Reduced Levels of hsa-miR-342-5p in Plasma Are Associated With Worse Cognitive Evolution in Patients With Mild Alzheimer's Disease

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Research

Keywords: Alzheimer's disease, Cognitive decline, miRNA, miR-342-5p

Posted Date: December 16th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-125973/v1

Abstract

Background: Progressive cognitive decline is the most relevant clinical symptom of Alzheimer's disease (AD). However, the rate of cognitive decline is highly variable between patients. Synaptic deficits are the neuropathological event most correlated with cognitive impairment in AD. Considering the important role of microRNAs (miRNAs) in regulating synaptic plasticity, our objective was to identify the plasma miRNAs associated with the rate of cognitive decline in patients with mild AD.

Methods: To discover the miRNAs related to the rate of cognitive impairment, we analysed 754 plasma miRNAs from 19 women diagnosed with mild AD using TaqMan low-density array cards. The patients were grouped based on the rate of decline in the MMSE score after two years (<4 points (N=11) and ≥ 4 points (N=8)). The differentially expressed miRNAs between the two groups were validated in an independent cohort of men and women (N=53) with mild AD using RT-qPCR.

Results: In the discovery cohort, 17 miRNAs were differentially expressed according to the fold change between patients with faster declines in cognition and those with slower declines. miR-342-5p demonstrated differential expression between the groups and a good correlation with the rate of cognitive decline in the validation cohort (r=-0.28; p=0.026). This miRNA had a lower expression level in patients who suffered from more severe decline than in those who were cognitively more stable after two years (p=0.049).

Conclusion: Lower levels of miR-342-5p in plasma were associated with faster cognitive decline in patients with mild AD after two years of follow-up.

Introduction

Alzheimer's disease (AD) is an irreversible, progressive brain disorder that gradually destroys memory and other thinking skills and, eventually, leads to complete dependency in daily life activities [1]. The rate of cognitive decline is highly variable among patients, with some having a faster course than others. Studies have demonstrated a strong association between the rate of cognitive decline and mortality in AD patients [2]. In addition, understanding the physiopathological processes underlying this variability is highly important because of the great potential benefits for the development of effective therapeutic approaches.

Extracellular amyloid plaques (accumulation of amyloid-β (Aβ) protein) and intracellular neurofibrillary tangles (aggregations of hyperphosphorylated tau protein, P-tau) are two main pathological hallmarks of AD. Although both of these pathological characteristics are considered specific to AD, none of them have demonstrated a good correlation with the clinical symptoms [3]. For example, the accumulation of Aβ plaques, known as the first pathological event of AD according to the "amyloid cascade hypothesis", peaks at the asymptomatic stage of the disease [4]. It is widely accepted that neuronal injury, particularly synaptic loss, is the AD neuropathological alteration that most correlates with cognitive dysfunction [3, 5]. The measurement of some proteins released in CSF, such as tau and neurofilament light chain (NfL), can

be used to assess neurodegeneration and has shown a good correlation with cognitive decline [3, 6]. However, they are not specific to neuronal damage due to AD. In addition, the method for obtaining CSF is invasive, which limits its use for the concurrent monitoring of therapeutic trials and drug efficacy and for longitudinal studies where multiple lumbar punctures are needed. Therefore, searching for new biomarkers in the circulatory system that can predict the rate of cognitive decline and reveal neuropathological alterations specific to AD is of great importance.

MicroRNAs (miRNAs) are small (typically 22 nt in size) noncoding RNA molecules that regulate the activity of specific messenger RNA (mRNA) targets by binding to their 3´-untranslated regions (UTRs). This union suppresses the translation of the mRNA or induces its degradation. miRNAs are present in tissues and bodily fluids and play important roles in a wide range of physiological and pathological processes, including AD [7–9]. Circulatory miRNAs have shown high stability [10, 11], making them ideal biomarker targets. Increasing evidence indicates that miRNAs play a pivotal role in the regulation of synapses and synaptic plasticity [12]. Therefore, miRNAs are importantly involved in cognitive functions such as learning and memory [13]. On the other hand, some studies have revealed that deregulation of several miRNAs contributes to synaptic and memory deficits in AD mouse models [14–16].

