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Experimental validation of candidate schizophrenia gene *ZNF804A*as target for hsa-miR-137

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that mainly function as negative regulators of gene expression (Lai, 2002) and have been shown to be involved in schizophrenia etiology through genetic and expression studies (Burmistrova et al., 2007; Hansen et al., 2007a; Perkins et al., 2007; Beveridge et al., 2010; Kim et al., 2010). In a mega analysis of genome-wide association study (GWAS) of schizophrenia (SZ) and bipolar disorders (BP), a polymorphism (rs1625579) located in the primary transcript of a miRNA gene, hsa-miR-137, was reported to be strongly associated with SZ. Four SZ loci (*CACNA1C, TCF4, CSMD1, C10orf26*) achieving genome-wide significance in the same study were predicted and later experimentally validated (Kwon et al., 2011) as hsa-miR-137 targets.

Here, using *in silico*, cellular and luciferase based approaches we also provide evidence that another well replicated candidate schizophrenia gene, *ZNF804A*, is also target for hsa-miR-137.

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Contributors

A.H.K., E. K.P. and G. M. performed the experimental work. V.W. performed the in silico analyses. V.I.V. designed the study and wrote the first draft of the manuscript. A.H.F. contributed to the final writing of the manuscript. All authors contributed to and have approved the final manuscript.

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Keywords

miRNA; Gene expression; Luciferase; Real-time PCR; GWAS; Schizophrenia

1. Introduction

Both SZ and BP disorders are debilitating psychiatric illnesses that pose a major burden on public health due to early onset and for many patients, the need for long-term care. While the etiology of these disorders is still unknown, in conjunction with environmental and developmental factors (Kendler et al., 1985; Kety, 1987; Jablensky et al., 1992a, 1992b; Kendler and Diehl, 1993a, 1993b; Kendler et al., 1994a, 1994b; Kendler KS, 1996; Hansen et al., 2007b) there is consistent evidence for a substantial genetic component (Sullivan et al., 2003; Kato et al., 2005) (heritability ~80%) with some shared between both diseases (Berrettini, 2003; Purcell et al., 2009a).

GWAS are a powerful, systematic and unbiased genetic approach to study the common disease/common variant (CDCV) hypothesis of complex disorders like schizophrenia. In a recent mega GWAS analysis (Ripke et al., 2011), performed by the Psychiatric GWAS Consortium (PGC), the strongest finding for association with schizophrenia was to a variant within the primary transcript of miRNA gene, hsa-miR-137 (miR-137). This miRNA has been previously implicated in regulation of adult neurogenesis (Szulwach et al., 2010), dendritic development, and neuronal maturation (Smrt et al., 2010). In a recent study, using mouse embryonic neural stem cells, miR-137 was shown to control the dynamics between neural stem cell proliferation and differentiation during neural development (Sun et al., 2011). Further, a study integrating GWAS genetic data with brain imaging as a quantitative trait (Potkin et al., 2010) found miR-137 gene targets to be significantly enriched for association with schizophrenia. Additionally, this study also provided evidence for genome-wide significance of association with schizophrenia for four other loci (transcription factor 4 (*TCF4*), calcium channel, voltage-dependent, L type, alpha 1C subunit (*CACNA1C*), cub and sushi multiple domains 1(*CSMD1*) and chromosome 10 open reading frame 26 (*C10orf26*)) which were also predicted and validated (Kwon et al., 2011) as miR-137 gene targets. MiRNAs mainly function to down-regulate gene expression (Lai, 2002) and genetic studies have found genetic variants within miRNA genes to be associated with schizophrenia as well as expression data performed in postmortem brain tissue have demonstrated dysregulated expression of miRNAs schizophrenic subjects (Burmistrova et al., 2007; Hansen et al., 2007a; Perkins et al., 2007; Beveridge et al., 2010; Kim et al., 2010).

