Epigenetic dysregulation of \textit{SHANK3} in brain tissues from individuals with autism spectrum disorders

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Received July 21, 2013; Revised October 21, 2013; Accepted October 28, 2013

The molecular basis for the majority of cases of autism spectrum disorders (ASD) remains unknown. We tested the hypothesis that ASD have an epigenetic cause by performing DNA methylation profiling of five CpG islands (CGI-1 to CGI-5) in the \textit{SHANK3} gene in postmortem brain tissues from 54 ASD patients and 43 controls. We found significantly increased overall DNA methylation (epimutation) in three intragenic CGIs (CGI-2, CGI-3 and CGI-4). The increased methylation was clustered in the CGI-2 and CGI-4 in ∼15% of ASD brain tissues. \textit{SHANK3} has an extensive array of mRNA splice variants resulting from combinations of five intragenic promoters and alternative splicing of coding exons. Altered expression and alternative splicing of \textit{SHANK3} isoforms were observed in brain tissues with increased methylation of \textit{SHANK3} CGIs in ASD brain tissues. A DNA methylation inhibitor modified the methylation of CGIs and altered the isoform-specific expression of \textit{SHANK3} in cultured cells. This study is the first to find altered methylation patterns in \textit{SHANK3} in ASD brain samples. Our finding provides evidence to support an alternative approach to investigating the molecular basis of ASD. The ability to alter the epigenetic modification and expression of \textit{SHANK3} by environmental factors suggests that \textit{SHANK3} may be a valuable biomarker for dissecting the role of gene and environment interaction in the etiology of ASD.

INTRODUCTION

Although a genetic component is strongly implicated in the etiology of autism spectrum disorders (ASD), the molecular basis remains poorly understood in the majority of cases; single gene mutations and chromosomal microdeletions or duplications are found only in ∼10–20% of idiopathic ASD cases (1–4). The increasing prevalence of ASD also points to the role of gene and environment interaction in ASD susceptibility (5,6). Through analysis of the Angelman and Prader–Willi syndrome imprinting region in brain tissue from individuals with ASD, we proposed a mixed genetic and epigenetic model for the etiology of ASD (7). A similar model has also been reported by others (8–12). Evidence from recent genetic studies suggests that dysfunction of brain synapses underlies the pathogenesis of ASD (13,14) which led us to hypothesize that epigenetic dysregulation of synaptic genes may be implicated in the molecular basis of ASD. Among the synaptic proteins implicated in human ASD, SHANK3/PROSAP2 is of particular interest. The SHANK3 protein contains multiple conserved protein domain including ANK domain (ankyrin repeat), SH3 domain (SRC homology 3 domain), PDZ domain [postsynaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1) and zonula occludens-1 protein (zo-1)], proline-rich cluster/Homer-binding domain and SAM (sterile alpha motif) domain. Each of these domains has the capacity to bind different proteins and mediate different functions at the postsynaptic density of excitatory synapses (15,16). The \textit{SHANK3} gene plays a key role in the chromosome 22q13.3 microdeletion syndrome (Phelan-McDermid syndrome, PMS), a condition in which autistic features are prominent (17). Furthermore, small microdeletions, \textit{de novo} missense and frame-shift mutations in \textit{SHANK3} have been identified in ASD cases (18–20) as well as in families with schizophrenia and mild intellectual disability (21).

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SHANK3 has five CpG islands (CGIs) in the 5′ promoter region and within the gene body (intragenic CGIs) that display a brain region-specific DNA methylation pattern (22,23). The intragenic CGIs are implicated in isoform and tissue-specific expression of SHANK3 (22–24). This raises the tantalizing possibility that changes in methylation of intragenic CGIs might affect transcription of the SHANK3 gene in human brain and thus contribute to ASD susceptibility.

We tested the hypothesis that epigenetic dysregulation of SHANK3 contributes to the etiology of ASD by performing an extensive DNA methylation profile of the five CGIs (CGI-1 to CGI-5) of SHANK3 in brain tissues from 54 cases of ASD and 43 controls. We found a significant increase in overall DNA methylation in SHANK3 intragenic CGIs in ASD brain tissues. A complex pattern of transcriptional dysregulation, including reduced expression and altered alternative splicing of specific SHANK3 mRNA isoforms, was observed in ASD brain tissues.

**Figure 1.** Increased DNA methylation in SHANK3 intragenic CGIs in ASD postmortem brain tissues. (A) The alternatively spliced exons are marked in red and by asterisks. SHANK3a–f transcripts initiated from six promoters as described in text are shown by arrows (see also Supplementary Material, Fig. S10). The CGIs are shown by solid bars in blue. CGI-X is displayed in Ensembl but not in the UCSC genome browser, probably due to the use of slightly different computational programs for the prediction of CGIs. We were not able to design primers to analyze CGI-X. (B) Heat map of the percentage of methylation of CGIs in ASD cerebellum (all available samples were analyzed but only cases with data for all CGIs are shown in the heat map). The heat map was generated using the calculated percentage of methylation from the data of bisulfite genomic sequencing for each CGI. The color bar indicates the level of methylation from lowest (red) to highest (green). The ASD case numbers are in red and the controls are in black on the Y-axis. ASD cerebellum samples AN17777 (14), AN16115 (17), AN07948 (14), UMB1174 (17), UMB1182 (17) (the number in parenthesis indicates the hypermethylated sites out of a total of 17 CG sites covered) were significantly hypermethylated in CGI-2 and ASD samples AN03345 (29), AN19511 (30), AN09730 (31), UMB1174 (32), UMB1182 (33) and AN11989 (33) were significantly hypermethylated in CGI-4 (a total of 40 CG sites analyzed). (C) Heat map of the methylation percentage of SHANK3 CGIs in BA19 tissues. ASD samples AN03632 (16), AN03935 (17), AN14829 (17) and AN17138 (17) have a significantly higher percentage of methylation compared with controls and other ASD samples (Cluster I). (D and E) Representative methylation plots for control and ASD cerebellum with significantly increased methylation in CGI-2 (D) and CGI-4 (E). (F and G) Representative methylation plots for control and ASD BA19 samples with significantly increased methylation in CGI-2 (F) and CGI-4 (G). The closed circles indicate methylated CG sites and open circles indicate unmethylated CG sites. The ASD cases with significantly increased methylation of CGI-3 are shown in Supplementary Material, Dataset D1 (y, year; M, male; F, female).
with increased methylation of \textit{SHANK3}. Treatment with the methylation inhibitor 5-azacytidine (5-AZA) in cultured cells resulted in demethylation of \textit{SHANK3} CGIs, and altered the isoform-specific expression of \textit{SHANK3}. Together with the synaptic dysfunction and ASD-like behaviors in mutant mice with isoform-specific \textit{Shank3} disruptions (25–28), our findings

Figure 1. Continued
strongly support a pathological association between altered DNA methylation and expression of \textit{SHANK3} in brain, and susceptibility to ASD.

