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MicroRNA-382 expression is elevated in the olfactory neuroepithelium of schizophrenia patients

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Abstract

Schizophrenia is a common neuropsychiatric disorder that has a strong genetic component. MicroRNAs (miRNAs) have been implicated in neurodevelopmental and psychiatric disorders including schizophrenia, as indicated by their dysregulation in post-mortem brain tissues and in peripheral blood of schizophrenia patients. The Olfactory Epithelium (OE) is one of the few accessible neural tissues that contain neurons and their stem cells. Previous studies showed that OE-derived tissues and cells can be safely and easily collected from live human subjects and may provide a “window” into neuronal processes involved in disorders such as schizophrenia, while avoiding the limitations of using postmortem brain samples or non-neuronal tissues. In this study, we found that the brain-enriched miR-382 (miR-382-5p) expression was elevated in *in vitro* cultured olfactory cells, in a cohort of seven schizophrenia patients compared to seven non-schizophrenic controls. MiR-382 elevation was further confirmed in laser-capture microdissected OE neuronal tissue (LCM-OE), enriched for mature olfactory neurons, in a cohort of 18 schizophrenia patients and 18 non-schizophrenic controls. In sharp contrast, miR-382 expression could not be detected in lymphoblastoid cell lines generated from schizophrenic or non-schizophrenic individuals. We further found that miR-382 directly regulates the expression of two genes, *FGFR1* and *SPRY4*, which are downregulated in both the cultured olfactory cells and LCM-OE derived from schizophrenia patients. These genes are involved in the Fibroblast Growth Factor (FGF) signaling pathway, while impairment of this pathway may underlie abnormal brain development and function associated with schizophrenia. Our data suggest that miR-382 elevation detected in patients’ OE-derived samples might serve to strengthen current biomarker studies in schizophrenia. This study also illustrates the potential utility of OE-derived tissues and cells as surrogate samples for the brain.

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Conflict of interest

The authors declare no conflict of interest.

Keywords

olfactory epithelium; olfactory neuroepithelium; microRNA; miRNA; Schizophrenia

Introduction

Schizophrenia is a common neuropsychiatric disorder affecting about 1% of the general population worldwide. The disorder is characterized by a diverse range of symptoms and neurocognitive impairments although the exact pathogenesis remains obscure. Schizophrenia is considered to be neurodevelopmental in origin (Harrison, 1997) and it is clear that it has a strong genetic component involving multiple genetic loci interacting with one another (Harrison, 1997). Schizophrenia has been studied extensively using linkage and association studies, although no definitive genetic risk factors for the disease have been determined. In recent years, a number of expression studies performed on postmortem brain samples of schizophrenics and psychiatrically unaffected individuals have shown alternation in the expression of a large number of genes (e.g. (Aston et al., 2004; Benes et al., 2007; Bowden et al., 2008; Choi et al., 2009; Hashimoto et al., 2003; Iwamoto et al., 2005; Kano et al., 2011; Kim et al., 2007; Lin et al., 2012; Mirnics et al., 2000; Perez-Santiago et al., 2012; Roussos et al., 2012; Vawter et al., 2006; Weickert et al., 2004)). These alterations may indicate a systematic dysregulation at the transcriptional or post-transcriptional level.

Small non-coding RNAs, termed microRNAs (miRNAs), form a regulatory layer which plays a central role during gene expression. miRNAs are ~19 to 24 nucleotides (nt) long and are derived from longer RNA precursors (pre-miRNA) of ~70 to 100 nt, which are processed from primary transcripts (pri-miRNA). MiRNAs control gene expression via the regulation of translation efficiency and mRNA stability, by binding to the 3' untranslated region (UTR) of the mRNA (Bushati and Cohen, 2007). The over 1000 reported human miRNAs (miRbase database (Griffiths-Jones et al., 2008)) are predicted to control the expression of more than half of our genes. miRNAs, that are abundantly expressed in brain tissue, have emerged as important in a diverse range of biological processes such as differentiation (Hornstein et al., 2005), cell cycle (Linsley et al., 2007), apoptosis (Cimmino et al., 2005), neurite outgrowth (Vo et al., 2005), dendrite morphology (Schratt et al., 2006), and brain insults (Mor et al., 2011). MiRNAs have also attracted considerable attention as regulators of neuronal development and synaptic activity.

Several recent studies on postmortem cortices identified multiple miRNAs that are differentially expressed in schizophrenia patients (e.g. (Beveridge et al., 2010; Perkins et al., 2007)). The underlying miRNA biogenesis machinery and miRNA genes themselves are also subject to disease-associated genetic mutations and epigenetic influence (reviewed in (Beveridge and Cairns, 2011)). Notably, the dysregulated miRNAs were shown to target genes associated with schizophrenia (e.g. (Kocerha et al., 2009; Miller et al., 2012)).

Cellular, molecular and gene expression studies of schizophrenia have almost exclusively utilized postmortem samples of brain regions despite many limitations: the difficulty of obtaining these samples; the long delay between death and sample collection, during which rapid cellular and molecular changes occur; and the often suboptimal retrospective assignment of diagnostic and demographic data.

Gardiner et al. (Gardiner et al., 2011) and Lai et al. (Lai et al., 2011) have recently speculated that schizophrenia-associated miRNA expression signatures may also be detected in non-neuronal tissue. They performed a miRNA expression profiling of peripheral blood mononuclear cells (PBMCs) and found a group of dysregulated miRNAs in schizophrenia

patients. The authors pointed out that the miRNA expression signature observed in PBMCs may have the potential to serve as biomarkers of schizophrenia.

