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A MicroRNA Gene is Hosted in an Intron of a Schizophrenia-susceptibility Gene

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Abstract

Schizophrenia (SZ) is a neuropsychiatric disorder that affects about 1% of the adult population. Numerous genes have been implicated in SZ susceptibility. MicroRNAs (miRNA) are small RNA molecules that regulate the translation of mRNAs via interactions with their 3' untranslated regions. Identification of known miRNA targets on all human genes indicated that miRNA-346 targets SZ susceptibility genes listed in the SchizophreniaGene database, twice as frequently than expected relative to other genes in the genome. The gene encoding this miRNA, *miR-346*, is located in intron 2 of the *glutamate receptor ionotropic delta 1 (GRID1)* gene, which has been previously implicated in SZ susceptibility. We used quantitative real-time PCR to determine the expression levels of *miR-346* and *GRID1* using brain RNA samples from the Stanley Array Collection, Stanley Medical Research Institute. Expression of both *miR-346* and *GRID1* is lower in SZ patients than that in normal controls ($P=0.017$ and 0.086 , respectively). However, the expression of *miR-346* and *GRID1* is less correlated in SZ patients than in bipolar patients or in normal controls. This study implicates the importance of a miRNA in SZ.

Keywords

miR-346; GRID1; Schizophrenia; Bipolar

Introduction

Schizophrenia (SZ) is a severe neuropsychiatric illness affecting an estimated 1% of the adult population. SZ is a “complex genetic disease” whose etiology involves multiple genetic and environmental components (Kirov et al., 2005; Owen et al., 2005). The genetic contribution is significant, as SZ shows heritability of approximately 80% and a reduction of risk by about 50% for each degree of relationship. Large-scale whole-genome scans for SZ susceptibility genes have met with a measure of success, and a variety of replication and follow up studies support the assumption that particular loci are important in certain populations (O'Donovan et al., 2008; Stefansson et al., 2008). However, only a few of the genes that are dysregulated in SZ correspond with susceptibility loci. In some cases, there are compelling biological rationales for the involvement of particular proteins in the pathology of SZ, but there is little or no evidence supporting the hypothesis that coding variation in the corresponding genes is important.

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MicroRNAs (miRNA) are endogenous RNAs of about 22 nucleotides in length that play important regulatory roles by targeting mRNAs for degradation or translational repression. These RNA molecules are transcribed in the nucleus as larger primary transcripts (pri-miRNA). Pri-miRNAs are first cropped to release ~65 nt of hairpin-shaped precursor (pre-miRNAs) by an enzyme complex that is composed of Drosha and its cofactor, DiGeorge syndrome critical region gene 8 (DGCR8). Drosha generally needs the stem regions of the pre-miRNA and its flanking segments (~40bp) for efficient processing (Han et al., 2006). Following this initial processing, the resulting pre-miRNAs are exported by the nuclear transport factor, exportin-5 (Exp5). In the cytoplasm, Dicer, a cytoplasmic RNase III-like protein, dices the transported pre-miRNAs to generate 22 nt miRNA duplexes. One strand of the Dicer product remains as a mature miRNA and is then assembled into the effector complex called miRNP or miRNA-induced silencing complex (miRISC). The final products (mature miRNAs and RISC) bind to specific target mRNAs through partially complementary sequences that are predominantly located in the 3'-UTR. The bound mRNAs either remain untranslated or are degraded by the RNA-induced silencing complex (RISC) [depending on their degree of complementarity], resulting in lower level of protein products [reviewed in (Bartel, 2004)].

Over 500 human miRNA genes have been identified, yet the physiological function of a limited number of them has been experimentally determined. It is likely that some of these miRNAs may play pivotal roles in the etiology of SZ (Perkins et al., 2007). In this study, we report that one miRNA that preferentially targets genes which might be involved in SZ, is down-regulated in SZ, and is located in an intronic region of a known SZ-susceptibility gene.

Methods

RNA samples and genomic DNAs of SZ patients were obtained from the Brain Array Collection of the Stanley Medical Research Institute (Chevy Chase, MD), which includes samples extracted from Brodmann's area 46 (dorsolateral prefrontal cortex) of 35 SZ patients, 32 bipolar patients, and 34 unaffected controls (we excluded the only African American sample). Real-time PCR of miRNA and mRNA analyses were performed with Taqman® gene expression assays (Applied Biosystems Inc., Foster City, CA). DNA sequencing was performed by Polymorphic DNA Technologies (Alameda, CA). Our analyses suggested that the miR-346 expression level has no correlation with the time intervals from death to refrigeration, sample extraction, refrigeration time span, or medication dosage in all samples (data not shown).

