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Hypermethylation of the Reelin (*RELN*) Promoter in the Brain of Schizophrenic Patients: A Preliminary Report

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DNA methylation changes could provide a mechanism for DNA plasticity and dynamism for short-term adaptation, enabling a type of cell memory to register cellular history under different environmental conditions. Some environmental insults may also result in pathological methylation with corresponding alteration of gene expression patterns. Evidence from several studies has suggested that in schizophrenia and bipolar disorder, mRNA of the reelin gene (*RELN*), which encodes a protein necessary for neuronal migration, axonal branching, synaptogenesis, and cell signaling, is severely reduced in post-mortem brains. Therefore, we investigated the methylation status of the *RELN* promoter region in schizophrenic patients and normal controls as a potential mechanism for down regulation of its expression. Ten post-mortem frontal lobe brain samples from male schizophrenic patients and normal controls were obtained from the Harvard Brain Tissue Resources Center. DNA was extracted using a standard phenol–chloroform DNA extraction protocol. To evaluate differences between patients and controls, we applied methylation specific PCR (MSP) using primers localized to CpG islands flanking a potential cyclic AMP response element (CRE) and a stimulating protein-1 (SP1) binding site located in the promoter region. For each sample, DNA extraction, bisulfite treatment, and MSP were independently repeated at least four times to accurately determine the methylation status of the target region. Forty-three PCR trials were performed on the test and control samples. MSP analysis of the *RELN* promoter revealed an unmethylated signal in all reactions (43 of 43) using DNA from the frontal brain tissue, derived from either the schizophrenic patients or normal controls indicating that this region of the *RELN* promoter is predominantly unmethylated. However, we observed a distinct methylated signal in 73% of the trials (16 of 22) in schizophrenic patients compared with 24% (5 of 21) of controls. Thus, the hypermethylation of the CpG islands flanking a CRE and SP1 binding site observed at a significantly higher level ($t = -5.07$, $P = 0.001$) may provide a mechanism for the decreased *RELN* expression, frequently observed in post-mortem brains of schizophrenic patients. We also found an inverse relationship between the level of DNA methylation using MSP analysis and the expression of the *RELN* gene using semi-quantitative RT-PCR. Despite the small sample size, these studies indicate that promoter hypermethylation of the *RELN* gene could be a significant contributor in effecting epigenetic alterations and provides a molecular basis for the *RELN* gene hypoactivity in schizophrenia. Further studies with a larger sample set would be required to validate these preliminary observations.

KEY WORDS: DNA methylation; schizophrenia; reelin; gene expression; frontal lobe; post-mortem; brain; human; methylomics

INTRODUCTION

A direct relationship between the environment and the adaptation of genes resulting in altered gene expression patterns has been well established [for a review see Abdolmaleky et al., 2004]. In general, the inactive genes are legitimate

Sam Thiagalingam and Ming T. Tsuang contributed equally and co-directed this work.

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candidates for silencing of gene expression due to DNA methylation [Bird, 2002]. The addition of methyl groups to the cytosine (C) of CpG dinucleotides is mediated by several DNA methyltransferases in an interactive manner [Singal and Ginder, 1999; Bestor, 2000; Kim et al., 2002]. In vertebrates, although 4%–8% of cytosines [Attwood et al., 2002; Bird, 2002], and 70% of all CpGs are methylated, less than 30% of CpGs in the promoter regions of active genes are methylated [Naveh-Many and Cedar, 1981; Kress et al., 2001]. There are about 29,000 CpG islands in the human genome [Costello and Plass, 2001] and 50%–60% of genes contain CpG islands that regulate the gene's activity depending upon their state of methylation [Singal and Ginder, 1999]. Despite the maintenance of a specific pattern of DNA methylation throughout cell divisions, it may become altered due to global or local demethylation and de novo methylation [Kress et al., 2001]. The activity of methyltransferase enzymes, and the presence of folic acid and *S*-adenosyl methionine are necessary components in de novo methylation [Kim, 2000]. Any imbalance in the levels, or activity, of these components may cause developmental defects or cell death due to aberrant DNA methylation changes [Watson and Goodman, 2002]. Additionally, several animal studies suggest that the epigenetic germline DNA modifications and cytosine methylation patterns are inherited [Russo et al., 1996; Roemer et al., 1997; Wolff et al., 1998; Cooney et al., 2002].

