Distinct phenotypes distinguish the molecular classes of Angelman syndrome


Abstract

Background—Angelman syndrome (AS) is a severe neurobehavioural disorder caused by defects in the maternally derived imprinted domain located on 15q11-q13. Most patients acquire AS by one of five mechanisms: (1) a large interstitial deletion of 15q11-q13; (2) paternal uniparental disomy (UPD) of chromosome 15; (3) an imprinting defect (ID); (4) a mutation in the E3 ubiquitin protein ligase gene (UBE3A); or (5) unidentified mechanisms. All classical patients from these classes exhibit four cardinal features, including severe developmental delay and/or mental retardation, profound speech impairment, a movement and balance disorder, and AS specific behaviour typified by an easily excitable personality with an inappropriately happy affect. In addition, patients can display other characteristics, including microcephaly, hypopigmentation, and seizures.

Methods—We restricted the present study to 104 patients (93 families) with a classical AS phenotype. All of our patients were evaluated for 22 clinical variables including growth parameters, acquisition of motor skills, and history of seizures. In addition, molecular and cytogenetic analyses were used to assign a molecular class (I-V) to each patient for genotype-phenotype correlations.

Results—In our patient repository, 22% of our families had normal DNA methylation patterns in several patients with normal DNA methylation along 15q11-q13. Of these, 44% of sporadic patients had mutations within UBE3A, the largest percentage found to date. Our data indicate that the five molecular classes can be divided into four phenotypic groups: deletions, UPD and ID patients, and subjects with unknown aetiology. Deletion patients are the most severely affected, while UPD and ID patients are the least. Differences in body mass index, head circumference, and seizure activity are the most pronounced among the classes.

Conclusions—Clinically, we were unable to distinguish between UPD and ID patients, suggesting that 15q11-q13 contains the only significant maternally expressed imprinted genes on chromosome 15.

Keywords: Angelman syndrome; genotype-phenotype correlations; DNA methylation; 15q11-q13

Angelman syndrome (AS) (MIM 105830) is a severe neurobehavioural disorder that occurs with a frequency of ~1/15 000. First documented by Dr Harry Angelman,1 AS is now diagnosed by four cardinal features: (1) severe developmental delay; (2) profound speech impairment; (3) a movement and balance disorder; and (4) a characteristic behavioural profile that includes frequent, inappropriate laughter, a happy affect, and an easily excitable personality. Other common features include seizures, microcephaly, abnormal EEG patterns, sleep disturbances, hypopigmentation, and strabismus.2–4

Four major molecular mechanisms are known to cause Angelman syndrome. Most patients with AS (class I, deletion) have an ~4 Mb maternally derived interstitial deletion of 15q11-q13. Some patients (class II, UPD) have paternal uniparental disomy of the entire chromosome 15, while others (class III, ID) show defects in the imprinting process. In addition, intragenic mutations in the E6AP-E3 ubiquitin protein ligase gene (UBE3A) occur in several subjects with AS (class IV, UBE3A). Furthermore, a diagnosis of Angelman syndrome has been established in a large number of patients who have no known molecular lesion. These patients (class V) comprise a fifth group of subjects with AS. Although patients in classes I-III can be easily diagnosed by DNA methylation analyses along the 15q11-q13 imprinted domain,5 subjects from classes IV and V show normal DNA methylation in this region.

The discovery of maternally inherited mutations in several patients with normal DNA methylation along 15q11-q13 first established a role for UBE3A in the aetiology of AS.6 7 Subsequent studies identified an overall UBE3A mutation rate of 14-38% in sporadic patients.8–12 Although cell lines from subjects with AS failed to show imprinted expression of UBE3A,13 demonstration of brain specific imprinting by RT-PCR in the human confirmed the role of UBE3A in the pathogenesis of AS.14

Phenotypic analyses of several deletion (class I) patients have generated a profile considered by many to typify the classical AS phenotype. These patients showed a high incidence of severe, early onset seizures, microcephaly, and hypopigmentation.15 16 However, patients from classes II and III often exhibited atypical features that ameliorated the classical phenotype. Patients with UPD and imprinting defects (ID) were less likely to present with...
hypopigmentation, microcephaly,17–21 or seizures,18 20 21 and often showed atypical features that were much milder than the deletion patients.18 20 21 Analysis of 14 patients with mutations in UBE3A also suggested that class IV patients had a phenotype distinct from UPD and ID patients,22 as they were more likely to present with microcephaly and seizures.

In the present study, we sought to conduct a large scale genotype-phenotype correlation of all five molecular classes in order to: (1) determine the accurate frequency of each molecular class; (2) provide detailed genotype based clinical data on each class; and (3) examine the molecular basis of the patients who have no known molecular defect. We report the highest UBE3A mutation frequency to date, show that mutations in UBE3A do not account for all patients with normal DNA methylation along the AS/PWS imprinted domain, and examine alternative mechanisms for the aetiology of AS in class V patients. In addition, we present the first comprehensive analysis of genotype-phenotype correlations among all five molecular classes of AS and show significant differences among the five classes in growth parameters, achievement of developmental milestones, as well as severity, frequency, and onset of seizures.