In addition to the PGC study, other GWAS have also provided compelling evidence for association of the ZNF804A gene located at chromosome 2q32.1 with schizophrenia. In the original GWAS an intronic polymorphism, rs1344706, in *ZNF804A* achieved genome wide significance of association with schizophrenia and bipolar disorder in a combined SZ and BP samples (O'Donovan et al., 2008). This was later corroborated by a meta-analysis of over 21,000 cases and 38,000 controls, which found an odds ratio (OR) of 1.10, $P=2.5\times10^{-11}$ for

schizophrenia alone, and OR 1.11, $P=4\times10^{-13}$ for schizophrenia and bipolar disorder combined (Williams et al., 2011).

Given that animal miRNAs bind with imperfect complementarity to their targets and considering the labor intensive approaches to experimentally verify such miRNA targets, much effort has been put toward devising a genome-wide computational search that captures most of the regulatory targets without inflating the rate of false-positive predictions. While, the prediction methods are diverse and all have room for improvement, a general agreement has emerged on three important criteria (Lewis et al., 2003; Brennecke et al., 2005; Bartel, 2009; Huang et al., 2010). First, strong binding of the 5′ seed sequence (nucleotides 2–7) of the mature miRNA to the 3′-UTR sequence of the target gene, second assessing the thermodynamic properties of the miRNA/mRNA duplex by calculating the free-energy ($_{\Delta}G$) of the putative interaction, i.e. a lower $_{\Delta}G$ indicating stronger miRNA/mRNA binding and third evolutionary conservation of the miRNA target sequences. Based on these criteria for computational prediction of miRNA/mRNA interactions, many algorithms have been developed and eleven of these well-established algorithms have been compiled into a single database, miRecords (Xiao et al., 2009).

Regardless of the computational algorithms used, the experimental approaches are still the best option to unequivocally establish if a given miRNA interacts with its predicted gene target. Therefore here, using both in silico and cellular based approaches we provide strong evidence that in addition to other schizophrenia implicated genes,*TCF4*, *CACNA1C*, *CSMD1* and *C10orf26*, *ZNF804A* is another target for hsa-mi-137.

2. Material and methods

2.1. In silico analysis

Since the accuracy of in silico predictions is shown to be considerably improved by integrating multiple prediction programs, we used miRecords to predict *ZNF804A* as a miR-137 gene target. The predicted targets module in miRecords is an integration of 11 established miRNA target prediction programs. Within miRecords database only one prediction algorithm (PITA) (Kertesz et al., 2007) predicted *ZNF804A* as a gene target for miR-137.

2.2. Cell cultures

Be2C (neuroblastoma) cells (ATCC # CRL-2268) were propagated at 37 °C and 5% CO_2, in a 1:1 mixture of Eagle's Minimum Essential Medium, F12 Medium, supplemented with 10% fetal bovine serum (FBS), and 1% non-essential amino acids. HEK293 (human embryonic kidney cells) cells (ATCC # CRL 1573) were maintained at 37 °C and 5% CO_2, in a 1:1 mixture of Dulbecco's Modified Essential Medium supplemented with 10% FBS and 1% glutamine. Both cell lines were subcultured at ratio 1:3 every 2 to 3 days following the supplier protocols. Briefly, after cells were rinsed with PBS, 2 ml of 0.25% (w/v) trypsin was added and cells were incubating at 37 °C for 3 min. After cells detachment, 2 ml of full growth media was added, cells were aspirated and aliquoted at a 1:3 ratio in a 10 cm culture dish.

2.3. Transfections

2.3.1. Gene transfection assays—One day before transfection, HEK293 and Be2C cells were seeded in 6-well plates containing media without antibiotics at 600,000 cells density per well to allow 90–95% confluency at the time of transfection. 4 μg of either mir-137 precursor or scrambled hairpin (SH) control plasmid (Genecopoeia Inc, Rockville, MD) was diluted into 250 μL of Opti-MEM without serum. In a separate tube, 10 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was diluted into 250 μL Opti-MEM without serum and incubated for 5 min at room temperature. After incubation, the DNA and Lipofectamine dilutions were combined and incubated for 20 min at room temperature to allow complex formation to occur. The DNA/Lipofectamine complex was then added to each well containing cells and 3 mL of media without antibiotic. The reactions were incubated at 37 °C and harvested at 24 h. To increase accuracy and to reduce assay variability, each sample was transfected in triplicate.