In the present study, we aimed to detect and validate circulating miRNAs that can be associated with the rate of cognitive decline in patients with AD. To this end, we selected a discovery cohort of women with mild AD and assessed their cognitive loss during a two-year follow-up by the Mini-Mental State Examination (MMSE). The miRNAs present in the plasma of these patients were subjected to high-throughput miRNA expression profiling. Subsequently, the candidate miRNAs were validated in an independent cohort of men and women with mild AD via individual RT-qPCR methods.

Material And Methods

Study population

The subjects were prospectively recruited from a sample of outpatients who visited the Cognitive Disorders Unit at Hospital Universitari Santa Maria in Lleida (HUSM) and Hospital Clínic de Barcelona (HCB). The discovery cohort consisted of 19 women diagnosed with mild AD (MMSE score ≥ 20) and with abnormal Aβ42 levels (≤ 600 pg/mL) from HUSM. The validation study consisted of 53 subjects with mild AD and abnormal Aβ42 levels from HUSM (N = 41) and HCB (N = 12). AD was diagnosed according to the clinical criteria of the National Institute on Ageing and Alzheimer’s Disease Association (NIA-AA) [17]. Patients with cognitive impairment caused by other conditions, such as stroke, brain tumour, other neurodegenerative diseases, etc. were excluded from the study. We also excluded male patients from the discovery cohort to eliminate the sex effect in this small population.

Demographic data and general medical aspects such as hypertension, diabetes mellitus, hypercholesterolemia, stroke, depression, and APOE4 status were also evaluated in all subjects.

The cognitive evolution of the patients was measured by MMSE at baseline and after one and two years. The MMSE is a screening questionnaire for the detection of cognitive impairment [18]. It has 30 questions, and each question to be answered is scored with points, with a maximum possible score of 30 points. This questionnaire can be used to estimate the severity of cognitive impairment and to follow the course of cognitive changes in an individual over time. Based on the rate of cognitive decline, each cohort was divided into two groups: patients who had lost less than 4 points (named slow decline in cognition, SDC) and those who had lost four or more points (named fast decline in cognition, FDC) after a two-year follow-up [19].

Sample collection, RNA extraction and reverse transcription

Blood samples were collected by venipuncture into EDTA-containing tubes between 8:00 and 10:00 A. M in fasting condition. The samples were centrifuged at 2500 g for 10 min, and plasma was separated, aliquoted and stored at − 80 °C until use. Total RNA was extracted from plasma samples by using the *mir*Vana PARIS RNA and Native Protein Purification Kit (Cat. No. AM1556, Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, 300 µl plasma was added to an equal volume of 2x denaturing solution and then spiked with 10 µl of 100 pM synthetic cel-miR-39-3p (478293_mir, Thermo Fisher Scientific). The phenol extraction was applied, and finally, total RNA was eluted in 40 µl of 95 °C nuclease-free water following the recommended protocol. Two microlitres of RNA was reverse-transcribed to cDNA template using the TaqMan Advanced miRNA cDNA Synthesis Kit (Cat No. A25576, Applied Biosystems) and according to the corresponding user guide (publication number MAN0016122, revision C.0). The amplified cDNA product was stored at − 20 °C until use.