Materials and methods

PATIENT REPOSITORY

Clinical definition of AS

Over the last 11 years, we have accumulated a large AS repository totalling 146 patients from 134 Angelman families. Each of our patients was extensively examined by clinical geneticists at the University of Florida (UF). Before laboratory testing, each patient was evaluated for 22 distinct criteria and given an AS rating of 1-5 based on clinical impression. We restricted this study to the 104/146 patients (93 families) who had an AS clinical score of 1 or 2. Patients who scored 1 showed an absolutely “classical” phenotype in every aspect. A score of 2 meant that the patient was fairly classical, but showed one or two features (for example, mild or absent seizures or obesity) that were atypical for AS. However, these patients were extremely likely to have AS, since they fulfilled all of the four main AS criteria,34 including the “AS specific” behaviour. The remaining 42 “AS-like” patients, all of whom had normal laboratory testing (DNA methylation, DNA polymorphism, and cytogenetic analyses) within 15q11-q13, exhibited some but not all of the four cardinal features of AS and were excluded from further investigation for the purposes of this study. Blood and tissue were procured with

### Table 1  BESS-T primers

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer (5’→3’)</th>
<th>Direction</th>
<th>Location</th>
<th>Size (bp)</th>
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<tr>
<td>7</td>
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<tr>
<td>8</td>
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<td>284</td>
</tr>
<tr>
<td>10*</td>
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<td>Intron</td>
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</tr>
<tr>
<td>11</td>
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<td>Forward</td>
<td>Exon</td>
<td>192</td>
</tr>
<tr>
<td>12</td>
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<td>Forward</td>
<td>Exon</td>
<td>155</td>
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<tr>
<td>13</td>
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<td>Forward</td>
<td>Exon</td>
<td>155</td>
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<tr>
<td>14</td>
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<tr>
<td>15</td>
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<tr>
<td>16*</td>
<td>ACCCATGACTACAGTTTCTCT TGGGCTCATACACACAA</td>
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<td>189</td>
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</table>

*Kishino et al.6

### Table 2  Sequencing primers

<table>
<thead>
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<th>Location</th>
<th>Size (bp)</th>
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<td>345</td>
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<tr>
<td>8</td>
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<td>9</td>
<td>GAGGCTGAAAGACCATTAAT TCATTCTAGGCCCTTCTT</td>
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<td>Exon</td>
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<tr>
<td>10</td>
<td>CAGATTCTGGCAGGATGTT TCGATATTACAGCATGTAAG</td>
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<td>Exon</td>
<td>1401</td>
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<tr>
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<td>TTGGCTTCTACATCCTCCTCCTG ACCAAATCTCTTTTGCTG</td>
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<td>Exon</td>
<td>352</td>
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<td>12</td>
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<td>559</td>
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<tr>
<td>13/14</td>
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<td>257</td>
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<tr>
<td>15</td>
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<td>Forward</td>
<td>Intron</td>
<td>416</td>
</tr>
</tbody>
</table>

*Katsuura et al.7
†Malzac et al.8
the approval of the families and the University of Florida Institutional Review Board.

Molecular diagnostics

Once a clinical diagnosis of AS was determined, the patient and family members were analysed by both molecular and cytogenetic tests in order to place each patient into the appropriate molecular class. Patients in classes I-III had uniparental DNA methylation at several loci along 15q11-q13, including *MKRN3* (formerly *ZNF127*, *PW71*, and 5' *SNURF-SNRPN*, while patients from classes IV and V showed normal biparental methylation at these loci.23–28 DNA dosing, 29 high resolution chromosome and fluorescence in situ hybridisation (FISH) studies detected patients with large interstitial deletions (class I), while microsatellite and RFLP analyses separated patients in class II from those in class III.30 31 Patients with normal DNA methylation were then tested for *UBE3A* mutations by a combination of Southern blot, BESS-T scan, and direct sequence analyses. Patients with intragenic *UBE3A* mutations were placed into class IV, while those with no detectable chromosome 15 abnormalities were put into class V.

### Table 3 Molecular classes of Angelman syndrome

<table>
<thead>
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<th>Class</th>
<th>Molecular defect</th>
<th>Families</th>
<th>Patients</th>
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<tr>
<td>I</td>
<td>15q11-13 deletion</td>
<td>63 68 64 61</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Uniparental disomy (UPD)</td>
<td>7 7 7 7</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Imprinting defect (ID)</td>
<td>3 3 7 7</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td><em>UBE3A</em> mutation</td>
<td>10 11 15 14</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>Unknown</td>
<td>10 11 11 11</td>
<td></td>
</tr>
</tbody>
</table>

**Seizure criteria**

According to our definitions, severe seizures occurred when at least two drugs were administered for epileptic episodes. When one anticonvulsant effectively controlled grand mal seizure activity, patients were considered to have moderate seizures. Mild seizures were defined by very rare grand mal, petit mal, or multiple febrile seizures. Patients with fewer than four febrile or a complete absence of seizures were considered to have no history of seizure activity.

