Abstract

Aims: MicroRNAs (miRNAs) are small noncoding RNAs that regulate translational repression of target mRNAs. Accumulating evidence indicate that various miRNAs, expressed in a spatially and temporally controlled manner in the brain, play a key role in neuronal development. However, at present, the pathological implication of aberrant miRNA expression in neurodegenerative events remains largely unknown. To identify miRNAs closely associated with neurodegeneration, we performed miRNA expression profiling of brain tissues of various neurodegenerative diseases. **Methods:** We initially studied the frontal cortex derived from three amyotrophic lateral sclerosis (ALS) patients by using a microarray of 723 human miRNAs. This was followed by enlargement of study population with quantitative RT-PCR (qRT-PCR) analysis (n = 21). **Results:** By microarray analysis, we identified upregulation of miR-29a, miR-29b, and miR-338-3p in ALS brains. However, due to a great interindividual variation, we could not validate these results by qRT-PCR, but found significant downregulation of miR-29a in Alzheimer disease (AD) brains. The database search on TargetScan, PicTar, and miRBase Target identified neuron navigator 3 (NAV3), a regulator of axon guidance, as a principal target of miR-29a, and actually NAV3 mRNA levels were elevated in AD brains. MiR-29a-mediated downregulation of NAV3 was verified by the luciferase reporter assay. By immunohistochemistry, NAV3 expression was most evidently enhanced in degenerating pyramidal neurons in the cerebral cortex of AD. **Conclusions:** These observations suggest the hypothesis that underexpression of miR-29a affects neurodegenerative processes by enhancing neuronal NAV3 expression in AD brains.

Keywords: Alzheimer disease, bioinformatics, microarray, miR-29a, neuron navigator 3, real-time RT-PCR

Introduction

MicroRNAs (miRNAs) constitute a class of endogenous small noncoding RNAs conserved through the evolution [1]. miRNAs mediate posttranscriptional regulation of protein-coding genes by binding to the 3' untranslated region (3'UTR) of target mRNAs, leading to translational inhibition or mRNA degradation, depending on the degree of sequence complementarity. The primary miRNAs (pri-miRNAs) are transcribed from the intra- and inter-genetic regions of the genome by RNA polymerase II, followed by processing by the RNase II enzyme Drosha into pre-miRNAs. After nuclear export, they are cleaved by the RNAse III enzyme Dicer into mature miRNAs consisting of approximately 22 nucleotides. Finally, a single-stranded miRNA is loaded onto the RNA-induced silencing complex (RISC), where the seed sequence located at positions 2-7 from the 5' end of the miRNA plays a pivotal role in binding to the target mRNA. The miRNAs in a whole cell regulate approximately 30% of all protein-coding genes [2]. A single miRNA is capable of reducing the production of hundreds of proteins [3]. Thus, by targeting multiple transcripts and affecting expression of numerous proteins, miRNAs play a key role in cellular development, differentiation, proliferation, apoptosis and metabolism.

Increasing evidence indicated that various miRNAs, expressed in a spatially and temporally controlled manner in the brain, are involved in neuronal development, differentiation, and synaptic plasticity [4]. miR-134 localized to the synaptodendritic compartment of hippocampal neurons regulates synaptic plasticity by inhibiting translation of Lim-domain-containing protein kinase 1 (LIMK1) [5]. miR-30a-5p, a miRNA enriched in layer III pyramidal neurons in the human prefrontal cortex, decreases BDNF protein levels [6]. Because a single miRNA has a great impact on the expression of numerous downstream mRNA targets, deregulation of miRNA function in the brain affects diverse cellular signaling pathways essential for neuronal survival and protection against neurodegeneration [7].

The transcription factor Pitx3 indispensable for differentiation of dopaminergic neurons transcribes miR-133b, which in turn suppresses Pitx3 expression [8]. The levels of expression of miR-133b are substantially reduced in dopaminergic neurons in Parkinson disease (PD) brains [8]. The expression of miR-107 that inhibits the production of the β-site amyloid precursor protein cleaving enzyme 1 (BACE1) is reduced in the cerebral cortex of the patients with Alzheimer disease (AD) in the earliest stage [9]. BACE1 is also a target gene for miR-29, and the expression of miR-29a/b-1 cluster is reduced, inversely correlated with BACE1 protein levels, in the anterior temporal cortex of a subgroup of AD patients [10]. The levels of miR-298 and miR-328, both of which decrease the expression of mouse BACE1 protein, are reduced in the hippocampus of aged APPSwe/PS1 transgenic mice [11]. The expression of miR-106b that targets amyloid precursor protein (APP) is decreased in the anterior temporal cortex of AD patients [12]. Upregulation of the nuclear factor NFκB-responsive miR-146a induces downregulation of complement factor H (CFH), an anti-inflammatory mediator in AD brains [13]. Although approximately 70% of presently identified miRNAs are expressed in the brain, physiological and pathogenetic roles of most of these remain unknown [14].