The methylation rate of CpG sites is determined by the proportional availability of DNA methyl transferases (DNMT) and other factors, and their relative affinity for a given CpG site on the DNA [Russo et al., 1996]. Cytosine methylation can mediate silencing of gene expression through the actions of proteins such as MeCP2, MBD1, MBD2, MBD3, and MBD4, which bind directly to methylated DNA and/or through other factors that bind directly or indirectly to methylated CpGs. This binding inhibits transcription from these sites due to the establishment of a silenced histone code [Hendrich et al., 1999; Attwood et al., 2002; Bird, 2002; Thiagalingam et al., 2003]. Thus, the amount and the specific sites of DNA methylation directly correlates with gene expression in specific cells at specific times [Ngo et al., 1996; Russo et al., 1996; Kress et al., 2001; Kim et al., 2002].

Interestingly, some nutritional derivatives such as butyrate (one of milk products) through inhibition of histone deacetylases [Zhu and Otterson, 2003], or tea polyphenol (-)-epigallocatechin-3-gallate that inhibits DNMT [Fang et al., 2003] may also influence genes DNA methylation level. Additionally, there is accumulating evidence that suggests that the activation of genes may have a feedback effect impacting DNA methylation patterns. In a recent animal study, neuronal depolarization reduced the methylation level of the BDNF gene, dissociated MeCP2 from the promoter region, and increased BDNF gene transcription [Martinowich et al., 2003].

Although, in general, dense methylation results in irreversible silencing of gene expression, strong activators may overcome partial methylation. Suspension of the process of DNA methylation, due to the direct effect of transcription factors involved in gene activation, has been reported [Kress et al., 2001; Bird, 2002]. Successive binding of transcription factors to a gene's regulatory region may alter the nucleosome structure leading to decreases in the methylation rate and retention of the capability for gene expression for a prolonged period of time [Murray et al., 2000]. For example, in rat liver, glucocorticoid induces DNA demethylation in the tyrosine aminotransferase gene that is stable after hormone withdrawal and chromatin remodeling. Subsequent hormonal stimulation evokes a much stronger response by the gene, suggesting a potential mechanism for cell memory [Thomassin et al., 2001]. Thus, incremental differential DNA methylation of the genome could play a critical role in allowing DNA plasticity, enabling gradual adaptation to variable environments in each generation. Thus, adaptation to the environment may occur at the individual level due to DNA methylation changes in response to specific environments, and at the species level due to natural selection [Monk, 1995]. By the same token, these findings also suggest that environmental factors, by altering methylation states of genes, may participate in the pathogenesis of multi-factorial diseases, and that epigenetic modification may play a role in schizophrenia and other neuronal/ brain disorders [Costa et al., 2001].

Reelin, an extra-cellular matrix protein mainly expressed by GABAergic inter-neurons, binds to lipoprotein receptors and activates a tyrosine kinase cascade leading to Dab 1 phosphorylation [Pesold et al., 1999]. These signaling events are essential for neuronal migration, axonal branching, and synaptogenesis throughout brain development. Additionally, reelin activates a second-messenger cascade that influences gene expression required for long-lasting structural changes in the neurons [Pesold et al., 1999]. The reelin gene (*RELN*) localizes to chromosome 7, and harbors a long CpG rich promoter region [DeSilva et al., 1997]. Bisulfite treated DNA sequencing in the NT2 neuronal precursor cell line to analyze the effects of demethylating agents demonstrated that DNA methylation of the *RELN* promoter region could be responsible for silencing *RELN* gene expression [Chen et al., 2002].

Because the mRNA and protein levels of reelin are severely reduced in post-mortem brains of schizophrenia and bipolar patients, some investigators have hypothesized that reduced reelin levels may increase susceptibility to these disorders [Fatemi et al., 2000; Costa et al., 2001]. Therefore, we decided to investigate whether there is a direct relationship between differential promoter methylation of the *RELN* gene and schizophrenia, by comparing the methylation status of brain samples from patients who had been diagnosed with schizophrenia to samples from controls.

MATERIALS AND METHODS

Patient and Control Samples

Five post-mortem brain samples from male patients with schizophrenia and five male normal controls of the same age at similar post-mortem interval were obtained from the Harvard brain tissue resources center. All ten donors were between the age of 42 and 50 years, as shown in Table I. Autopsies were carried out between 14 and 29 hr after death. Brain tissues were stored at −80°C until further analysis.