As schizophrenia is a neural disorder, it would be ideal to perform gene expression studies on samples obtained from the central nervous system (CNS) of live patients. The olfactory epithelium (OE) contains neurons and their stem cells. This tissue uniquely undergoes regeneration throughout life (Cascella et al., 2007). Gene expression profiling suggests a certain degree of similarity between the CNS and the OE (including mucosal tissue) (Arnold et al., 2001; Genter et al., 2003). Evidence of the relevance of this tissue to schizophrenia is seen in the structural and functional olfactory deficits reported in schizophrenia patients (for additional information see (Cascella et al., 2007; Moberg and Turetsky, 2003; Sawa and Cascella, 2009)) that correlates with severity of negative symptoms (Brewer et al., 1996; Brewer et al., 2001; Coleman et al., 2002; Corcoran et al., 2005; Good et al., 2006; Ishizuka et al., 2010; Malaspina and Coleman, 2003; Moberg et al., 2006; Stedman and Clair, 1998). Additionally, adhesion, proliferation and maturation abnormalities of OE cells were observed in schizophrenia patients (Arnold et al., 2001; Feron et al., 1999). Importantly, OE tissues can be safely and easily obtained from live human subjects (Cascella et al., 2007; Kano et al., 2012; Tajinda et al., 2010) and may provide a “window” into neuronal processes involved in disorders such as schizophrenia, while avoiding the limitations of using postmortem brain samples or non-neuronal tissues. Furthermore, in this methodology, we can obtain cells carrying neuronal traits without reprogramming and converting cells with exogenous genetic factors. Samples from olfactory epithelium tissues are potentially heterogeneous cell populations that include both neuronal and non-neuronal cells although recent modified methods for the collection of either LCM-OE or olfactory cells have successfully minimized this possibility (Kano et al., 2012; Tajinda et al., 2010). Future studies using single cell purification combined with small-scale molecular profiling (e.g. (Qiu et al., 2012)) may eventually overcome all these limitations in cellular heterogeneity and greatly advance the research using OE tissues.

In this study we profiled global miRNA expression in the schizophrenic OE-derived cells. Our overall aim was to identify differentially expressed miRNAs in tissue derived from diseased versus healthy individuals. We expect that these miRNAs may pave the road towards development of potential biomarkers and that their further study may reveal the mechanism of neuronal miRNAs-mediated dysregulation of gene expression in schizophrenia patients.

Materials and Methods

Subjects and clinical assessment

Patients with schizophrenia were recruited from the outpatient psychiatric clinics of the Johns Hopkins Medical Institutions. The diagnosis was performed according to criteria of the Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition (DSM-IV). Normal controls were recruited from the general population through flyers posted at the Hopkins Hospital and an ad hoc ad placed in a local magazine. All subjects were administered the Structured Clinical Interview for DSM-IV Axis I Disorders-Clinician Version (SCID-IV). All patients were assessed with the Scales for the Assessment of Positive and Negative Symptoms (SAPS and SANS) by a study psychiatrist that specializes in schizophrenia. Subjects were excluded from the study if they had a history of traumatic brain injury with loss of consciousness for >1 h, a history of drug abuse within 6 months of the study or drug dependence within 12 months of the study, a history of untreated major medical illnesses. The study was approved by the Johns Hopkins Institutional Review Board and all subjects gave their written consent for their participation.

Nasal biopsy

The nasal biopsy was performed at the Johns Hopkins Otolaryngology Clinic as previously described (Kano et al., 2012; Sattler et al., 2011; Tajinda et al., 2010). The procedure, which takes 5 min, was performed with local anesthesia to the nasal cavity provided by lidocaine liquid 4% and oxymetazoline HCl 0.05% sprayed in the nose. It was followed by injection of 1% lidocaine with 1/100,000 epinephrine, to provide both anesthesia and vasoconstriction. The biopsy procedure was performed under endoscopic control and used either a small curette or a biting forceps for tissue removal. To avoid trauma to the cribriform plate, the biopsies were usually taken from the upper nasal septum. Occasionally, a small piece of superior turbinate (which is usually lined with olfactory tissue) was removed from the lateral nasal wall. The tissue was removed from either the front or the back of the olfactory cleft, and sometimes both. Four 1-mm tissue blocks were removed from each nostril. After the biopsy, no packing or treatment was needed. After the biopsy, subjects were observed in the clinic for 15 to 30 min.

Olfactory cell culture

Dissociated olfactory cells were prepared as previously described (Kano et al., 2012). Briefly, OE tissue pieces were incubated with 2.4 U/mL Dispase II for 45 min at 37°C, and mechanically minced into small pieces. Then, the tissue pieces were further treated with 0.25 mg/mL collagenase A for 10 min at 37°C. Cells were gently suspended, and centrifuged to obtain pellets. Cell pellets were resuspended in D-MEM/F12 supplemented with 10% FBS and antibiotics (D-MEM/F12 medium), and tissue debris were removed by transferring only the cell suspension into a new tube. Cells were then plated on 6-well plate in fresh D-MEM/F12 medium. Cells floating or loosely attached to the plate were collected on days 2 and 7, and plated on 6-well plate, which was further incubated until cells reached confluence. Finally, cells were harvested by a gentle trypsinization and stored in liquid N₂ for further use. After recovery, cells were maintained in D-MEM/F12 medium and supplemented with fresh medium every 2–3 days. Almost all the resultant cells (designated as olfactory cells) are well stained with the beta-III tubulin (a representative marker for immature neurons) (Kano et al., 2012).