In the present study, to identify miRNAs aberrantly expressed in the brains of human neurodegenerative diseases, we initially studied miRNA expression profiles of the frontal cortex of three amyotrophic lateral sclerosis (ALS) patients on a miRNA microarray. Following enlargement of the study population, we found significant downregulation of miR-29a in AD brains, being consistent with the previous observations [10]. We identified neuron navigator 3 (NAV3) as a principal target of miR-29a by bioinformatics database search and luciferase reporter assay. NAV3 expression was most evidently enhanced in degenerating pyramidal neurons in the cerebral cortex of AD. These results suggest that the interaction between miR-29a and NAV3 might play a role in neurodegenerative processes of AD.

Materials and methods

Human brain tissues

All the brain tissues of the frontal cortex were obtained from Research Resource Network (RRN), Japan. Written informed consent was obtained at autopsy form all the cases examined. The Ethics Committee of both National Center of Neurology and Psychiatry and International Medical Center of Japan approved the present study. The present study includes seven AD patients composed of four men and three women with the mean age of 69.1±9.2, and 14 non-AD patients composed of eight men and six women with the mean age of 73.1±10.1. The latter include four patients with Parkinson disease (PD), six patients with amyotrophic lateral sclerosis (ALS), and four subjects who died of non-neurological causes (NC). The neuropsychiatric disease patients were clinically diagnosed as AD, PD, or ALS by board-certified neurologists and psychiatrists, and the clinical diagnosis was verified by comprehensive examination of autopsied brains by three certified neuropathologists (KA, YS, TI). All AD cases were satisfied with the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria for diagnosis of definite AD [15], and were categorized into the stage C or B of amyloid deposition and the stage VI or IV of neurofibrillary degeneration, following the Braak staging system [16]. The postmortem interval (PMI) of the cases ranges from 1.1 to 14 hours with the average interval of 5.5 ± 4.1 hours prior to freezing the brain tissues for storage at -80℃. Although a recent study showed limited stability of specific brain-enriched miRNAs [17], we found that the quality of RNA of frozen brain tissues with PMI longer than 10 hours is still sufficient for microarray analysis (data not shown). The characteristics of the study population are summarized in Supplementary Table 1 online.

MicroRNA expression profiling

Total RNA enriched in miRNA was isolated from frozen brain tissues by using mirVana miRNA isolation kit (Ambion, Austin, TX, USA). We initially studied RNA samples isolated from the frontal cortex of three ALS patients. They were individually processed for miRNA expression profiling on a human miRNA microarray version 2 (Agilent Technologies, Palo Alto, CA, USA). The array includes 723 human miRNAs based on the miRBase release 10.1 (microrna.sanger.ac.uk). The human frontal cortex total RNA of a 79 year-old Caucasian man who died of bladder cancer (AM6810, Ambion) was utilized as a universal reference. The quality of RNA was validated by identification of 18S rRNA and 28S rRNA peaks on Agilent 2100 Bioanalyzer with a RNA 6000 nano kit (Agilent Technologies). Microarray analysis was performed according to the manufacteurer's instruction. In brief, one hundred nanogram of total RNA was dephosphorylated by calf intestine alkaline phosphatase (CIP) (Takara Bio, Shiga, Japan). Three prime end of dephosphorylated RNA was labeled with Cyanine 3-cytidine bisphosphate (pCp-Cy3) by T4 RNA ligase. The microarray was hybridized with labeled RNA at 55℃ for 20 hours, washed, and processed for scanning on an Agilent microarray scanner. The signal intensity was quantified by using Feature Extraction Software version 9.9.3.1 (Agilent Technologies).

Quantitative real-time RT-PCR

To quantify miRNA expression levels, TaqMan microRNA assay-based quantitative real-time RT-PCR (qRT-PCR) was performed on the 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), following the Delta-Delta Ct method. RNU6B was utilized for an endogenous reference to standardize miRNA expression levels. All the data were calibrated by the universal reference data.