DNA Extraction

The post-mortem brain tissue was dissected to obtain pure gray matter through removal of the pia, visible blood vessels, and white matter. DNA was extracted using a standard phenol–chloroform DNA extraction protocol.

Bisulfite Treatment

For the MSP assay, genomic DNA was isolated from the brain samples and subjected to bisulfite modification. Bisulfite treatment converts unmethylated cytosine nucleotides to uracils, while methylated cytosine nucleotides remains unchanged [Frommer et al., 1992; Herman et al., 1996; Singal and Ginder, 1999]. In brief, approximately 1μg of genomic DNA was denatured with 2M NaOH for 15 min, followed by bisulfite treatment with freshly prepared 10 mM hydroquinone and 3M sodium bisulfite, pH 5.0 (Sigma, St. Louis, MO). Each reaction was overlaid with mineral oil and incubated at 50°C for 16–20 hr. After treatment, the modified DNA was purified using a Wizard DNA purification kit (Promega, Madison, WI), followed by desulfonation treatment with 3M NaOH, which changes uracil

TABLE I. Summary of Selected Clinical and Demographic Data of the Controls and Patients

Brain sample no.	Group	Sex	Age (years)	Post-mortem interval (hr)	Brodmann's area (BA)	Laterality: left/center frontal lobe
1	Control	Male	50	26.50	BA 10	Right
2	Control	Male	44	23.00	BA 9	Right
3	Control	Male	46	25.90	BA 10	Left
4	Control	Male	47	18.80	BA 10	Left
5	Control	Male	43	23.00	BA 9	Left
Average (SD)			46 (2.74)	23.44 (3.05)		
6	Schizophrenia	Male	44	17.80	BA 10	Left
7	Schizophrenia	Male	42	14.20	BA 10/9	Right
8	Schizophrenia	Male	46	29.06	BA 10	Left
9	Schizophrenia	Male	46	29.50	BA 10	Right
10	Schizophrenia	Male	49	24.50	BA 10	Left
Average (SD)			45.4 (2.61)	23.01 (6.81)		

to thymine (T). The DNA was ethanol precipitated, and the purified DNA pellet was resuspended in 30 μl of distilled water.

Sequencing of Bisulfite Treated DNA

The primers used for the amplification of the *RELN* promoter region from the bisulfite modified genomic DNA were as follows. The forward primer: 5′-GTATTTTTTTAG-GAAAAATAGGGTATATTGA-3′ (−687 to −656) and the reverse primer: 5′-ACTCCCAAAATTACTTTAAACC-3′ (−180 to −202). The PCR product was sequenced using the following nested primer: 5′-GTTAAAGGGGTTGGTTTTT-3′ (−659 to −640).

Methylation Specific PCR (MSP) Primer Design

MSP was used to determine the differences in the DNA methylation status [Herman et al., 1996] of the *RELN* promoter region, between the patients and controls. The sequences of the forward and reverse MSP primers to distinguish between the methylated (M) and unmethylated (U) genomic DNA in the *RELN* promoter used in this study were as follows: methylated DNA specific primers: forward primer: 5′-CGGGGTTTTGACGTTTTTCG-3′ (−602 to −582), reverse primer: 5′-CGCCCTCTCGAACTAACTCGACG-3′ (−418 to −441). Unmethylated DNA specific primers: forward primer: 5′-GTGGGGTTTTGATGTTTTTTG-3′ (−603 to −582), reverse primer: 5′-CACCCTCTCAAACTAACTCAACA-3′ (−418 to −441). The site corresponding to the forward primer included a putative cyclic AMP response element (CRE), TGACGT, which when methylated has been shown to reduce gene expression in several genes [Moens et al., 1993]. On the other hand, the reverse primer included a core sequence of a putative stimulating protein-1 (SP1) binding site (GGCGGG).

MSP-PCR Setup and Cycling Conditions

The PCR reaction was performed in a total volume of 25 μl, consisting of the following components: 2.5 μl of 10× buffer (670 mM Tris-HCl, pH 8.8, 166 mM ammonium sulfate, 67 mM magnesium chloride, 10 mM β-mercaptoethanol), 0.4 μl of 10 μM dNTPs (Invitrogen, Carlsbad, CA), 1.5 μl of dimethyl sulphoxide (Sigma), 2.5 U of platinium *Taq* DNA polymerase (Invitrogen), 25 pmol each of methylated specific or unmethylated specific primers, and 50 ng bisulfite modified genomic DNA.