OE neuronal layer-containing tissue obtained by laser-capture microdissection (LCM-OE)

Olfactory neuron layers were excised from OE blocks by laser capture microdissection (LCM) as previously described (Tajinda et al., 2010). Freshly collected OE blocks were mounted with O.C.T. compound (Sakura Finetek) into small tissue cups and frozen at –80°C until used. The frozen OE blocks were then cryosectioned and slices with 20 µm thickness were placed onto PALM MembraneSlide (Zeiss). The slices were treated with diethylpyrocarbonate (DEPC)-treated water to remove O.C.T.™ compound and air-dried with heat for 1 min. Olfactory neuron layers were excised with a laser-capture microdissection system (PALM MicroLaser Systems) and collected into RNAase-free microtubes. Enrichment of olfactory neuron layers was verified by qRT-PCR for several marker genes including olfactory marker protein (OMP, a marker for olfactory receptor neurons); tubulin beta 3 (TUBB3, a marker for immature olfactory receptor neurons); aldehyde dehydrogenase 1A3 (ALDH1A3, a marker for nasal submucosa); and regenerating islet-derived 3-gamma (REG3G, a marker for respiratory epithelium) as previously described (Tajinda et al., 2010).

Lymphoblastoid cells

Immortalized lymphoblastoid cells were generated from peripheral blood lymphocytes (Penno, 1993; Sawa et al., 1999) by Epstein-Barr virus (EBV) infection. The establishment of these cells was carried out at the Johns Hopkins Genetic Resources Core Facility. Briefly,

lymphocytes purified from subject's blood samples were washed with PBS and resuspended in complete RPMI medium. One million cells in 2 ml were mixed with 2 ml of fresh filtered marmoset supernatant containing EBV. The cells were then mixed with 20 μ l of PHA-M, and supplemented with 10 μ l of recombinant IL-2 (10,000 units/ml). Typically, the cells grew and reached confluence in 20–25 days. At confluency the cells were transferred to a new flask, and incubated in 2 ml of complete RPMI medium for further use.

RNA extraction

Total RNA was purified with either miRNeasy Mini Kit (Qiagen; for olfactory cells and lymphoblastoid cells), Paradise Reagent System (Arcturus; for LCM-OE) or TRIzol® (Invitrogen; for SH-SY5Y cells). RNA quality was assessed by use of RNA integrity number (RIN) score determined with Bioanalyzer RNA 6000 Nano Chip (Agilent Technologies). All the RNA from olfactory cells and lymphoblastoid cells were RIN 9.0. Among the RNA from LCM-OE, only the samples with RIN score ≥ 6.1 were used for the study. NanoDrop ND-1000 spectrophotometer (Thermo Scientific) was used for the measurement of RNA extracted from SH-SY5Y cells.

MiRNA profiling

First-strand cDNA was synthesized from cultured olfactory cells' total RNA using Megaplex reverse transcriptase reaction with the High Capacity cDNA kit (Applied Biosystems, CA, USA). cDNA and TaqMan Universal PCR Master Mix (No AmpErase UNG; Applied Biosystems) was then transferred into a loading port on Human TLDA card A according to the manufacturer's instructions. PCR amplification was carried using ABI Prism 7900HT Sequence Detection System under the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of (30 sec at 95°C and 1 min at 60°C). MiRNA relative levels were calculated based on the comparative threshold cycle (Ct) method. In short, the Ct for each miRNA and endogenous control RNU48 in each sample, were used to create Δ Ct values ($C_{t_{miRNA}} - C_{t_{RNU48}}$). Thereafter, $\Delta\Delta$ Ct values were calculated by subtracting the average Δ Ct of the non-schizophrenic controls from the Ct value of schizophrenia patients. The RQs were calculated using the equation: $RQ = 2^{-\Delta\Delta C_t}$.

MiR-382 expression analysis by quantitative Real-Time Polymerase Chain Reaction

First-strand cDNA was synthesized from LCM-OE total RNA using a MultiScribe reverse transcriptase reaction with the High Capacity cDNA kit (Applied Biosystems, CA, USA) and TaqMan MicroRNA Assay RT primer (Applied Biosystems) for miR-382 or U6 snRNA. Mixtures containing cDNA, TaqMan Universal PCR Master Mix (No AmpErase UNG; Applied Biosystems) and TaqMan MicroRNA Assay Real Time probe (Applied Biosystems) for each miRNA, were loaded on 96 well plates while PCR amplification and results analysis were done as described under 'miRNA profiling' in Materials and Methods (Thermal cycler conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of (15 sec at 95°C and 1 min at 60°C)).

mRNA expression analysis by quantitative Real-Time Polymerase Chain Reaction

cDNA synthesis of RNA isolated from LCM-OE was performed using the RNEasy kit with oligo d(T)₂₀ primer (both from Invitrogen). cDNA synthesis of RNA isolated from SH-SY5Y cells was performed using a MultiScribe reverse transcriptase reaction with the High Capacity cDNA kit (Applied Biosystems). Mixtures containing cDNA, specific primers (Sigma) (see below) and Power SYBR green PCR master mix (Applied Biosystems) were loaded on 96 well plates and PCR amplification was done as described under 'miRNA profiling' in Materials and Methods (Thermal cycler conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of (15 sec at 95°C and 1 min at 60°C) and dissociation curve cycle of 15 sec

at 95°C, 15 sec at 60°C and 15 sec at 95°C. The dissociation curve is required to show a united peak for all samples, meaning that only one product was created. Standard curve was first created for each pair of primers to determine proper primer concentration for linear amplification. Result analysis was done as described under 'miRNA profiling', using *GAPDH* as endogenous control. The primers used were: *GAPDH*-Fwd: 5' AAA GTG GAT GTC GTC GCC ATC AAT GAT 3', *GAPDH*-Rev: 5' CTG GAA GAT GGT GAT GGG ATT TCC ATT 3'; *FGFR1*-Fwd: 5'GGC AGC ATC AAC CAC ACA TA 3', *FGFR1*-Rev: 5' TAC CCA GGG CCA CTG TTT T 3'; *SPRY4*-Fwd: 5' CCT GCA GCT CCT CAA AGG 3', *SPRY4*-Rev: 5' TGA CTG AGT TGG GAG TCA AGG 3'.

miR-382 transfection into SH-SY5Y cells

The expression vector for pre-miR-382 (the miRVec plasmid) was provided by Prof. R. Agami (Voorhoeve et al., 2006). SH-SY5Y cells were seeded in 12-well plates in DMEM supplemented with 10% FBS. Transfection of miR-382 was performed 24 hours later using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions, with 1.6 µg miRVec plasmid containing the pre-miR-382 or an empty miRVec plasmid. Cells were harvested for RNA purification 24 hours later.