2.4. ZNF804A expression assays

cDNA was made from 1.5 μg of RNA using the High Capacity cDNA Kit (ABI) according to manufacturer's recommendations. Gene expression assays were performed by adding 0.25 μL dH_2O, 0.25 μL 20× Taqman Assay, 5.0 μL Gene Expression Master Mix and 4.5 μL cDNA diluted 1:10. The reactions were run in triplicate in a 384-well format on the ABI 7900HT according to manufacturer's recommendations. PCR-efficiency for each reaction was assessed using the LinRegPCR (Ramakers et al., 2003) program, which uses the raw real-time PCR data of each individual sample and performs a linear regression analysis to calculate starting concentrations of mRNAs and individual PCR efficiencies for each sample. *ZNF804A* expression for each sample was normalized with the $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001) using the geometric mean of the *IPO8*, *HMBS* and *PPIA* reference genes.

2.5. 3′-UTR target site cloning and mutagenesis

Approximately 100 base pairs (50 bp up- and downstream of the predicted target site) from the *ZNF804A* 3′ UTR was cloned into the pEZX-MT01 vector (Genecopoeia) using the AsisI and Mpe restriction sites in the multiple cloning region downstream of the luciferase reporter gene. All target-site sequence cloning was performed by Genecopoeia, Inc. Mutagenesis was performed using the QuickChange II Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to manufacturer's protocol. The sequence accuracy of all clones was verified by sequencing.

2.6. Luciferase transfection assays

The luciferase transfection assays were accomplished in 96-well plates by following the alternate rapid protocol without pre-plating as outlined in Invitrogen's Lipofectamine 2000 manual. The following combination of miRNAs and targets were used to assess specificity of miRNA binding: 1) 180 ng of mir-137 precursor (Genecopoeia) with 120 ng of *ZNF804A* target wild type (WT) sequence, 2) 180 ng of mir-137 precursor with 120 ng of *ZNF804A* mutated target (MT) sequence, 3) 180 ng of mir-137 precursor with 120 ng of *ZNF804A* target WT sequence and 90 nmol anti-miR-137 oligo (Ambion), 4) 180 ng of miR-377

precursor, an off-targeting miRNA precursor serving as a negative control, with 120 ng *ZNF804A* WT sequence and 5) 180 ng of miR-125a precursor with 120 ng of *lin-4*, a known miR-125a target gene, serving as positive transfection control. The respective reactions were then diluted into 20 μL of Opti-MEM without serum. Next, for each well, 0.8 μL of Lipofectamine 2000 (Invitrogen) was diluted into 24.2 μL Opti-MEM without serum and incubated for 5 min at room temperature. After incubation, the DNA and Lipofectamine dilutions were combined and incubated for 20 min at room temperature to allow complex formation to occur. Meanwhile, suspensions of HEK293 cells were prepared to contain 120,000 cells in 100 μL of media without antibiotics. The DNA-Lipofectamine complex in each well was then mixed with 100 μL of cells and incubated at 37 °C and harvested after 24 h. To increase accuracy and to reduce assay variability, each sample was transfected in quadruplicate.

2.7. Luciferase assay

The luciferase assays were accomplished using the Luc-Pair miR Luciferase Assay (Genecopoeia) microplate procedure. First, media was aspirated and 100 μL of Working Solution I (Solution I:Substrate I in a 1:200 ratio) was added to each well. After 10 min, firefly luciferase activity was measured in a Wallac Victor II luminometer. Next, 100 μL of Working Solution II (Solution II:Substrate II in a 1:200 ratio) was added to each well. After 10 min, *Renilla* luciferase activity was measured. The ratio of firefly to *Renilla* luciferase was calculated (F/R) for each well and the average ratio for each quadruplicate was taken. The average ratios were then normalized to the mock transfection, i.e. cells only.