Bisulfite modified placental DNA (PD) and in vitro methylated DNA (ID) was used as negative and positive controls, respectively. In addition, water blanks were used as a control for the PCR reactions to detect any potential contamination events.

PCR cycling conditions were as follows: one cycle each of 94°C for 2 min, 60°C for 1 min, 70°C for 1 min, 31 cycles of 94°C for 30 sec, 60°C for 40 sec, 70°C for 40 sec, followed by a final extension at 70°C for 10 min. A 10 μl sample of each PCR product was analyzed by electrophoresis on a non-denaturing 6% polyacrylamide gel, stained with ethidium bromide, and visualized under UV light.

Recognizing that the conditions of the bisulfite treatment, and the location of the cells from the original tissue blocks, could have confounding effects on the pattern of methylation detected we repeated the DNA extraction, bisulfite treatment, and PCR for each of the samples analyzed in this study at least four times. In summary, we performed 21 trials for controls and 22 trials for patients as shown in Table II.

TABLE II. Frequency of Methylated/Unmethylated Signals During MSP Analysis of the Samples From Controls and Schizophrenic Patients

Sample no.	Group	No. of trials	No. of negative methylated signals	No. of positive methylated signals	Percent of positive methylated signals
1	Control	4	2	2	50
2	Control	4	3	1	25
3	Control	4	4	0	0
4	Control	5	4	1	20
5	Control	4	3	1	25
Average (SD)		4.2 (0.45)	3.2	1 (0.71)	24 (0.18)
6	Schizophrenia	4	1	3	75
7	Schizophrenia	5	1	4	80
8	Schizophrenia	4	1	3	75
9	Schizophrenia	5	2	3	60
10	Schizophrenia	4	1	3	75
Average (SD)		4.4 (0.55)	1.2	3.2 (0.84)	73 (0.55)

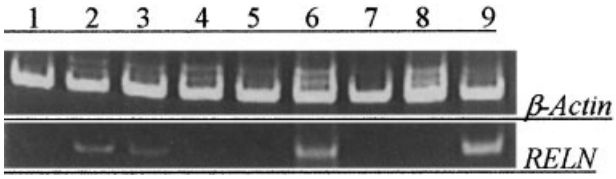

Fig. 1. Differential expression of *RELN* in various normal human tissues. **Lanes 1–9** correspond to samples from lung, liver, stomach, kidney, heart, breast, bone marrow, colon, and brain tissues, respectively. RT-PCR analysis of the *RELN* expression was normalized with the levels of β-actin from the same samples.

Analysis of *RELN* Expression Using Semi-Quantitative RT-PCR

Total RNA was prepared from brain samples, using TRIzol (Life Technologies, Inc., Grand Island, NY) according to manufacturer instructions. We also used total RNA from several human tissues obtained through Clontech to set up the accurate conditions for further experiments (Fig. 1). cDNA synthesis and PCR amplification was performed essentially as previously described [Cheng et al., 2004].

The gene specific primer pair used in the analysis of the *RELN* gene expression are as follows: forward primer, 5′-CGGCATGGAGCGCAGTGGCTGGGC-3′ located in exon 1 and reverse primer, 5′-GTGTATAGTCCTGTCACCAGCAAGC-3′ located in exon 2 of the variant 1 *RELN*. The β-actin gene was used as an internal standard to normalize the abundance of the RT-PCR product derived from the *RELN* gene [Cheng et al., 2004]. Note that the extracted RNA from the tissue of seven brains was qualified for RT-PCR.

Data Analysis

A *t*-test was used to compare the arcsine of the proportion of trials that showed methylation in the case and control groups. The Cochran test for the equality of variances showed that the variance for the proportion of trials that were methylated in the cases was not different from the variance in the controls ($F = 1.90, P = 0.55$). This allowed us to proceed with the pooled variance test using all of the available data in this small sample.

RESULTS

We surveyed the expression of *RELN* in different human tissues using semi-quantitative RT-PCR (Fig. 1). The level of expression was highest in the brain followed by breast, liver, stomach, kidney, and lung in our experiment. We considered the possibility that down regulation of *RELN* expression in the schizophrenia patients could be correlated to promoter methylation. We evaluated the methylation status of the *RELN* promoter by both bisulfite modified DNA sequencing and MSP analysis using the genomic DNA focusing at the sequences around the CRE and the SP1 elements.

