Quantitation of GAD67 Gene Expression in Prefrontal Cortex of Schizophrenia Patients Using the iCycler iQ™ Detection System and Molecular Beacons

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Introduction

Schizophrenia is a severe mental illness that afflicts about 1% of the population (Strange 1992). Patients experience a variety of symptoms that have been divided into subgroups of positive symptoms (e.g., thought disorder, abnormal beliefs and experiences) and negative symptoms (e.g., deficiency of speech, loss of emotional response, reduced motor function). The etiology and pathophysiology of schizophrenia have been the focus of intensive research for decades. Convergent lines of evidence indicate that dysfunction of a specific area of the cerebral cortex — the dorsolateral prefrontal cortex — represents one of the central features of the pathophysiology of schizophrenia (Bunney and Bunney 2000). A synthesis of results from in vivo imaging and postmortem studies (Lewis et al. 1999) also suggests that prefrontal cortex dysfunction may be related to abnormalities in the connectivity between neurons in this region that are likely to involve GABAergic interneurons (neurons that synthesize and release the major inhibitory neurotransmitter in the brain, γ-aminobutyric acid or GABA). The synthesis of GABA is catalyzed by the enzyme glutamic acid decarboxylase (GAD). Molecular cloning studies have shown that in the adult brain, GAD exists in 2 isoforms, called GAD65 and GAD67, which are the products of 2 independently regulated genes. GAD65 and GAD67 are coexpressed in GABAergic neurons. Gene lesioning studies in mice (Condie et al. 1997, Kash et al. 1997) suggest that GAD67 is the major isoform and is responsible for the maintenance of basal GABA levels in cells, whereas GAD65 can be rapidly activated in times of high GABA demand.

The goal of our study was to compare GAD67 gene expression in dorsolateral prefrontal cortex of postmortem specimens from chronically ill elderly schizophrenia patients to normal controls matched for age and postmortem interval (PMI). Real-time PCR* was applied to quantitate the expression of the gene. Real-time PCR necessitates the use of fluorogenic probes. Reactions are quantitated by the point in time during cycling when amplification of PCR product is first detected (the threshold cycle, or C_T) rather than by the amount of PCR product accumulated after a fixed number of cycles. The early cycles of PCR are characterized by an exponential increase in target amplification. As reaction components become limiting, the rate of target amplification decreases until a plateau is reached and there is little or no net increase in the PCR product. The iCycler iQ detection system allows for product formation to be visualized by monitoring fluorescent reporter molecules in the PCR. In this way the exponential phase of the amplification can be determined. The point at which fluorescence significantly increases above background is described as the threshold cycle (C_T). At the C_T, the PCR amplification is still in the exponential phase and none of the reaction components are limiting. C_T values are very reproducible for reactions with the same starting copy number. This leads to greatly improved precision in the quantitation of DNA.

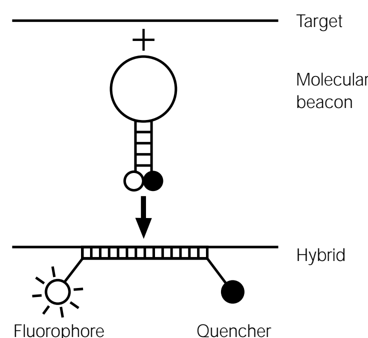

Fig. 1. Operation of molecular beacons.

Molecular beacons were used as fluorogenic probes in our real-time PCR experiments. Molecular beacons (Marras et al. 1999, Tyagi and Kramer 1996) are hairpin-shaped molecules with a terminally quenched fluorophore whose fluorescence is restored when they bind to a target nucleic acid (Figure 1). They are designed in such a way that the loop portion is a probe sequence complementary to a target DNA molecule. The stem is formed by the annealing of complementary arm sequences on the ends of the probe molecule. A fluorescent moiety is attached to the end of one arm and a quenching moiety is attached to the end of the other arm. The stem-loop structure keeps these 2 moieties in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by energy transfer.