RESULTS

Significantly increased DNA methylation in select intragenic CGIs of \textit{SHANK3} in ASD brain tissues

We used bisulfite genomic sequencing and pyrosequencing methods to determine the level of DNA methylation of \textit{SHANK3} CGIs in postmortem brain tissues from individuals with ASD and controls. The methylation of CGI-1, CGI-3 and CGI-5 of \textit{SHANK3} was determined primarily by the pyrosequencing method, while the CGI-2 and CGI-4 was determined by bisulfite genomic sequencing method only because of the technical issue of designing the pyrosequencing experiment. The quality of bisulfite genomic sequencing data was monitored by several control experiments as described in Supplementary Material. The structure of the \textit{SHANK3} gene and the positions of the CGIs are shown in Figure 1A. A more detailed description of each CGI and the primers and conditions used in this study are included in the Supplementary Material, D12. Using these methods, we first confirmed previous reports (22–24) showing tissue-specific methylation of \textit{SHANK3} CGIs in various brain regions (Supplementary Material, Fig. S1).

We then compared the level of DNA methylation for CGI-1 to CGI-5 in cerebellum and cerebral cortex Brodmann region 19 (BA19) in postmortem brain tissues from 54 individuals with ASD and 43 controls. The basic demographic information for the individuals from whom these brain tissues were obtained is summarized in the Supplementary Material, clinical information. There were no significant differences in age, gender, ethnicity or postmortem interval (PMI) between the ASD and control groups. Although the specific brain regions and neural circuits involved in the pathogenesis of ASD have not been clearly defined by neuropathological and neuroimaging studies, involvement of the prefrontal and temporal lobes as well as the cerebellum have been frequently reported (31,32,34–36). Many other brain regions have also been implicated but the results are less consistent (36,37). BA19 and cerebellum were chosen for this study because of the availability of a large number of samples from brain tissue banks and to maintain relative homogenous tissue sampling. The diagnosis of ASD in 49 cases (90.7%) was confirmed by clinical evaluation using both DSM IV and ADI-R (autism diagnostic interview revised) andADOS (autism diagnosis observation schedule). For the remaining cases, the diagnosis was made based on clinical history but detailed clinical records were not available for review. Comprehensive clinical data were available on 35 individuals with ASD. Two clinicians with extensive experience in ASD evaluation reviewed these data and classified 19 of the cases (54.3%) as severe, 13 cases (37.1%) as moderate and 3 (8.6%) cases as mild (Supplementary Material, Table S1). Reports from neuropathological studies were available for 25 ASD cases. The pertinent findings are summarized in Supplementary Material, Table S1 and detailed reports can be obtained from the brain tissue banks. Genetic profiles for 25 of the ASD cases, including copy number variant (CNV) analysis using the Affymetrix Genome-Wide SNP array and the Illumina human 1M-Duo DNA Analysis BeadChip microarray platforms, have been reported previously (38) (Supplementary Material, Table S1).

Compared with controls, ASD cerebellum samples had a significantly higher percentage of overall methylation in CGI-2 (methylated CG sites/total number of CG sites analyzed) (control: 3.8 ± 6.5%; ASD: 8.8 ± 17.8%, \( P = 3.6 \times 10^{-4} \)), CGI-3 (control: 14.6 ± 10.1%; ASD: 52.4 ± 12.6%, \( P = 5.4 \times 0.09 \)) and CGI-4 (control: 3.1 ± 6.0%; ASD: 9.2 ± 17.3%, \( P = 1.9 \times 10^{-4} \)) (mean ± SD, thereafter) using a t-test (Table 1). These statistically significant differences were confirmed by two additional analytic methods: t-test with 10 000 permutations (permutation t-test), and generalized estimating equation (GEE) method that takes into account within the subject correlation of all CG sites for each CGI and covariate adjustments (Supplementary Material, Table S2). The differences in methylation between ASD and controls for CGI-2 (\( P < 0.0001 \)) and CGI-4 (\( P = 0.0009 \)) remained significant after adjustment for race, sex, age, PMI and brain tissue resource.

A sex-specific difference was noted for the methylation level of CGI-2 in cerebellum. The difference in methylation between ASD and controls was statically significant in females (ASD = 13 and control = 10, \( P = 0.0018 \)) but not in males (ASD = 38 and controls = 25, \( P = 0.388 \)). The reason for this difference is not known. The overall methylation of CGI-5 was slightly lower in ASD cerebellum tissue (67.0 ± 19%) than in controls (69.2 ± 18.9%), which did not reach statistical significance by direct comparison through t-tests but was significant in GEE analysis (\( P = 0.028 \)) where covariates were adjusted and the significant age effect was found (\( P = 0.0003 \)). CGI-5 overlapped with the last coding exon and the 3’ UTR region of \textit{SHANK3}. The biological effect of slightly reduced methylation in CGI-5 on the expression of \textit{SHANK3} is not clear. The difference in methylation in ASD and control cerebellum samples for each CG site within the CGIs varied and is summarized in Supplementary Material, Dataset D1.

To further confirm the observed differences, we performed hierarchical clustering analysis to generate a heat map of methylation patterns for ASD cases and controls. A heat map of the percentage of methylation at each CG site in ASD cerebellum samples showed a striking subcluster pattern (Fig. 1B, only cases with methylation data for all CGIs available are shown in the heat map). To identify the ASD cases with significantly increased methylation, we used the beta distribution \( P \)-value = 0.01 and a probability of \( P < 0.01 \) that at least 50% of CG sites within a CGI in a given sample were methylated. The significantly increased methylation was clustered in 5 out of 53 (9.4%) ASD cerebellum samples for CGI-2 and 6 out of 54 for CGI-4 (11.1%). Seven out of 51 ASD cases were classified as having increased methylation of CGI-3 (15.7%). However, it should be noted that the distribution of the methylation level of CGI-3 in ASD and control samples is continuous. The percentage of methylation at each CG site in control cerebellum, and in ASD samples with normal and increased methylation in CGI-2 to CGI-4 is shown in Supplementary Material, Figure S2. Representative plots from bisulfite sequencing of ASD cerebellum samples with increased methylation are shown in Figure 1D and E. Bisulfite sequencing plots of all ASD samples with increased methylation in CGI-2 and CGI-4 are shown in Supplementary Material, Figure S3 and those with increased methylation in CGI-3 are shown in...
Supplementary Material, Dataset D1. The methylation levels determined by bisulfite sequencing were verified in select samples, when sufficient DNA was available, by Southern blot analysis of genomic DNA using digestion by methylation sensitive restriction enzymes (data not shown).