**SOUTHERN ANALYSIS**

Southern blots were done according to standard conditions.32 Patients were examined with genomic probes from the 5' end of *UBE3A*, as well as cDNA probes that covered exons 3-16. Genomic probes were generated by restriction digestion of cosmids 24 and 34.25 cDNA probes were amplified using previously published primers for exons 3-97 and exons 9-16.67 DNA methylation analyses of *UBE3A* were performed by hybridisation with a 2.7 kb *UBE3A* CpG island probe, which was generated by EcoRI to NotI digestion of cosmid 34.

**METHYLATION SPECIFIC PCR**

Sodium bisulphite treatment of genomic DNA and subsequent methylation analysis of *SNRPN* was done according to established protocols.32 33 Two sets of primers were designed to assess the methylation status of both NotI sites in the *UBE3A* CpG island. At the proximal end of the CpG island (more 5'), the

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**Figure 1**  *UBE3A* mutations in patients from the UF repository. (A) Genomic organisation of *UBE3A*. UBE3A spans 120 kb of genomic DNA. The gene is shown to scale. Exons are numbered numerically from 1-16. Transcription (arrow) and translation (ATG) initiation sites are depicted. The primary translation initiation site begins in exon 8. The location of each mutation is indicated (A-K). (B) Description of mutations. The mutation from each family (A-K) is shown in column 2. Superscripts indicate previously identified mutations. Column 3 describes the predicted protein changes, while column 4 shows the inheritance of the mutation.
Figure 2 DNA methylation analysis of 5′ UBE3A. (A) Genomic structure of UBE3A and the 5′ CpG island drawn to scale. Locations of selected restriction enzyme sites are depicted: EcoRI (E), NotI (N), BosHI (B), EagI (G). Localisations of the 2.7 kb NotI-EcoRI probe as well as the 5.1 kb region containing the CpG island are indicated. The extent of overlap of the antisense transcript is also shown. If the CpG island is methylated, the 2.7 kb probe will hybridise to the 5.1 kb fragment on genomic DNA digested with EcoRI and NotI. If the CpG island is unmethylated, the probe will hybridise to itself, showing a 2.7 kb band on Southern blots. (B) Southern blot of normal (NL), AS (A), and PWS (P) genomic DNA from peripheral blood leucocytes (PBL); AS brain samples consisting of frontal cortex (F), occipital lobe (Oc), cerebellum (C), and coronal section (Sx); frontal cortex from normal brain (NB); adult testis (T); sperm (S); and fetal ovary (Ov) digested with EcoRI and NotI. DNA from normal PBL was also digested with EcoRI alone as a control (E). When digested with EcoRI and NotI, the 2.7 kb probe only hybridised to a 2.7 kb fragment, indicating that this site was unmethylated. (C) Southern blot analysis of PBL genomic DNA from class V patients digested with EcoRI and NotI shows that this site is unmethylated in all patients.

**Primers** Collectively, primers for BESS-T (table 1) and direct sequence analysis (table 2) were designed to amplify the entire UBE3A coding region (exons 6-16). Novel primers were designed using the Primer 3 program from the Whitehead Institute.

**Statistical analyses** Descriptive statistics were calculated as a percentage or by the mean (SD). All outcome variables were modelled as a function of age at evaluation, birth weight, molecular class (I, II, III, IV, and V), gestational age, maternal age, paternal age, race (white, Hispanic, and other), and sex. It should be noted that 93% of the UF patients and 56% of the total patients were white. Outcome variables consisted of continuous, binary, and ordinal measures, and were modelled using analysis of covariance (ANCOVA), logistic regression, and ordinal logistic regression, respectively. For binary and ordinal outcome variables with limited sample size, we employed the exact chi-square test. Odds ratios were used to summarise the binary and ordinal outcomes. All tests were two sided and considered significant if alpha=0.05. All statistical analyses were performed using SAS 6.12 (SAS Institute, Cary, NC).

**Results** MOLECULAR CLASSES OF ANGELMAN SYNDROME Each patient was assigned a molecular class based upon results from high resolution chromosome, FISH, UBE3A mutation analysis, and DNA dosing, methylation, and polymorphism analyses along 15q11-q13 (table 3). The majority (63) of our classical families were deleted for the entire 4 Mb AS/PWS imprinted domain (class I). In seven cases, AS occurred via paternal UPD of chromosome 15 (class II), and three families had imprinting defects (class III). Intragenic mutations in UBE3A occurred in 10 of our classical patients (class IV), while the remaining 10 had no detectable chromosome 15 abnormalities (class V).