At the annealing temperature of the probe, it forms a hybrid with the target molecule that is longer and more stable than the stem, causing the fluorophore and the quencher to move away from each other and leading to increased fluorescence. This can be measured by the iCycler iQ detection system. Molecular beacons that do not bind to the target return to the stem-loop structure and are dark. Detailed directions on molecular beacon synthesis and design are available at http://www.molecular-beacons.org.

Table 1. Postmortem characteristics of schizophrenic patients and controls.

Group	Male:Female	Age (yr)	PMI (hr)	Tissue pH
Schizophrenic				
Overall group	17:9	72.3 ± 2.4	14.6 ± 1.9	6.33 ± 0.06
Matched group				
For PMI	8:3	72.4 ± 3.1	10.9 ± 2.6	6.28 ± 0.08
For age	4:6	81.1 ± 3.1	14.2 ± 2.8	6.36 ± 0.10
Control				
Overall group	5:8	82.8 ± 2.9	8.0 ± 1.6	6.29 ± 0.09
Matched group				
For PMI	3:7	82.7 ± 3.5	11.5 ± 1.9	6.34 ± 0.07
For age	2:8	81.5 ± 3.7	8.0 ± 1.9	6.23 ± 0.11

Values represent means ± SEM.

Methods

Human Postmortem Tissue

Frozen postmortem brain samples of subjects diagnosed antemortem with schizophrenia (n = 26) by DSM-IV criteria, and of normal elderly controls (n = 13), were obtained from the Mount Sinai/Bronx Veterans Administration Medical Center Department of Psychiatry Brain Bank. The mean age, PMI, tissue pH, and sex distribution of the cohorts used in this study are shown in Table 1. Gray matter from the frozen dorsolateral prefrontal cortex, Brodmann area 46 (Rajkowska and Goldman-Rakic 1995), was dissected from coronal sections of frozen (-80°C) brain. The dissected tissues were pulverized at -190°C into a fine powder and aliquotted into individual micro test tubes and stored at -80°C until use.

RNA Isolation

Total RNA was isolated from 50 mg of tissue by the guanidinium isothiocyanate method, using a ToTALLY RNA kit (Ambion) according to the manufacturer's protocol. To remove genomic DNA contamination, isolated RNA samples were then treated with 40 U DNase I (Ambion) for 1 hr at 37°C in a 200 µl reaction mixture containing 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM Tris-HCl (pH 7.5), and 120 U of RNaseOUT enzyme (Invitrogen). RNA samples were then extracted with phenol/chloroform/isoamyl alcohol, precipitated with 100% ethanol, and washed twice with cold 70% ethanol. Total RNA concentration was determined by absorbance at 260 nm. Yield of total RNA ranged from 15 to 30 µg per 50 mg of brain tissue. The A_{260}/A_{280} ratios of the samples were >2.1. The yield and quality of total RNA was also analyzed by agarose gel electrophoresis.

Reverse Transcription (RT) Reaction

Total RNA (~2 µg) was used in a 20 µl RT reaction to synthesize cDNA using a ThermoScript RT-PCR system kit (LTI-Invitrogen) and random hexamers as primers. RT reactions were performed for 1 hr at 52°C, followed by RNase H treatment to remove RNA template, as described in the commercial protocol.

Primer and Molecular Beacon Design

The PCR primers and molecular beacons were synthesized commercially (IDT Corporation, Coralville, IA). PCR primers were designed using Vector NTI software (InforMax, North Bethesda, MD). The melting temperatures (T_m) of the primer and probe sequences were determined by Oligo Analyzer 2.5 software (IDT Corporation). Because the sensitivity and reliability of real-time PCR quantitation are dependent on the specificity of PCR, several different primer pairs were initially designed for GAD67 mRNA detection and tested for specificity. PCR was performed with each of these primer pairs using cDNA derived from a pooled sample of human cortical specimens from 10 randomly selected cases (see below for cycling conditions). The PCR products were analyzed on ethidium bromide-stained agarose gels. The primer pair that generated the highest yield of specific product without any nonspecific bands was chosen for the real-time PCR experiments. A similar approach was undertaken to select primers for β-actin detection.