Profiling of miRNAs using TaqMan low-density array (TLDA) cards

The expression profiling of miRNAs in 19 plasma samples was carried out by loading a 1:10 dilution of amplified cDNA and TaqMan Fast Advanced Master Mix into two microarray cards (TaqMan Advanced miRNA Human A and B cards, Applied Biosystems), each containing 384 assays. The cards were run on a QuantStudio 7 Flex RT-PCR system (Life Technologies) and amplified based on the corresponding user guide (publication number MAN0016122, revision C.0). The threshold values were determined by QuantStudio software v-1.3. The data were processed with the Relative Quantification tool (powered by Thermo Fisher cloud), and their quality was evaluated based on the following criteria: 1) RT-PCR products were considered below the detection threshold and deleted if $Ct \geq 35$ or if the Ct value were reported as "Undetected" and 2) RT-PCR products with an acceptable Ct range but an irregular amplification curve was censored. After evaluation of the quality of the raw data (Supplementary Figs. 1 and 2), they were normalized based on the mean-centering method, which is the gold standard when a high number of miRNAs are evaluated [20, 21].

Validation of differentially expressed miRNAs by RT-qPCR

The differentially expressed miRNAs from the microarray experiment were validated in a new and independent cohort that consisted of 53 AD patients. RT-qPCR was carried out by using individual TaqMan Advanced miRNA Assays and TaqMan Fast Advanced Master Mix that were loaded on 384-well plates (Applied Biosystems). The samples were run in duplicate for each assay. We normalized these data using four endogenous controls (EC) (miR-103a-2-5p, miR-22-5p, miR-1301-3p, and miR-425-3p) and cel-miR-39 as an exogenous control. These ECs were shown to be stable in the plasma samples of controls and patients with or without pathophysiological changes in AD (Data under publication/Supplementary Fig. 3).

Target analysis

We used TargetScan (www.targetscan.org/vert_7.2) and miRDB (www.mirdb.org) tools to search for the possible target genes of the differentially expressed miRNAs. The biological targets of microRNAs in TargetScan are predicted by searching for the presence of sites that match the seed region of each miRNA [22]. In miRDB, miRNA targets are predicted from interactive modelling of miRNA binding and overexpression data [23].

Statistical analysis

Quantitative variables are shown as the mean (standard deviation) or median [interquartile range] according to the normality of the data. Absolute and relative frequencies were used to describe qualitative variables. We compared patient characteristics according to the study groups (FDC and SDC) in the discovery and validation cohorts. The Kruskal-Wallis test was used to compare quantitative variables, and the chi-squared test was used for qualitative variables. The differences in miRNA expression between groups were evaluated using linear models for arrays [24]. Given the age differences between study groups in the validation cohort, the linear models were age-adjusted in this cohort. The p-value threshold defining statistical differential expression was set at < 0.05. All statistical analyses and data processing procedures were performed using R software, version 3.5.2 (Vienna, Austria).

Results

Patient characteristics

The discovery and validation cohorts consisted of 19 and 53 patients with mild AD, respectively. In this regard, the pilot study consisted of 11 patients with SDC and 8 patients with FDC, while the validation cohort consisted of 32 SDC and 21 FDC patients. In the validation cohort, patients in the FDC group were older than those in the SDC group. There was no other significant difference regarding demographic data, comorbidities, AD core biomarker level or MMSE at baseline between patients included in each cohort (Table 1).

Identification of miRNAs related to the rate of cognitive impairment (TLDA experiment)

We identified 17 miRNAs that were differentially expressed between the two groups (Fig. 1A). The expression profile of these 17 miRNAs was able to completely discriminate between the two groups of the study (Fig. 1B). Among these 17 miRNAs, hsa-miR-25-3p, hsa-miR-496, hsa-miR-342-5p, hsa-miR-193a-3p, hsa-miR-483-5p, and let-7c-5p were upregulated in the FDC group (Fig. 1A, blue dots). Eleven miRNAs, including hsa-miR-30e-5p, hsa-miR-153-3p, hsa-miR-497-5p, hsa-miR-196b-3p, hsa-miR-148a-5p, hsa-miR-191-3p, hsa-miR-652-3p, hsa-miR-431-3p, hsa-miR-30d-3p, hsa-miR-744-3p, and hsa-miR-27b-5p, were downregulated in the FDC group (Fig. 1A, red dots). Furthermore, all 17 miRNAs had a good correlation with the rate of cognitive decline (Supplementary Table 1).

