BRIEF REPORT

Methylation Status of the Reelin Promoter Region in the Brain of Schizophrenic Patients

Mamoru Tochigi, Kazuya Iwamoto, Miki Bundo, Atsuko Komori, Tsukasa Sasaki, Nobumasa Kato, and Tadafumi Kato

Background: Hypermethylation of the reelin (RELN) promoter region and the reduced levels of its messenger RNA and protein have been implicated in the pathophysiology of schizophrenia. We intended a technical replication of recent studies that observed hypermethylation of CpG or CpNpG sites in the RELN promoter region in the brain of schizophrenic patients.

Methods: The DNA methylation status of the promoter region of RELN was examined by using the pyrosequencing method in the prefrontal cortices of 14 patients with schizophrenia and 13 control subjects.

Results: All of the CpG and two proposed CpNpG sites analyzed showed no detectable DNA methylation (< 5%) in both control subjects and patients with schizophrenia. No detectable DNA methylation was observed in both gray and white matter, excluding the possibility of cellular heterogeneity of start materials.

Conclusions: We did not confirm the hypermethylation of the RELN promoter region in the brains of schizophrenic patients, suggested in the previous studies.

Key Words: DNA methylation, epigenetics, postmortem brain, pyrosequencing, reelin, schizophrenia

The etiological importance of inherited and acquired epigenetic defects has been suggested in schizophrenia (1). Recently, Grayson *et al.* (2) observed hypermethylation of the promoter region of the reelin (*RELN*) gene. There was increased methylation of cytosines at positions −139 and −134 (complementary strand) in the occipital or prefrontal cortices of schizophrenic patients. The DNA methylation status of the region was suggested to play an important role for regulation of the *RELN* expression, although they are nonstandard methylation sites, having the form of CpNpG (2). *RELN* is a glycoprotein controlling cell–cell interactions critical for cell positioning and neuronal migration during brain development (3). Several lines of studies have observed that the *RELN* messenger (m)RNA and protein levels are reduced in various cortical structures of postmortem brain from schizophrenic patients (4). Thus, *RELN* has been suggested as one of the most promising candidates, whose DNA methylation level might be increased in the promoter region and reduce the expression of its mRNA or protein in schizophrenic patients. Abdolmaleky *et al.* (5) also observed hypermethylation of the CpG islands, which are located upstream of the positions −139 and −134, in the prefrontal cortices of schizophrenic patients. At positions +131, +227, and +229, no significant difference was observed in the DNA methylation status among schizophrenic, bipolar patients, and control subjects, although a correlation between age and the DNA methylation status was detected in the control subjects (6). No other study has investigated the DNA methylation status of the region, and further investigation might be needed to confirm the results.

Here, we examined the DNA methylation status of the promoter region of *RELN* in the brains of patients with schizophrenia by using the pyrosequencing method (7), a high-throughput and reliable method for quantification of DNA methylation status.

Methods and Materials

Postmortem prefrontal cortices (Brodmann area [BA] 10) of 15 patients with schizophrenia and 15 control subjects, matched for age, gender, postmortem interval, and sample pH, were provided by the Stanley Foundation Brain Collection (The Stanley Medical Research Institute, Bethesda, Maryland) (8). Fourteen patients with schizophrenia and 13 control subjects were examined in the present study, because genomic DNA from the rest of the sample did not show sufficient quality for the *RELN* methylation analysis. Three additional brain samples (BA10), including two control subjects and one with psychotic disorder, were also provided by the Stanley Foundation Brain Collection. These three specimens were divided into gray and white matter portions. Two micrograms of genomic DNA derived from postmortem brains was treated with sodium bisulfite as described previously (9). The DNA loss during the bisulfite modification was quantified by measuring the amount of genomic DNA after the treatment. To validate the assay method, two positive control samples were processed in the same way with the brain samples; one is various proportions (0%, 2.5%, 5%, 7.5%, 10%, 25%, 50%, 75%, and 100%) of mixtures of CpGenome Universal Methylated and Unmethylated DNA (Chemicon, Temecula, California). The other is genomic DNA of a pancreatic cancer cell line, BxPC3 (American Type Culture Collection, Manassas, Virginia), in which the promoter region of *RELN* was observed to be methylated (10).