The molecular beacons were designed according to the guidelines available at http://www.molecular-beacons.org. They consisted of probe sequences 24–25 nucleotides long, 6-nucleotide arm sequences, the fluorophore FAM covalently linked to the 5' end, and the quencher DABCYL linked to the 3' end. The melting temperatures of the hairpin stems and probe sequences were 63–66°C. To estimate the stability of the molecular beacon's hairpin stem structure, the DNA folding program m-fold (available at http://www.bioinfo.math.rpi.edu/~mfold/dna/form1.cgi) was utilized. Selected primer and probe sequences for this study are shown in Table 2.

Real-Time PCR

Real-time PCR analysis was performed using the iCycler iQ detection system. The conditions of the reaction were carefully optimized by varying molecular beacon (50–250 nM), primer (200–1,000 nM), and Mg^{2+} (3–5 mM) concentrations and the annealing temperature (55–60°C). The optimal 25 µl PCR contained 5 µl of the relevant cDNA (diluted 25-fold with water), 200 nM of molecular beacon for GAD67 or 100 nM for β-actin, 500 nM of forward and reverse primers, 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), 250 µM of each dNTP, 4 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl (pH 8.3). The thermal cycling program consisted of 10 min at 95°C to activate the polymerase, followed by 10 cycles of 15 sec at 95°C, 30 sec at 68–59°C (touchdown PCR, annealing temperature was decreased 1°C after each cycle), and 30 sec at 72°C. This touchdown step was followed by 35 cycles of 15 sec at 95°C, 30 sec at 58°C, and 30 sec at 72°C. Fluorescence was monitored during the 58°C annealing steps.

Table 2. Primers and molecular beacons used in real-time RT-PCR to assess the expression of GAD67 and the endogenous reference β-actin in postmortem brain specimens from human subjects.

Gene	Primer and Probe Sequences	Gene Accession Number	PCR Product Size
GAD67	Forward primer: 5'–CGAGGACTCTGGACAGTAGAGG– 3' Reverse primer: 5'–GATCTTGAGCCCCAGTTTTCTG–3' Molecular beacon: FAM-5'–CGGTGCGGACCCCAATACCACTAACCTGCGGCACCG–3'-DABCYL	NM_000817	182 bp
β-Actin	Forward primer: 5'–TCACCCACACTGTGCCCATCTACGA–3' Reverse primer: 5'–CAGCGGAACCGCTCATTGCCAATGG–3' Molecular beacon: FAM-5'–CCGGTCAGCCGTGGCCATCTCTTGCTCGAAGGACCGG–3'-DABCYL	NM_001101	295 bp

Assessment of DNA Contamination in RNA Samples

In order to measure the level of contaminating chromosomal DNA, all RNA samples not treated with reverse transcriptase and their respective cDNA samples were subjected to PCR using GAD67 primers. The products of the PCR reactions were analyzed on ethidium bromide-stained agarose gels. In contrast to their respective cDNA templates, no PCR products could be detected with gel electrophoresis when RNA samples were used. Random RNA samples (n = 10) were also subjected to real-time PCR using β-actin primers and a molecular beacon. The RNA samples showed at least 3 orders of magnitude lower amounts of the initial template molecules than their respective cDNAs (the difference in C_T between the RNA and cDNA in each sample was at least 10 cycles), demonstrating negligible amounts of genomic DNA contamination (not shown).