The level of methylation of the C residues in the CpG islands determined from the MSP analysis showed direct concordance with the relative heights of the DNA sequencing traces derived from the C and T residues (Fig. 2). These results suggested that the high throughput MSP analysis could be used to determine the level of methylation of the *RELN* promoter. The expression patterns of these samples also showed concordance with methylation status of the *RELN* promoter determined by both DNA sequencing and the MSP analysis (Figs. 2–4). Thus, we analyzed and compared the levels of *RELN* promoter methylation in samples from the schizophrenia patients and matched

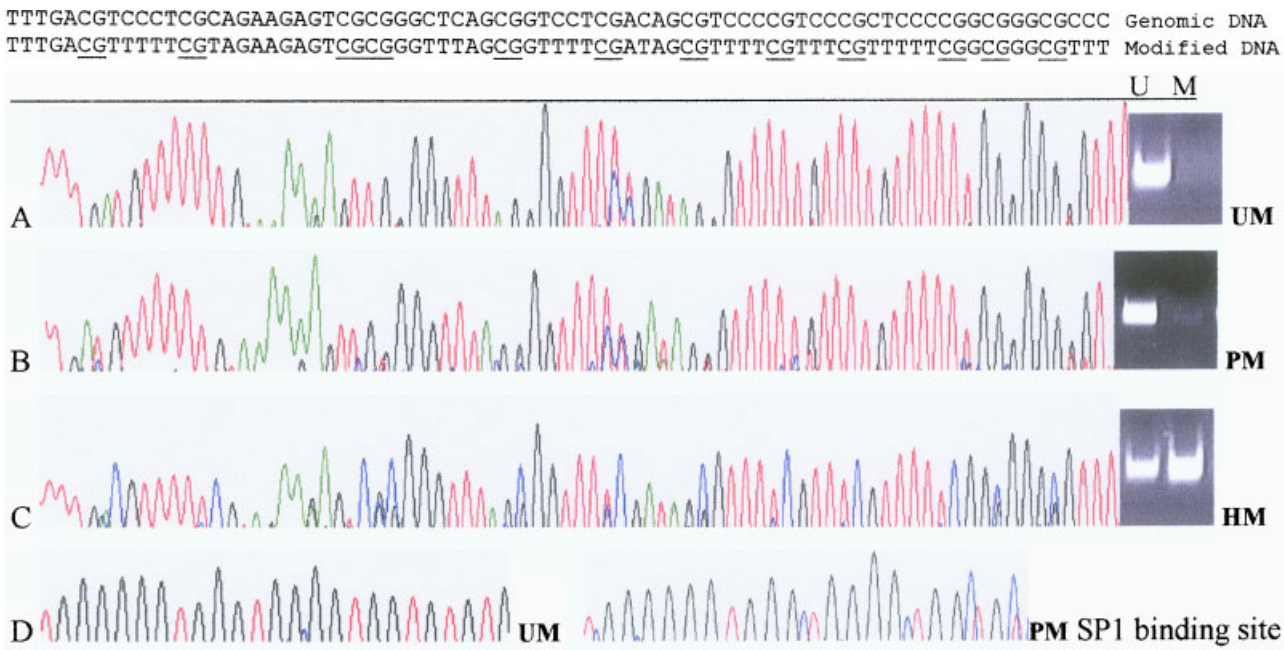

Fig. 2. Bisulfite modified DNA sequencing of the *RELN* promoter in schizophrenia patients and controls. At the top of the figure, the unmodified genomic DNA sequence and the bisulfite modified DNA sequence (methylated) of the promoter region corresponding to −595 to −520 at a CRE binding site and its down stream region is shown. Underlined Cs in the CpGs are candidates for methylation. **Panels A, B,** and **C** show the sequence traces and the corresponding MSP analyses data for the unmethylated (UM), partially methylated (PM), and highly methylated (HM) templates, respectively. **Panel D** shows the sequence traces of three successive SP1 binding sites (−420 to −398) derived from a control (left; template from sample shown in panel A) and a patient (right; template from sample shown in panel B). U and M indicate the use of primers that amplify unmethylated and methylated DNA templates. In the sequence traces red, black, blue, and green indicate T, G, C, and A, respectively. When the template was methylated, most of the Cs in CpGs gave a blue trace as opposed to a red trace indicating the conversion of C to T. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

