

*Forum Minireview***MicroRNAs and Their Therapeutic Potential for Human Diseases:
Aberrant MicroRNA Expression in Alzheimer's Disease Brains**Jun-ichi Satoh^{1,*}¹*Department of Bioinformatics and Molecular Neuropathology, Meiji Pharmaceutical University,
2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan**Received May 27, 2010; Accepted June 25, 2010*

Abstract. MicroRNAs (miRNAs) are a group of small noncoding RNAs that regulate translational repression of multiple target mRNAs. The miRNAs in a whole cell regulate greater than 30% of all protein-coding genes. The vast majority of presently identified miRNAs are expressed in the brain in a spatially and temporally controlled manner. They play a key role in neuronal development, differentiation, and synaptic plasticity. However, at present, the pathological implications of deregulated miRNA expression in neurodegenerative diseases remain largely unknown. This review will briefly summarize recent studies that focus attention on aberrant miRNA expression in Alzheimer's disease brains.

Keywords: Alzheimer's disease, bioinformatics, microRNA, neuropathology

1. Introduction

MicroRNAs (miRNAs) constitute a class of endogenous small noncoding RNAs conserved through evolution (1). They 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 III 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 – 8 from the 5' end of the miRNA plays a pivotal role in binding to the target mRNA. At present, more than 900 human miRNAs have been identified (miRBase Release 15). A single miRNA reduces the production of hundreds of proteins (2). The miRNAs in a whole cell regulate approximately 30% of all protein-coding genes (3). Furthermore, some miRNAs activate

transcription and translation of the targets (4, 5). 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 indicates that a battery of miRNAs, expressed in a spatially and temporally controlled manner in the brain, are involved in neuronal development and differentiation (6). miR-134, localized to the synaptodendritic compartment of hippocampal neurons, regulates synaptic plasticity by inhibiting translation of Lim-domain-containing protein kinase 1 (LIMK1) (7). miR-30a-5p, enriched in layer III pyramidal neurons in the human prefrontal cortex, decreases brain-derived neurotrophic factor (BDNF) protein levels (8). Because a single miRNA has a great impact on the expression of numerous downstream mRNA targets, deregulated expression of even a small set of miRNAs in the brain affects diverse cellular signaling pathways essential for neuronal survival and protection against neurodegeneration (9). Importantly, approximately 70% of presently identified miRNAs are expressed in the brain, but the pathological implications of deregulated miRNA expression in neurodegenerative diseases remain largely unknown (10). The present review will briefly summarize recent studies that focus attention on aberrant miRNA expression in the brains of Alzheimer's disease (AD).

*Corresponding author. satoj@my-pharm.ac.jp

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2. Aberrant miRNA expression in AD brains

AD is the most common cause of dementia worldwide, affecting the elderly population, characterized by the hallmark pathology of amyloid- β ($A\beta$) deposition and neurofibrillary tangle (NFT) formation in the brain. Although the precise mechanisms underlying neurodegeneration in AD remain mostly unknown, previous studies support a role of the complex interaction between genetic and environmental factors (11). Furthermore, recent studies indicate the cardinal involvement of deregulated miRNA expression in the pathogenesis of AD (Table 1).

By using a nylon membrane-bound DNA array, a previous study identified upregulated expression of miR-9 and miR-128 in the hippocampus of AD brains, although they did not characterize the target mRNAs (12). More recently, the same group showed that the levels of miR-146a expression are elevated in the hippocampus and the superior temporal cortex of AD (13, 14). Importantly, the expression of miR-146a is directly regulated

by nuclear factor-kappa B (NF- κ B), and it targets the mRNA of complement factor H (CFH), a negative regulator of the inflammatory response in the brain. They validated upregulation of NF- κ B in the neocortex of AD by gel shift assay, suggesting that activation of NF- κ B induces miR-146a that amplifies inflammatory neurodegeneration via reducing CFH in AD brains. Recently, the same group revealed the instability of brain-enriched miRNAs that contain a high content of AU and UA dinucleotides (14).