We performed the same analysis on the cerebral cortex from ASD samples and controls. The level of DNA methylation for CGI-2 and CGI-4 among different Brodmann regions in a single control cerebral cortex sample was variable (Supplementary Material, Fig. S1). This emphasizes the importance of using matched brain regions as controls. The BA19 region was chosen for this study due to availability of tissues. For CGI-2 and CGI-4, the percentage of methylation was significantly higher in ASD BA19 samples than controls (CGI-2, control: 6.6 ± 10.6%; ASD: 25.4 ± 22.3%, \( P = 1.2 \times 10^{-4} \); CGI-4, control: 14.9 ± 9.9%; ASD: 19.5 ± 15.1%, \( P = 5.5 \times 10^{-1} \)) using a \( t \)-test. The difference in the methylation level of CGI-2 between the two groups was significant when compared by permutation \( t \)-test (\( P = 0.0011 \)) and GEE analysis without covariate adjustments (\( P = 0.005 \) (Supplementary Material, Table S2). However, when we adjusted for race, sex, age, PMI and brain tissue source in the GEE analysis, only borderline \( P \)-value was detected (\( P = 0.069 \), which may be due to significant reduction of total sample size due to missing data in the covariates that was considered. The difference in methylation of CGI-4 in BA19 samples from ASD cases and controls was not significant by all tests (Supplementary Material, Table S2). These discrepancies may also be due to the small number of BA19 tissues analyzed. A comparison of methylation for each CGI site in BA19 samples from ASD cases and controls is shown in Supplementary Material, Figure S2 and Dataset D2. A heat map from hierarchical clustering analysis showed a subcluster of increased methylation for CGI-2 in ASD BA19 samples (Fig. 1C). Four out of 29 ASD samples (13.7%, Cluster I) had a significantly higher percentage of methylation (65.6 ± 6.9%). In addition, three cases (Cluster II) had mild to moderately increased methylation (44.8 ± 2.5%) compared with controls. In CGI-4, only one case had significantly increased methylation in BA19. Representative plots for ASD samples with increased methylation and controls are shown in Figure 1F and G and for all ASD samples in Supplementary Material, Figure S3.

A comparison of clinical information for ASD cases with and without increased methylation is summarized in Supplementary Material, Table S3. The increased methylation in cerebellum and BA19 is not completely concordant in ASD cases suggesting that increased methylation is brain region specific in ASD brain tissues. The significantly increased methylation in at least one CGI in either cerebellum or BA19 strengthens the association between increased methylation of \( SHANK3 \) and ASD (12/53 ASD versus 0/43 in controls \( P = 0.0028 \), Fisher’s exact test). The association remains significant after Bonferroni correction (\( P < 0.01 \)). However, as the cut-off value for classifying a sample as hypermethylated was chosen subjectively (50% of CG sites) without knowledge of functional correlation, it is possible that this analysis may overestimate the significance of increased methylation in cases where it is only mildly or moderately increased, such as CGI-2 in BA19 relative to controls, or the pattern of increased methylation is continuous as seen in CGI-3 in ASD cerebellum. In addition, the caveat of the bisulfite genomic sequencing by cloning and sequencing method that only a limited number of clones were sequenced for each PCR product may introduce the amplification bias and thereby could enter into the interpretation of methylation levels.

We were not able to determine whether differences in methylation level were allele specific due to the absence of informative single nucleotide polymorphisms (SNPs) in the bisulfite-sequenced regions of CGI-2 or CGI-4. The pattern of increased methylation suggests that changes in methylation may be cell type specific in ASD brain tissue. However, further investigation is warranted when reliable techniques for analysis of single cell DNA methylation become available. Neurohistological reports were available for 11 ASD cases with increased methylation and 14 cases without increased methylation (Supplementary Material, Table S1). Cellular defects, such as cortical dysplasia and heterotopia, were observed in five ASD cases with increased methylation (5/11) and four cases without increased methylation (4/14). This difference is not statistically significant (\( P = 0.48 \)). Although these data do not support an association between cellular defects and increased \( SHANK3 \) methylation, the possibility that a defect in a certain cell type may contribute to increased \( SHANK3 \) methylation, or the increased \( SHANK3 \) methylation may contribute to cellular defect cannot be

### Table 1. The percentage of methylation for CGIs \( SHANK3 \) in ASD and controls

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CGI</th>
<th>Controls</th>
<th>ASD Total</th>
<th>Normal methylation</th>
<th>Increased methylation</th>
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<tr>
<td></td>
<td>( N )</td>
<td>( M ) ( \pm SD )</td>
<td>( N )</td>
<td>( M ) ( \pm SD )</td>
<td>( N )</td>
</tr>
<tr>
<td>Cere</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CGI-1</td>
<td>31</td>
<td>4.9 ± 2.4</td>
<td>52</td>
<td>4.6 ± 2.1</td>
<td>53</td>
</tr>
<tr>
<td>CGI-2</td>
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<td>3.8 ± 6.5</td>
<td>48</td>
<td>8.8 ± 17.8</td>
<td>43</td>
</tr>
<tr>
<td>CGI-3</td>
<td>32</td>
<td>45.6 ± 10.1</td>
<td>43</td>
<td>52.3 ± 12.6</td>
<td>35</td>
</tr>
<tr>
<td>CGI-4</td>
<td>38</td>
<td>3.1 ± 6.0</td>
<td>48</td>
<td>9.2 ± 17.3</td>
<td>42</td>
</tr>
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<td>BA19</td>
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<tr>
<td>CGI-5</td>
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<td>69.2 ± 18.9</td>
<td>53</td>
<td>67.0 ± 19.0</td>
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<td>23</td>
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<td>23</td>
<td>19.5 ± 15.1</td>
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<tr>
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<td>68.9 ± 12.2</td>
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<td>69.0 ± 12.7</td>
<td>23</td>
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</table>

Cere, cerebellum; \( M \) \( \pm SD \), mean \( \pm \) standard deviation; \( * \) \( P < 0.001 \) between ASD with increased methylation and controls. \%\%, percentage of ASD cases with increased methylation/total ASD cases. I and II, ASD cases with increased methylation in Class I and Class II.

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completely ruled out. In addition, we did not find any evidence that the increased methylation in any of the CGIs was associated with age, race, co-morbidity of seizures or PMI. Although a sex-specific effect was seen in the methylation of CGI-2 (Supplementary Material, Table S2), this association was not observed when the methylation of all CGIs of SHANK3 was considered (Supplementary Material, Table S3). Interestingly, the methylation level of CGI-3 is inversely correlated to age, regardless of disease status (Supplementary Material, Fig. S4).