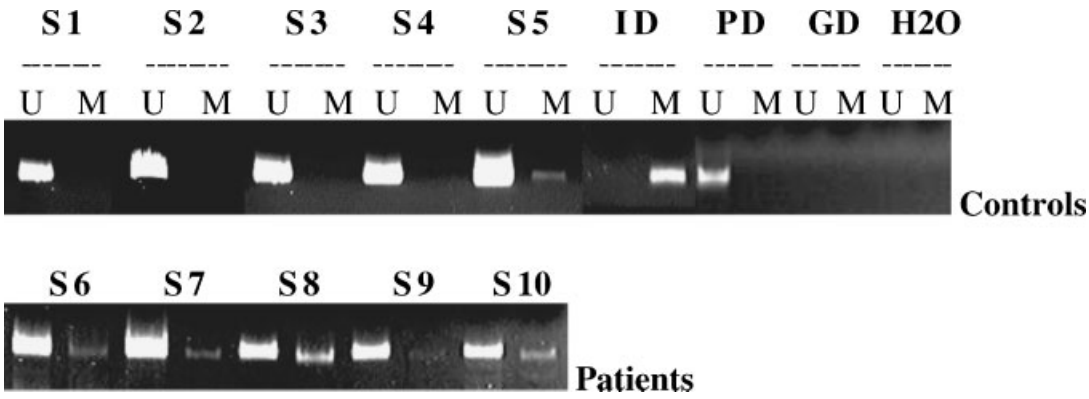

Fig. 3. MSP analysis of the CpG islands of the promoter of the *RELN* gene in the indicated brain samples from schizophrenic patients and controls. In vitro methylated DNA (ID) and placental DNA (PD) serve as positive and negative controls, respectively. Genomic DNA (GD) serves as an additional control to indicate that the same primers will not amplify the unmodified DNA. Water (H_2O) was used to detect contamination. **Lanes U** and **M** indicate the presence of unmethylated and methylated template, respectively.

controls using the MSP analysis. Our results consist of ten observations with a maximum of five measures of methylation each. Table I shows the sample composition. All subjects were male. There were no significant differences between the case and control groups with respect to age, post-mortem interval, or number of trials (Table I). Table II shows the data for each subject.

From 22 trials for patients, we obtained 16 methylated signals (73%). In contrast, from 21 trials for controls we found five methylated signals (24%). The pooled *t*-test showed that the samples from brains of schizophrenic subjects were significantly more likely to be methylated than those of the control subjects ($t = -5.07$, $P = 0.001$) (Table III and Fig. 3).

Furthermore, the expression pattern of the *RELN* gene in selected samples, as determined using semi-quantitative RT-PCR, also exhibited a high level of concordance to the degree of methylation determined by the MSP analysis (Fig. 4).

DISCUSSION

From an etiological point of view, there is strong evidence to suggest that both L-methionine as well as environmental insults can induce pathologic DNA hypermethylation, which silences gene expression. It is known that transient ischemia increases DNA methylation and endangers the survival of CNS neurons, and that prevention of DNA methylation prevents brain damage [Endres et al., 2000]. Since "Fetal hypoxia is associated with greater structural brain abnormality among schizophrenic patients and their non-schizophrenic siblings than controls" [Cannon et al., 2002], DNA methylation processing may be abnormal in schizophrenic patients and their family members. Nitric oxide (NO), which is believed to play a role in schizophrenia, has neurodegenerative effects in focal ischemia and could also be a mediator of pathological hypermethylation [Hmadcha et al., 1999].