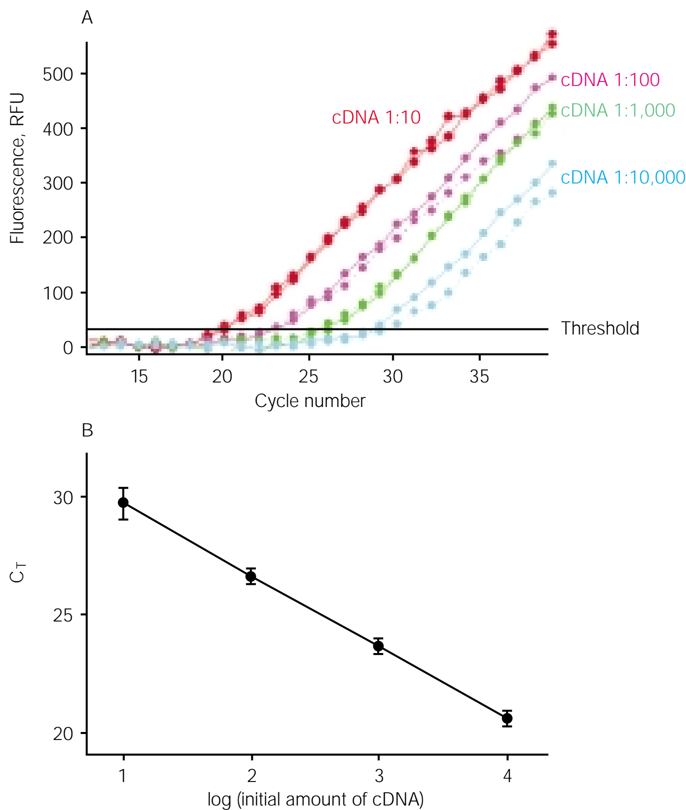

Fig. 2. Dynamic range of GAD67 mRNA quantitation assay. A, amplification of GAD67 mRNA in 10-fold dilutions of the pooled cDNA; B, C_T of GAD67 mRNA amplification plotted against the log of the relative initial amount of the pooled cDNA. The slope was -3.03, and r^2 = 0.999.

Dynamic Range of Real-Time PCR

To determine sensitivity of the assays, the amplification of each mRNA in serial dilutions of cDNA derived from a pooled sample (see above) was measured (Figure 2A). Figure 2B shows C_T values of these amplifications plotted against the logarithm of the relative initial amount of the cDNA. Theoretically, there should be an inverse linear relationship between the C_T and the logarithm of the number of target molecules that were present initially; the threshold cycles of each replicate group should be separated by exactly 3.32 cycles (antilog$_2$ 10). In the experimental data shown in Figure 2, a linear relationship between C_T and the initial amount of GAD67 mRNA was demonstrated for 3 orders of magnitude. The mean separation between the groups (the slope of the curve) was -3.03, which is very close to the theoretical value. These data demonstrate the broad dynamic range of GAD67 mRNA quantitation. Similar results were obtained for the β-actin mRNA amplification assay.

Quantitation of GAD67 Gene Expression

The PCRs were quantitated by selecting the amplification cycle where the PCR product of interest was first detected (C_T). To account for different degrees of RNA degradation and other technical artifacts, the relative quantitation of GAD67 mRNA expression was performed as described in User Manual #2 (comparative C_T method in separate tubes; Applied Biosystems). The expression level of GAD67 was normalized to the expression level of the endogenous reference (β-actin) in each sample. This relative value was further normalized to the relative expression of GAD67 in the pooled sample. Pooled cDNAs were run in every plate simultaneously with experimental samples. To avoid competition, GAD67 and β-actin mRNAs were amplified in 2 separate PCR reactions. All samples were run in quadruplicate. A representative example of the real-time RT-PCR quantitation of GAD67 and β-actin mRNAs expression in one individual case is shown in Figure 3. The mean threshold cycles for GAD67 and β-actin were 21.9 and 15.8 with standard deviations of 0.12 and 0.22, respectively.

Statistical Tests of Significance

Analysis of variance (ANOVA) followed by Newman-Keuls tests and Student's t tests were used to analyze the results of these studies. Statistical analyses were performed using Statistica for Windows (release 5.5, Statsoft Inc., Tulsa, OK) or SPSS for Windows (version 10, SPSS Inc., Chicago, IL).

