listed in Table I, which were not present in 120 randomized normal chromosomes. Six of these mutations are novel.

Maternally inherited mutations were identified in 3/6 (50%) familial cases (Table I). In AS families, 'H' and 'W,' the mutations cause frameshifts, predicted to result in premature amino-acid chain termination with resultant loss of the substrate binding domain, the HECT domain and carboxy terminus. This would cause loss of ubiquitination activity in all E6-AP isoforms [Huibregtse et al., 1993]. In family 'A,' a 3 bp deletion resulting in loss of a phenylalanine residue at codon 782ECT domain within the HECT domain was identified in the mother and two affected siblings. This mutation has previously been identified in an unrelated AS family [Fang et al., 1999], indicating that this hydrophobic residue is functionally important.

Heterozygous mutations were identified in 4/39 (10.3%) sporadically occurring AS patients (Table I). A

TABLE I. *UBE3A* Mutations in Angelman Syndrome (AS) Cases and Families

Family/proband	Recurrence	Exon	Primers	Sequence alteration	Predicted effect	Mutation carriers
Family H	Familial	9	E6-9C[1], E6-9D[1]	1993del5	Frameshift; chain termination at amino acid 473	Mother and both affected siblings
Family W	Familial	9	E63-C[1], E63-D[1]	1161dup4	Frameshift; chain termination at amino acid 194	Mother and confirmed in two of three AS siblings
Family A	Familial	15	15F, 15R	2930del3	F782Δ	Mother and both affected siblings
Proband 13798	Isolated	14	E6-14F[1], E6-14R[1]	2855del12	YTRD757Δ	Mother
Proband 5954	Isolated	9	3F5, 3R5	1965delT	Frameshift; chain termination at amino acid 490	De novo
Proband 5173	Isolated	16	E6ZA[2], E6ZB[2]	3093del4	Frameshift; chain termination at amino acid 839	De novo
Proband 18244	Isolated	16	E6ZA[2], E6ZB[2]	3094dup5	Frameshift; chain termination at amino acid 842	De novo

Nucleotide and amino acid positions are according to GenBank accession number U84404. Previously published primer sequences: [1]Malzac et al., [1998], [2]Kishino et al. [1997].

12 bp deletion resulting in loss of 4 amino acids within the HECT domain was identified in sporadic patient 13798 and his mother, but not in an unaffected sibling, indicating a recurrence risk of 50% in this family. This mutation is likely to disrupt the main functions of the HECT domain, including thioester formation and ubiquitin transfer. In the remaining three sporadic cases, the mutations identified were de novo frameshift errors predicted to cause protein truncation, resulting in loss of the final 10–13 carboxy-terminal amino acids of E6-AP. Loss of the last six amino acids would render the mutant E6-AP incapable of transferring ubiquitin to target substrates [Huibregtse et al., 1993].

Disparity in Frequency of *UBE3A* Mutations in Familial and Sporadic AS Is Statistically Significant

To determine whether the higher frequency of *UBE3A* mutations observed in familial compared to sporadic AS patients was statistically significant, we combined our data with those from the literature, and analyzed them using the χ^2 (2×2) contingency test. Data was collated from studies which report (i) strict clinical ascertainment, (ii) a thorough screen of the *UBE3A* gene in familial and sporadic cases of AS, and (iii) the total number of cases screened (Table II) [Malzac et al., 1998; Baumer et al., 1999; Fang et al., 1999; van den Ouweland et al., 1999]. Only cases meeting the clinical diagnostic criteria for AS were included. These were categorized as 'familial' if there was more than one affected sibling, and as 'sporadic' if there was an isolated occurrence of AS within the family, irrespective of whether the mutation was de novo or maternally inherited in the latter group (Table II). The frequency of *UBE3A* mutations was found to be significantly different between the familial and sporadically occurring AS cases, ($\chi^2 = 36.03 \gg 10.83$

TABLE II. Frequency of *UBE3A* Mutation Detection Amongst Familial and Sporadic AS Cases

	Familial	Sporadic	Total
UBE3A mutation detected	20	24	44
No UBE3A mutation detected	8	123	131
Total (columns)	28	147	175

$\chi^2_{(1\,df)} = \frac{175(20 \times 123 - 24 \times 8)^2}{44 \times 28 \times 131 \times 147}$.

$\chi^2_{(1\,df)} = 37.94$.

$\chi^2_{(1\,df)} \gg 10.83, P \ll 0.001$.

The number of familial and sporadic index cases of AS reported with and without a mutation of *UBE3A* are given in a χ^2 (2×2) contingency table. The data from Malzac et al. [1998], Fang et al. [1999], van den Ouweland et al. [1999], Baumer et al. [1999], and our study were combined, and only included patients with a working diagnosis of AS. Cases of *UBE3A* mutations described by Kishino et al. [1997] and Moncla et al. [1999] were included in the study by Malzac et al. [1998], and those originally reported by Matsuura et al. [1997] and Tsai et al. [1998] were included in Fang et al. [1999], so there was no redundancy in numbers. Patients were categorized as familial if more than one sibling was affected, or sporadically occurring. Sporadic cases were grouped together irrespective of whether the *UBE3A* mutation identified was de novo or maternally inherited. The χ^2 contingency test was performed to determine whether the rate of *UBE3A* mutation detection was the same in the two AS subsets. $\chi^2_{(1)} = 37 \gg 10.8, P \ll 0.001$, indicating that the two patient categories differ significantly.