2.8. Statistical analyses

Each set of miRNA and gene expression and luciferase gene target experiments was performed in triplicate from at least three independent experiments. The Student's t-test was used to evaluate significant mean *ZNF804A* expression differences in the two cell lines. The expression differences in the luciferase assays were analyzed using the nonparametric Kruskal–Wallis (KW) test. The KW test provides an overall, already corrected for the number of tests, significance level. Once the KW test was significant, the individual intergroup comparisons were performed by the Dunn's post-hoc multiple comparison test and these comparisons were deemed significant for pb0.05. All analyses were performed in GraphPad (GraphPad Software v.5.04, San Diego CA).

3. Results

3.1. Computational prediction of ZNF804A as hsa-miR-137 target

ZNF804A was predicted as miR-137 gene target using the miRecords database. Within miRecord, one miR-137 binding site at nucleotide (nt) position 4660 was predicted for *ZNF804A* (NM_194250.1) by PITA and therefore was used as the major target site for the subsequent experiments.

3.2. ZNF804A expression in HEK293 and Be2c cell lines

To assess whether miR-137 down-regulates the native expression of *ZNF804A*, expression vectors containing the miR-137 hairpin and a SH control were transfected independently

into HEK293 and Be2C cell lines. After 24 h, total RNA was isolated and the *ZNF804A* gene expression was evaluated via real-time PCR. *ZNF804A* showed significant down-regulation at the mRNA level in the miR-137 transfected versus scrambled hairpin transfected conditions in both cell lines (Fig. 1A and B).

3.3. Luciferase assays

Given that miR-137 overexpression significantly down-regulates *ZNF804A*, we next sought to demonstrate that this repression of expression is indeed mediated by the specific interaction between hsa-miR-137 and *ZNF804A*. Briefly, approximately 100 bp fragment (~50 bp up- and down-stream of the predicted target site) was cloned into a reporter construct behind luciferase gene. The mutant constructs were generated similarly by site-directed mutagenesis, yielding a 4 bp mutation in the target site (Fig. 2).

Next, we transfected the following combination of miRNA vectors and gene targets in quadruplicate in a 96-well plate to assess spec-ificity of miRNA binding: i) mir-137 with *ZNF804A* WT target sequence, ii) miR-137 with *ZNF804A* MT target sequence, iii) miR-137 with *ZNF804A* WT target sequence and anti-miR-137 oligo, and iv) miR-377, as an off-targeting miRNA with *ZNF804A* WT target sequence. All of these reactions were performed in HEK293 only and assayed 24 h post transfection. HEK293 was chosen due to its high transfection efficiency and very low endogenous expression of hsa-miR-137. By comparing the ratio of *Renilla* to Firefly luciferase activity, the predicted target showed a 25–50% reduction in luciferase activity in the cells co-transfected with hsa-miR-137 and *ZNF804A* WT target sequence compared to the empty vector transfected cells(KW $p=0.0042$, Dunn's post testb0.01; Fig. 2). Likewise, in cells co-transfected with the mutated *ZNF804A* target site (Fig. 2) and hsa-miR-137, the Renilla/Firefly luciferase ratio did not show significant differences to that of the empty vector control (Dunn's post test $p>0.05$ KW). Further, to demonstrate that hsa-miR-137/*ZNF804A* interaction is indeed specific to miR-137, the *ZNF804A* WT target site was co-transfected with hsa-miR-377, which is not predicted to target *ZNF804A*. No significant differences between the mock (empty vector) control and miR-377 were observed either (Dunn's post-hoc $p>0.05$ KW). Thus, these results demonstrate that miR-137 interacts with the *ZNF804A* target sequence in a site-specific manner.