From these 17 miRNAs, 16 were selected for validation in an independent cohort. The sequences of all 17 miRNAs are shown in Supplementary Table 1. We eliminated the miRNA hsa-miR-193a-3p from the list of validation because it had a similar expression pattern between two groups except for 3 outlier patients in SDC group that had a lower expression than the other members of group.

Validation of differentially expressed miRNAs

Individual RT-qPCR probes were used for validation of the 16-miRNA signature in an independent cohort of patients with mild AD (N = 53). After evaluation of the quality of the data (Supplementary Fig. 4) and normalization, as explained in the Methods section, hsa-miR-342-5p showed significant differences in expression between the SDC and FDC groups (Supplementary Fig. 5). Then, we dichotomized the patients into high and low expression of hsa-miR-342-5p groups, and our results revealed that patients with low expression of hsa-miR-342-5p had a worse cognitive evolution after two years of follow-up ($p = 0.026$) (Fig. 2).

Predicted target genes for hsa-miR-342-5p

We investigated the potential role of hsa-miR-342-5p in the pathological processes of memory loss in AD by searching through its predicted targets. Interestingly, not only proteins directly related to AD, such as BACE1 and 2 (beta-site amyloid precursor protein cleaving enzyme 1 and 2), MAP1A (microtubule associated protein 1A) and TTBK1 (tau tubulin kinase 1) but also the genes for many synaptic proteins, including ephrins, syntaxin 1A, neurogranin, shank proteins, several synaptotagmins, synaptobrevin2, synaptic Ras GTPase activating protein 1, synaptic vesicle associated glycoprotein, synaptosomal associated protein, synapsin, synaptopodin and synaptojanin, were among targets that may be regulated by this miRNA. Importantly, the ephrin A2 gene had the best target rank in miRDB and the second best target rank in TargetScan for hsa-miR-342-5p.

Discussion

This study was designed to detect the association of miRNAs with the rate of cognitive decline measured by the MMSE in patients with mild AD using a hypothesis-free approach. We identified 17 miRNAs that were differentially expressed between women with AD who suffered from a faster cognitive decline over two years and those with slower cognitive decline. We validated these results in an independent cohort of men and women with AD. Our results revealed that hsa-miR-342-5p had a good correlation with the rate

of cognitive decline, and the patients with lower expression levels of this miRNA had worse cognitive evolution after 2 years of follow-up.

Importantly, many differential miRNAs in the discovery study were previously associated with AD. For instance, hsa-miR-431-3p has been reported to prevent Aβ-mediated synaptic loss [25], and hsa-let-7c-5p, hsa-miR-483-5p, hsa-miR-342-5p, and hsa-miR-191-3p have been associated with AD in several studies [26]. In addition, miR-153-3p was reported to inhibit the expression of amyloid precursor protein (APP) [27, 28].

It is widely accepted that memory and cognitive impairment in AD primarily result from synaptic failure. Gene array experiments have shown alterations in genes involved in neurotransmitter receptors and receptor trafficking, synaptic vesicle trafficking and release, cell adhesion regulating synaptic stability, postsynaptic density scaffolding, and neuromodulatory systems in the early stages of AD [29–31]. Although the association of miRNAs with AD has been widely studied, there is scarce information about the miRNAs related to the rate of cognitive impairment and their role in the processes that underlie cognitive decline in these patients. To date, some studies have been conducted to determine the miRNAs important for the regulation of synaptic structure and function and to study their dysregulation and their effect on cognitive impairment in animal models of AD [14–16, 32]. For example, Song et al. found that upregulation of miR-30b causes synaptic and cognitive deficits in 5XFAD APP transgenic mice. miR-30b targets molecules such as ephrin type-B receptor 2 (ephB2), sirtuin1 (sirt1), and glutamate receptor subunit 2 (GluA2) that are important for maintaining synaptic integrity. These investigators observed that WT mice treated with miR-30b showed impaired spatial learning and memory retention measured by the Morris water maze and novel object recognition tests [16]. In a study by Borras-Viegas and collaborators, overexpression of miR-31-5p in a 3xTg-AD model resulted in a better performance of animals in the T-maze, novel object recognition and Barnes maze, which were used for assessing spatial memory and short-term and long-term memory, respectively [32].