Results and Discussion

miR-346 is predicted to target genes listed in the SchizophreniaGene database with high frequency

We performed a comprehensive analysis of all known human miRNAs by comparing their frequencies of targeting SZ-associated genes to the targeting frequencies for all known human genes. For this, we downloaded all miRNA data from the miRBase 9.2 and analyzed the predicted targets of all human transcripts and those of the SZ-susceptibility genes listed in the SchizophreniaGene database (SZGene, [<http://www.schizophreniaforum.org/res/sczgene/default.asp>]) by miRNAs. Though the predicted miRNA:target interaction in miRBase is experimentally untested and there is as yet no methodology to systematically assess the accuracy of the computational output of miRBase or similar computationally-based predictions, it does allow an objective comparison of targeting frequency for all reported human transcripts and that for genes in the SZgene database. The genes from SZGene include several categories of genes. Some are now widely accepted as playing a role in SZ susceptibility; some have limited genetic evidence in support of their

role; and others have good biological rationale but minimal or no genetic evidence for a role in susceptibility.

Next, we identified the miRNAs predicted to target putative SZ-genes with the highest frequencies. The ten miRNAs with the highest predicted targeting frequencies are listed in Table 1. Most of these miRNAs target the 3'-UTRs of SZ genes with frequencies similar to those for expected based on a random cross section of human genes. In fact, eight out of ten of these miRNAs (miR-181a, -769, -768, -299, -30a, -515, -369, and 199a) are also among those that most frequently target all human transcripts. However, two of the frequently targeting miRNAs, miR-566 and miR-346 target genes from SZgene more frequently than expected.

We analyzed the SZ genes targeted by miR-346 according to the prediction in miRBase. The genetic association studies on these genes and odds ratios if available are provided in Supplemental Table S1. With the exception of *CSF2RA* all the genes predicted to be targeted by miR-346 do not have undisputed positive results from genetic association studies, though all of them have certain biological rationale for roles in brain function and pathology. We also performed a search to determine whether these genes are dysregulated at the mRNA level using brain RNA samples of SZ patients and found mRNA levels of these genes are not significantly dysregulated in SZ patients in four extensive microarray studies (Glatt et al., 2005; Hakak et al., 2001; Mirnics et al., 2000; Perkins et al., 2007). However, this result is by far the most common findings even for genes with well-documented roles in SZ (e.g. neuregulin). Of course, lack of an effect at the RNA level does not mean levels of protein products for these genes are not altered in SZ, as most miRNAs down-regulate target expression through translational repression rather than mRNA turnover (Baek et al., 2008; Mourelatos, 2008).

Interestingly, the *mir-346* gene is a unique miRNA in that it is located in the intron of a gene previously associated with SZ. The gene lies in intron 2 of the *GRID1* gene, which has been proposed to be important in SZ susceptibility (Fallin et al., 2005; Guo et al., 2007). In contrast, *mir-566* is located in the antisense region of the *SEMA3F* gene, which has not been reported to be related to SZ or any other mental disorder. We did not analyze the expression of miR-566 in this study. Given that about 50% of miRNA genes are located in the intron regions of protein-coding genes (Saini et al., 2007), we analyzed whether any other miRNA is in an intron of a gene from the SZgene database. Of the all miRNA genes evaluated, no other miRNA than miR-346 is located in an intronic region of a SZgene. Six SNPs in the host *GRID1* gene were found to be significantly associated with SZ in Ashkenazi Jewish (Fallin et al., 2005) and Northern-Han Chinese patients (Guo et al., 2007). Notably, all these SNPs in the *GRID1* gene showing highly suggestive association with SZ are in the noncoding regions of the *GRID1-miR346* transcript, which is over 700kb.

***miR-346* and *GRID1* are down-regulated in brains of Caucasian patients with SZ and bipolar disorder**

We wanted to determine whether miR-346 levels are altered in SZ. To test this, we evaluated *miR-346* and *GRID1* expression levels in brain RNA samples from the Stanley Medical Research Institute by real-time PCR (Fig. 1). The RNA collection also includes samples from bipolar disorder patients, which can be used as a control. The mean miR-346 expression levels were significantly lower in SZ patients compared to controls (57% vs. control; $P=0.017$). There was a similar, though non-significant trend for bipolar patients (68% vs. control; $P=0.086$). This result indicates that lower mean miR-346 expression may relate to altered regulation of important mRNA targets in SZ and bipolar disorder.