DNase-treated total RNA was processed for cDNA synthesis using oligo$(dT)_{20}$ primers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). To

quantify mRNA expression levels, cDNA was amplified by qRT-PCR on the LightCycler ST300 (Roche Diagnostics, Tokyo, Japan) using SYBR Green I and the neuron navigator-3 (NAV3) primer sets consisting of 5'tgacccagagttgtggtctccaag3' and 5'gtccagtttggctatcccatgtgc3'. The expression levels of target genes were standardized against those of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene detected in identical cDNA samples. All the assays were performed in triplicate.

MicroRNA target prediction

The target mRNAs that have the potential binding sites for individual miRNAs were identified by searching them on public databases endowed with prediction algorithms, such as TargetScan (targetscan.org), PicTar (pictar.mdc-berlin.de), and miRBase Target (www.mirbase.org) [18].

Reporter assay

The precursor of hsa-miR-29a (pre-miR-29a) (GeneBank Accession No. AF017104) was amplified by PCR with PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA) and a set of sense and antisense primers composed of 5'cgggatcccgctggatttagtaagatttgggccct3' and 5'cccaagcttgggaacggtcaccaatacatttcctc3'. Then, it was cloned in the expression vector pBApo-CMV-Neo (Takara Bio) at the BamHI/HindIII cloning site. The 3'UTR sequence of the human NAV3 gene spanning the nucleotide position 379727-380353 that surrounds a conserved miR-29a target sequence 5'aggaacatttcctatggtgctg3'(GenBank Accession No. NC_000012) was amplified by PCR with a set of sense and antisense primers composed of 5'ctaggcgatcgcacaaatccaagaggccagtcctc3' and 5'ctaggtttaaacctctttcacttagaactggatgg3'. Then, it was cloned in the dual luciferase reporter vector psiCHECK2 (Promega, Madison, WI, USA) at the SgfI/PmeI cloning site. In this construct, a 6 bp-deletion was

introduced in the miR-29a seed sequence 5'atggtgctg3' of the NAV3 3'UTR by using QuikChange II XL site-directed mutagenesis kit (Stratagene). The psiCHECK2 vector contains the *Renilla* luciferase gene to monitor expression changes of the target gene in addition to the firefly luciferase gene controlled by a HSV-TK promoter to normalize the transfection efficacy. At 24 hours after cotransfection of the miR-29a expression vector and the luciferase reporter vector in HEK293 cells, cell lysate was processed for dual luciferase assay on a 20/20 Luminometer (Promega). All the assays were performed in triplicate.

Western blot analysis

To prepare total protein extract, frozen brain tissues were homogenized in RIPA lysis buffer composed of a cocktail of protease inhibitors (Sigma, St. Louis, MO, USA), followed by centrifugation at 12,000 rpm for 5 min at room temperature (RT). The supernatant was collected for separation on a 12% SDS-PAGE gel. The protein concentration was determined by a Bradford assay kit (BioRad, Hercules, CA, USA). After gel electrophoresis, the protein was transferred onto nitrocellulose membranes, which were immunolabeled at RT overnight with rabbit anti-NAV3 antibody (ab69868; Abcam Japan, Tokyo, Japan). We validated the specificity of the ab69868 antibody by western blot of a truncated NAV3 protein spanning amino acid residues 1366-1688 (data not shown). The membranes were incubated at RT for 30 min with HRP-conjugated anti-rabbit or goat IgG (Santa Cruz Biotechnology). The specific reaction was visualized by using a chemiluminescent substrate (Pierce, Rockford, IL, USA). After the antibodies were stripped by incubating the membranes at 50℃ for 30 min in stripping buffer composed of 62.5 mM Tris-HCl, pH 6.7, 2% SDS and 100 mM 2-mercaptoethanol, they were processed for relabeling with rabbit antibody against 14-3-3 protein (sc-629; Santa Cruz Biotechnology), an internal control for protein loading.

Immunohistochemistry

After deparaffination, tissue sections were heated in 10 mM citrate sodium buffer, pH 6.0 by autoclave at 125℃ for 30 sec in a temperature-controlled pressure chamber (Dako, Tokyo, Japan). They were exposed to 3% hydrogen peroxide-containing methanol at RT for 15 min to block the endogenous peroxidase activity. The tissue sections were then incubated with phosphate-buffered saline (PBS) containing 10% normal goat serum at RT for 15 min to block non-specific staining. The tissue sections