To examine whether the increased methylation of SHANK3 found in ASD cases was associated with a small deletion within the CGIs, similar to an imprinting center mutation (39), we performed PCR amplification followed by Sanger sequencing of SHANK3 CGI-1, CGI-2 and CGI-4 in ASD cases with increased methylation. No small deletions, that could suggest a defect in a regulatory element, were identified. Various single nucleotide variants (SNVs) and single base pair insertions and deletions were identified (Supplementary Material, Table S4). We searched the SNP database (dbSNP135), 1000 genomes and NHLBI exome variant server, and found that two SNVs in CGI-2 and CGI-4 were listed in the dbSNP135 database (rs9616818 and rs813690). Whether these sequence variants may affect the methylation level of SHANK3 remains to be investigated. CNV in the SHANK3 region was not identified in ASD cases with increased methylation in this study or a previous report using Affymetrix Genome-Wide Human SNP Array 6.0 platform (38). Interestingly, three ASD cases (AN03935, AN14829, AN17138) with significantly increased methylation in BA19 also have a copy number gain in the chromosome 15q11–q13 region (38). The CNVs in the AN17138 is about ~5 Mb that cover the typical Angelman and Prader–Willi syndrome (AS and PWS) region. The CNVs for AN03935 and AN14829 are ~10 Mb and covered more distally of the typical PWS and AS region. The exact genomic locations of these copy number gains are shown in Supplementary Material, Table S1. The parental origin of the 15q11–q13 duplications in these ASD cases has not been determined.

We also looked for global differences in methylation in ASD brain samples by examining methylation of LINE repetitive elements and the CGI of the imprinted SNRPN gene from the chromosome 15q11–q13 imprinting domain. No significant alterations in these regions were found (Supplementary Material, Fig. S5). Similarly, we did not find aberrant methylation in a select group of ASD candidate genes, including A2BP1 and CANC1A (Supplementary Material, Fig. S6).

**Correlation between clinical severity and increased SHANK3 methylation**

There was no significant difference in overall severity between ASD cases with increased and normal SHANK3 methylation (\( \chi^2 = 0.85 \) and \( P = 0.65 \)). The ASD cases with increased methylation of SHANK3 in brain shared behavioral and developmental profiles, including severe language delay and seizures, that significantly overlap with patients with genetic deletions of SHANK3 in the PMS (30). To further examine whether the level of SHANK3 methylation correlated with the clinical features of ASD, we used the scores of the ADI-R, a relative objective indicator for behavioral presentation, for this analysis (29). The only significant difference was observed between the scores for section D of the ADR-I for ASD cases with increased SHANK3 methylation in CGI-2 (5 ± 0.0, \( n = 11 \)) and normal methylation (4.3 ± 0.7) (\( n = 12, P = 0.009 \)) in cerebellum. This indicates an earlier onset and more severe developmental delay in ASD cases with increased SHANK3 methylation. In BA19, the only statistically significant correlation was between the level of methylation in CGI-2 and the scores for section A of the ADR-I (\( r = 0.74, P = 0.04 \)) (Supplementary Material, Fig. S7). The non-verbal ASD group had a significantly higher level of methylation in CGI-4 of SHANK3 than the verbal group (44.3 ± 14.0% versus 10.3 ± 4.0%, \( P = 0.015 \)). This is consistent with the clinical reports of severe speech delay in patients with SHANK3 genetic deletions. Further investigation in a large number of cases with better clinical data is warranted to extend this observation.

**SHANK3 intragenic CGIs are associated with promoter activity**

We hypothesized that the intragenic CGIs of SHANK3 harbor promoter or regulatory elements involved in SHANK3 isoform-specific expression (Fig. 1A). We used a luciferase reporter assay in HEK293 cells to test the activity of computer-predicted promoter elements from CGI-2, CGI-3, CGI-4 and CGI-X (red bars in Fig. 2A and B). The luciferase reporter assay showed that CGI-2, CGI-3 and CGI-4 harbored promoter activity (Fig. 2C) and that CGI-2 and CGI-4 also have possible enhancer activity (Supplementary Material, Fig. S8). The finding of promoter activity in CGI-3 and CGI-4 is consistent with a previous report (24). The sequences of the genomic fragments harboring promoter activities have been deposited in Genbank (HQ848666–HQ848668). *In vitro* DNA methylation of genomic fragments from CGI-2 and CGI-4 significantly inhibited promoter (Fig. 2D) and enhancer activity (Supplementary Material, Fig. S8). The electrophoretic mobility shift assay showed that nuclear proteins bind to these potential promoters or regulatory elements (Supplementary Material, Fig. S9). These data support that methylation plays a role in regulating the transcriptional activity of SHANK3. The presence of multiple intragenic promoters (promoters 2–5) is also supported by the finding of mRNA transcripts initiated from the intronic regions of the SHANK3 gene as diagramed (Fig. 3A). The activity of promoter 6, which is associated with CGI-4, was weak in a reporter assay in HEK293 cells. We have confirmed the presence of a transcriptional start site (TSS) in CGI-4 by 5′ RACE (rapid amplification of cDNA ends) in mouse brain tissue (28). The TSS maps to the genomic fragment harboring promoter activity within the human CGI-4. The presence of multiple promoters is consistent with in silico analysis of predicting transcriptional starting sites using chromatin immunoprecipitation sequencing (ChIP-seq) data of different chromatin markers by ENCODE project (Supplementary Material, Fig. S10).

**Isoform-specific expression of SHANK3 is altered in ASD brain tissue with increased methylation**

Previous reports suggest that SHANK3 pre-mRNA transcripts undergo alternative splicing. However, many of these transcripts have not been fully characterized (18,33). Using different combinations of primers anchoring at different exons, we amplified more than 20 mRNA isoforms of SHANK3 from human and...
moters of the and 22. The mRNA transcripts initiated from six different promoters, and alternative splicing of coding exons 11, 11a, 12, 18, 21 transcripts was resulted from the combination of six promoters SHANK3 mouse brains (data not shown). The large number of SHANK3 transcripts was resulted from the combination of six promoters and alternative splicing of coding exons 11, 11a, 12, 18, 21 and 22. The mRNA transcripts initiated from six different promoters of the SHANK3 gene were named SHANK3a–f, respectively (Fig. 3A). The SHANK3b–e isoforms are transcribed from intronic regions as diagramed and confirmed by RT–PCR in human brain. SHANK3f is initiated within the exon 21 (28). We also identified a new exon, exon 11a, as described in detail in Supplementary Material, Figure S11. We found that the alternative splicing pattern of exon 18 is tissue specific (Fig. 3J). The inclusion of exon 18 is predominant in cerebellum while the exclusion of exon 18 is seen in liver and heart. SHANK3 exon 21, which is also alternatively spliced (Fig. 3M), contains a Homer-binding site that is predicted to be important for mGluR5 interaction (15). These data suggested the functional relevance of SHANK3 from these alternatively spliced exons. Western blot analysis of control human brain tissues using a SHANK3 antibody did not produce reliable data. The same antibody, however, revealed multiple protein bands in mouse brain confirming the presence of multiple SHANK3 protein isoforms (28). SHANK3a represented a full length of SHANK3 protein. SHANK3b lacks the alternatively spliced exons 18 and 21 that encode the proline-rich region containing the Homer-binding site. However, we were not able to fully characterize the mRNAs for other isoforms because they are typically longer than 4–6 kb in size. Northern blot analysis of SHANK3 using total RNA isolated from human brain tissues was not successful due to the poor quality of RNA isolated from postmortem tissues.