A previous study showed that miR-107 targets the β -site amyloid precursor protein (APP)-cleaving enzyme 1 (BACE1), a rate-limiting enzyme for $A\beta$ production (15). By analyzing a microarray, miR-107 levels are substantially reduced in the temporal cortex of the patients affected with mild cognitive impairment (MCI) and those with AD (15). These observations suggest that downregulation of miR-107 begins at the very early stage of AD. A different study showed that miR-29 also targets BACE1 (16). By using a microarray containing 328 miRNAs, they identified reduced expression of the miR-

Table 1. Aberrant expression of microRNAs in Alzheimer disease (AD) brains

Authors, years, and reference No.	Brains of AD patients	MicroRNAs aberrantly expressed	Upregulation or downregulation	Target mRNAs characterized	Target prediction and validation	Possible pathological implications
Lukiw, 2007 (12)	Hippocampus of 5 AD patients, 5 age-matched controls, and 5 fetal brains	miR-9, miR-128	up	ND	ND	general neuropathology of AD
Lukiw et al., 2008 (13)	Hippocampus and superior temporal lobe of 23 AD patients and 23 age-matched controls	miR-146a	up	CFH	ND; introduction of an anti-miR-146a oligonucleotide	suppression of anti-inflammatory response
Wang et al., 2008 (15)	Temporal cortex of 6 AD and 6 MCI patients and 11 non-demented controls	miR-107	down	BACE1	miRanda, TargetScan, PicTar; luciferase reporter assay	increased production of $A\beta$
Hébert et al., 2008 (16)	Anterior temporal cortex of 5 AD patients and 5 age-matched controls	miR-29a/b-1	down	BACE1	miRanda, TargetScan, PicTar, miRBase; luciferase reporter assay	increased production of $A\beta$
		miR-15a, miR-9, miR-19b	down	BACE1	miRanda, TargetScan, PicTar, miRBase; not validated	
		let-7i, miR-15a, miR-101, miR-106b	down	APP	miRanda, TargetScan, PicTar, miRBase; not validated	
		miR-22, miR-26b, miR-93, miR-181c, miR-210, miR-363	down	ND	ND	
		miR-197, miR-511, miR-320	up	ND	ND	

Authors, years, and reference No.	Brains of AD patients	MicroRNAs aberrantly expressed	Upregulation or downregulation	Target mRNAs characterized	Target prediction and validation	Possible pathological implications
Cogswell et al., 2008 (21)	Cerebellum of 15 AD patients and 12 non-demented controls	miR-27a, miR-27b, miR-34a, miR-100, miR-125b, miR-381, miR-422a	up	ND	miRanda, RNAhybrid	general neuropathology of AD
		miR-9, miR-98, miR-132, miR-146b, miR-212, miR-425	down	ARHGAP32 by miR-132		
	Hippocampus of 15 AD patients and 12 non-demented controls	miR-26a, miR-27a, miR-27b, miR-30e-5p, miR-34a, miR-92, miR-125b, miR-145, miR-200c, miR-381, miR-422a, miR-423	up	ND		
		miR-9, miR-30c, miR-132, miR-146b, miR-210, miR-212, miR-425	down	ARHGAP32 by miR-132		
		miR-27a, miR-27b, miR-29a, miR-29b, miR-30c, miR-30e-5p, miR-34a, miR-92, miR-100, miR-125b, miR-145, miR-148a, miR-381, miR-422a, miR-423	up	ND		
Hébert et al., 2009 (17)	Anterior temporal cortex of 19 AD patients and 11 non-demented controls	miR-9, miR-26a, miR-132, miR-146b, miR-200c, miR-210, miR-212, miR-425	down	ARHGAP32 for miR-132	miRanda, TargetScan, PicTar, miRBase; luciferase reporter assay	increased production of A β
		miR-106b	down	APP		
Sethi and Lukiw, 2009 (14)	Temporal lobe cortex of 6 AD and 13 non-AD patients and 6 controls	miR-9, miR-125b, miR-146a	up	ND	ND	general neuropathology of AD
Nunez-Iglesias et al., 2010 (22)	Parietal lobe cortex of 5 AD patients and 5 age-matched controls	miR-18b, miR-34c, miR-615, miR-629, miR-637, miR-657, miR-661, mir-09369, mir-15903, mir-44691	ND	positively correlated with target mRNAs	miRanda, TargetScan, PicTar; not validated	general neuropathology of AD
		miR-211, miR-216, miR-325, miR-506, miR-515-3p, miR-612, miR-768-3p, mir-06164, mir-32339, mir-45496	ND	negatively correlated with target mRNAs	miRanda, TargetScan, PicTar; not validated	
Shioya et al., 2010 (23)	Frontal lobe of 7 AD patients and 4 non-neurological controls	miR-29a	down	NAV3	TargetScan, PicTar, miRBase; luciferase reporter assay	a putative compensatory mechanism against neurodegenerative events

AD, Alzheimer's disease; MCI, mild cognitive impairment; ND, not described; BACE1, beta-site APP-cleaving enzyme 1; APP, amyloid precursor protein; NAV3, neuron navigator 3; A β , amyloid-beta; and ARHGAP32, Rho GTPase activating protein 32 (p250GAP).