Choice of reference genes

U6 snRNA was primarily used in this study as a reference for miRNA expression, and *GAPDH* mRNA as a reference for mRNA expression. We chose U6 snRNA because it is widely used by many other researchers and studies as a reference for non-coding RNA (e.g. (Long et al., 2011; McCall et al., 2011; Perkins et al., 2007)). Using U6 snRNA, we successfully normalized miR-382 expression in LCM-OE, lymphoblastoid cells and SH-SY5Y cells. However, U6 snRNA expression was not stable in olfactory cells, and thus we selected RNU48 as a reference only in olfactory cells as it exhibited highly stable expression in these cells.

3' UTR constructs

3' UTR constructs were generated as previously described (Mor et al., 2011). Fragments of ~400 bp of *FGFR1* and *SPRY4* 3' UTR spanning the miRNAs binding sites were cloned into the XhoI-NotI restriction site downstream of the Renilla Luciferase Reporter gene of the psiCHECK™-2 plasmid (see Promega website: http://www.promega.com/products/rna-analysis/rna-interference/psicheck_1-and-psicheck_2-vectors/) that also contains a Firefly Luciferase Reporter (used as control) under a different promoter. For this purpose, the 3' UTR fragments were PCR-amplified from human genomic DNA and XhoI-NotI restriction sites were added (*italics*), using the primers: *FGFR1*-Fwd: 5' *ACA CTC GAG CCA TCG ACC ATG GAT GGT TT* 3', *FGFR1*-Rev: 5' *AAG GTC AAG CGG CCG CGT TTC AGT TTC TGC AGA CCT* 3'; *SPRY4*-Fwd 5' *ACA CTC GAG CTA CGT GTC CTG GGT TCT CT* 3', *SPRY4*-Rev 5' *AAG GTC AAG CGG CCG CTG ACA GTG AGC AGC AGA ATC* 3'. The miRNAs binding sites were site-directed mutated (4 bases in the seed region) by PCR reaction of the plasmid using the enzyme PfuUltra II Fusion HS DNA Polymerase (Genex), and the PCR reaction: 1) 95°C for 2min, 2) (95°C for 20sec, 58°C for 20sec, 72°C for 2min)X16, 3) 72°C for 3min. The primers used for mutagenesis were the ones indicated below (target nucleotides in *italics*) and a complementary Reverse primer: *FGFR1*: 5' GAG ACC AGC CTG GCC *GTG CTA* GTG AAA CCC CAT C 3', *SPRY4*: 5' AAA TAA TAA TAA AAC *GGT GTG TTT CCT TTT* GGC C 3'. Products were then incubated with DpnI (New England BioLabs) to digest the methylated source plasmid and the mutated plasmid was sequenced to confirm mutagenesis prior to use.

Dual Luciferase assay

HEK293T cells were seeded in 24-well plates in DMEM supplemented with 10% FBS and 1% Pen/Strep (penicillin/streptomycin). Cells were transfected using the TransIT-LT1 Transfection Reagent (Mirus), according to the manufacturer's instructions, with psiCHECKTM-2 containing the desired 3' UTR with or without site-directed mutations and miRVec plasmid containing the pre-miR-382 (provided by Prof. R. Agami (Voorhoeve et al., 2006)) or an empty miRVec plasmid. After 48 h firefly and renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System kit (Promega) and a Veritas microplate luminometer, according to Promega's instructions.

Microarray analysis

Total RNA of cultured olfactory cells was submitted to the Johns Hopkins Microarray Core Facility. Biotin-labeled cRNA, preparation by the Affymetrix 1-cycle amplification kit, hybridization and scanning were conducted at the facility. Fragmented biotin-labeled cRNA was hybridized on Affymetrix U133Plus2.0 chip at 45°C overnight. Post-hybridization was done according to the Affymetrix instructions. The chips were stained with R-Phycoerythrin streptavidin (Invitrogen) and scanned for signal detection with the Affymetrix scanner. Microarray data analysis was performed using custom code in the R statistical language (<http://www.r-project.org/>) and additional contributing packages within Bioconductor (<http://www.bioconductor.org/>). After quality control analysis and normalization, differential expression was determined by standard and moderated t-statistics (Significance Analysis of Microarrays, SAM in the "siggenes" R package) along with False Discovery Rate (FDR) analysis.

Statistics

P-values were calculated using an unpaired one/two-tailed student's t-test.

Results

MicroRNA-382 expression is elevated in the olfactory epithelium-derived samples of schizophrenia patients

Using OE-derived primary cells with immature neuronal traits (olfactory cells) (Kano et al., 2012) from schizophrenia and control groups (Table 1), we assessed the expression of 378 human miRNAs using TaqMan low density Array cards (Supplementary Table 1).