**Mutation analysis** Initially, a molecular confirmation of AS could not be made in 20/93 families using cytogenetic, DNA methylation, or DNA polymorphism tests. Hybridisation of 5′ and 3′ UBE3A cDNA probes to Southern blots did not detect genomic rearrangements in these patients (data not shown). However, sequence analysis of the UBE3A coding region in these 20 families showed intragenic mutations in 10 families (15 patients). We detected six deletions that resulted in frameshifts, three nonsense mutations, and...
one missense mutation (fig 1). We found a 50% mutation detection rate with 3/4 familial (75%) and 7/16 sporadic cases (44%) containing mutations within the coding region of \textit{UBE3A}. In our examination of published reports, we found that six of our patients had lesions in \textit{UBE3A} that were identical with previously published mutation sites. Further studies indicated that these sites were not mutation hotspots, but independent reports of mutations in patients A, B, C, D, E, and G.

In addition to disease associated mutations, we also found three novel polymorphisms. A maternally inherited silent transition (A1144G) occurred in exon 9 in one subject. No other mutations were found in this patient. A common polymorphism was detected in a poly T tract within intron 6. Sequence analysis of 67 subjects showed that 17% of the chromosomes (p=0.83, q=0.17) contained a 1 bp thymidine insertion at nucleotide position –47, relative to exon 7. One AS-like patient had a paternally inherited 14 bp deletion of the 3’ UTR. No disease associated mutation was found in this person. In addition, BESS-T scan detected one subject who was homozygous for a previously identified expressed polymorphism in exon 6.

\textbf{DNA METHYLATION OF 5' UBE3A}

Although we identified disease causing mutations in 50% of our patients who had normal DNA methylation along 15q11-q13, the aetiology of AS in the remainder of our classical Angelman syndrome patients was unknown. We postulated that disruption of the normal \textit{UBE3A} DNA methylation pattern in class V patients could be causative of AS in these patients.

Using rare cutting restriction enzymes, we isolated a 2.7 kb \textit{NotI–EcoRI} fragment from the CpG island at 5’ \textit{UBE3A} to test for parent of

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\textbf{Table 4 Clinical and growth data} & & & & & & & \\
\hline
\textbf{p=} & \textbf{Class I} & \textbf{Class II} & \textbf{Class III} & \textbf{Class IV} & \textbf{Class V} \\
\hline
\textbf{Speech} & 4.1\times10^{-3} & 2.0\times10^{-3} & (21) & (7) & (27) & (7) & (20) & (15) & (46) & (11) \\
0 words & 71 & * & 44 & 43 & 40 & 60 & 33 & 82 \\
<5 words & 29 & 14 & 15 & 14 & 15 & 27 & 26 & 9 \\
3–10 words & * & 43 & 33 & 43 & 30 & 13 & 41 & 9 \\
\textgreater{}10 words & * & 14 & 8 & * & 15 & * & * & * \\
\hline
\textbf{Seizure onset\textsuperscript{+}} & 6.7\times10^{-4} & 7.6\times10^{-4} & (20) & (3) & (14) & (2) & (9) & (12) & (24) & (5) \\
1–2 y & 10 & * & 7 & * & 33 & 17 & 13 & 40 \\
2–3 y & 25 & * & 14 & 50 & 11 & 25 & 29 & 40 \\
3–4 y & 25 & * & * & * & 50 & 20 & 29 & * \\
\textgreater{}4 y & * & 67 & 65 & 50 & 33 & * & 25 & * \\
\hline
\textbf{Seizure severity} & 2.0\times10^{-3} & 1.0\times10^{-3} & (21) & (7) & (26) & (7) & (18) & (15) & (32) & (10) \\
Severe & 29 & * & 4 & 14 & 6 & 33 & 19 & 10 \\
Moderate & 62 & 14 & 8 & * & 11 & 20 & 22 & 30 \\
Mild & 9 & 29 & 23 & 14 & 22 & 33 & 44 & 20 \\
None & * & 57 & 65 & 72 & 61 & 14 & 15 & 40 \\
\hline
\textbf{Height\textsuperscript{†} (centile)} & ND & 2.9\times10^{-2} & (21) & (7) & (28) & (7) & (17) & (15) & (30) & (9) \\
<5 & 42 & * & 7 & 30 & 12 & 7 & 10 & * \\
5–20 & 5 & * & 11 & 14 & 12 & 20 & 10 & 56 \\
21–40 & 14 & 43 & 18 & 14 & 18 & 46 & 27 & 22 \\
41–60 & 19 & 14 & 14 & 14 & 23 & 13 & 33 & * \\
61–80 & 10 & * & 18 & 14 & 23 & 7 & 17 & 22 \\
81–95 & 10 & 14 & 21 & 14 & 6 & 7 & 3 & * \\
\textgreater{}95 & * & 29 & 11 & * & 6 & * & * & * \\
\hline
\textbf{Weight (centile)} & 3.4\times10^{-3} & 3.7\times10^{-3} & (21) & (7) & (27) & (7) & (15) & (15) & (28) & (9) \\
<5 & 18 & * & 4 & * & * & * & * & 11 \\
5–20 & 14 & * & 7 & 29 & 13 & 13 & 7 & 33 \\
21–40 & 24 & 14 & 4 & * & * & * & * & 11 \\
41–60 & 24 & 14 & 15 & * & * & 27 & 14 & 34 \\
61–80 & 10 & 14 & 15 & 29 & 13 & 13 & 18 & * \\
81–95 & * & 14 & 22 & * & 20 & 27 & 18 & * \\
\textgreater{}95 & 10 & 44 & 33 & 42 & 54 & 20 & 43 & 11 \\
\hline
\textbf{BMI (kg/m\textsuperscript{2}) (centile)} & 4.2\times10^{-3} & 1.7\times10^{-2} & (21) & (7) & (17) & (7) & (14) & (13) & (13) & (9) \\
<5 & 10 & * & * & * & * & 8 & 8 & 11 \\
5–20 & 10 & * & * & * & * & 8 & 8 & 33 \\
21–40 & 24 & * & 6 & * & * & * & * & 11 \\
41–60 & 18 & 14 & 6 & * & * & 8 & 8 & 34 \\
61–80 & 18 & 14 & 12 & 57 & 29 & 31 & 31 & 11 \\
81–95 & 10 & 29 & 29 & * & 7 & 14 & 14 & 11 \\
\textgreater{}95 & 10 & 43 & 47 & 43 & 64 & 31 & 31 & * \\
\hline
\textbf{Head circum (centile)} & 3.2\times10^{-2} & 4.2\times10^{-2} & (20) & (6) & (27) & (7) & (19) & (15) & (32) & (9) \\
<5 & 60 & * & 15 & * & 11 & 53 & 56 & 60 \\
5–20 & 10 & 33 & 11 & 14 & 5 & * & * & 10 \\
21–40 & 20 & 17 & 30 & 29 & 26 & 27 & 16 & 20 \\
41–60 & 10 & 33 & 14 & 21 & 20 & 12 & 10 \\
61–80 & * & 17 & 15 & 29 & 21 & * & * & * \\
81–95 & * & * & 4 & 14 & 5 & * & 16 & * \\
\textgreater{}95 & * & * & 7 & * & 11 & * & * & * \\
\hline
\end{tabular}
\end{table}