were incubated in a moist chamber at 4℃ overnight with rabbit anti-NAV3 antibody (ab69868). After washing with PBS, they were labeled at RT for 30 min with a horseradish peroxidase (HRP)-conjugated secondary antibody (Nichirei, Tokyo, Japan), followed by incubation with a colorizing solution containing diaminobenzidine tetrahydrochloride (DAB). For double immunolabeling, after inactivating all the antibodies by autoclaving the sections in the citrate sodium buffer, the tissue sections were incubated with mouse anti-amyloid-beta (Aβ) 11-28 peptide antibody (12B2; Immuno-Biological Laboratories, Gunma, Japan) or rabbit anti-tau antibody (paired 356; AnaSpec, San Jose, CA, USA) at 4℃ overnight, followed by incubation with alkaline phosphatase (AP)-conjugated secondary antibody (Nichirei), and colorized with New Fuchsin substrate. All the sections were exposed to a counterstain with hematoxylin. For negative controls, the step of incubation with primary antibodies was omitted.

Results

The expression of miR-29a is reduced in the frontal cortex of Alzheimer's disease

To identify miRNAs aberrantly expressed in the brains of human neurodegenerative diseases, we initially conducted microRNA expression profiling of frozen brain tissues derived from the frontal cortex of three ALS patients on a microarray. All microarray data are shown in Supplementary Table 2 online. They were filtered through the following stringent criteria, i.e. the detection of all signals above the threshold, the reference signal value exceeding 100, and the fold change expressed as the signal of ALS divided by the signal of the universal reference greater than 5. After filtration, we identified only three miRNAs, including miR-29a, miR-29b and miR-338-3p, as a group of miRNAs whose expression is substantially upregulated in all three ALS brains

(Table 1). Importantly, rodent cortical neurons express both miR-29a and miR-338-3p [19]. Since miR-29a and miR-29b are located on the identical MIRN29B/MIRN29A gene cluster located on chromosome 7q32.3, their putative biological functions are similar [10]. Thereafter, we focused our attention solely on miR-29a and miR-338-3p.

Next, we increased the number of the cases to validate microarray data by qRT-PCR. They include four non-neurological controls (NC), six patients with ALS, seven with AD, and four with PD. All qPCR data of individual subjects are shown in Supplementary Table 3 online. Although we observed a trend for upregulation in the levels of miR-29a expression in ALS versus NC, the difference did not reach the statistical significance ($p = 0.263$), due to a great interindividual variation (Figure 1a). Nevertheless, we found that miR-29a expression levels were significantly reduced in AD ($p = 0.041$), when compared with NC, while the levels of miR-29a expression were not substantially decreased in PD ($p = 0.470$) (Figure 1a). On the other hand, the levels of miR-338-3p expression were varied among the cases, and not significantly different among ALS ($p = 0.956$), AD ($p = 0.676$), and PD ($p = 0.578$), when compared with NC (Figure 1b).

Database search suggests neuron navigator-3 as one of miR-29a targets

Next, we explored putative miR-29a target genes by searching them on three distinct web-accessible microRNA target databases, including TargetScan, PicTar, and miRBase Target [18], all of which did not always suggest an identical list of target genes. They identified numerous candidates, which are arranged in order of the highest probability. When top 200 most reliable miR-29a targets identified by each program were compared, we found 11 genes shared among the three programs (Table 2). They include fibrillin 1 (FBN1), neuron navigator 3 (NAV3), collagen, type V, alpha 3 (COL5A3), collagen, type XI, alpha 1 (COL11A1), collagen, type I, alpha 2 (COL1A2), nuclear autoantigenic sperm protein (NASP), tripartite motif-containing 37 (TRIM37),

post-GPI attachment to proteins 2 (PGAP2), collagen, type VI, alpha 3 (COL6A3), inducible T-cell co-stimulator (ICOS), and mediator complex subunit 12-like (MED12L). Thus, the genes encoding extracellular matrix (ECM) components are enriched in the list of miR-29a targets. Among them, we focused our attention on NAV3 for further investigations, because NAV3, alternatively named pore membrane and/or filament interacting like protein 1 (POMFIL1), is predominantly expressed in the nervous system [20].

MiR-29a directly downregulates NAV3 expression

The TargetScan search indicated that the 3'UTR of the human NAV3 gene contains two separate miR-29a-binding seed sequences that are conserved through evolution. They are located in the nucleotide position 807-813 with TargetScan context score -0.24 and the position 1831-1837 with TargetScan context score -0.33. We cloned the former with the higher score in the luciferase reporter vector. Then, it was cotransfected with a miR-29a expression vector in HEK293 cells. Overexpression of miR-29a significantly suppressed the expression of the luciferase reporter containing the wild-type target sequence with a % reduction = 24.4 ($p = 0.014$; Figure 2, left panels). In contrast, miR-29a did not affect the expression of the vector containing a 6-bp deletion in the seed sequence ($p = 0.138$; Figure 2, right panels).