To determine if increased DNA methylation of CGI-2, CGI-3 and CGI-4 in ASD brain tissues is associated with a change in isoform-specific expression of SHANK3 from the corresponding promoters, we performed expression analysis using regular and real-time RT–PCR for ASD cases with increased methylation (n = 10), ASD with normal methylation (n = 7) and controls (n = 11) that had passed an RNA quality check and had as similar a PMI, age and gender as possible (Supplementary Material, Table S5). We first examined the expression of promoter 3- and 5-specific transcripts, SHANK3c and SHANK3e, which are associated with CGI-2 and CGI-3, respectively. The expression level of SHANK3c in brain tissues could not be assessed by real-time PCR because of the extremely high GC content requiring a special polymerase and conditions unsuitable for real-time PCR amplification. The expression of SHANK3e was significantly reduced in ASD cerebellum (n = 4) and BA19 samples (n = 9) (P = 0.001) (Fig. 3B) with increased methylation of CGI-2 when compared with controls (n = 4 for cerebellum and n = 11 for BA19) and ASD samples with normal methylation (n = 4 for cerebellum and n = 7 for BA19). Spearman’s rank correlation revealed a significant negative correlation between the levels of expression of SHANK3e and methylation for CGI-3 (−0.527) but not for CGI-1 (0.064) or CGI-2 (−0.283). We were unable to determine the expression level of SHANK3f, which is transcribed from promoter 6 in CGI-4, because the promoter is embedded in the alternatively spliced exon 21.

We also examined the pattern of alternative splicing of SHANK3 in ASD cases with increased methylation. We used RT–PCR with one pair of primers to semi-quantitatively evaluate the expression level of three splice variants, SHANK3c1–3, which result from alternative splicing of exons in the S′ region of the gene (red arrows in Fig. 3D). All three splice variants were detected in control cerebellum (Fig. 3E). Increased methylation of CGI-2 was associated with either reduced or absent expression of alternative splice variants SHANK3c1–3 in cerebellum (Fig. 3E) and BA19 (Fig. 3F). The expression of SHANK3c1–3 in ASD cases with normal methylation level is similar to controls. The methylation level of CGI-2 for the ASD cases analyzed is summarized in Supplementary Material, Table S5. Using primers that anneal in exons
10 and 14 (red arrows in Fig. 3G), we found that the expression of splice variants \textit{SHANK3}s1–s3 was also reduced or altered in ASD cerebellum and BA19 with increased methylation of CGI-2 (Fig. 3H and I). Similarly, we examined the pattern of alternative splicing of exon 18 in ASD brain tissues (Fig. 3J). The splicing out of exon 18 (E18 – ) was increased in ASD cerebellum and BA19 tissues with significantly increased methylation (Fig. 3K and L). Using primer pairs diagramed in Figure 3M, we found that the expression of \textit{SHANK3} exon 21 splice variants (E21 + and E21 – ) is decreased in ASD tissues with increased methylation of CGI-4 (Fig. 3C). Quantification of the ratio of E21+/E21 – splice variants is complicated by the fact that the transcription of \textit{SHANK3} is also initiated within exon 21. Quantification of \textit{SHANK3}c1–c3, \textit{SHANK3}s1–s3, and the ratio of exons 18+/18 – splicing are summarized in Supplementary Material, Figure S12. The altered alternative splicing of \textit{SHANK3} exon 18 in ASD cases with increased methylation may indicate an association between increased methylation of CGIs and enhanced alternative splicing.

Figure 3. Altered isoform-specific expression of \textit{SHANK3} in ASD brain tissues with increased DNA methylation. (A) Diagram of multiple intragenic promoters and promoter specific isoforms of \textit{SHANK3}a–f. \textit{SHANK3}b–e are transcribed from intronic regions as diagramed. These transcripts were confirmed by RT–PCR using isoform-specific primers in human brains. The full-length mRNA for each isoform have not been characterized. The exons highlighted in red are alternatively spliced exons. (B) The expression of the \textit{SHANK3}e in ASD cerebellum with increased methylation was significantly lower than in controls (\#P = 0.0006) and ASD with normal methylation (\*P = 0.02). Expression of \textit{SHANK3}e was also reduced in ASD BA19 with increased methylation compared with ASD with low methylation (\#P = 0.004) and controls (\*P = 0.001). (C) The expression of both E21+ and E21 – variants was decreased in ASD tissues with increased methylation (n = 4) compared with controls (n = 5) and ASD with normal methylation (n = 5). For the E21 + variant, \#P < 0.01 for ASD samples with increased methylation versus controls, and \*P < 0.03 for increased methylation versus normal methylation. For the E21 – variant, \#P < 0.003 for E21+ variant, \*P < 0.001 for ASD samples with increased methylation versus controls, \#P < 0.0006 for ASD samples with increased methylation versus normal methylation. (D) The structure of \textit{SHANK3}c1–c3 splice variants and primer design for RT–PCR (red arrows). (E) RT–PCR analysis of \textit{SHANK3}c1–c3 in cerebellum. The reduced expression of \textit{SHANK3}c1–c3 isoforms in ASD UMB1182, UMB1174 and AN16115 cerebellum with significantly increased methylation in CGI-2 compared with controls (N) or ASD with a lower level of increased methylation in AN07948. An extra band (red arrow) was sometimes seen in both ASD and control samples but the sequencing of this band revealed either \textit{SHANK3}c1 or \textit{SHANK3}c2, indicating that this band reflected an artifact of gel electrophoresis. (F) \textit{SHANK3}c1 and c2 are significantly decreased in BA19 of ASD with increased methylation. (G) The structure of \textit{SHANK3}s1–s3 splice variants and primer design for RT–PCR (red arrows). (H) Reduced expression of \textit{SHANK3}s1–s3 splice variant in ASD cerebellum with increased methylation in CGI-2. (I) Altered \textit{SHANK3}s1–s3 expression in ASD BA19 with increased methylation in CGI-2. (J) The structure of exon 18 splice variants and tissue-specific alternative splicing. The primers for RT–PCR are shown as red arrows. (K and L) Altered alternative splicing of exon 18 in ASD brain tissues with increased methylation in ASD cerebellum and BA19 tissues. (M) The structure of exon 21 splice variants and primer locations. Primer pair of P1 and P3 was used to detect the E21 – variant. The condition would not amplify the E21+ variant because the product is > 3 kb. Primer pair P1 and P2 was used to detect E21 + variant. The information of age and gender for brain tissues used for RT–PCR are listed in the Supplementary Material, Table S5.
Figure 3. Continued
A role for DNA methylation in alternative splicing was reported in a recent study which showed that DNA methylation of the CD45 gene inhibits alternative splicing mediated by CTCF (40). However, using the ChIP assay, we did not find evidence to support CTCF binding in SHANK3 (data not shown). Altogether, we have shown a disruption of SHANK3 isoform-specific expression in select ASD brain tissues with increased methylation of SHANK3 CGIs. The pattern of altered SHANK3 expression varied between cases. This may reflect the variable pattern of increased methylation in these brain tissues.