To our knowledge, this is the first study in which the association of miRNAs has been evaluated with cognitive evolution in patients with mild AD. In a targeted study by Tan et al., the serum levels of several miRNAs related to AD were evaluated in patients with AD and healthy controls. They observed that serum levels of miR-125b had a negative correlation with MMSE score in AD patients [33]. Wiedrick and collaborators assessed the correlation of the CSF levels of 14 miRNAs that were differentially expressed between AD and controls with MMSE. They observed that in these profiles, miR-193a-5p showed a higher correlation with MMSE [34]. However, in contrast to our study, in none of these studies assessed the association of miRNAs with the cognitive evolution of AD during a given time of follow-up. In a study by Mengel-From et al., the association between plasma miRNAs and MMSE and Cognitive Composite Score (CCS) was evaluated in healthy aged twins followed-up for ten years. They observed that miR-151a-3p, miR-212-3p and miR-1274b were associated with CCS in both the individual and paired analyses. hsa-miR-548c-3p, hsa-miR-539-5p, hsa-miR-532-3p, hsa-miR-369-3p, hsa-miR-548a-3p, and hsa-miR-27a-5p were associated with the MMSE score [35].

In the present study, we validated the differential expression of plasma hsa-miR-342-5p between AD patients with FDC and SDC. This miRNA had a good correlation with the rate of cognitive decline evaluated by the MMSE. Interestingly, we found the genes for many synaptic proteins, including ephrin A2, syntaxin, synaptotagmin, synaptojanin, and neurogranin, among possible targets of miR-342-5p. Some of these targets, such as neurogranin, have been shown to have a good correlation with the rate of cognitive decline in patients with AD [36, 37]. Therefore, it is possible that miR-342-5p plays a part in the cognitive alteration of AD via the regulation of some synaptic genes. However, this hypothesis should be tested in cellular experiments and animal models of AD.

miR-342-5p was reported to regulate the proliferation and differentiation of neuronal stem cells [38]. Sun et al. reported higher levels of miR-342-5p in APP/PS1, PS1DE9, and PS1-M146 V mouse models than in the wild-type mouse brain and suggested that miR-342-5p plays a role in AD axonopathy by hampering the function of the axon initial segment via downregulation of ankyrin G [39]. Although it is challenging to compare our results with the results of other studies in which different tissues and species and study designs were used, our result is not in accordance with the results presented by Sun et al. because we detected higher levels of miR-342-5p in patients who were cognitively more stable than those who were suffering from a more severe decline. However, our results are in agreement with the study by Lugli et al. [40], who reported downregulation of miR-342-5p in plasma exosomal samples of patients with AD compared with nondemented controls.

This study has several strengths. We identified and validated miRNAs related to the rate of cognitive evolution in two independent cohorts. We only included mild AD patients with pathological levels of Aβ42 in both cohorts to assure that assessed cognitive decline is due to AD. Furthermore, we evaluated a high number of miRNAs in the discovery study to identify all possible candidates. Finally, the cognitive alterations of the patients were followed-up for two years, while in previous studies [33, 34], the association of miRNAs was assessed with the cognitive status of the patients at baseline.