29a/b-1 cluster, which inversely correlated with BACE1 protein levels, in the anterior temporal cortex of AD (16). The database search on miRanda, TargetScan, PicTar, and miRBase (MicroCosm) predicted the presence of several binding sites in the human BACE1 3'UTR for miR-9, miR-15a, miR-19b, and miR-29a/b-1 and in the human APP 3'UTR for let-7, miR-15a, miR-101, and miR-106b, all of which are downregulated in AD brains. They validated miR-29a-mediated downregulation of

BACE1 by the luciferase reporter assay. Furthermore, an introduction of the miR-29a/29b-1 precursors reduced secretion of A β from HEK293 cells stably expressing APP Swedish (APPswe) (16). Subsequently, the same group reported reduced expression of miR-106b capable of targeting APP in the anterior temporal cortex of AD, although they did not find a clear-cut inverse correlation between the levels of miR-106b and APP protein expression (17).

Thus, different studies identified various miRNAs in AD brains. This variability is in part attributable to disease-specific and nonspecific interindividual differences, including differences in age, sex, the brain region, the pathological stage, and the postmortem interval (PMI), since most studies are performed on a fairly small number of samples and controls, complicated by variable confounding factors (Table 1). With respect to animal models of AD, a recent study showed that the expression levels of a noncoding BACE1-antisense (BACE1-AS) RNA that enhances BACE1 mRNA stability are elevated in the brains of Tg19959 APP transgenic mice and those of AD (18). Furthermore, the levels of miR-298 and miR-328, both of which reduce the expression of mouse BACE1 protein, are decreased in the hippocampus of aged APPswe/PS1 transgenic mice (19). All of these observations suggest that abnormally reduced expression of a set of miRNAs accelerates A β deposition via overproduction of BACE1, the enzyme and/or APP, the substrate in AD brains. It is worthy to note that genetic variability involving miRNA-binding sites in both BACE1 and APP 3'UTRs does not serve as a major risk factor for development of AD (20), suggesting that minor variations in miRNA-binding sequences do not play a central role in upregulation of BACE1 and APP in AD brains. These observations sound reasonable because miRNAs generally induce translational inhibition without requiring the perfect match in the binding sequences of target mRNAs.

By the TaqMan microRNA assay-based semi-quantitative RT-PCR method, a previous study intensively characterized miRNA expression profiles of the brains and CSF samples derived from AD patients and nondemented controls (21). They found that a wide variety of miRNAs are either upregulated or downregulated in specific regions of AD brains at defined pathological stages, and the levels of all miR-30 family members are coordinately elevated in CSF samples of AD. They predicted miRNA-binding sites of the targets and then identified a relevant biological pathway by the hypergeometric enrichment method named miRNAPath. As a result, the analysis identified a meaningful relationship between upregulated miRNAs and metabolic pathways in AD brains such as insulin signaling, glycolysis, and glycogen metabolism (21). By combining microarray-based miRNA expression profiling and transcriptome analysis of the brains of AD patients and age-matched control subjects, a recent study showed that the levels of various miRNAs are not only negatively but also positively correlated with those of the potential target mRNAs (22). The expression of miR-211 shows a negative correlation with mRNA levels of BACE1, RAB43, LMNA, MAP2K7, and TADA2L, whereas the expression of mir-

44691 has a positive correlation with mRNA levels of CYR61, CASR, POU3F2, GGPR68, DPF3, STK38, and BCL2L2 in AD brains, supporting the previous observations that certain miRNAs activate transcription and translation of targets (4, 5).

3. miR-29a decreased in AD brains targets NAV3

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 microarray containing 723 miRNAs (23). 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 microarray data were filtered through the following stringent criteria, that is, 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 (23).

Next, we increased the number of the cases to validate microarray data by TaqMan microRNA assay-based quantitative real-time RT-PCR (qRT-PCR). They include four non-neurological controls (NC), six patients with ALS, seven with AD, and four with PD. Most importantly, all the brains were carefully evaluated by pathological examination of the corresponding paraffin sections, following the Braak staging system to characterize the stage of AD pathology (24). Although we observed a trend for upregulation of miR-29a expression levels in ALS versus NC, the difference did not reach statistical significance due to a great interindividual variation. However, unexpectedly, we found that miR-29a expression levels were significantly reduced in AD when compared with NC ($P = 0.041$) (Fig. 1a). On the other hand, the levels of miR-338-3p were not significantly different among the study groups, although a larger cohort is required to obtain a statistical power enough to make a definitive conclusion (23). Since miR-29a and miR-29b are located on the identical MIRN29B/MIRN29A gene cluster on chromosome 7q32.3, their biological functions are presumably similar. Therefore, we further focused our attention solely on miR-29a.