Percentage of patients with each trait is indicated, with the sample size in parentheses.
ND = not determined.
UF = University of Florida data.
All = combined data from UF and published reports.
*No patients in category.
†Statistically significant in combined data set only.
All other parameters are statistically significant in UF and combined data sets.
origin DNA methylation imprints in DNA from peripheral blood leucocytes (PBL), AS brain, normal brain, and germ cells from both sexes. The 2.7 kb genomic probe detected a 5.1 kb fragment when the DNA was digested with EcoRI alone. However, when DNA was digested with both EcoRI and NotI, the probe only hybridised to a 2.7 kb fragment (fig 2B). In addition, we used methylation specific PCR (MSP) to assess the DNA methylation status of UBE3A in DNA from peripheral blood leucocytes and skin fibroblast cell lines. We analysed two regions of the CpG island. In both regions, only the unmethylated allele amplified, indicating that the regions covered by our primers are unmethylated (data not shown). Using these two independent methods, we found no evidence for methylation within these sites. We next examined PBL DNA from class V patients. The 2.7 kb probe only detected the unmethylated fragment, indicating that de novo methylation at this site did not occur in these subjects (fig 2C).

GENOTYPE-PHENOTYPE CORRELATIONS
We analysed phenotypic data on 61 patients from the University of Florida AS repository. We included all of our patients from classes II-V and randomly chose 20 class I (deletion) patients for clinical comparisons. Also included in our statistical models were 77 additional patients (classes II, III, and IV) gathered from published reports.10 11 18–22 35–56

We compared the results of statistical models established between our patients and the combined data set (tables 4, 5, and 6). Statistical analysis of body mass index (BMI) showed that patients from classes II, III, and IV were taller and heavier than the deletion and class V patients (fig 3A, table 4), for both UF patients (p=0.0042) and the combined data set (p=0.0167). The data also showed that less than 15% of subjects from classes II and III had microcephaly, whereas more than 55% of patients from the other three classes had a head circumference below the 3rd centile (fig 3B, table 4, p=0.0415).

Although all AS patients showed delayed development of gross motor skills, the deletion patients as a group were more severely affected. One of the most striking observations was that 50% of deletion patients were non-ambulatory at 5 years, while over 95% of patients from the other classes had the ability to walk unassisted by 5 years (table 6, p=1.1 × 10–5). The mean age to walk also differed significantly among the classes. Patients in class I walked at a mean age of 4.6 (SD 3.4) years (p=0.0001), while patients in classes II-V walked much earlier, with mean ages varying from 2.4 (SD 0.9) (class III) to 2.8 (SD 1.4) years (class IV, fig 3C, table 5).