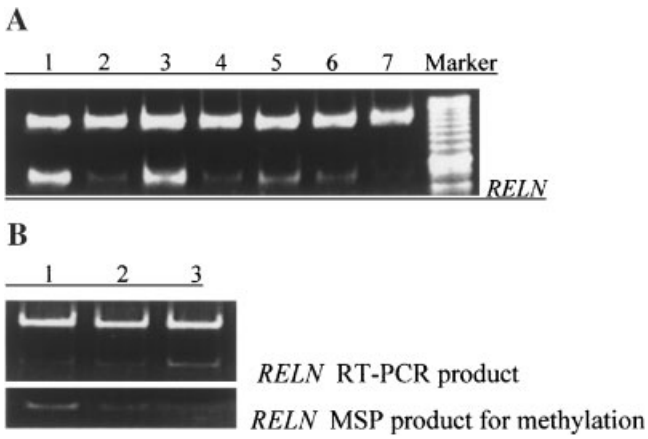

Fig. 4. Analysis of *RELN* expression in schizophrenia. **A**: Total RNA prepared from indicated post-mortem brain samples and analyzed by semi-quantitative RT-PCR (**Lanes 1–7**). Lanes 1–3 and 4–7 correspond to normal and patient brains, respectively. Multiplex RT-PCR analysis for β-actin (upper band) and *RELN* (lower band), respectively as described under Methods. **B**: **Lanes 1–3**, samples with decreasing level of methylation (**lower panel**) with corresponding increase in the level of *RELN* expression (**upper panel**: upper band, β-actin; lower band, *RELN* RT-PCR product).

In an intriguing connection to psychiatric diseases, severely reduced mRNA and protein levels of reelin was seen in the examination of post-mortem brains from schizophrenia and bipolar disorder patients [Fatemi et al., 2000; Costa et al., 2001]. A recent study reported an over-expression of DNMT1 (an enzyme that primarily acts to maintain DNA methylation states) in the GABAergic interneurons of schizophrenic patients assayed by RNA in situ hybridization [Veldic et al., 2004]. Concurrent with DNMT1 over-expression, the *RELN* mRNA was reduced in the same layers of BA 10 in schizophrenic patients [Veldic et al., 2004]. Studies using animal models and cells in culture also showed that the expression of reelin is influenced by methylation of the *RELN* promoter region [Chen et al., 2002]. Furthermore, L-methionine, a known methyl donor is also known to increase the amount of cytosine methylation in the CpG islands of the *RELN* promoter region resulting in a decrease in the reelin mRNA levels that may exacerbate symptoms in most schizophrenic patients [Tremolizzo et al., 2002].

We have followed up on these important observations and report for the first time that brain samples from patients who had been diagnosed with schizophrenia show increased DNA methylation of the CpG islands localized to the *RELN* promoter regulatory regions. In this study, first we confirmed the reliability of the use of a high throughput MSP assay for determining the methylation status of the *RELN* promoter (Fig. 2). We used MSP analysis to evaluate the methylation status of the *RELN* promoter and show that there is a significantly higher level of the methylation of the *RELN* promoter region in brain samples from patients who had been diagnosed with schizophrenia when compared to the matched controls (Fig. 3 and Tables I–III). The expression of the *RELN* gene in the samples analyzed also exhibited a high level of concordance to the degree of DNA methylation suggesting that promoter methylation is responsible for silencing of *RELN*

TABLE III. Sample Composition and Pooled *t*-Test Analysis of the Methylated Signals

	N	Average number of trials (range)	No. of left hemisphere	No. of right hemisphere	Area no. 9	Area no. 10	Average % methylated
Total sample	10						
No. of controls	5	4.2 (0.45)	3	2	2	3	24% (0.18)
No. of cases	5	4.4 (0.55)	3	2	1 mixed	4	73% (0.55)
							$t = -5.07$, $P = 0.001$
% Male	100%						

expression in schizophrenia (Figs. 3 and 4). The MSP analysis used in our studies considered CpG methylation in a putative c-AMP response element localized to the *RELN* gene promoter region. Interestingly, it has also been previously reported that methylation of the c-AMP response element in the promoter region of genes could cause a decrease in the level of gene expression [Moens et al., 1993].

Our control and patient samples were matched for age, sex, race, and post-mortem interval to reduce the likelihood that these variables would confound our analyses. Furthermore, our review of medical records showed that the patients had not used any drugs known to change DNA methylation status, at least in the last eight weeks of their lives. Thus, it is reasonable to predict that the useful effects of sodium valproate in schizophrenia and bipolar disorder could be due to its inhibition of the histone deacetylases, which ultimately results in the removal of the methyl groups from the methylated cytosine of the DNA [Alonso-Aperte et al., 1999; Manev and Uz, 2002; Tremolizzo et al., 2002]. Given our small sample size, it is possible that our results are an artifact of some unknown confound. Thus, replication of these findings in a larger sample set will be required to verify whether the DNA methylation of the putative CRE and SP1 binding sites in the *RELN* gene promoter are responsible for gene silencing in the frontal lobe of schizophrenic patients.

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