Then, we explored putative miR-29a target genes by searching them on web-accessible microRNA target databases, including TargetScan, PicTar, and miRBase (MicroCosm). When the top 200 most reliable miR-29a targets were extracted by each program, we found 11

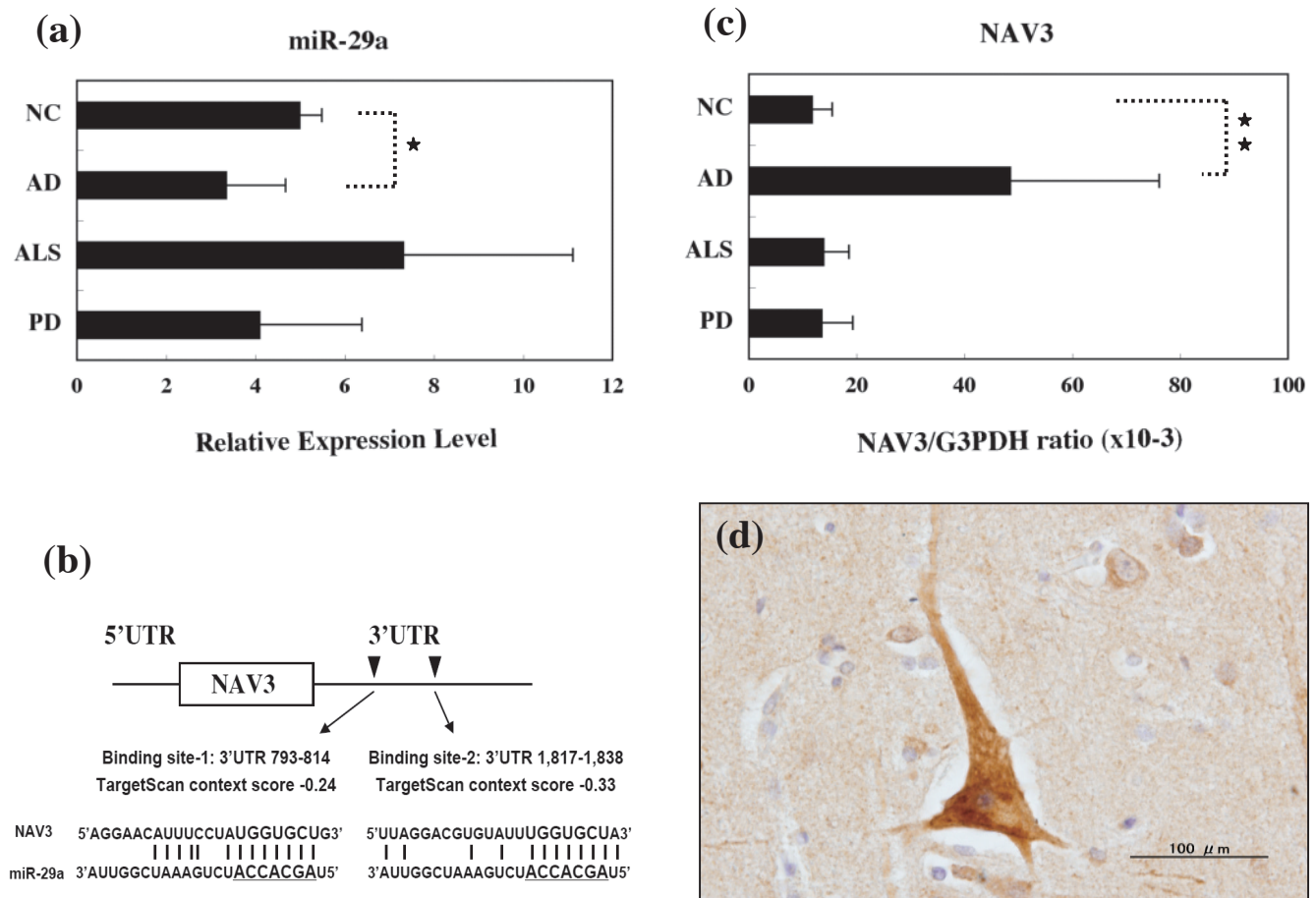


Fig. 1. MicroRNA-29a decreased in AD brain targets NAV3. a) qRT-PCR of miR-29a expression. The expression of miR-29a was studied in frozen frontal cortex tissues of non-neurological controls (NC) (n = 4), Alzheimer's disease (AD) (n = 7), amyotrophic lateral sclerosis (ALS) (n = 6), and Parkinson disease (PD) (n = 4) by TaqMan microRNA assay-based quantitative RT-PCR (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 (AM6810, Ambion) data. The star indicates $P = 0.041$ by Student's t -test. b) Two miR-29a-binding sites located in 3'UTR of the NAV3 gene. The 3'UTR of the human NAV3 gene contains two separate, evolutionarily conserved miR-29a-binding sites, located in the nucleotide positions 793 – 814 and 1,817 – 1,838. The seeding sequence of miR-29a is underlined. c) qRT-PCR of NAV3 mRNA expression. The expression of NAV3 mRNA was studied in frozen frontal cortex tissues of NC (n = 4), AD (n = 7), ALS (n = 6), and PD (n = 4) by qRT-PCR. The levels of NAV3 mRNA are standardized against those of G3PDH mRNA detected in identical cDNA samples. The double stars indicate $P = 0.028$ by Student's t -test. d) Enhanced NAV3 immunoreactivity in degenerating pyramidal neurons in AD brains. The expression of NAV3 was studied in the frontal cortex tissue sections of AD by immunohistochemistry using anti-NAV3 antibody (ab69868, Abcam).

genes shared among the three programs: 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) (23). Among them, NAV3, alternatively named pore membrane and/or filament interacting like protein 1 (POMFIL1), was selected for

further investigations because it is predominantly expressed in the nervous system (25). Although the precise biological function of NAV3 in the human brain remains unknown, a *Caenorhabditis elegans* gene named unc-53, highly homologous to NAV3, plays a key role in axon guidance (26). Although a previous study identified BACE1 as the most important target of miR-29a (16), we found BACE1 as one of miR-29a targets ranking 750th of 850 candidates on TargetScan and 197th of 326 candidates on PicTar.

The TargetScan search indicated that the 3'UTR of the