Aberrant chromatin modification in the CGIs of SHANK3 with increased methylation in brain

We then asked whether increased methylation of CGIs in the SHANK3 gene is associated with changes in chromatin modification. We performed ChIP assays to analyze histone modifications that have been linked to DNA methylation, including H3K4me3 and H3K27me3, which are associated with active and repressed chromatin, respectively (41). Experiments to quantify the ChIP products by real-time PCR analysis were not successful because of the high GC content of CGI-2 and CGI-4 (75–90% of CG sites), which requires a special polymerase and conditions for PCR (AccuPrime™, Invitrogen, CA, USA). Instead, we performed amplification with a lower amount of template and reduced cycles, and conducted a semi-quantitative analysis by measuring the intensity of the ChIP products. Compared with control cerebellum, the level of H3K4me3 in CGI-2 was significantly decreased in ASD cases with increased methylation. The binding of H3K27me3 in CGI-2 was low and no significant difference was seen in ASD tissues with increased DNA methylation in CGI-2 (Fig. 4A and C). No significant difference...
in the level of H3K36me3 in ASD tissues with increased DNA methylation at CGI-2 (Fig. 4B and D). In addition, no consistent and significant difference was observed for H3K4me3 and H3K27me3 between the ASD case with increased methylation and controls in the CGI-4 of SHANK3. The level of methylation for cases used in the ChIP assays is summarized in Figure 4E and other clinical information in Supplementary Material, Table S5. We were not able to obtain consistent results for normal BA19 tissue samples for ChIP experiments because the tissue samples were small and had a low density of nuclei.

**DNA methylation inhibitor modified the methylation of the CGIs and altered the isoform-specific expression of SHANK3 in cultured cells**

We wanted to determine whether there is a causative relationship between changes in methylation and the expression of the SHANK3 gene observed in human brain tissues. An *in vitro* promoter activity assay showed that methylation inhibited the promoter activity of SHANK3 (Fig. 2). We then used 5-AZA, a potent DNA methylation inhibitor, to treat cultured HEK293 cells and examined the DNA methylation of CGIs and expression of SHANK3. Treatment with 5-AZA at different concentrations (1, 3, 5 μM) for 14 days significantly demethylated CGI-2 and CGI-3 at a dosage dependent manner. We found that different durations of 5-AZA treatment (3, 5 and 14 days at 5 μM) also correlated with the level of demethylation in the CGIs of SHANK3. The results of 14-day treatment at 5 μM are shown in Figure 5A–D (data not shown for 1 and 3 μM) while the results of 3 and 5 days of treatment at 5 μM are shown in Supplementary Material, Figure S13. No significant difference in cellular toxicity was observed for different concentrations and durations of treatment. The ratio of SHANK3c1-c3 was altered in HEK293 cells treated with 5 μM of 5-AZA. After 3 days of treatment, the expression of SHANK3c2 and SHANK3c3 was reduced. Interestingly, the expression of the SHANK3c1 transcript was increased (Fig. 5I). In contrast, 5-AZA treatment did not alter the expression of SHANK3s1--s3 or the exon 18 splice variants (Fig. 5J). The expression of the SHANK3e transcript was increased from the CGI-3 promoter in which significant demethylation occurred (Fig. 5K). The altered expression of only select SHANK3 isoforms after 5-AZA treatment suggests that the effect is more likely caused by altered methylation than interfering RNAs or cellular toxicity. These results suggested a different sensitivity of isoform-specific expression in response to methylation changes.

Analysis of the effect of 5-AZA on SH-SY5Y neuronal cells was not successful because of poor growth of the cells. We then treated human SH-SY5Y neuronal cells with S-adenosyl methionine, a major methyl donor promoting methylation that may mimic the increased methylation found in ASD brain tissues. S-Adeosyl methionine treatment only slightly increased the methylation of CGI-2 (from 1.9 to 8.2%) (Fig. 5E and G) and had no effect on the level of methylation of CGI-3 because CGI-3 is fully methylated in this cell line under normal culture conditions (Fig. 5F and H). The expression of SHANK3c1-c3 and SHANK3s1--s3 splice variants was not significantly altered after S-adenosyl methionine treatment (data not shown). The results of 5-AZA treatment support a plausible correlation between the methylation of CGIs and isoform-specific expression, although the possibility of a genome-wide or off-target effect related to these treatments cannot be excluded.

**DISCUSSION**

Through analysis of a cohort of ASD and control brain tissues, we have found significantly increased DNA methylation in intragenic CGIs and altered isoform-specific expression of the SHANK3 gene in ∼15% of ASD brain tissues. Several pieces of evidence support the clinical relevance of increased SHANK3 methylation found in ASD brain tissues. First, there is strong evidence that mutations in the SHANK3 gene, including whole gene deletions and point mutations disrupting select mRNA isoforms, can cause ASD (18–20,42). Similar to observations in ASD patients with point mutations or small intragenic deletion of SHANK3, we have found that the isoform-specific expression of SHANK3 is altered in ASD brain tissue with increased SHANK3 methylation. The pattern of altered transcription of SHANK3 in ASD brain tissues varied among ASD cases with increased methylation, reflecting the different patterns of increased methylation in different individuals. Second, we found that the promoter activity associated with specific SHANK3 CGIs was inhibited by *in vitro* methylation, and that a DNA methylation inhibitor could modify the level of DNA methylation and isoform-specific expression of SHANK3 in cultured cells. This indirectly supports a causal role for DNA methylation in altering the isoform-specific expression of SHANK3 in ASD brain tissue. Finally, the complex transcriptional regulation of human SHANK3 is highly conserved in mice (28). Several lines of Shank3 mutant mice with different mutations have been reported (25–28). These Shank3 mutant mice lack exons 4–7, exons 4–9, exon 11 and exons 13–16, respectively. In each line of mutant mice, different Shank3 isoforms are disrupted, but none is a complete knockout of Shank3. Distinct biochemical, synaptic and behavioral phenotypes were reported in different lines of Shank3 mutant mice which indicates that individual Shank3 isoforms harbor distinct functions *in vivo* (16). These observations together strongly support a pathological association between changes in methylation and altered isoform-specific expression of SHANK3, and thereby the susceptibility to ASD.