The levels of NAV3 mRNA but not of protein are elevated in AD brains

By qRT-PCR analysis, we found that the levels of NAV3 mRNA expression in the frontal cortex were much higher in AD patients, compared with NC subjects, PD patients, and ALS patients (Figure 3). By western blot analysis, the levels of expression of NAV3 protein, composed of two major bands of 100-kDa and 46-kDa, were varied among the cases and not elevated in AD brains, while the levels of 14-3-3 protein, an

internal control for protein loading, were almost constant (Figure 4a and 4b, lanes 1-21). There did not exist a clear correlation between NAV3 mRNA and protein levels in each case.

Pyramidal neurons express intense NAV3 immunoreactivity in the frontal cortex of AD brains

Finally, by immunohistochemistry, we investigated the expression of NAV3 in the frontal cortex of AD, ALS or PD. In all the brains examined, large and medium-sized pyramidal neurons in layers III and V of the cerebral cortex expressed strong NAV3 immunoreactivity located chiefly in the cytoplasm, axons and dendrites (Figure 5a-d). Notably, NAV3 immunolabeling was the most intense in neurons presenting with degenerating morphology bearing pyknotic nuclei in AD brains (Figure 5a, 5b, 5f). A population of non-pyramidal neurons also expressed much weaker NAV3 immunoreactivity, while the great majority of reactive astrocytes, microglia and oligodendrocytes are devoid of NAV3. In AD brains, a substantial population (<20%) of pyramidal neurons containing tau-immunolabeled neurofibrillary tangles (NFT) coexpressed intense NAV3 immunoreactivity (Figure 5e). In contrast, Aβ plaques did not typically express NAV3 immunoreactivity in AD brains (Figure 5f).

Discussion

To identify miRNAs aberrantly regulated in the brains of human neurodegenerative diseases, we initially studied miRNA expression profiles of frozen frontal cortex tissues of three ALS patients by using a miRNA microarray. We identified upregulation of miR-29a, miR-29b, and miR-338-3p in ALS brains. Among them, miR-338 is a biomarker candidate for neurodegeneration, because it acts as a negative regulator of neuronal differentiation by suppressing apoptosis-associated tyrosine kinase (AATK) and cytochrome oxidase complex IV (COXIV) [21, 22]. However, following enlargement of the study population by qRT-PCR, we could not verify upregulation of miR-338-3p in ALS brains. On the other hand, we identified significant downregulation of miR-29a in AD brains, being consistent with previous observations that miR-29 expression levels are reduced in the anterior temporal cortex of AD patients, associated with substantial elevation of BACE1 protein levels [10]. However, in the present study, the search on three distinct miRNA target databases, such as TargetScan, PicTar, and miRBase Target operating on different algorithms, did not identify BACE1 within top 200 miR-29a targets. These programs commonly identified NAV3 as one of top-ranking miR-29a targets. We verified the miR-29a-mediated suppression of NAV3 by luciferase reporter assay.

Previous studies showed that miR-29 is involved in translational repression of a wide range of target genes. Downregulation of miR-29 in the area surrounding the myocardial infarct core enhances fibrosis by derepressing the expression of a battery of collagen genes [23]. miR-29a represses translation of osteonectin, the most abundant non-collagenous matrix protein in the bone [24]. In line with these, we identified a panel of ECM components, such as FBN1, COL5A3, COL11A1 and COL1A2, as a group of top-ranking miR-29a target genes. The levels of expression of the miR-29 family are reduced in lung cancer, cholangiocarcinoma, chronic lymphocytic leukemia, and neuroblastoma, suggesting that miR-29 acts as a proapoptotic anti-oncomir [25-27].

The oncogenic transcription factor cMyc reduces the expression of miR-29 in cancer cells by binding directly to the miR-29 promoter region [28]. By suppressing the expression of PI3 kinase p85α and CDC42, miR-29 activates p53 and induces apoptosis in HeLa cells [29]. miR-29 acts as an enhancer of myogenic differentiation and a suppressor of rhabdomyosarcoma by targeting YY1 transcription factor [30]. miR-29a promotes the epithelial-to-mesenchymal transition of breast cancer cells by suppressing the expression of tristetraprolin [31]. All of these observations suggest that miR-29a modulates cellular differentiation and survival by regulating a wide range of target mRNAs.

