Promoter Variant in the GRK3 Gene Associated with Bipolar Disorder Alters Gene Expression

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Background: We have previously reported a single nucleotide polymorphism (P-5, G-384A) in the proximal promoter of the gene for G protein receptor kinase 3 (GRK3) that was associated with bipolar disorder in two independent samples. In this study, we examined whether the G-384A variant has a functional effect on GRK3 transcription.

Methods: Electrophoretic mobility shift assays were conducted using nuclear extracts from both Hela cells and adult mouse cortex. Transcriptional function was also examined using a dual luciferase reporter system transfected into in vitro human neuroblastoma cells and cultured mouse cortical neurons.

Results: The G-384A variant abolished or reduced the formation of DNA-protein complexes using nuclear extract from both HeLa cells and adult mouse cortical neuron cells. However, gene expression was significantly enhanced by G-384A in both in vitro human neuroblastoma cells and cultured mouse cortical neurons.

Conclusions: These data suggest that the G-384A SNP in the promoter of human GRK3 gene represents an important functional variant. The G-384A variant may alter binding of Sp1/Sp4 transcription factors resulting in an increase in gene transcription and an increase in vulnerability to bipolar disorder.

Key Words: Bipolar disorder, cortical neuron, EMSA, G-protein receptor kinase 3, gene expression, Sp1 family transcription factors, SNP

G-protein-coupled receptors (GPCRs) are the most numerous superfamily of cell surface receptors and play essential roles for a variety of physiologic functions. Most of the GPCRs are expressed in brain, which mediate the signal transduction of various neuromodulators such as dopamine, noradrenaline, serotonin, glutamate, γ-aminobutyric acid, and others (1). Neuropharmacologic studies have demonstrated that administration of agonists and antagonists for GPCRs in brain result in a range of animal behavioral abnormalities that mimic some human psychiatric disorders such as mania and depression (2–4). Signaling of GPCRs is sensitive and dynamic, in part because of the modulation of receptor function by homologous desensitization mediated by a family of G protein receptor kinases (GRKs) (5). The GRKs can discriminate between the inactive and agonist-activated states of the receptors, and phosphorylate the activated receptors for subsequent receptor internalization. The GRKs thus serve as gatekeepers for receptors, modulating their signaling pathways. GRK3, one of the six known GRKs, is abundantly expressed in several brain regions including cortex, hippocampus, and ventral striatum, suggesting an important role for the GRK3 gene in the modulation of neurotransmission signaling in these regions (6,7).

Two independent genomewide linkage analyses, one including 20 extended families from our lab and one including Wave 1 families from the National Institutes of Mental Health Bipolar Consortium, have suggested a susceptibility locus for bipolar disorder located on chromosome 22q12 near the GRK3 gene (8,9) around the microsatellite markers D22S419 and D22S533. These markers are in the immediate vicinity of G-protein receptor kinase 3. A study of two inhibitory electrophysiologic endophenotypes in families with schizophrenia also found genomewide maximum evidence of linkage at a marker (D22S315) in intron 1 of the GRK3 gene (10). After conducting a detailed examination of GRK3 in bipolar subjects in search of potential pathogenic mutations and examining several resulting single nucleotide polymorphisms (SNPs) for association to bipolar disorder, we found that two (P-5 and P-6) of six SNPs identified in the proximal promoter of the GRK3 gene displayed association with the disease in transmission disequilibrium tests in two independent sets of family triads (11). Interestingly, the P-5 variant GaGAGGG, which is −384 bp upstream from the ATG, appears to disrupt the Sp1/Sp4 binding consensus sequence GGGAGGG found in the normal GRK3 allele. For clarity, the variant designated P-5 in our earlier article is referred to as G-384A here. To examine whether the G-384A variant indeed interrupted the binding of Sp1 family transcription factors, we conducted electrophoretic mobility shift assays (EMSA) by using nuclear extracts from both HeLa cells and adult mouse cortex. Dramatic reduction of a binding complex was observed for G-384A variant. The function of the G-384A variant in the promoter of human GRK3 gene was finally analyzed in human neuroblastoma cells and cultured mouse cortical neurons.