Our findings are significant because they not only further support the involvement of SHANK3 in ASD but also indicate an epigenetic mechanism underlying the susceptibility to ASD. Such an epigenetic mechanism would not be detected by current genetic studies of ASD which typically look for causative genetic mutations (43). The limitations of our study include the small number of ASD cases despite the use of the best tissue source currently available (Jan Pickett, personal communication). The clinical records for the ASD cases were collected retrospectively and were incomplete in some cases. A future study with a larger cohort is warranted to strengthen our findings but the tissue-specific change may post a technical challenge for a direct comparison. The increased methylation observed in intragenic CGIs, but not in the major 5' CGI (CGI-1), was unexpected but not surprising considering recent reports supporting an important role for intragenic CGIs in SHANK3 and other genes in regulating tissue-specific gene expression and alternative splicing (24,44). Several previous
reports, involving small numbers of brain tissue or blood samples from monozygotic twins, have implicated an epigenetic factor in the susceptibility to ASD (7,9–12,45,46). Our study provides strong support for epigenetic profiling of additional candidate genes or genome-wide epigenetic analysis of ASD brain tissues. Our findings also emphasize the importance of examining CGIs in the gene body in future epigenetic studies of ASD as this has not been a focus of previous studies.

The cause of increased methylation in select SHANK3 CGIs in ASD brain tissues is not known. The abnormal methylation could be the result of stochastic errors occurring during the epigenetic reprogramming in the early developing brain (47,48), associated with a genetic susceptibility allele in the genome (49), or less likely, caused by a defect similar to an imprinting center mutation (50). The observation that a DNA methylation inhibitor could modify the methylation and isoform-specific expression of SHANK3 in the brain during early development. It is interesting to note that methylation in offspring can be modified by maternal supplementation of methyl-donors in mice (51–53). Similarly in humans, prenatal nutrition can change global DNA methylation postnatally (54,55). The pattern of increased methylation in SHANK3 CGIs points to the possibility of a cell lineage-specific change.

Figure 5. A DNA methylation inhibitor and methyl donor modified methylation of CGIs and altered the isoform-specific expression of SHANK3 in cultured cells. (A–D) The methylation level of CGI-2 and CGI-3 SHANK3 in HEK293 cells with or without 5-AZA treatment (5 μM) for 14 days. (E–H) The methylation level in CGI-2 and CGI-3 of SHANK3 in SH-SY5Y neuronal cells with or without treatment of S-adenosyl methionine (100 μM). The methylation of CG site 11 and 12 of CGI-2 were more affected by the treatment. (I) SHANK3c1 is increased in HEK293 cells treated with 5 μM 5-AZA. (J) The ratio of exon 18 splice variants was not altered after 5-AZA treatment and only E18− variant was present. No change for SHANK3s1–s3. (K) The significantly increased expression of SHANK3e from CGI-3 promoter in HEK293 cells after 5-AZA treatment by real-time PCR analysis (n = 3 for each concentration; *P < 0.001 treated versus not treated) (see also Supplementary Material, Fig. S13).
in methylation found in ASD brain tissues. Further investigations of cell type-specific DNA methylation at the single cell level may provide clues to understand the changes in DNA methylation in the SHANK3 gene. In addition, a genome-wide DNA methylation and whole genome sequencing analyses may be considered in these brain samples to further delineate a molecular interaction between epigenetic and genetic factors implicated in the susceptibility of ASD.

One interesting observation is that three ASD cases with increased SHANK3 methylation also carry a duplication of the chromosome 15q11–q13 AS and PWS region (38). The parental origins of these duplications could not be determined. Maternal duplication of 15q11–q13 was reported to associate with ASD in the initial report (56). Recent studies have suggested an association between maternal 15q11–q13 duplication and much broader phenotypes including schizophrenia, epilepsy and developmental delay (57–59). The clinical significance of paternal duplication of the same region is less clear (60). The clinical presentation of cases AN03935 and AN17138 appear more severe than those of the cases caused by either maternal 15q11–q13 duplication or SHANK3 deletion alone (61–64). These observations raise the possibility that the combination of the SHANK3 epigenetic defects and 15q11–q13 duplication may contribute to more severe clinical phenotypes, or that epigenetic defects of SHANK3 may be a major modifier of the clinical phenotypes related to 15q11–q13 duplication or vice versa. In this scenario, genetic or epigenetic modifiers may serve as a second hit and contribute to the penetrance of different clinical features associated with a defined molecular defect as was suggested in studies of SHANK2 and CNV on chromosome 15q11.2 in ASD, and other CNVs in other neurodevelopmental disorders (65,66).

One of the major challenges in studying epigenetic causes of disease is to establish causality between changes in methylation and molecular changes in gene expression, as well as disease susceptibility. We have shown that methylation of SHANK3 CGIs suppresses the activity of intragenic promoters in vitro. Treatment with 5-AZA significantly demethylated CGIs in the SHANK3 gene and changed the isoform-specific expression of SHANK3 in cultured cells. Treatment with 5-adenosyl methionine had a minimal effect on the methylation of SHANK3 CGIs under the conditions used. Consistent with reports of a link between methylation of intragenic CGIs and isoform-specific expression of Shank3 from previous studies using cultured neurons from rodents (22–24,67), our data support, indirectly, that changes in methylation alter expression of SHANK3 in ASD brain tissues. Interestingly, we observed differences in the alternative splicing pattern of specific exons in ASD brain with increased methylation and in cultured cells treated with 5-AZA. These observations support the hypothesis that changes in methylation levels of gene body regions may influence the alternative splicing of coding exons as suggested from other recent studies (40,68,69).