Analysis of the miRNAs differently expressed between schizophrenia patients group and control group revealed three miRNAs with a significant dysregulation (Figure 1A; p-value < 0.05, two-tailed student's t-test): miR-382 (miR-382-5p; relative expression 2.04, p-value = 0.036), miR-532-3p (relative expression 0.67, p-value = 0.028) and miR-660 (miR-660-5p; relative expression 0.66, p-value = 0.041).

We chose to focus on miR-382 for the following reasons: (i) miR-382 resides in a miRNA cluster in the imprinted DLK1-DIO3 region on the 14q32 locus that was implicated in schizophrenia in a previous study (Gardiner et al., 2011); (ii) the miR-382 expression induction we see is similar to its reported increase in postmortem dorsolateral prefrontal cortex of schizophrenia subjects (Santarelli et al., 2011); (iii) miR-382 is an exceptionally conserved miRNA in mammals, with only rare mutations in its mature form throughout mammalian evolution (Supplementary Figure 1); and (iv) miR-382 is expressed mainly in brain tissues (Supplementary Figure 2).

Given the potential function of miR-382 in brains of schizophrenia patients, we sought to validate our findings in a larger cohort of laser-capture microdissected OE neuronal tissues

(LCM-OE), which are enriched for olfactory neurons (18 schizophrenia patients and 18 non-schizophrenia controls) (Table 1) (Tajinda et al., 2010). As in the initial cohort, the average expression level of miR-382 was 1.64 fold higher in the schizophrenia patients compared with the controls (p-value = 0.023, one-tailed student's t-test). The fold change of each individual relative to all controls is presented in Figure 1B. The average fold change was 2.3 (p-value = 0.021, one-tailed student's t-test).

We then asked whether miR-382 dysregulation in schizophrenia can be also detected in the peripheral blood-derived samples. We extracted blood from five schizophrenic or non-schizophrenic individuals and generated lymphoblastoid cell lines (see Materials and Methods). Notably, expression of miR-382 could not be detected even at large amounts of starting materials (100 ng total RNA) despite the abundant expression of U6 snRNA (positive control) (Figure 1C; see Methods). Thus, increased miR-382 expression was observed in OE tissue-derived samples, but not in non-neuronal samples, from live patients with schizophrenia.

MiR-382 regulates the expression of FGF signaling genes

In order to gain insight of the potential role of miR-382 in normal brain function and in schizophrenic brain, we used the TargetScan 6.0 target prediction web-tool (Grimson et al., 2007) to predict gene targets for this miRNA. We analyzed the list of all potential target genes, irrespective of binding site conservation and found that the potential miR-382 targets were enriched with genes related to neuronal connectivity and synaptic plasticity terms such as “MAPK signaling”, “Axon guidance” and “Endocytosis” (Figure 2A). We decided to focus on the Mitogen-Activated Protein Kinase (MAPK) pathway as it was the most significant term (Figure 2A) and since abnormal MAPK signaling pathway activity is associated with schizophrenia (Funk et al., 2012). We then asked which of the putative miR-382 targets can be genuine targets in the context of OE. As these genes should have reciprocal expression to miR-382, we searched for those downregulated in the olfactory cells of schizophrenia patients (Materials and Methods; data not shown), given the elevated expression level of miR-382. We crossed this gene list with the list of miR-382 putative targets and the list of MAPK signaling related genes (Figure 2B). Three genes were present in all three lists: *SPRY4*, *NLK* and *FGFR1*. Importantly, the three binding sites for miR-382 on these genes are conserved at least among primates. Two of these genes, *FGFR1* and *SPRY4* are related to the Fibroblast Growth Factor (FGF) signaling, that when impaired could underlie abnormal brain development and function associated with schizophrenia (Gaughran et al., 2006; Terwisscha van Scheltinga et al., 2010). *FGFR1* encodes fibroblast growth factor receptor 1 (FGFR1), one of the FGF signaling receptors that was implicated in schizophrenia and possibly in a wider range of psychiatric disorders (Terwisscha van Scheltinga et al., 2010). Sprouty homolog 4 (encoded by *SPRY4*) is a negative-feedback regulator of the FGF signaling that was previously correlated with schizophrenia, in screening of chromosome 5q31-32 (Zaharieva et al., 2008). The expression of these two genes in the schizophrenic olfactory cells microarray data was 0.806 fold (p-value = 0.011) for *FGFR1* and 0.738 (p-value = 0.022) for *SPRY4* (data not shown). We validated these findings by real-time PCR analysis of the 18 LCM-OE samples of schizophrenia patients used to test miR-382 expression, compared with 14 of the non-schizophrenic controls (see Table 1). The average expression level of *FGFR1* and *SPRY4* was 0.511 (p-value = 0.016) and 0.759 (p-value = 0.024) fold lower, respectively, in the schizophrenia patients (one-tailed student's t-test). The fold change of each individual relative to all controls is presented in Figure 3. The average fold change for *FGFR1* and *SPRY4* was 0.637 (p-value = 0.016) and 0.805 (p-value = 0.023), respectively (one-tailed student's t-test).

We then asked whether the higher levels of miR-382 expression in schizophrenia patients might be associated with the lower levels of *FGFR1* and *SPRY4* expression. We transfected a plasmid that harbors the miR-382 precursor or a control plasmid into SH-SY5Y neuroblastoma cells and evaluated the expression of *FGFR1* and *SPRY4* by real-time PCR 24 hours later. Both *FGFR1* and *SPRY4* showed reduced expression in the samples transfected with miR-382 (Figure 4A).