(1 df), $P \ll 0.001$ that there is no difference between the two patient categories) (Table II). This indicates that the clinical phenotype in the majority of sporadic cases is caused by an independent genetic mechanism, or a 'phenocopy,' which has reduced penetrance or a low recurrence risk.

Screen for *MECP2* Mutations in Sporadic AS Patients

To determine whether RS might represent a 'phenocopy' for AS, 24/35 of the sporadic AS patients for whom no *UBE3A* mutation was identified were screened for *MECP2* mutations. No DNA was available for 11 patients. No mutations were identified, suggesting that the remaining sporadic patients with a clear AS diagnosis but unknown molecular aetiology, are not attributable to a clinical overlap between RS and AS.

Screen for *MECP2* Mutations in Atypical Patients With Features of AS

In addition, we screened *MECP2* for mutations in a separate cohort of 43 patients with some features of AS, to investigate whether mutations of this gene might account for a broader phenotype with features that overlapped both AS and RS. One male patient, 14118, was identified with a mosaic 241del2 frameshift mutation within exon 2 of *MECP2* (GenBank accession number X99686), predicted to introduce a stop signal at codon 89. The level of mosaicism was estimated to be below 50% in PB lymphocytes on the basis of the relative heights of the peaks representing the mutated and normal alleles in the heteroduplex and electropherogram traces. The *MECP2* mutation in this patient has independently been identified by Watson et al. [2001]. There was no further overlap between our patients and the group studied by Watson et al. [2001]. Patient 14118 presented with mental retardation and delayed motor milestones, seizures, a broad ataxic gait, absent speech, hand-flapping, and inappropriate laughter, but did not have the typical AS EEG pattern. Repeated EEG examination showed an excess of slow wave activity. No mutations were identified in the remaining 42 patients.

DISCUSSION

We report disease-causing mutations of *UBE3A* in 3/6 familial cases and 4/39 sporadic patients with a clinical diagnosis of AS but no 15q11-13 lesion. Since the mutations in sporadic patients 18244, 5954, and 5173 occurred de novo, there is a low risk of recurrence, assuming there is no maternal gonadal mosaicism for the *UBE3A* mutation in these cases. It is likely that some *UBE3A* mutations were missed, due to the reduced sensitivity of SSCP compared to direct sequencing, or that mutations exist in regions of the gene not screened, including the promoter region, 3'-UTR, introns or the two <50 bp exons omitted. While there are likely to be as yet undetected mutations within the *UBE3A* locus in AS families where linkage to 15q12 has been established,

the lack of identification of *UBE3A* mutations in sporadic cases further complicates genetic counseling in these families.

Although the rate of *UBE3A* mutation detection has varied between individual studies depending on clinical ascertainment and technology employed for screening, a common notable factor is that mutations are more prevalent in AS families than in sporadically occurring patients. The bias in mutation rate between the two subsets in our AS cohort was consistent with previous surveys in other groups of patients. Reported mutation rates in familial and sporadic groups, respectively, range from 75–80 to 14–23% [Malzac et al., 1998; Fang et al., 1999], 1/3 (33%) to 4/49 (8%) in a further study [Baumer et al., 1999], and 50% familial to 10.3% sporadic in our study. We have now shown by combining the data from these studies with ours, that the higher prevalence of *UBE3A* mutations in familial cases of AS is statistically significant, providing evidence for a distinct genetic aetiology for disease in the sporadic group. It is possible that an alternative mechanism disrupting the regulation of *UBE3A* expression may exist in this category of patients, such as localized epigenetic defects or altered expression of the *UBE3A* antisense RNA. Alternatively, a 'phenocopy' may exist that is predominantly sporadic. Two reports have shown that mutations of *MECP2* can elicit a phenotype resembling AS in a small number of patients, including some who met the clinical diagnostic criteria for AS [Imessaoudene et al., 2001; Watson et al., 2001]. However, we did not identify any *MECP2* mutations in 24 sporadic cases with a definitive diagnosis of AS. Thus mutations of this gene failed to account for the excess number of sporadic AS cases with no detectable 15q11-13 lesion or *UBE3A* mutation in our cohort. Out of a separate group of 43 patients presenting with a marginal phenotype including some features of AS and RS, we identified a de novo mosaic frameshift mutation of *MECP2* in one male patient previously described as "AS-like" [Watson et al., 2001]. The frequency of *MECP2* mutations might be expected to be low in our second group of patients due to the inclusion of 14 male subjects. However, recent reports suggested that *MECP2* mutations elicit a broad clinical spectrum in males, including a phenotype resembling AS [Imessaoudene et al., 2001; Watson et al., 2001]. Our strategy of heteroduplex analysis of *MECP2* was designed to detect both hemizygous and mosaic mutations in the male patients. The precise clinical classification and genetic aetiology of the remaining 42 patients in this group remain unknown. Anomalies of 15q11-13, 22q13.3 → qter [De Vries et al., 2002], and now *MECP2* mutations have been excluded. In clinical practice, the marginal cases, which present with only some of the features of a syndrome pose the most difficulty in diagnosis and therefore, genetic counseling. Other clinical diagnoses with overlapping features of AS will need to be considered in this group of patients, such as the X-linked aplha-thalassaemia retardation syndrome, Gurrieri syndrome, methylene tetrahydrofolate reductase deficiency, Mowat–Wilson syndrome, and mitochondrial encephalopathy (reviewed by Williams et al., 2001). This will be the subject of further study.

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