NAV3 is a member of the neuron navigator protein family expressed predominantly in the central and peripheral nervous systems [20]. In adult mouse brain, NAV3 is expressed chiefly in nuclear membranes of neurons in the cerebral cortex, midbrain, cerebellum, and the hippocampal formation [20]. The NAV3 protein structure is characterized by an N-terminal calponin homology domain, several coiled-coil regions, an actin-binding domain, a GTP/ATP-binding domain, and an AAA-type ATPase domain [32]. Although the biological function of mammalian NAV3 protein in the brain remains totally unknown, a *Caenorhabditis elegans* gene named unc-53 highly homologous to NAV3, plays a key role in axon guidance [33]. Importantly, NAV2, a paralog of NAV3, plays a central role in neurite outgrowth and axonal elongation in human neuroblastoma cells [34]. Furthermore, the NAV3 gene is occasionally disrupted in primary cutaneous T-cell lymphomas by chromosomal translocation, suggesting that NAV3 acts as a tumor suppressor gene [35].

We found that the levels of NAV3 mRNA were elevated in AD brains, although we did not find a correlation between NAV3 mRNA levels by real-time RT-PCR and protein levels by western blot. The lack of the correlation between mRNA levels and protein abundance might be in part attributable to the differential stability and turnover of mRNA and protein via various post-transcriptional mechanisms, including the selective degradation of proteins by proteasome and autophagosome machineries [36].

Nevertheless, we found that pyramidal neurons in the cerebral cortex expressed strong NAV3 immunoreactivity in both AD and non-AD brains. Furthermore, a substantial population of cortical pyramidal neurons coexpressed NAV3 and NFT in AD brains, where NAV3 immunoreactivity was the most intense in neurons with degenerating morphology bearing pyknotic nuclei. A previous study performed on mouse brains showed that NAV3 immunoreactivity is constitutively expressed at the outer nuclear membrane of neurons, and it is induced in reactive astrocytes after brain injury [20]. In contrast, we did not detect NAV3 immunoreactivity in reactive astrocytes in the brains of AD, PD, and ALS. At present, it remains unknown whether enhanced expression of NAV3 in a subpopulation of cortical pyramidal neurons in AD brains reflects some pathogenetic changes or it is attributable to a compensatory mechanism against neurodegenerative events. By miRNA target database search, we found that not only miR-29, but also miR-19, miR-34, and miR-449, potentially downregulate NAV3 expression (unpublished data), suggesting that multiple miRNAs converge on the regulation of NAV3 expression. Overall, the results of the present study could propose a possible scenario that underexpression of miR-29a affects neurodegenerative processes by upregulating NAV3 and other miR-29a targets in AD brains. Further studies using large cohorts are required to clarify an active involvement of the miR-29a and NAV3 circuit in progression of neurodegeneration in AD.

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References

1. Kosik KS. The neuronal microRNA system. *Nat Rev Neurosci* 2006; **7**: 911-20
2. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* 2008; **9**: 102-14
3. Selbach M, Schwanhäusser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. Widespread changes in protein synthesis induced by microRNAs. *Nature* 2008; **455**: 58-63
4. Fineberg SK, Kosik KS, Davidson BL. MicroRNAs potentiate neural development. *Neuron* 2009; **64**: 303-9
5. Schratt GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, Kiebler M, Greenberg ME. A brain-specific microRNA regulates dendritic spine development. *Nature* 2006; **439**: 283-9
6. Mellios N, Huang HS, Grigorenko A, Rogaev E, Akbarian S. A set of differentially expressed miRNAs, including miR-30a-5p, act as post-transcriptional inhibitors of BDNF in prefrontal cortex. *Hum Mol Genet* 2008; **17**: 3030-42
7. Nelson PT, Wang WX, Rajeev BW. MicroRNAs (miRNAs) in neurodegenerative diseases. *Brain Pathol* 2008; **18**: 130-8
8. Kim J, Inoue K, Ishii J, Vanti WB, Voronov SV, Murchison E, Hannon G, Abeliovich A. A MicroRNA feedback circuit in midbrain dopamine neurons. *Science* 2007; **317**: 1220-4
9. Wang WX, Rajeev BW, Stromberg AJ, Ren N, Tang G, Huang Q, Rigoutsos I, Nelson PT. The expression of microRNA miR-107 decreases early in Alzheimer's disease and may accelerate disease progression through regulation of beta-site amyloid precursor protein-cleaving enzyme 1. *J Neurosci* 2008; **28**: 1213-23
10. Hébert SS, Horré K, Nicolaï L, Papadopoulou AS, Mandemakers W, Silahtaroglu AN, Kauppinen S, Delacourte A, De Strooper B. Loss of microRNA cluster

miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/beta-secretase expression. *Proc Natl Acad Sci USA* 2008; **105**: 6415-20