In order to test the direct interaction between miR-382 and these targets, we employed the Luciferase reporter assay. The regions of the human target gene 3'-UTRs spanning miR-382 binding sites were cloned downstream to a Renilla Luciferase reporter gene. Reporter plasmids and miR-382 were co-transfected into HEK293T cells. Relative expression of the Renilla Luciferase reporter was measured compared to that of a Firefly Luciferase reporter, a control transfection and the 3'-UTRs mutated in the miRNA binding site (see Materials and Methods). MiR-382 significantly reduced the Renilla Luciferase-*FGFR1* and the Renilla Luciferase-*SPRY4* activity to 0.80 and 0.87, respectively (Figure 4B), indicating a direct regulation by this miRNA.

Discussion

MiRNAs have been implicated in control of gene expression during neurodevelopmental and in psychiatric disorders including schizophrenia. Studies that have evaluated gene and specifically miRNA dysregulation in schizophrenia, have primarily utilized postmortem brain samples. The use of postmortem brain tissue has limitations, including availability, potential for long delay between death and sample collection, during which rapid cellular and molecular changes occur, the possible lack of medication history and the difficulty in separating the effect of disease from progression of aging (Popova et al., 2008). Thus, there is a need for samples that can be obtained in a non-invasive procedure and that can reflect the brain miRNA signature. Based on the assumption that gene expression alterations in the CNS can be identified in blood lymphocytes, two studies have recently attempted to detect miRNAs dysregulation in peripheral blood (Gardiner et al., 2011; Lai et al., 2011). Several miRNAs were reported to be dysregulated in both peripheral blood and postmortem brain samples. These miRNAs include miR-34a (Kim et al., 2010; Lai et al., 2011), miR-134 (Gardiner et al., 2011; Santarelli et al., 2011) and miR-181b (Beveridge et al., 2008; Gardiner et al., 2011). Nevertheless, it is not clear whether miRNA dysregulation in the schizophrenic brain is indeed truly reflected in the blood. We therefore attempted to detect miRNAs dysregulation in the schizophrenic brain using OE tissue-derived samples (LCM-OE and olfactory cells). Given the gene expression similarity of the OE to the CNS, and since the OE is an accessible tissue that can be biopsied without significantly invasive procedures, we believe that OE is of great promise for elucidating miRNA alterations that occur in brains of living individuals. To our knowledge, this is the first study of human miRNAs in the OE tissue.

The most abundant miRNAs identified in the in the olfactory cells of the control group are presented in Supplementary Table 2. Three miRNAs were found to be dysregulated in the olfactory cells of schizophrenia patients: miR-382 (miR-382-5p), miR-532-3p and miR-660. Interestingly, miR-660 and miR-532-3p both reside in the same miRNA cluster on chromosome X, which suggests that their downregulation is due to transcriptional arrest of their common promoter. Previously, a point mutation in the mature form of miR-660 was associated with schizophrenia (Feng et al., 2009). MiR-532-5p (rather than miR-532-3p) was found to be upregulated in the postmortem dorsolateral prefrontal cortex of schizophrenia subjects (Santarelli et al., 2011).

We found that miR-382 increase in olfactory cells is apparent also in LCM-OE in a cohort of schizophrenia patients compared with non-schizophrenic controls.

We note that one possible limitation of our findings is that antipsychotic medications that schizophrenic patients receive at the time of sample collection might influence the expression of miRNAs (and/or target genes) in the OE tissue. The fact that our findings are similar in both cultured olfactory cells and LCM-OE might alleviate this concern to some degree. MiR-382 elevation was apparent in most but not all of the schizophrenic patients, when compared to the average in the control group. We did not observe a correlation of miR-382 elevation in specific patients with any of the parameters that are demonstrated in Table 1, including demographic variables, smoking and consumption of medications.

Given that miR-382 was not elevated in all patient samples, its power as a single biomarker needs further investigation. However, a possible combination of miRNAs, target genes and other features might serve as a more effective approach for diagnostic purposes.

Our observations regarding miR-382 elevation in the schizophrenic OE-derived brain surrogate samples add onto previous findings regarding the association of this brain-enriched miRNA to neuronal disorders. Interestingly, miR-382 was previously shown to be increased in the schizophrenic postmortem dorsolateral prefrontal cortex (Santarelli et al., 2011). Other studies found it to be upregulated in brains of the Rett syndrome mouse model (Urdinguio et al., 2010) as well as in the amygdala of rats under acute stress (Meerson et al., 2010). In contrast, miR-382 is downregulated in Alzheimer's disease patients with enriched expression in the gray matter (Wang et al., 2011).

The miR-382 precursor resides in a miRNA cluster in the imprinted *DLK1-DIO3* region on the long arm of chromosome 14q32. This loci harbors the pri-form of at least 48 human miRNAs (Gardiner et al., 2011). The expression of these miRNAs is mostly restricted to the adult mouse brain (Seitz et al., 2004), is upregulated in neuronal differentiation (Huang et al., 2009) (including miR-382), and is important for brain function and development (Fiore et al., 2009). Moreover, miRNAs from this cluster may have facilitated evolution of higher brain functions in eutherian (placental) mammals (Glazov et al., 2008).

