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MicroRNAs in Alzheimer's disease: differential expression in hippocampus and cell-free cerebrospinal fluid

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ABSTRACT

MicroRNAs (miRNAs) are small, noncoding RNAs that function in complex networks to regulate protein expression. In the brain, they are involved in development and synaptic plasticity. In this study, we aimed to identify miRNAs with a differential expression in either hippocampus or cerebrospinal fluid (CSF) from Alzheimer's disease (AD) patients and age-matched nondemented control subjects using quantitative polymerase chain reaction. In hippocampus, we also differentiated between AD patients with an intermediate stage, according to Braak III/IV stage, and a late stage, characterized according to Braak VI stage. Eight selected miRNAs were analyzed in hippocampus, and the expression of miR-16, miR-34c, miR-107, miR-128a, and miR-146a were differentially regulated. In CSF, out of 8 selected miRNAs only miR-16 and miR-146a could be reliably detected. In addition, we identified an effect of blood contamination on the CSF levels of miR-16, miR-24, and miR-146a. For group comparisons, we therefore selected CSF samples absent of, or containing only low numbers of blood cells. Levels of miR-146a were significantly decreased in CSF of AD patients. In conclusion, the abnormal expression of several miRNAs in hippocampus of intermediate- and late-stage AD patients suggests their involvement in AD pathogenesis, and low levels of miR-146a in CSF were associated with AD.

1. Introduction

Alzheimer's disease (AD) is the most common type of dementia with more than 13% of the elderly older than the age of 65 suffering from its devastating symptoms. Although incidence of the disease keeps increasing, no cures have been developed. Effective treatment is not only hampered by the lack of curing medicine, but also by the lack of knowledge on the disease mechanisms and the difficulties of accurately diagnosing AD in its earliest stages before clinical symptoms occur. Current cerebrospinal fluid (CSF) biomarkers, including amyloid-β $(Aβ)_{42}$, total tau, and phosphorylated tau, can support the clinical diagnosis of AD with a sensitivity and specificity of 85%–90%, if AD patients are compared with nondemented control subjects (Shaw et al., 2009; Welge et al., 2009). However, in differentiating AD from other types of dementia, the current biomarkers have their limitations.

In the past years, microRNAs (miRNAs) have gained increasing attention in studies on neurodegenerative diseases. An estimated 70% of these small, approximately 20 nucleotides long, nonprotein-coding RNAs are expressed in the brain and many are brain-specific or brain-enriched. miRNAs act by binding to the 3′ untranslated region of messenger RNAs, and inhibit translation, causing downregulation of specific targets. A single miRNA can bind multiple messenger RNAs and can therefore fine-tune the expression and function of numerous proteins. Vice versa, several miRNAs can be involved in the regulation of 1 single protein. The first study that described abnormal expression of miRNAs in AD was published in 2007 (Lukiw, 2007). Three miRNAs, miR-9, miR-125b, and miR-128, were upregulated in the hippocampus of AD patients compared with age-matched control subjects.

Furthermore, some miRNAs might inhibit expression of amyloid precursor protein (APP) in mice (e.g., miR-16; Liu et al., 2012) or β-site APP cleaving enzyme 1 (BACE1) (e.g., miR-107; Wang et al., 2008) in humans. Both proteins are involved in the production of Aβ, which accumulates in AD brains. Higher Aβ production could be explained by decreases in miRNAs that regulate APP or BACE1.

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Other miRNA expression studies demonstrated that the expression of several miRNAs was up- or downregulated in the hippocampus and cortex of AD patients (Hébert et al., 2008; Wang et al., 2008). Therefore, miRNAs might be added to the list of targets with potential to be used in treatment or diagnosis. Two studies focused on miRNA quantification in body fluids of AD patients (Cogswell et al., 2008; Schipper et al., 2007), and in 1 of them, CSF was analyzed in parallel with hippocampus, medial frontal gyrus, and cerebellum tissue. Several differentially expressed miRNAs in AD were identified in these studies, but these results have not been confirmed.

The present investigation was designed to assess differential expression of miRNAs in brain tissue and translate these observations into potential new CSF biomarkers for AD. We show that some miRNAs are indeed differentially regulated in the hippocampus of AD patients compared with nondemented control subjects, the degree of which is dependent on the Braak stage. In CSF, we demonstrate that miRNA expression levels might strongly depend on the number of blood-derived cells in the sample, which we took into account for comparative measurements. Of 8 miRNA targets, only 2 are detected in CSF, and concentrations of miR-146a are lower in AD compared with control subjects.

2. Methods

2.1. Subjects

Tissue samples from hippocampus, containing all 4 cornu ammonis regions and the dentate gyrus, were obtained postmortem from patients that were all scored according to Consortium to Establish a Registry for Alzheimer's Disease and Braak stage (Table 1). Postmortem delay was between 3 and 6 hours and similar in the AD and control groups. Groups were age- and sex-matched. AD samples with Braak stages VI ($n = 10$) and control samples ($n = 6$) were obtained from the Radboud University Medical Centre, Nijmegen. Additional control hippocampus samples ($n = 5$) and samples with Braak stages III ($n = 5$) and IV ($n = 5$) were obtained from the Netherlands Brain Bank, Amsterdam. CSF of control subjects ($n = 22$) and AD patients ($n = 20$) were obtained from Radboud University Medical Centre, Nijmegen. AD patients had been diagnosed according to clinical National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association Alzheimer's criteria (McKhann et al., 1984) and Mini Mental State Examination (Table 2). Control subjects were identified as not having dementia.