To test if the lower levels of this RNA can be entirely accounted for by changes in the level of expression for the *GRID1* gene, we performed real-time RT-PCR to quantitatively assess the *GRID1* RNA levels. Our results revealed that the mean *GRID1* expression level in SZ patients

is somewhat lower than in controls (54%; $P=0.086$), and is significantly lower in bipolar patients (48%; $P=0.061$) (Fig. 1). This result indicates that lower mean *GRID1* expression is associated with SZ and bipolar disorder and that the whole transcriptional unit of *GRID1-miR-346* is down-regulated in brains of patients compared to normal controls. It further implies that *miR-346* (in addition to *GRID1*) is important to SZ etiology supported by both genetic association studies by other groups (Fallin et al., 2005; Guo et al., 2007) and expression analysis in this report.

We examined the correlation between *miR-346* and *GRID1* RNA levels in individuals. Interestingly *miR-346* showed stronger correlation with *GRID1* RNA in normal controls ($r^2=0.53$) and bipolar patients ($r^2=0.47$) than in SZ patients ($r^2=0.17$) (Fig. 2). Since the two RNAs (*miR-346* and *GRID1* mRNA) presumably are derived from the same primary transcript, the most likely explanation for the difference of correlation is altered post-transcriptional processing or decreased stability of the *GRID1-miR-346* transcript in SZ.

We hypothesized that genetic variation in and around the genomic sequence encoding *miR-346* might account for the putative posttranscriptional differences. To test this, we sequenced a 200 bp region centered on the *miR-346* sequence for all of the genomic DNA samples from Stanley Array Selection as Droscha processing pri-miRNA is reported to be dependent on ~150bp *miRNA* flanking region (Han et al., 2006). We observed no novel genetic mutations in these samples. Though the sample size was small, there were no frequency differences observed for (SZ or bipolar disorder) cases and controls for the two known SNPs in the region (rs2607863 and rs10887569), which are located 87bp and 23bp, respectively, away from the mature *miR-346* sequence. Furthermore, we did not observe any correlation between expression levels of *miR-346* and *GRID1* and the genotype of these two SNPs. As we did not observe measurable differences in the sequence immediately flanking the *miR-346* gene, trans-acting elements, such as the posttranscriptional *miRNA* processing machinery, may be involved. However, we cannot rule out the possibility that flanking sequence in the primary transcript lying farther away from the *miR-346* sequences might account for altered processing or stability of one or both RNAs. Such a scenario is currently under investigation.

In summary, *miR-346*, a *miRNA* located in an intron of *GRID1* that has been associated with SZ (Fallin et al., 2005; Guo et al., 2007), is predicted to target genes listed in SZgene with high frequency and is down-regulated in dorsolateral prefrontal cortex in SZ patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

- Baek D, Villen J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. *Nature* 2008;455:64–71. [PubMed: 18668037]
- Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281–297. [PubMed: 14744438]
- Fallin MD, Lasseter VK, Avramopoulos D, Nicodemus KK, Wolyniec PS, McGrath JA, et al. Bipolar I disorder and schizophrenia: a 440-single-nucleotide polymorphism screen of 64 candidate genes among Ashkenazi Jewish case-parent trios. *Am J Hum Genet* 2005;77:918–936. [PubMed: 16380905]
- Glatt SJ, Everall IP, Kremen WS, Corbeil J, Sasik R, Khanlou N, et al. Comparative gene expression analysis of blood and brain provides concurrent validation of *SELENBP1* up-regulation in schizophrenia. *Proc Natl Acad Sci USA* 2005;102:15533–15538. [PubMed: 16223876]
- Guo SZ, Huang K, Shi YY, Tang W, Zhou J, Feng GY, et al. A Case-control association study between the *GRID1* gene and schizophrenia in the Chinese Northern Han population. *Schizophr Res* 2007;93:385–390. [PubMed: 17490860]