The expression of many members of this cluster is down-regulated in PBMCs from schizophrenia patients compared with normal controls. MiR-382 is an exception as it was not detected in PBMCs, in agreement with our results that indicated that miR-382 is not expressed in lymphoblastoid cell lines. This result is another indication that blood lymphocytes do not always reflect dysregulation in the CNS. Around 80% of the miRNAs in the cluster harboring miR-382 are found on the miRNA array used in our miRNA profiling, 92% of those are expressed in olfactory cells with differential intensities (data not shown), yet only miR-382 was found to be dysregulated in schizophrenia patients. These data suggest that the members of this miRNA cluster are differentially regulated in PMBC and in the OE. A possible explanation for this phenomenon may be post-transcriptional factors that govern global miRNA biogenesis with differential sensitivity to miRNA precursors (Volk and Shomron, 2011). Schizophrenia is associated with a global miRNA expression elevation in the cortex. This phenomenon was explained by excessive miRNA maturation as a result of expression elevation of the microprocessor component *DGCR8* and *DICER* (Beveridge et al., 2010; Santarelli et al., 2011). We identified a trend of a global increased expression of miRNAs in the schizophrenic olfactory cells compared with non-schizophrenic controls, yet this observation fell beneath statistical significance (p-value = 0.092; two-tailed student's t-test). The mRNA levels of miRNA biogenesis factors like *DICER1*, *DROSHA*, *DGCR8*, *EXPO5* and *EIF2C2* were not constantly and significantly altered in the schizophrenic olfactory cells (data not shown). The pri-form of miR-382 was not detected in the LCM-OE

neurons using real-time PCR (data not shown). This might be due to rapid shift towards maturation of all miR-382 precursor transcripts. However pri-miR-382 might not have been detected due to technical issues.

The miRNA influence on the expression of its target genes is very often limited to a mild effect. However, this regulation, sometimes referred to as ‘fine tuning’ can exert a substantial and significant outcome (Nielsen et al., 2007). We found that miR-382 regulate the expression of two FGF/MAPK signaling related genes, *FGFR1* and *SPRY4*, and we suggest that this regulation is associated with the decreased expression of these genes in the schizophrenic olfactory cells and LCM-OE. *FGFR1* was previously implicated in schizophrenia (Jungerius et al., 2008). Two different *FGFR1* knockout mice models display “schizophrenia-like” characteristics ((Terwisscha van Scheltinga et al., 2010) and ref. within). The expression of *FGFR1* mRNA is downregulated in the hippocampus after social defeat in rats (Terwisscha van Scheltinga et al., 2010) whereas *FGFR1* mRNA is higher in subjects with depression (hippocampal CA1 and CA4) and schizophrenia (CA4) than in controls (Gaughran et al., 2006). *FGFR1* is also known to regulate neurogenesis in the olfactory epithelium (Hebert et al., 2003; Hsu et al., 2001; Layman et al., 2011). The FGF signaling inhibitor *SPRY4* was previously correlated with schizophrenia, in screening of chromosome 5q31-32 (Zaharieva et al., 2008). This region is one of the five regions most consistently associated with schizophrenia as found in a meta-analysis of genome-wide linkage studies (Lewis et al., 2003). *SPRY4* has a role in cerebellum development (Yu et al., 2011), and is required for hindbrain patterning (Labalette et al., 2011).

Thus, decreased *FGFR1* expression can reduce the overall FGF signaling while decreased expression of *SPRY4*, which is an FGF signaling inhibitor, can potentially lead to contradictory findings (Furthauer et al., 2001). Nevertheless, both increased and decreased FGF levels could cause aberrations in the brain (Terwisscha van Scheltinga et al., 2010).

Currently, the diagnosis of schizophrenia is challenging, and is based solely on questionnaires (such as the DSM-IV) that allow diagnosis only if symptoms have been manifested over several months. Our findings may not only provide the first neuronal miRNA biomarker for schizophrenia in live patients, but may also advance our understanding of the pathogenesis of the disease. Future experiments will clarify whether miR-382 could serve as an early neuronal biomarker of schizophrenia in schizophrenic-prone families.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

MiR-382 expression is elevated in the olfactory epithelium-derived samples of schizophrenia patients. (A) MiRNA dysregulation in the olfactory cells (Kano et al., 2012) of schizophrenia patients. A volcano plot of miRNAs expression in schizophrenia patients (N=7) relative to non-schizophrenic controls (N=7). Values are presented relative to a non-related small non-coding RNA (RNU48). Fold-change values were calculated by $2^{(-\Delta\Delta\text{ct})}$. Three miRNAs are found above the horizontal line (p-value = 0.05). Only miRNAs with a detectable expression in all samples were considered. (B) MiR-382 expression is elevated in olfactory neuronal layer tissues of schizophrenia patients enriched by laser capture microdissection (LCM-OE) (Tajinda et al., 2010). MiR-382 expression in LCM-OE of schizophrenia patients (N=18) relative to non-schizophrenic controls (N=18). Values are presented relative to a non-related small non-coding RNA (U6 snRNA). MiR-382 fold change of each schizophrenia patient (see Table 1) was calculated by $2^{(-\Delta\Delta\text{ct})}$ relative to the mean of all non-schizophrenic controls. Average fold change is presented $\pm$ SEM. (C) Real-Time PCR expression of miR-382 and U6 snRNA, which served as a positive control, in lymphoblastoid cell lines generated from blood of five schizophrenic (SZ) or non-schizophrenic (C) individuals (see Table 1). Values represent raw expression (threshold cycle) of equal number of starting material (RNA and cells). MiR-382 could not be detected even at large amounts of starting materials (100 ng total RNA).

Figure 2.

FGFR1 and *SPRY4* are relevant putative miR-382 targets. (A) KEGG pathway terms enrichment among miR-382 targets. Data obtained from DAVID Bioinformatics Resources (Huang da et al., 2009). In bold: MAPK signaling pathway that was used for further research. * Number of genes that are targets of miR-382 and are also associated with the pathway. (B) A Venn diagram for prioritizing relevant miR-382 targets. In blue: all miR-382 putative targets according to TargetScan 6.0; In yellow: MAPK signaling related genes; In green: olfactory cells' down-regulated genes with fold-change <-1.2 and p-value <0.03 .

Figure 3. *FGFR1* and *SPRY4* expression is decreased in LCM-OE of schizophrenia patients. Expression of *FGFR1* and *SPRY4* in schizophrenia patients (N=18) relative to non-schizophrenic controls (N=14). Values are presented relative to *GAPDH* endogenous control. Each gene fold change for each schizophrenia patient was calculated by $2^{(-\Delta\Delta ct)}$ relative to the mean of all non-schizophrenic controls. Average fold change is presented $\pm$ SEM.