Our data support a model of dysregulation of transcription of synaptic protein genes due to epimutation as one of the molecular causes of ASD susceptibility. This model predicts that epimutations of DNA methylation, resulting from errors of an intrinsic or extrinsic nature, lead to cell-type and isoform-specific aberrant expression and alternative splicing of synaptic protein genes during a critical window of brain development. Our findings provide the first evidence to support the proposed model. In this regard, the results of our study are significant because methylation of SHANK3 CGIs could be used as a molecular biomarker for monitoring gene environment interactions contributing to ASD susceptibility as shown by the treatment of 5-AZA in cultured cells. Further investigation in this direction and animals and humans would be interesting to pursue. The ability to modify DNA methylation and expression of SHANK3 by environmental agents also supports use of SHANK3 as a biomarker to dissect the role of gene and environment interactions in susceptibility to ASD. As epigenetic modifications are plastic in nature, this discovery may also open an exciting new avenue for exploring intervention strategies for ASD.

MATERIALS AND METHODS

DNA extraction and RNA isolation

DNA and RNA were extracted using standard methods as described (7). For DNA extraction, frozen brain tissues were digested with proteinase K overnight followed by phenol/ chloroform extraction, and isopropanol precipitation. Total RNA was isolated using the Trizol method (Invitrogen, CA). DNase treatment was performed prior to cDNA synthesis to remove contaminating genomic DNA.

Bisulfite genomic sequencing analysis

Bisulfite genomic sequencing analysis was carried out using the EpiTect bisulfite kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol. Briefly, ~2 μg DNA for each sample was used for bisulfite conversion in 140 μl of reaction. The bisulfite conversion was conducted in the thermal cycler conditions with the following protocol: 95°C 5 min, 60°C 25 min, 95°C 5 min, 60°C 85 min, 95°C 5 min, 60°C 175 min. About 50 ng bisulfite-converted DNA were used for PCR reaction using Hot Star-Taq DNA polymerase (Qiagen). Genomic fragments from individual CGIs of SHANK3 were amplified with specific primers listed in the Supplementary Material D. The annealing temperature for each primer pair for SHANK3 CGI is listed in Table 1. An average of 35–45 cycles was used for PCR applications. PCR fragments were recovered from gel and cloned into the pGEM-T easy vector (Promega, Madison, WI, USA). Typically, 14–16 clones from each PCR product were sequenced with M13F or M13R primers at Beckman Coulter Genomics (Danvers, MA, USA). A minimum of 10–12 clones with good quality of sequence was obtained for each ASD or control sample (see additional detail in Supplementary Material D).

SHANK3 isoform expression analysis by regular RT–PCR and real-time RT–PCR

A total of 5 μg of RNA was reverse transcribed using SuperScript III reverse transcriptase and random hexamers according to the manufacturer’s protocol (Invitrogen). The cDNA template was used for regular RT–PCR and real-time RT–PCR analysis. All primers for RT–PCR were designed to anneal to exonic sequences separated by large introns to avoid amplification of genomic DNA. RNA quality was checked prior to the experiments. Primer sequences and annealing temperatures are provided in Supplementary Material D10. Real-time quantitative RT–PCR was carried out using a LightCycler 480 Instrument.
(Roche Diagnostics, Mannheim, Germany) and QuantiFast SYBR green PCR kit (Qiagen), following the manufacturer’s recommendations. All samples were run in triplicate. Each PCR run also included three negative controls containing no template. Human 18S rRNA or beta actin was used as an internal reference to normalize RNA input across samples when performing relative quantification analysis.

PCR amplification of GC-rich DNA templates

For the RT–PCR and regular PCR of DNA and cDNA in the CGI-2 region, AccuPrime™ GC-Rich DNA Polymerase (Invitrogen) was used because of the extremely high GC content around CGI-2. Special GC-rich PCR buffers were provided with AccuPrime Polymerase and contained 300 mM Tris–HCl (pH 9.2), MgSO₄ at 10 mM (Buffer A) or 7.5 mM (Buffer B), 150 mM NaCl, 1 mM dGTP, 1 mM dATP, 1 mM dTTP, 1 mM dCTP, thermostable AccuPrime™ proteins and enhancers. The RT–PCR and PCR condition for individual pairs of primers are described in Supplementary Material.

Statistical analysis

We used both the t-test and GEE to examine the differences in methylation between ASD cases and controls. The t-test assumed the observed percentage of methylation for all CGI sites per subject are independent and the data follow one distribution. Considering the relatively small sample size, we also conducted permutation tests with 10,000 repeats for t-test to obtain the empirical P-values. However, t-test may not reflect our data as multiple CGI sites may be more correlated within a subject than between subjects. To address this concern, we applied GEE, which takes into account the repeated measure (CGI sites) within each subject. To test the methylation differences between ASD cases and controls, we performed two models within GEE, one without covariate adjustment and one with covariate adjustment. The covariates included are age, gender, race (Caucasian versus African American), PMI and brain tissue resource source (i.e. Harvard versus NICHD brain tissue bank). We drew our conclusions based on the similarity of these three approaches.

In addition, we generated a heatmap for the percentage of methylation at each CGI site for ASD and control samples to visualize the differences between ASD cases and controls. Only samples for which we have data for CGI-1 to CGI-5 are shown in the heat map, but all cases were included for statistical analysis.

To test if a sub-set of ASD samples has a significantly increased methylation percentage compared with that of control samples, we assumed that the methylation percentage in control samples at each CGI site within the same CGI has the same methylation percentage distribution. Using control samples, we derived a null distribution for each CGI. Using beta distribution and type I error of 0.01 we determined if the methylation percentage in an ASD sample was different from the null distribution. For each CGI, we identified samples with an abnormal methylation pattern at >50% of CGI sites.

Single CpG site methylation raw data for each sample were exported from Pryo Q-CpG software (Qiagen). We summarized the methylation rate per sample by calculating the average of methylation across all the CpG sites detected.

All statistical analyses were performed with R version 2.12.0 and SAS (Cary, NC, USA). For regular bisulfite sequencing, we used QUMA (http://quma.cdb.riken.jp/) software and Microsoft PowerPoint to draw the dot plots (see additional methods in Supplementary Material D).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We thank Yoonji Lee, Xiaodong Zhai, Zhiqing Huang and Richard Person for technical assistance. We also thank Catherine Rehder for assisting CNV analysis. We would like to acknowledge the Autism Tissue Program, Harvard Brain Tissue Bank and NICHD Brain Tissue Bank for providing the brain tissues for this study. We also thank Jane Picket from the Autism Tissue Program and Robert Johnson from NICHD brain tissue bank for their assistance. We thank David Goldstein, Jan Bressler and Susan Murphy for critical reading of the manuscript. Y.H.J. is supported by an Autism Speaks grant and National Institute of Health grant R01MH098114-01. X.W. is supported by Phelan-McDermid syndrome foundation.

Conflict of Interest statement. None declared.

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