human NAV3 gene contains two separate miR-29a-binding sequences, highly conserved through evolution, located in the nucleotide positions 793–814 and 1,817–1,838 (Fig. 1b). We cloned the former in the luciferase reporter vector, which was cotransfected with a miR-29a expression vector in HEK293 cells. The expression of miR-29a significantly suppressed activation of the luciferase reporter following the wild-type target sequence, whereas miR-29a did not affect the expression of the reporter following the target sequence with a 6-bp deletion corresponding to the seed sequence (23). Importantly, qRT-PCR indicated that the levels of NAV3 mRNA expression were significantly higher in the frontal cortex of AD compared with NC ($P = 0.028$) (Fig. 1c), suggesting that NAV3 is indeed a candidate for an miR-29a target in vivo in AD brains. However, we could not validate elevation of NAV3 protein levels in AD brains by western blot analysis due to a great interindividual variation (23). The lack of the correlation between mRNA levels and protein abundance might be in part attributable to the complexity of brain tissues composed of various cellular constituents with differential expression of target proteins that affects the efficacy of purification, or alternatively 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 in individual cells (27).

Finally, we investigated NAV3 expression in the frontal cortex of AD, ALS, or PD by immunohistochemistry. In all the brains examined, large and medium-sized pyramidal neurons in layers III and V of the cerebral cortex expressed NAV3 immunoreactivity located chiefly in the cytoplasm, axons, and dendrites (23). Notably, NAV3 immunolabeling was the most intense in neurons presenting with degenerating morphology in AD brains (Fig. 1d). In AD brains, a substantial population (<20%) of pyramidal neurons containing tau-immunolabeled NFT coexpressed intense NAV3 immunoreactivity. However, at present, it remains unknown whether enhanced expression of NAV3 in a subpopulation of cortical pyramidal neurons in AD brains reflects a pathogenetic change or a compensatory mechanism against neurodegenerative events. A recent study by *in situ* hybridization (ISH) showed that each subpopulation of neurons in different cerebral cortical layers expresses a distinct set of miRNAs in the human transentorhinal cortex (TEX), and the expression pattern is greatly affected by AD pathology from the earliest stage of the disease (28).

In conclusion, increasing evidence indicates that aberrant expression of various miRNAs, both upregulated and downregulated species, plays an active role in the

pathological processes of AD (Table 1). A single miRNA has a great impact on the cellular function by mostly suppressing and occasionally activating numerous downstream mRNA targets. Therefore, even the small-scale turbulence occurring in the miRNA-mediated gene regulation affects various biological pathways involved in both neurodegeneration and neuroprotection in AD brains. We need to further elucidate the entire picture of the pathophysiological interaction among miRNAs, mRNAs, and proteins in AD brains.

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