After bisulfite modification, the strands (designated as the top and bottom strands) are no longer complementary. Therefore, each strand requires a different set of primers: 5'-GGGGTTTTAAGAAGGTGTGGAG-3' and 5'-ACTCCCAAAATTACTTTAAACC-3'Å for the top strand, and 5'-CTAACCTTCCCCTTCAAACAACTA-3' and 5'-GGTTTTTAAAGTTATTTTGGGT-3' for the bottom strand (Figure 1). The polymerase chain reaction (PCR) product of the top strand includes −141 and −136 cytosines,

From the Laboratory for Molecular Dynamics of Mental Disorders (MT, KI, MB, AK, TK), RIKEN Brain Science Institute, Saitama; Department of Neuropsychiatry (MT, TS, NK), Graduate School of Medicine; Health Service Center (TS), University of Tokyo, Tokyo, Japan.

Address reprint requests to Tadafumi Kato, M.D., Ph.D., Director, Aging and Psychiatric Research Group, Head, Laboratory for Molecular Dynamics of Mental Disorders, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama, 351-0198 Japan; E-mail: kato@brain.riken.jp.

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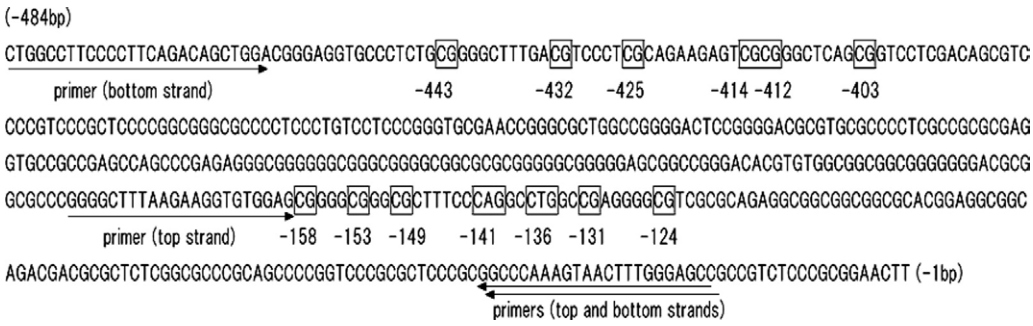

Figure 1. Amplified region of the *RELN* gene. Sequences from −484 to −1, not modified with bisulfite treatment (position number followed as described by Chen *et al.* [11]). Used primers were indicated by underlines. Those for the bottom strand were designed for complementary sequence. The top and bottom strand primers start from different sites, because the specificity of the corresponding sequence of the same region become different between the top and bottom strands after bisulfite modification. Boxed CpG/CpNpG sites were analyzed in the present study. Those located from −443 to −403 were analyzed in the bottom strand; those located from −158 to −124 were in both top and bottom strands. Cytosines −443, −432, and −425 were included in the forward primer used in methylation specific PCR conducted in the previous study (5). Cytosines −141 and −136, which belong to the same CpNpG sites with −139 and −134 cytosines in the bottom strand, respectively, were suggested to be hypermethylated in the brain of schizophrenic patients (2).

which belong to the same CpNpG sites with −139 and −134 cytosines, respectively, in the bottom strand. That of the bottom strand includes −139 and −134 cytosines as well as the upstream CpG region located from −442 to −424, whose corresponding region in the top strand were observed to be hypermethylated (5). The 50-μL PCR mixture contained 2.5 μL of bisulfite-modified DNA, 5 μL 10× PCRx Amplification Buffer (Invitrogen, Carlsbad, California), 3 μL 50 mmol/L magnesium sulfate, 4 μL 10 mmol/L deoxyribonucleoside triphosphate (dNTP), 10 μL 5 mol/L betaine, 2 ng Single-Stranded DNA Binding Protein (Promega, Madison, Wisconsin), 1 μL each of 10 μmol/L primers, and 5 U Platinum *Taq* DNA Polymerase (Invitrogen). The PCR conditions included an initial denaturation step for 3 min at 95°C, 50 cycles of 10 sec at 98°C, 30 sec at 55°C (for the top strand) or 58°C (for the bottom strand), and 30 sec at 72°C.