Onset of seizures also depended upon molecular class (fig 3D, table 5). Seizures began very early in class I and V patients (1.9 (SD 1.1) and 1.4 (SD 0.9) years, respectively) compared to classes II, III, and IV (4.9 (SD 3.4, 5.5 (SD 5.0), and 2.7 (SD 1.4) years, respectively; p=0.0028). In the UF patient population, significant seizures (that is, grand mal seizures requiring the use of at least one
anticonvulsant) occurred in 19/21 deletion patients, but only in 1/7 UPD, 1/7 ID, 8/15 UBE3A mutation, and 4/10 class V patients \((p=9.96\times10^{-5})\). Similar findings were observed in the combined data set \((p=8.26\times10^{-8})\). In addition, 90% of class I patients were hypopigmented compared to family members \((p=3.33\times10^{-8})\). However, less than 25% of classes II-IV and 44% of patients in class V showed evidence of hypopigmentation.

**Discussion**

**MOLECULAR BASIS OF AS IN CLASSES IV AND V**

Molecular analyses showed that 20 of the 104 classical patients with AS (93 families) we studied had normal DNA methylation at several imprinted 15q11-q13 loci, excluding large deletions, UPD, or imprinting defects from the aetiologies of AS in these subjects. We found mutations in 50% of these patients, with 44% \((7/16)\) of our sporadic and 75% \((3/4)\) of our familial cases having mutations within \(UBE3A\). Recent mutation analyses by other laboratories showed a lower \(UBE3A\) mutation frequency \((5-38\%)\) in their sporadic patients.8–12

Including our patients, \(UBE3A\) mutations have been identified in 51 AS families.81 25 45 7 Fourty-five are unique mutations (fig 4), with no more than three unrelated subjects sharing a common error. Most defects are nonsense mutations that are predicted to result in premature termination during translation, and therefore may not represent true null mutations. However, the last six amino acids of \(UBE3A\) are crucial for normal protein function in the presence of human papillomavirus,58 and it is possible that these mutations produce unstable message owing to nonsense mediated decay.59 60

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<th>Class IV</th>
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Percentages of patients with each phenotype are indicated under category, with the sample size shown in parentheses.

UF = University of Florida set only.
All = combined data from UF and published reports.
*Significant in both data sets.
NS = not significant.

Table 6 Clinical data II

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<td>0 (7)</td>
<td>14 (22)</td>
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Discussion

MOLECULAR BASIS OF AS IN CLASSES IV AND V

Molecular analyses showed that 20 of the 104 classical patients with AS (93 families) we studied had normal DNA methylation at several imprinted 15q11-q13 loci, excluding large deletions, UPD, or imprinting defects from the aetiologies of AS in these subjects. We found mutations in 50% of these patients, with 44% \((7/16)\) of our sporadic and 75% \((3/4)\) of our familial cases having mutations within \(UBE3A\). Recent mutation analyses by other laboratories showed a lower \(UBE3A\) mutation frequency \((5-38\%)\) in their sporadic patients.8–12

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Genotype-phenotype correlation in Angelman syndrome

Figure 4 - Summary of all published mutations in UBE3A. The gene is shown to scale. Exons are numbered numerically from 1-16. Transcription (arrow) and translation (ATG) start sites are noted. The primary translation initiation site begins in exon 8. The location of each mutation is indicated by a symbol. Filled symbols indicate protein truncating mutations. Symbols are stacked at positions where two unrelated subjects share a common mutation. There are 51 total mutations in unrelated subjects. Forty-five mutations are unique. Familial mutations are counted once. Most mutations (38/45) are protein truncating and cluster in exons 9 and 16.

Out of frame insertion
Out of frame deletion
Splicing mutation
Out of frame deletion/insertion
Nonsense mutation
Missense mutation
In frame insertion
In frame deletion
Putative splicing mutation

virtually all protein coding exons, most cluster in exons 9 and 16 (fig 4). Exon 16 is within the HECT domain, a region of high conservation between different E3 protein ligases.

Although our study has shown the highest UBE3A mutation frequency found to date in sporadic cases, our results indicate that approximately 11% of patients with AS have an unknown aetiology. Alternative explanations must account for the molecular basis of AS in this group. Five possible explanations follow. (1) Many UBE3A mutations occur in non-coding regions; (2) UBE3A can be inactivated by other mechanisms; (3) other genes, some of which may reside in the ubiquitin pathway (genome wide), can also cause AS; (4) AS associated mutations can occur in other 15q11-q13 genes; (5) these patients do not have AS, but instead have disorders that mimic AS. However, our rigorous clinical criteria and extensive clinical expertise with AS suggest that the last explanation is unlikely.

Although the relative lack of UBE3A mutations in patients with normal DNA methylation could be accounted for if various mutations occurred outside the UBE3A coding region, we did not detect genomic rearrangements in any of our 20 families, and only one genomic rearrangement has been identified to date. Furthermore, no mutations have been reported in the promoter region or 3’ UTR of UBE3A.