- Hakak Y, Walker JR, Li C, Wong WH, Davis KL, Buxbaum JD, et al. Genome-wide expression analysis reveals dysregulation of myelination-related genes in chronic schizophrenia. *Proc Natl Acad Sci USA* 2001;98:4746–4751. [PubMed: 11296301]
- Han J, Lee Y, Yeom KH, Nam JW, Heo I, Rhee JK, et al. Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. *Cell* 2006;125:887–901. [PubMed: 16751099]
- Kirov G, O'Donovan MC, Owen MJ. Finding schizophrenia genes. *J Clin Invest* 2005;115:1440–1448. [PubMed: 15931379]
- Mirnics K, Middleton FA, Marquez A, Lewis DA, Levitt P. Molecular Characterization of Schizophrenia Viewed by Microarray Analysis of Gene Expression in Prefrontal Cortex. *Neuron* 2000;28:53–67. [PubMed: 11086983]
- Mourelatos Z. Small RNAs: The seeds of silence. *Nature* 2008;455:44–45. [PubMed: 18769430]
- O'Donovan MC, Craddock N, Norton N, Williams H, Peirce T, Moskvina V, et al. Identification of loci associated with schizophrenia by genome-wide association and follow-up. *Nat Genet* 2008;40:1083–1085.
- Owen MJ, Craddock N, O'Donovan MC. Schizophrenia: genes at last? *Trends Genet* 2005;21:518–525. [PubMed: 16009449]
- Perkins D, Jeffries C, Jarskog LF, Thomson JM, Woods K, Newman M, et al. microRNA expression in the prefrontal cortex of individuals with schizophrenia and schizoaffective disorder. *Genome Biology* 2007;8:R27. [PubMed: 17326821]
- Saini HK, Griffiths-Jones S, Enright AJ. Genomic analysis of human microRNA transcripts. *Proc Natl Acad Sci USA* 2007;104:17719–17724. [PubMed: 17965236]
- Stefansson H, Rujescu D, Cichon S, Pietilainen OP, Ingason A, Steinberg S, et al. Large recurrent microdeletions associated with schizophrenia. *Nature* 2008;455:232–236. [PubMed: 18668039]

Figure 1. miR-346 and *GRID1* expression levels in SZ, bipolar patients (BP) and normal unaffected controls (NC)

The expression levels of both miR-346 and *GRID1* are determined as the relative quantity ($2^{\Delta CT}$) using U6 snRNA as a reference. Whisker-box plot with the boxes indicate 25th and 75th percentile; thin lines in the boxes indicate 50th percentile and thick lines denote the mean values; whisker caps indicate 5th and 95th percentile; filled circles indicate outliers. A non-parametric student's *t* test was performed to calculate the P values.

Figure 2. Correlation of relative *GRID1* and miR-346 expression levels in SZ, BP, and NC samples
The relative quantity (RQ) of *GRID1* and miR-346 were determined by real-time PCR.

Table 1

Frequency of miRNA targeting 3'-UTRs of human transcripts

miRNA	Number of hits in 3'-UTRs of all transcripts [predicted] ^a	Number of hits in 3'-UTRs for SZ gene transcripts Observed [Expected if random] ^b
hsa-miR-566	1406	162 [94]
hsa-miR-181a	2682	159 [179]
hsa-miR-769	2661	131 [178]
hsa-miR-768	2524	129 [169]
hsa-miR-299	2450	127 [164]
hsa-miR-30a	3016	121 [201]
hsa-miR-515	2457	121 [164]
hsa-miR-346	903	121 [60]
hsa-miR-369	2455	119 [164]
hsa-miR-199a	2428	117 [162]

^aNumber of 3'-UTRs targeted based on a total of 37,431 distinct human transcripts representing 21,298 genes in the ensemble.org human genome database based on NCBI Assembly version 36.0 (www.ensembl.org) queried by miRBase Release 9.0 (<http://microrna.sanger.ac.uk>). We count the number of transcripts instead that of genes, as typically there are multiple transcripts per gene. If a 3'-UTR of a transcript is hit by a miRNA more than once, the program counts it multiple times. "Predicted" is the number of hits by all miRNAs as predicted in miRBase.

^bFor SZ genes we evaluated 2,501 transcripts from 455 genes listed in the SchizophreniaGene as of June, 2007 (www.szgene.org). These genes are candidate SZ susceptibility genes tested by others regardless whether there is a positive result from genetic association studies. "Expected if random" is the expected hits by a specific miRNA should all transcripts be randomly targeted by a miRNA. For instance, there are 903 hits on 37,431 transcripts in miRBase by miR-346 in miRBase. The "expected if random" hits for 2,501 SZ gene transcripts by miR-346 would be $2,501 \times (903/37,431) = 60$. "Observed" is the number of hits by the miRNA actually found, i.e. "predicted" in miRBase for 2,501 SZgene transcripts.