Figure 4.

FGFR1 and *SPRY4* are direct targets of miR-382. (A) *FGFR1* and *SPRY4* mRNA expression 24 hours following transfection of a plasmid containing the pre-miR-382 into SH-SY5Y neuroblastoma cells. Fold change was calculated by $2^{-(\Delta\Delta Ct)}$ relative to endogenous *GAPDH* and a control plasmid transfection. All values are presented as mean $\pm$ SEM (N>3). p-value<0.005 is indicated by an asterisk. (B) Regions from the miR-382 putative targets 3'-UTRs spanning the miRNA binding sites were cloned into the psiCHECK-2 plasmid downstream of a Renilla luciferase reporter gene. Each of these plasmids (or their mutated version; see Materials and Methods) was cotransfected along with a plasmid containing the pre-miR-382 into HEK293T cells. Renilla luciferase activity was measured 48 hours following transfections and normalized to firefly luciferase activity and transfections with a control plasmid and a mutated version (mut) of miR-382 binding site. Values are presented as mean $\pm$ SEM (N>3). p-value<0.05 is indicated by an asterisk.

Table 1

Demographic data of individuals that provided samples for this study.

Non-schizophrenic controls						
Label	Age	Gender	Race	Smoking	Medication type	Medication details
C1#	30	Male	Unknown	no	no medication	---
C2*#	29	Female	African American	no	no medication	---
C3*#	28	Male	Caucasian	no	no medication	---
C4*#	46	Male	Caucasian	no	no medication	---
C5#	48	Male	Caucasian	no	no medication	---
C6*#	42	Male	African American	no	no medication	---
C7	22	Male	African American	no	no medication	---
C8*#	49	Male	Caucasian	yes	no medication	---
C9	42	Male	African American	yes	no medication	---
C10*	48	Male	African American	yes	no medication	---
C11#	54	Male	Caucasian	no	no medication	---
C12#	48	Male	African American	no	no medication	---
C13	19	Female	Caucasian	no	no medication	---
C14#	38	Male	African American	no	no medication	---
C15#	57	Female	African American	no	Non-psychiatric	Hydrochlorothiazide, Felodipine
C16*#	27	Female	Caucasian	no	no medication	---
C17#	51	Female	African American	no	no medication	---
C18#	27	Male	Unknown	yes	no medication	---
Schizophrenia patients						
Label	Age	Gender	Race	Smoking	Age onset	Illness duration
SZ1#	52	Female	Caucasian	no	17	35
SZ2#	38	Male	African American	yes	22	16
						Medication type
						Clozapine, Aripiprazole, Venlafaxine, Benzotropine, Diphenhydramine, Atenolol, Glyburide, Metformin
						Psychiatric-others
						Psychiatric
						Olanzapine

Schizophrenia patients									
Label	Age	Gender	Race	Smoking	Age onset	Illness duration	Medication type	Medication details	
SZ3 [#]	45	Male	Caucasian	yes	17	28	Psychiatric	Olanzapine, Buspirone, Benzotropine, Diphenhydramine	
SZ4 [#]	54	Female	African American	yes	32	22	Psychiatric	Fluphenazine	
SZ5 [#]	45	Male	Caucasian	yes	27	18	Psychiatric	Haloperidol, Nortriptyline, Aripiprazole	
SZ6 [#]	53	Female	African American	yes	25-26	27-28	Psychiatric+others	Clozapine, Olanzapine, Levothyroxine, Amlodipine, Benazepril, Fluoxetine	
SZ7 [#]	55	Male	Caucasian	no	26	29	Psychiatric	Mirtazapine	
SZ8 [#]	30	Male	African American	yes	20	10	Psychiatric	Fluphenazine	
SZ9 [#]	48	Male	African American	yes	20-25	23-28	Psychiatric	Risperidone, Escitalopram, Diphenhydramine	
SZ10 ^{**#}	44	Male	African American	yes	25	19	Psychiatric	Trihexyphenidyl, Fluphenazine, Citalopram, Quetiapine, Lamotrigine	
SZ11 [#]	26	Male	Caucasian	no	20	6	Psychiatric	Clozapine	
SZ12 [#]	25	Female	Caucasian	yes	17	8	Psychiatric	Quetiapine, Lamotrigine, Diphenhydramine, Fluphenazine	
SZ13 [#]	25	Male	African American	no	19	6	Psychiatric	Risperidone, Valproate, Fluphenazine	
SZ14 ^{**#}	45	Male	African American	no	30	15	Psychiatric+others	Olanzapine, Diphenhydramine, Metformin, Lisinopril, Pravastatin	
SZ15 ^{**#}	19	Female	Caucasian	no	18	0.5	Psychiatric	Olanzapine, Clozapine	
SZ16 [#]	22	Male	African American	yes	18	4	Psychiatric	Haloperidol, Olanzapine	
SZ17 [#]	34	Male	African American	no	17	17	Psychiatric+others	Olanzapine, Atorvastatin	
SZ18 ^{**#}	50	Female	Caucasian	yes	13	37	Psychiatric	Thiothixene, Benzotropine	
SZ19 [*]	53	Male	African American	no	17	36	Unknown	Unknown	
SZ20 [*]	29	Female	African American	no	14	15	Unknown	Unknown	

* samples used for miRNA arrays of olfactory cells;

samples used for gene expression in LCM-OE by real-time PCR;

All samples were used for miR-382 expression in LCM-OE by real-time PCR except for SZ19 and SZ20.