11. Boissonneault V, Plante I, Rivest S, Provost P. MicroRNA-298 and microRNA-328 regulate expression of mouse β-amyloid precursor protein-converting enzyme 1. *J Biol Chem* 2009; **284**: 1971-81
12. Hébert SS, Horré K, Nicolaï L, Bergmans B, Papadopoulou AS, Delacourte A, De Strooper B. MicroRNA regulation of Alzheimer's amyloid precursor protein expression. *Neurobiol Dis* 2009; **33**: 422-8
13. Lukiw WJ, Zhao Y, Cui JG. An NF-κB-sensitive micro RNA-146a-mediated inflammatory circuit in Alzheimer disease and in stressed human brain cells. *J Biol Chem* 2008; **283**: 31315-22
14. Kocerha J, Kauppinen S, Wahlestedt C. microRNAs in CNS Disorders. *Neuromolecular Med* 2009; **11**: 162-72
15. Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel FS, Hughes JP, van Belle G, Berg L. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurology* 1991; **41**: 479-86
16. Braak H, Alafuzoff I, Arzberger T, Kretzschmar H, Del Tredici K. Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. *Acta Neuropathol* 2006; **112**: 389-404
17. Sethi P, Lukiw WJ. Micro-RNA abundance and stability in human brain: specific alterations in Alzheimer's disease temporal lobe neocortex. *Neurosci Lett* 2009; **459**: 100-4
18. Mendes ND, Freitas AT, Sagot MF. Current tools for the identification of miRNA genes and their targets. *Nucleic Acids Res* 2009; **37**: 2419-33
19. Kim J, Krichevsky A, Grad Y, Hayes GD, Kosik KS, Church GM, Ruvkun G. Identification of many microRNAs that copurify with polyribosomes in mammalian neurons. *Proc Natl Acad Sci USA* 2004; **101**: 360-5

20. Coy JF, Wiemann S, Bechmann I, Bächner D, Nitsch R, Kretz O, Christiansen H, Poustka A. Pore membrane and/or filament interacting like protein 1 (POMFIL1) is predominantly expressed in the nervous system and encodes different protein isoforms. *Gene* 2002; **290**: 73-94

21. Aschrafi A, Schwechter AD, Mameza MG, Natera-Naranjo O, Gioio AE, Kaplan BB. MicroRNA-338 regulates local cytochrome c oxidase IV mRNA levels and oxidative phosphorylation in the axons of sympathetic neurons. *J Neurosci* 2008; **28**: 12581-90

22. Barik S. An intronic microRNA silences genes that are functionally antagonistic to its host gene. *Nucleic Acids Res* 2008; **36**: 5232-41

23. van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, Hill JA, Olson EN. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. *Proc Natl Acad Sci USA* 2008; **105**: 13027-32

24. Kapinas K, Kessler CB, Delany AM. miR-29 suppression of osteonectin in osteoblasts: regulation during differentiation and by canonical Wnt signaling. *J Cell Biochem* 2009; **108**: 216-24

25. Fabbri M, Garzon R, Cimmino A, Liu Z, Zanesi N, Callegari E, Liu S, Alder H, Costinean S, Fernandez-Cymering C, Volinia S, Guler G, Morrison CD, Chan KK, Marcucci G, Calin GA, Huebner K, Croce CM. MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proc Natl Acad Sci USA* 2007; **104**: 15805-10

26. Mott JL, Kobayashi S, Bronk SF, Gores GJ. mir-29 regulates Mcl-1 protein expression and apoptosis. *Oncogene* 2007; **26**: 6133-40

27. Xu H, Cheung IY, Guo HF, Cheung NK. MicroRNA miR-29 modulates expression of immunoinhibitory molecule B7-H3: potential implications for immune based therapy of human solid tumors. *Cancer Res* 2009; **69**: 6275-81

28. Chang TC, Yu D, Lee YS, Wentzel EA, Arking DE, West KM, Dang CV,

Thomas-Tikhonenko A, Mendell JT. Widespread microRNA repression by Myc contributes to tumorigenesis. *Nat Genet* 2008; **40**: 43-50