4. Discussion

In the last few years, based on genetic and expression studies, there has been a steady increase in the number of reports demonstrating miRNAs involvement in schizophrenia and bipolar disorders as well as other psychiatric disorders including addiction (Dreyer, 2010; Miller and Wahlestedt, 2010; Xu et al., 2010). In regards to existing genetic studies, the recent mega GWAS study published last year by the PGC group is of special interest as insofar it provides the strongest genetic evidence for miRNAs involvement in schizophrenia. In that study the best genetic signal for association with schizophrenia was a polymorphism located in the primary transcript of hsa-miR-137. Mir-137 has been previously shown to be involved in adult neurogenesis (Szulwach et al., 2010), dendritic development, and neuronal maturation (Smrt et al., 2010). Further a study integrating genetic data from GWAS with

brain imaging results (Potkin et al., 2010) found significant enrichment for miR-137 gene targets in the schizophrenia samples. Additional targets of miR137, *TCF4*, *CACNA1C*, *CSMD1*, and *C10orf26* achieved genome-wide significance for association with schizophrenia in the PGC study and were later validated as miR-137 gene targets (Kwon et al., 2011).

In essence, *ZNF804A*, *CACNA1C* and *ANK3* could be considered the first GWAS success of major psychiatric disorders as these were first to pass genome wide significance level of association with schizophrenia and bipolar disorders (Ferreira et al., 2008; O'Donovan et al., 2008). Following on the original study from (O'Donovan et al., 2008) where they reported a SNP polymorphism (rs1344706) in the 3′ end of *ZNF804A* achieved genome wide significance of association with schizophrenia and bipolar disorders, the International Schizophrenia Consortium (ISC) (Purcell et al., 2009b) and a meta-analysis of approximately 60,000 individuals across several populations (Williams et al., 2011) also reported a positive SZ and BP associations with this gene.

Although we and others have attempted to establish a functional link between this gene and schizophrenia, currently little is known about the cellular functions of *ZNF804A*. However, several recent studies have provided evidence that *ZNF804A* participates in cellular functions that might be related to the etiology of neuropsychiatric disorders. First, *ZNF804A* has been shown to be expressed broadly throughout the brain with highest levels of expression in the developing hippocampus, cortex and in adult cerebellum (Johnson et al., 2009). Second, down regulation of *ZNF804A* was shown to affect expression of genes involved in cell adhesion, suggesting that *ZNF804A* might control processes such as neural migration, neurite outgrowth and synapse formation (Hill et al., 2012). Recently *ZNF804A* was also shown to modulate expression of four SZ candidate genes (Girgenti et al., 2012).

In healthy subjects the “risk” allele of rs1344706 has been linked to larger total white mater and reduced gray matter (Lencz et al., 2010). Additionally a study by (Hill and Bray, 2011) used electromobility shift assay (EMSA) to demonstrate that, when incubated in the presence of nuclear extracts, there is reduced binding of a yet unknown protein to DNA oligos carrying the rs1344706 risk allele. Using postmortem brain tissues from healthy subjects we have previously demonstrated significantly higher gene expression levels of *ZNF804A* in individuals with the risk allele of rs1344706 (Riley et al., 2010). In another study (Williams et al., 2011) a similar association between expression of *ZNF804A* and the risk allele of rs1344706 was observed. Further, due to the specific design of the study, a direct comparison between heterozygous and homozygous status of rs1344706, the authors were also able to conclude that rs1344706 is likely to affect *ZNF804A* expression indirectly. The later study would also suggest that, considering the intronic location of rs1344706 in the *ZNF804A* gene, possibility for this polymorphism being a tagging SNP rather than the true causative variant still remains open (Donohoe et al., 2010).

While, substantial work has been done to provide a functional impact of the disease associated polymorphism, our in silico analyzes did not identify any polymorphisms in the hsa-miR-137 target sequence, suggesting that hsa-miR-137 control on *ZNF804A* could be

considered as complementing and independent to rs1344706 mechanism of controlling *ZNF804A* expression.

In conclusion, we provide strong evidence that, *ZNF804A*, like other highly significant candidate SZ loci is also under the control of hsa-miR-137. While more studies will be needed to provide a better understanding for the disease related interplay between hsa-miR-137 and its targets, a possible mechanism for the etiology of psychiatric disorders is emerging, where the interplay between miRNAs and target genes could be pivotal for our understanding of schizophrenia and bipolar disorders pathophysiology.