Table 1
Hippocampus tissue sample data

	Control	Early AD	AD	p[a]
Number of patients[b]	11	10	10	
Sex (male/female)	4/7	5/5	3/7	NS ($p = 0.643$)
Mean age, y	82.5 ± 7.01	81.9 ± 6.1	77.8 ± 10.6	NS ($p = 0.38$)
Postmortem delay, h	4.5 ± 1.1	4.4 ± 0.7	3.7 ± 0.8	NS ($p = 0.144$)
CERAD score (0/A/B/C)	0 or A	B or C	B or C	NA
Braak stage (0–VI)	≤II	III or IV	VI	NA
RNA concentration (ng) per tissue weight (mg)	419 ± 162	433 ± 253	240 ± 78	$p = 0.011$

Values are expressed as mean ± SD.
Key: AD, Alzheimer's disease; CERAD, Consortium to Establish a Registry for Alzheimer's Disease; NA, not applicable; NS, not significant.
[a] p for differences in sex using χ^2 test, and for differences in age, postmortem delay, and RNA concentration/tissue weight using analysis of variance and Bonferroni's multiple comparison test for normally distributed data or Kruskal-Wallis test and Dunn's multiple comparison test for non-Gaussian distribution.
[b] All AD cases (Braak VI) were sporadic. Two early AD cases (Braak III/IV) had a family history in dementia, but for other early AD cases this was not known.

Table 2
CSF sample data

Variable	Control	AD	p[a]
Number of patients	20	20	
Sex (male/female)	10/10	10/10	NS
Mean age, y[b]	63.7 ± 9.8	69.7 ± 9.6	NS ($p = 0.058$)
MMSE score	ND	20.2 ± 3.4[c]	NA
Total protein, mg/L	486 ± 99	568 ± 148	$p = 0.126$
Number of cells per μL			
Erythrocytes	13.4 ± 29	15.9 ± 47	$p = 0.676$
Leukocytes	1.1 ± 0.8	0.6 ± 0.9	$p = 0.773$

Values are expressed as mean ± SD.
Key: AD, Alzheimer's disease; CSF, cerebrospinal fluid; MMSE, Mini Mental State Examination; NA, not applicable; ND, not determined; NS, not significant.
[a] p for differences in age and total protein using Student t test or Mann-Whitney U test.
[b] At the time of lumbar puncture.
[c] For 5 AD samples, the MMSE score was not determined, diagnosis of AD was based only on clinical National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association Alzheimer's criteria.

They had been assessed for another neurological disorder but were diagnosed with either a systemic disease without neurological manifestations or, for example, tension-type headache or burnout. CSF sample groups were matched for age and sex (Table 2), and were selected according to the absence of cells or presence of only low numbers of leukocytes (1–2 cells per μL) and erythrocytes (less than 200 cells per μL) (Table 2). CSF samples were obtained using lumbar puncture, collected in polypropylene tubes, and, after routine investigation, centrifuged, aliquoted, and stored at −80 °C. Use of lumbar CSF was approved by the Medical Ethical Committee, and informed consent was obtained from the subjects or their caregivers. Routine investigation also included manual counting of cells using a counting chamber.

2.2. Preparation of cell-spiked CSF samples

To evaluate the effects of blood contamination on miRNA levels in CSF, we used 2 blood samples, obtained using vena puncture from 2 healthy volunteers, and CSF samples that did not contain blood cells determined by manual counting. A concentration gradient of whole blood was spiked into 0.5-mL aliquots of the CSF samples, corresponding to final erythrocyte counts of 10 to 20,000 cells per μL CSF. Numbers of cells were confirmed using flow cytometry. CSF samples were left for 3 hours at room temperature, then 1 series (series 1) was stored at −80 °C, and the other series (series 2) was first centrifuged for 10 minutes at 3000 rpm at 10 °C to remove cells, and only the supernatant was retained at −80 °C. In this way, the various ways of processing CSF in clinical practice were simulated.

2.3. RNA isolation of tissue samples

Twenty milligrams of hippocampus tissue was used by cutting sections (each 7-μm thick) using a cryostat. Total RNA was isolated from hippocampus tissue and CSF samples using the *miR*Vana miRNA Isolation kit (Life Technologies, Carlsbad, CA, USA). In brief, hippocampus sections were lysed with lysis buffer supplied in the kit, and homogenated. Lysed samples were then subjected to a phenol-chloroform extraction step, followed by the addition of 100% ethanol. Next, samples were loaded onto a glass-fiber filter and subjected to several washing steps. Finally, total RNA was eluted from the filter cartridge with 100 μL sterile water, that had been heated to 95 °C. The RNA concentration of hippocampus extracts was determined using a Nanodrop ND 2000 instrument (Thermo Scientific, Rockford, IL, USA).