Methods and Materials

Electrophoretic Mobility Shift Assays

Two sets of complementary 29mer oligonucleotides, containing either control or G-384A variant, were synthesized. The complementary oligonucleotide pairs were annealed to form the double-stranded DNA and labeled with Digoxigenin by using DIG Gel Shift Kit (DIG Gel Shift Kit, 2nd Generation, cat. No. 3353591; Roche Applied Science, Indianapolis, Indiana); 100 ng of each double-stranded DNA was used for a labeling reaction, and the labeling efficiency was then determined according to the manufacturer's protocol. We used .4 ng of labeled DNA for incubation with the nuclear extract from either HeLa cell (Promega, Madison, Wisconsin) or mouse cortex. The binding reactions were separated by electrophoresis in nondenaturing 5%

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polyacrylamide gel (Criterion Precast Gel, cat. 345-0048; Bio-Rad, Hercules, California). After electrophoresis, the samples were transferred to NYTRAN nylon membranes (Schleicher & Schuell, Dassel, Germany) by contact blotting for at least 3 hours. After ultraviolet cross-linking, the DNA-protein complexes were visualized by chemiluminescent detection. For competition experiments, several transcription factor consensus oligonucleotides (AP1, AP2, CREB, SP1, OCT1) were obtained from Promega (Promega). In addition, double-stranded DNA containing GAGA factor binding site (5′ GAGAGAGAGAGAGAGAGAGAGAG 3′), and poly-(AT) (5′ ATATATATATATATATATATAT 3′) were also synthesized. Rabbit polyclonal antibodies for Sp1 (Upstate, Lake Placid, New York) and Sp4 (Santa Cruz Biotechnology, Santa Cruz, California) were used for supershift in the EMSA.

Nuclear Extract Preparation from Adult Mouse Cortex

The cortex was dissected from adult mouse with mixed S129/Black Swiss genetic background and immediately disrupted in homogenization medium (25 mmol/mL Sucrose, 25 mmol/mL KCl, 5 mmol/mL MgCl2, 20 mmol/mL Tris-Cl, pH 7.5) by using a Dounce Homogenizer (cat # KT885300-0002; VWR, West Chester, Pennsylvania) Pestle A (clearance for disruption of tissue) on ice. After 20 strokes, the homogenized mixture was placed on ice for 1 hour. Pestle B (clearance for the disruption of the cell, with the nuclei left intact) was then used to homogenize the mixture for about 20 strokes to release nuclei. Six volumes of 50% NycoPrep (50% NycoPrep, 25 mmol/mL KCl, 5 mmol/mL $MgCl_2$, 20 mmol/mL Tris-Cl, pH 7.5) were added to four volumes of cell lysis and were well mixed and centrifuged at 13,000 rpm for 2 min to pellet nuclei. One volume of Buffer A (10 mmol/mL 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 10 mmol/mL KCl, 1.5 mmol/mL MgCl2, 5 mmol/mL DTT, .5 mmol/mL NaF, .5 mmol/mL Na3VO4, 1X proteinase inhibitor cocktail (Sigma, St. Louis, Missouri) was used to suspend nuclei pellet, and 3 volumes of Buffer B (20 mmol/mL HEPES, pH 7.9, 420 mmol/mL NaCl, 1.5 mmol/mL MgCl2, .2 mmol/mL ethylenediamine tetraacetate, 5 mmol/mL dithiothreitol, .5 mmol/mL NaF, .5 mmol/mL Na3VO4, 1X proteinase inhibitor cocktail (Sigma) 20% Glycerol) was then added to release nuclear proteins, and the samples were incubated on ice for 30 min. After brief centrifugation, the nuclear extract was used immediately or frozen in liquid nitrogen and stored in −70°C.