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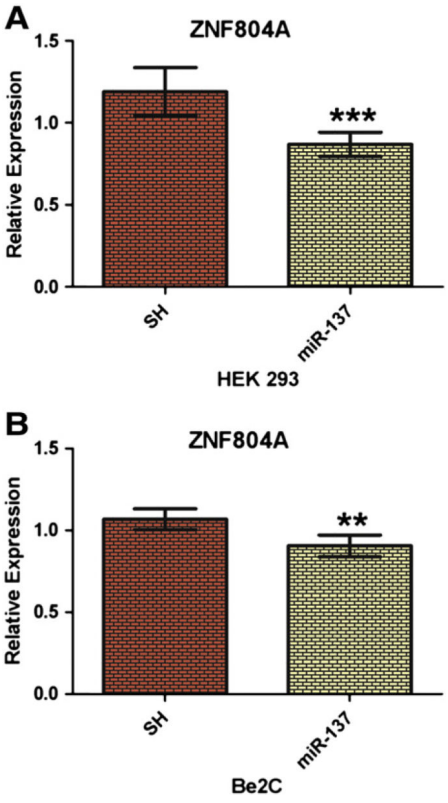

Fig. 1.

(A) Analysis of *ZNF804A* expression in HEK293 cells over expressing miR-137; (B) ZNF804A expression analysis in Be2C cells over expressing miR-137. The error bars represent the SE±95% CI from three independent experiments (***pb0.001; **pb0.01).

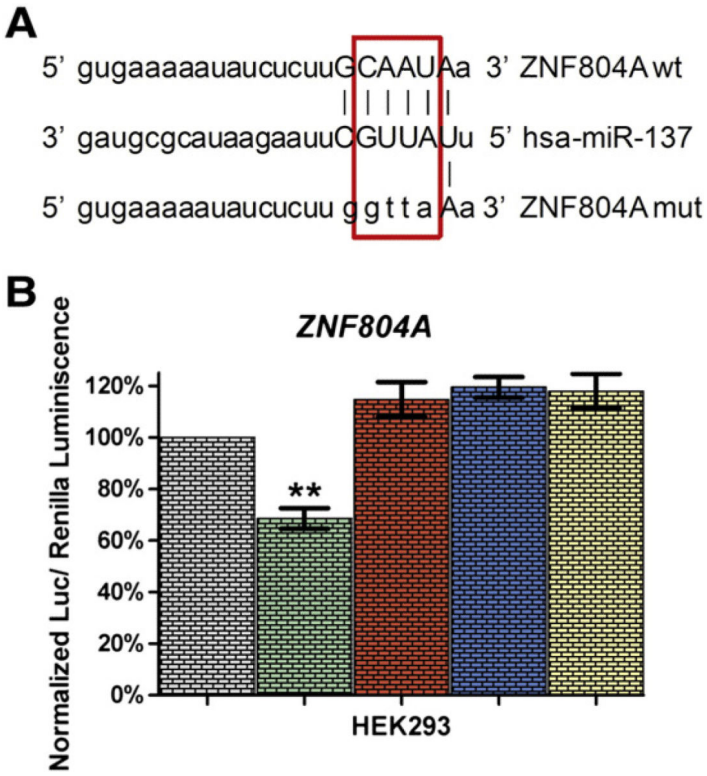

Fig. 2.

(A) The miR-137 mature sequence is aligned with wild type and mutant *ZNF804A* sequences. The alignment of nucleotide seed region (2–7nt) is shown with vertical lines. Mutated nucleotides in the seed region are boxed in the red rectangular. (B) Luciferase activity were measured in the empty vector (gray column) and compared to *ZNF804A* WT (green bar), *ZNF804A* MT (red bar), *ZNF804A* WT with anti-miR-137 oligo (blue bar), and *ZNF804A* WT in presence of miR-377 construct (yellow bar). Results were generated through the nonparametric Kruskal–Wallis test and are reported as average of four replicates from three independent experiments. Error bars represents SE±95% CI; **pb0.01.