After purification by using the Wizard PCR Preps DNA Purification System (Promega), the PCR product was processed for pyrosequencing analysis according to the manufacturer's standard protocol (Biotage, Uppsala, Sweden). The sequencing primers, the analyzed sequences, and the order of nucleotide dispensation are shown in Table 1. To confirm the result of pyrosequencing, the PCR product was TA cloned (TOPO TA cloning kit, Invitrogen). Single-bacterial colonies were subjected to sequencing analysis.

Table 1. Oligonucleotides, Reading Sequences, and Dispensation Orders Used in the Present Study

	Sequences
The Top Strand	
Sequencing Primer	5′-GGGGTTTTAAGAAGG-3′
Reading Sequence	5′-TGTGGAGC/TGGGGC/TGGGC/TGTTTTTC/TAGGTC/TTGGTC/TGAGGGGC/TGT-3′
Dispensation Order	CTGTGAGCTAGGCTAGCTCGTCTAGTCTCGTCTGAGGCTCG
The Bottom Strand	
Sequencing Primer	5′-AAAAATACCCTCTA-3′
Reading Sequence	5′- CG/AAAACTTTAACG/ATCCCTCG/ACAAAAAAATCG/ACG/AAACTCAACG/AATCCTCG/AAC-3′
Dispensation Order	TCAGAGCTACGAGTCTCGAGCAATCGATCAGAGCTCACGAGT
Sequencing Primer	5′-AAACTTTAAAAAAATATA-3′
Reading Sequence	5′-AAACG/AAAACG/AAACG/ACTTTCCCAG/AACCTG/AACCG/AAAAAACG/ATC-3′
Dispensation Order	GACAGATCAGATCGAGCTCAGAGCTGATCAGATCGAGT

Results

The DNA loss during the bisulfite modification was 84.5 ± 10.1%, which means the amount of degradation was not significantly larger than that in the previous bisulfite modification study (12). The strong linear correlation was observed between stepwise increments of methylated DNA and the measured methylation by pyrosequencing analysis in all CpG sites (Figure 2). The threshold of detection was > 5%, as observed in the previous pyrosequencing study (13). In a pancreatic cancer cell line (BxPC3) (10), methylated status of the region was observed except for the nonstandard CpNpG sites (Supplement 1).

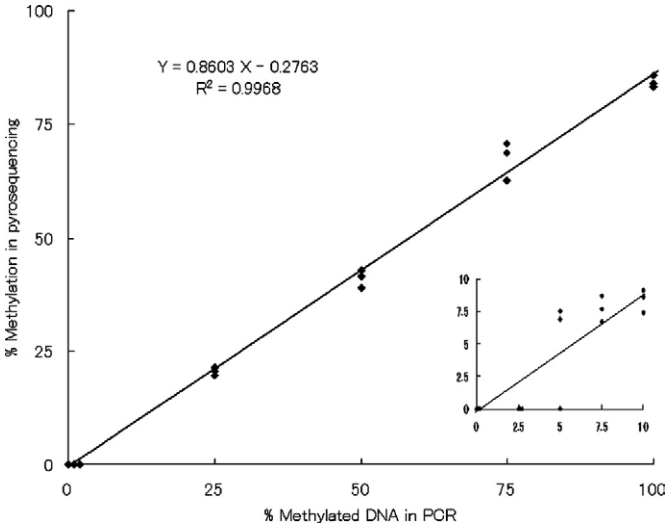

Figure 2. Linear response of the measured methylation of cytosine at position −158 in the top strand. The polymerase chain reaction (PCR)-amplified products generated from stepwise increments of methylated DNA were processed for pyrosequencing analysis. The DNA methylation status > 5% could be detected. Linearity and threshold of detection in other CpG sites was also confirmed ($R^2 > .99$ in the top strand and $.70 < R^2 < .96$ in the corresponding region of the bottom strand; data not shown).