Construction of Luciferase Reporter Genes

The promoter of human GRK3 gene is a GC-rich region, which is difficult to amplify. Two sets of primers were instead designed to amplify two overlapping fragments from both control and bipolar disorder patient DNA with G-384A variant, respectively. The two sets of primer pairs are as follows: set 1, forward: 5′ CCCATAACCCCTTGGGTTGTGGA 3′, reverse: 5′ CTGCGGGGAGCGCGCGTCCAACGGTCA 3′; set 2, forward: 5′ GGTCGGGAGGCGCCGGCCCAGCGA 3′, reverse: 5′ GGCGGCGGTTACTCCGGACCTGGAC 3′. The two overlapping fragments were then recombined using polymerase chain reaction (PCR) into full-length human GRK3 promoters. The final putative promoters, the 550 bp DNA fragments from −580 to −30 bp upstream of the start codon of human GRK3 gene were cloned upstream of firefly luciferase reporter gene in pGL3 basic vector (Promega).

Cell Culture and Transfection Experiments

HeLa cells (ATCC, CCL-2) were cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum and ampicilin/streptomycin. The phRL-TK (Promega) expressing *Renilla* luciferase was used as an internal control for normalization of transfection experiments. One or two micrograms of a firefly luciferase DNA reporter construct mixed with .1 μg phRL-TK constructs (ratio 10:1 or 20:1) were used to transfect cells at 70% confluence with Superfect (Invitrogen, Carlsbad, California) according to manufacturer's protocol. Six replicate 35-mm dishes were used for each firefly luciferase construct. The transfected cells were harvested 48 hours after transfection and lysed to measure both *Renilla* and firefly luciferase activities using a Dual Luciferase Kit (Promega).

Human neuroblastoma BE(2)-C cells were cultured in a 1:1 mixture of Eagle's minimal essential medium with nonessential amino acids and Ham's F12 medium containing 10% fetal bovine serum with ampicilin/streptomycin. Two micrograms of a firefly luciferase DNA reporter construct mixed with .2 μg phRL-TK constructs (ratio 10:1) were used to transfect cells at 70% confluence with Superfect (Invitrogen) as described earlier. Two double stranded decoy oligodeoxynucleotides (ODN), designated as G3 and P-5 decoy ODNs, were synthesized with the capping of both 3′ and 5′ ends of the two probes (Figure 1A) by phosphorothioate linkages to study the function of the G-384A variant in BE(2)-C cells. For decoy competition experiments, 2 μg of either G3 or P-5 ODNs were mixed with 2 μg of a firefly luciferase DNA reporter construct for the transfection of each well of 12-well plates. Five replicate wells were used for each treatment, and each experiment was repeated. The transfected cells were harvested 48 hours after transfection and lysed to measure both *Renilla* and firefly luciferase activities using a Dual Luciferase Kit (Promega).

Primary mouse cortical neurons were established form C57 BL/6 mouse embryos at E16 to E18 (Harlan, Indianapolis, Indiana). The entire cerebral cortex were dissected and cut into small pieces in Neurobasal A medium on ice. The tissue was titrated 10 times with a fire polished 9-inch Pasteur pipette and allowed to settle on ice for 1 min. The supernatant were then transferred to a new tube and centrifuged gently at 600–700 rpm for 5 min to pellet the cells. The cells were resuspended in B27/Neurobasal medium (B27/Neurobasal with .5 mmol/mL glutamine, no glutamate, 5 ng/mL FGF2) and counted. About 20 million cells were seeded in each well (12-well plates) coated with poly-D-lysine. The half of culture medium was changed every 3 days. The transfection experiments were performed at the sixth day of cortical neuron culture. Three micrograms of each firefly reporter construct per well, mixed with 1 μg of phRL-TK construct, was transfected using the NeuroPorter transfection system (Sigma) for the transfection of the primary cultured cortical neurons in each well. For each firefly DNA construct, five replicate wells were used for transfection. After overnight incubation, half of the medium was replaced. The cells were harvested 48 hours later for measurement of dual luciferase activities, and the firefly luciferase activity was normalized with the activity of *Renilla* luciferase. The Student's *t* test was used for statistical analysis.

Results

Attenuated Formation of DNA-Protein Complex by G-384A Variant

To examine the potential function of the G-384A variant in the promoter of human GRK3 gene, we conducted EMSA to investigate whether the G-384A variant altered the binding of any transcription factors. Two double-stranded DNA probes, differ-