We postulated that disruption of the normal DNA methylation pattern at 5’ UBE3A could inactivate the gene. With the exception of only a few genes, the promoter regions of most imprinted genes show differential methylation based on the parental origin of the chromosome. In the AS/PWS domain, several paternal only expressed genes display parent of origin DNA methylation imprints, with the promoters of the active allele being hypomethylated compared to the inactive allele. Since DNA methylation of promoter regions is closely associated with transcriptional repression, we hypothesised that methylation of the maternally derived 5’ CpG island of UBE3A could repress imprinted expression in class V patients. In addition, recent experiments suggested that in somatic cell hybrid cell lines, which are derived from skin fibroblasts, UBE3A was methylated on the maternal allele. However, in contrast to Meguro et al, we found that both parental alleles of UBE3A were completely unmethylated in PBL, skin fibroblasts, germ cells, and brain, which has been shown to demonstrate imprinted expression of UBE3A. In addition, class V patients were completely unmethylated at 5’ UBE3A in PBL DNA, indicating that they probably do not inactivate UBE3A by inappropriate methylation. To eliminate this possibility fully, brain DNA from class V patients should be examined.

Our data suggest that disruption of UBE3A may not be the only cause of AS and other factors may also be involved. The most obvious candidates are other genes in the ubiquitin pathway. Any protein that regulates or interacts with UBE3A in this cascade and all proteins that UBE3A targets for degradation are candidates for patients grouped in class V. So far, four proteins have been identified as substrates for UBE3A mediated degradation: p53, HHR23A, the Src family kinase Blk, and UBE3A itself.

Alternatively, other 15q11-q13 genes may play important roles in the aetiology of class V patients. The UBE3A antisense transcript is one candidate, as preliminary data suggest that the paternally expressed antisense transcript is restricted to the brain. It is possible that expression of the antisense transcript from the paternal chromosome specifically inactivates the paternal UBE3A allele in cis. According to this model, biallelic activation of the antisense transcript would result in repression of both UBE3A alleles. It would be important to know if class V patients show biallelic expression of the UBE3A antisense transcript in the regions of the brain that shows imprinted expression of
UBE3A. Another excellent candidate gene is the newly identified imprinted gene ATP10C. This gene is located approximately 200 kb distal to UBE3A and is preferentially expressed from the maternal allele in human brain. It would be interesting to determine if some of our class V patients have lesions within this new candidate gene.

GENOTYPE-PHENOTYPE CORRELATIONS

The second major goal of this study was to establish phenotypic profiles for each molecular class. Genotype-phenotype analyses of our patients indicated that all five classes had the four cardinal features of AS (that is, severe developmental delay, profound speech impairment, a movement and balance disorder, and characteristic behaviour). Statistical models showed that the five classes could be separated into four phenotypic groups: class I, classes II and III, class IV, and class V. The statistically significant clinical distinctions between the classes include 16 of 22 parameters (p<0.05), and were most striking for pigmentation, growth parameters, achievement of developmental milestones, and severity, frequency, and age of onset of seizures.

We found that the class I (deletion) patients as a group have the most classical and severe phenotype. They achieve developmental milestones later and to a lesser degree than the other classes. Statistical models show that deletion patients learn to sit and walk later than the other classes and are less likely to be able to follow simple commands. Class I patients have the highest incidence of severe seizures (90%) and hypopigmentation (90%) of all five classes. In addition, deletion patients typically have a complete absence of speech, a normal BMI distribution, and severe microcephaly.

In contrast, UPD and ID patients (whom we found as a group to have indistinguishable phenotypes) are much less severely affected. They have a low incidence of hypopigmentation, microcephaly, and severe seizures. In addition, almost half of UPD and ID patients have more than three words in their vocabulary. Patients in classes II and III are larger and heavier than class I patients. Analysis of body weight by BMI showed that more than 70% of these patients are above the 80th centile for their age group. Our data from these two classes correlate well with the combined data of others who also found fewer incidences of hypopigmentation, microcephaly, or seizures in UPD and ID patients.

UBE3A mutation patients fall somewhere in the middle. They are statistically similar to deletion patients with respect to seizures, absence of speech, and microcephaly. Moncla et al examined class IV patients individually for head circumference and seizures, and similarly concluded that UBE3A mutation patients had high incidences of seizures and microcephaly. Statistically, the class IV patients that we examined are similar to UPD and ID patients in the development of motor skills, ability to follow simple commands, pigmentation, and development of obesity. Class IV patients have a high incidence of early onset obesity. Perhaps UBE3A functions in the same pathway as the newly discovered ATP10C gene, whereby lesions in UBE3A also alter expression of ATP10C. In addition, UBE3A mutation patients differ from UPD and ID patients in that they were more likely to have a history of moderate to severe seizures and microcephaly. Although milder than deletion patients, class IV cases have a more typical phenotype than class II or III patients, indicating that lesions in UBE3A have pleiotropic consequences.