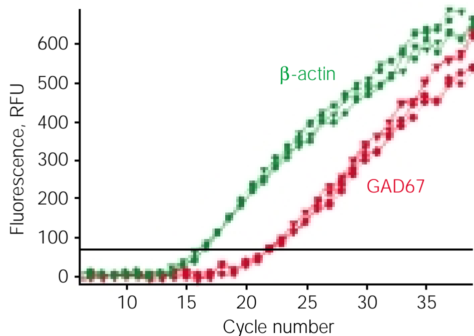

Fig. 3. Representative example of GAD67 and β-actin mRNAs quantitation in one individual case. The samples were run in quadruplicate. The mean threshold cycles for GAD67 and β-actin were 21.9 and 15.8 with standard deviations of 0.12 and 0.22, respectively.

Results and Discussion

The relative abundance of GAD67 in the dorsolateral prefrontal cortex is shown in Figure 4. Analysis revealed a significant increase in GAD67 expression in the schizophrenic cohort ($F(2,35) = 4.12$, $p = 0.02$; Newman-Keuls test, schizophrenia vs. normal-elderly, $p = 0.023$).

The schizophrenic cohort was significantly younger than the normal-elderly group (Table 1). To determine whether age at death affected the differences between groups, the schizophrenic and normal-elderly groups were subgrouped into 2 groups of 10 subjects each that were matched for age to within 1 year of each other. Differences in the expression of GAD67 were reassessed using t tests. The same significant ($p < 0.02$) group difference found when analyzing the entire cohort was observed when comparing the groups of schizophrenics and normal-elderly subjects that were matched for age (Figure 4). Similar results were obtained when 2 subgroups of 10 schizophrenics and 10 control subjects that were closely matched for PMI were compared (Figure 4).

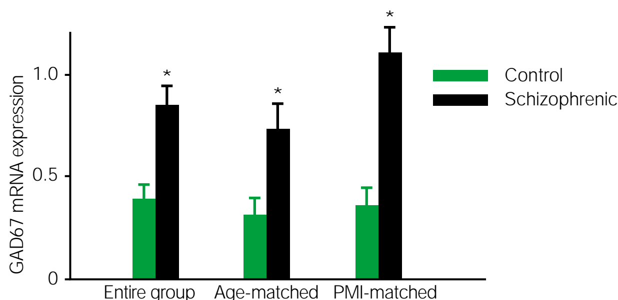

Fig. 4. Relative gene expression of GAD67 in the dorsolateral prefrontal cortex of normal control subjects and patients with schizophrenia. Asterisks indicate significant differences ($p < 0.015$) from the control group (Newman-Keuls tests for the entire group and Student's t tests for age-matched and PMI-matched groups).

All schizophrenics in this study had been exposed to neuroleptic drugs for decades. The history of neuroleptic exposure for each subject was assessed in detail by examination of his or her medical charts. Of the 26 schizophrenics, 13 had been exposed to neuroleptics within 1 week of death, while neuroleptic medications had been discontinued for the remaining 13 subjects from 1 week prior to death to as long as 124 weeks prior to death. To assess the possible influence of acute neuroleptic exposure on GAD67 gene expression, the schizophrenic group was subdivided into those who had been exposed to neuroleptics within 6 weeks of death ($n = 16$) vs. those who had been neuroleptic free for more than 6 weeks ($n = 9$). Comparison of GAD67 gene expression in the dorsolateral prefrontal cortex of these 2 groups of schizophrenics did not reveal any significant differences between the 2 groups ($p > 0.4$, data not shown). Matching of subjects for age and PMI and the examination of possible neuroleptic influences suggest that the detected abnormality was independent of obvious artifacts.

These results indicate that mRNA regulation of GAD67 is significantly changed in the dorsolateral prefrontal cortex of elderly chronically ill schizophrenics.

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