29. Park SY, Lee JH, Ha M, Nam JW, Kim VN. miR-29 miRNAs activate p53 by targeting p85 alpha and CDC42. *Nat Struct Mol Biol* 2009; **16**: 23-9
30. Wang H, Garzon R, Sun H, Ladner KJ, Singh R, Dahlman J, Cheng A, Hall BM, Qualman SJ, Chandler DS, Croce CM, Guttridge DC. NF-kappaB-YY1-miR-29 regulatory circuitry in skeletal myogenesis and rhabdomyosarcoma. *Cancer Cell* 2008; **14**: 369-81
31. Gebeshuber CA, Zatloukal K, Martinez J. miR-29a suppresses tristetraprolin, which is a regulator of epithelial polarity and metastasis. *EMBO Rep* 2009; **10**: 400-5
32. Maes T, Barceló A, Buesa C. Neuron navigator: a human gene family with homology to *unc-53*, a cell guidance gene from *Caenorhabditis elegans*. *Genomics* 2002; **80**: 21-30
33. Hekimi S, Kershaw D. Axonal guidance defects in a *Caenorhabditis elegans* mutant reveal cell-extrinsic determinants of neuronal morphology. *J Neurosci* 1993; **13**: 4254-71
34. Muley PD, McNeill EM, Marzinke MA, Knobel KM, Barr MM, Clagett-Dame M. The atRA-responsive gene neuron navigator 2 functions in neurite outgrowth and axonal elongation. *Dev Neurobiol* 2008; **68**: 1441-53
35. Karenko L, Hahtola S, Päivinen S, Karhu R, Syrjä S, Kähkönen M, Nedoszytko B, Kytölä S, Zhou Y, Blazevic V, Pesonen M, Nevala H, Nupponen N, Sihto H, Krebs I, Poustka A, Roszkiewicz J, Saksela K, Peterson P, Visakorpi T, Ranki A. Primary cutaneous T-cell lymphomas show a deletion or translocation affecting *NAV3*, the human *UNC-53* homologue. *Cancer Res* 2005; **65**: 8101-10
36. Laloo B, Simon D, Veilla V, Lauzel D, Guyonnet-Duperat V, Moreau-Gaudry F, Sagliocco F, Grosset C. Analysis of post-transcriptional regulations by a functional, integrated, and quantitative method. *Mol Cell Proteomics* 2009; **8**: 1777-88

Figure legends

Figure 1. MiR-29a and miR-338-3p expression levels in the brains of neurodegenerative diseases. The expression of miRNA was studied in frozen frontal cortex tissues of non-neurological controls (NC) (n = 4), ALS (n = 6), PD (n = 4), and AD (n = 7) by TaqMan microRNA assay-based qRT-PCR following the Delta-Delta Ct method. RNU6B was utilized for an endogenous reference to standardize miRNA expression levels. The results were expressed as relative expression levels after calibration with the universal reference data. The panels **(a, b)** represent **(a)** miR-29a and **(b)** miR-338-3p. The p-value by Student's t-test indicates (a) ★ 0.263, ★★ 0.041, ★★★ 0.470 and (b) ★ 0.956, ★★ 0.676, ★★★ 0.578.

Figure 2. Dual luciferase assay of miR29a-mediated downregualtion of NAV3. The pre-miR-29a sequence was cloned in the expression vector pBApo-CMV-Neo, while the 3'UTR sequence of the human NAV3 was cloned in the dual luciferase reporter vector psiCHECK2 that contains the *Renilla* (R) luciferase gene to monitor expression changes of NAV3 in addition to the firefly (F) luciferase gene to normalize the transfection efficacy. A 6 bp-deletion was introduced in the miR-29a seed sequence 5'ATGGTGCTG3' of the NAV3 3'UTR. At 24 hours after cotransfection of the miR-29a expression vector or the empty pBApo-CMV-Neo vector with the luciferase reporter vector in HEK293 cells, cell lysate was processed for dual luciferase assay. Two series of the experiments were performed. Each set represents the reporter vector containing **(the left)** the wild-type (WT) NAV3 3'UTR or **(the right)** the 6 bp-deletion mutant (MT) NAV3 3'UTR. The R/F signal ratio is shown. The p-value by Student's t-test indicates ★ 0.014, ★★ 0.138, ★★★ 0.037, and ★★★★ 0.289.

Figure 3. NAV3 mRNA expression levels in the brains of neurodegenerative diseases. The expression of NAV3 mRNA was studied in frozen frontal cortex tissues