Class V patients are very interesting. Although these patients have no detectable chromosome 15 abnormalities, statistically they are most similar to the deletion patients. Class V patients have normal height, weight, and BMI distributions, similar to those observed in class I patients. They also frequently present with a complete absence of speech, early onset of seizures, hypopigmentation, and microcephaly. The observation of hypopigmentation in these patients is unexpected. One possible explanation could be that the hypopigmented subjects are haploinsufficient for the P gene or for another pigmentation gene located elsewhere in the genome. However, class V patients differ from deletion patients in two important characteristics. They walk about two years earlier than deletion patients and they are less likely to have seizures compared to patients in class I.

In addition, class V patients tend to have a more severe phenotype than that observed in classes II, III, and IV. It is possible that disruption of more than one gene is causative of the class V phenotype in these patients. Perhaps these patients have mutations in a gene or "controlling region" in 15q11-q13 that affects additional loci within the domain, such as ATP10C, giving rise to the more severe phenotype. Alternatively, these patients may have defects upstream of UBE3A that alter additional genes within the ubiquitin pathway. However, some class V patients could suffer from a loss of function mutation in a UBE3A target protein. This could lead to a more severe phenotype than that observed in UBE3A mutation patients, who presumably experience an upregulation of a critical UBE3A target protein. In addition, two recent papers have shown a link between "AS-like" subjects and MECP2. Although at least one of our "AS-like" patients was later diagnosed with Rett syndrome, it remains to be seen if any of our class V patients have mutations in MECP2, the gene responsible for Rett syndrome.

Our data indicate that class I patients comprise a contiguous gene disorder, with haploinsufficiency of additional non-imprinted genes exacerbating the lack of the maternally expressed UBE3A gene. For example, hypopigmentation has been linked to haploinsufficiency of the P gene in PWS deletion patients, and the inheritance of a single copy of the GABRB3 gene has been implicated in seizure susceptibility. Our data clearly show that patients in classes II and III have the least severe and most atypical phenotype, suggesting that a “double dose” of the paternal only
expressed genes may ameliorate the AS phenotype in these patients. In addition, the finding that class II and III patients are larger and have better motor skills than the other patients with AS may have implications for the “parental conflict” hypothesis, which states that paternal expressed genes are involved in embryonic or postnatal growth.82 For our findings to support this hypothesis, it will be important to establish if the increased growth and obesity observed in UPD and ID patients occurs while the offspring is still capable of using maternal resources (for example, breast feeding).

Another important finding is that almost half of the UBE3A mutation patients and 70–75% of the UPD/ID patients have extremely high BMI ratios (above the 80th centile). Obesity is also observed in several mouse models of AS. Mice that have paternal UPD for the AS orthologous region, a 4 Mb maternally derived deletion of the ATXN15 region (Nicholls, unpublished data), or a smaller maternally derived deletion in this region,86–88 have adult onset obesity. Genetic studies in the mouse have mapped a maternally expressed “fat” gene (fptat) very close to the UBE3A gene.85 The human orthologue, ATPI0C, shows imprinted expression in human brain and maps very close to UBE3A.77 An integrated hypothesis would suggest that UBE3A and/or another closely linked gene (that is, ATPI0C) play a role in metabolism and/or feeding behaviour. The relative lack of obesity in class I patients could be caused by the severe underlying motor defects associated with deletion patients.

This comprehensive and extensive study of genotype-phenotype correlations provides additional delineation and enhances our understanding of the distinct clinical subgroups of AS. Our data indicate that patients with a high body mass index and a history of significant seizures would be good candidates for UBE3A mutation analysis. It is clear that although mutations in UBE3A are sufficient to cause the four cardinal features of Angelman syndrome, as well as recurrent seizures and microcephaly, other 15q11-q13 genes must contribute to the development of motor skills, severity of seizures, cognition, growth, and obesity found in the other classes of AS. Elucidating the role of these other 15q11-q13 genes will give us important insights into mammalian development and into an improved understanding of contiguous gene deletion disorders. Delineating all the UBE3A target proteins could promote the design of rational therapies for AS. Furthermore, discovery of the molecular mechanism(s) involved in the pathogenesis of class V patients will be crucial to our full understanding of Angelman syndrome and the molecular complexity governing phenotypic expression of imprinted genes.

We would like first to acknowledge the support of our AS families who have participated in our research over the last decade. We also thank Peter Howley, who provided us with valuable information about the UBE3A gene, and Josephine O’Hara, who kindly furnished us details of his UBE3A mutation analysis. We appreciate the excellent technical assistance of Amy MacNeill and Brian Savage, and we thank Edith and View Whidden for early contributions. This work was supported in part by NIH grants 2R01HD13191 (REN and JDD), R01HD36417 (JDD), K24HD1361 (JDD), and the University of Florida General Clinical Research Center grant RR00802, as well as funding from the Hayward Foundation and the R C Philips Unit.


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