This study has some limitations. First, the discovery cohort consisted of only female participants; however, we included male subjects in the validation cohort to overcome the bias that may have been caused by this issue. Second, the number of participants in the validation cohort was small, which may have affected the final result of our study. Finally, from 16 differential miRNAs selected for validation, we only validated one miRNA. All aforementioned limitations and variabilities related to the normalization method and analytical platforms between two cohorts may have caused this inconsistency. Therefore, this result does not rule out the importance of other differential miRNAs in the cognitive evolution of AD.

Conclusions

To our knowledge, this is the first study seeking to identify miRNAs related to the rate of cognitive decline in patients with mild AD among a large profile of miRNAs. We detected 17 miRNAs that were able to perfectly separate AD patients with FDC from those with SDC. From this panel, we validated that miRNA miR-342-5p was associated with the rate of cognitive decline in an independent cohort, suggesting that

uncovering the role of miRNAs in cognition may be of interest in seeking new biomarkers and furthering our understanding of the neuropathological processes underlying cognitive decline in AD.

Abbreviations

AD: Alzheimer's disease; Aβ: Amyloid-beta protein; APOE4: Apolipoprotein E epsilon 4 allele; APP: Amyloid precursor protein; BACE1 and 2: Beta-site amyloid precursor protein cleaving enzyme 1 and 2; CSF: Cerebrospinal fluid; cDNA: complementary DNA; Ct: Cycle threshold; EC: Endogenous control; FDC: Fast decline in cognition; MAP1A: Microtubule associated protein 1A; miRNA: MicroRNA; MMSE: Mini-mental state examination; mRNA: Messenger RNA; NfL: Neurofilament light chain; NIA-AA: National Institute on Ageing and Alzheimer's Disease Association; p-Tau: Phosphorylated tau protein; RT-qPCR: Quantitative real-time polymerase chain reaction; SDC: Slow decline in cognition; TLDA: TaqMan low-density array; TTBK1: Tau tubulin kinase 1; UTR: Untranslated region

Declarations

Ethics approval and consent to participate

The Clinical Investigation Ethical Committee (CEIC P16/109) of Arnau de Vilanova University Hospital of Lleida approved this study for the discovery cohort. All patients included in the confirmatory cohort signed an internal written regulatory document stating that residual samples used for diagnostic procedures can be used for research studies.

Acknowledgements

We would like to express our sincere gratitude to all the patients and all the members of the dementia unit at the Hospital Universitari Santa Maria. We were supported by the IRBLleida Biobank (B.0000682) and PLATAFORMA BIOBANCOS PT17/0015/0027/.

Funding

Generalitat of Catalonia, Department of Health (PERIS 2019 SLT008/18/00050) and "Fundació La Marató TV3" (464/C/2014) to G. Piñol-Ripoll. IRBLleida is a CERCA Programme/Generalitat of Catalonia. F. Dakterzada was supported by Agency for Management of University and Research Grants (FI-B100153). A. Lladó received funding from Generalitat of Catalonia, Department of Health (PERIS 2016-2020 SLT008/18/00061) and Spanish Ministry of Science and Innovation -Instituto de Salud Carlos III and Fondo Europeo de Desarrollo Regional (FEDER), European Union ('A way to build Europe') (PI19/00449).

Availability of data and materials

The data reported in this manuscript are available within the article and/or its supplementary data. Additional data will be shared by request from any qualified investigator.

Authors' contributions

FD, IDB, AT, and GPR designed the study. FD and AMM carried out the experiments. IDB, AT, GPR, AL, ATM, FB and FD analysed the data. FD, IDB, AT, GPR, AL, GT, LR, DGC, ATM, RH, MSDT, FB, and GPR interpreted the data. FD, IDB, and GPR wrote the manuscript draft. All authors revised the manuscript and approved it for submission.

Consent for publication

Not applicable.

Competing interests

David de Gonzalo-Calvo has filed a patent on miRNAs as biomarkers.

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Table

Due to technical limitations, table 1 xlsx is only available as a download in the Supplemental Files section.

Figures

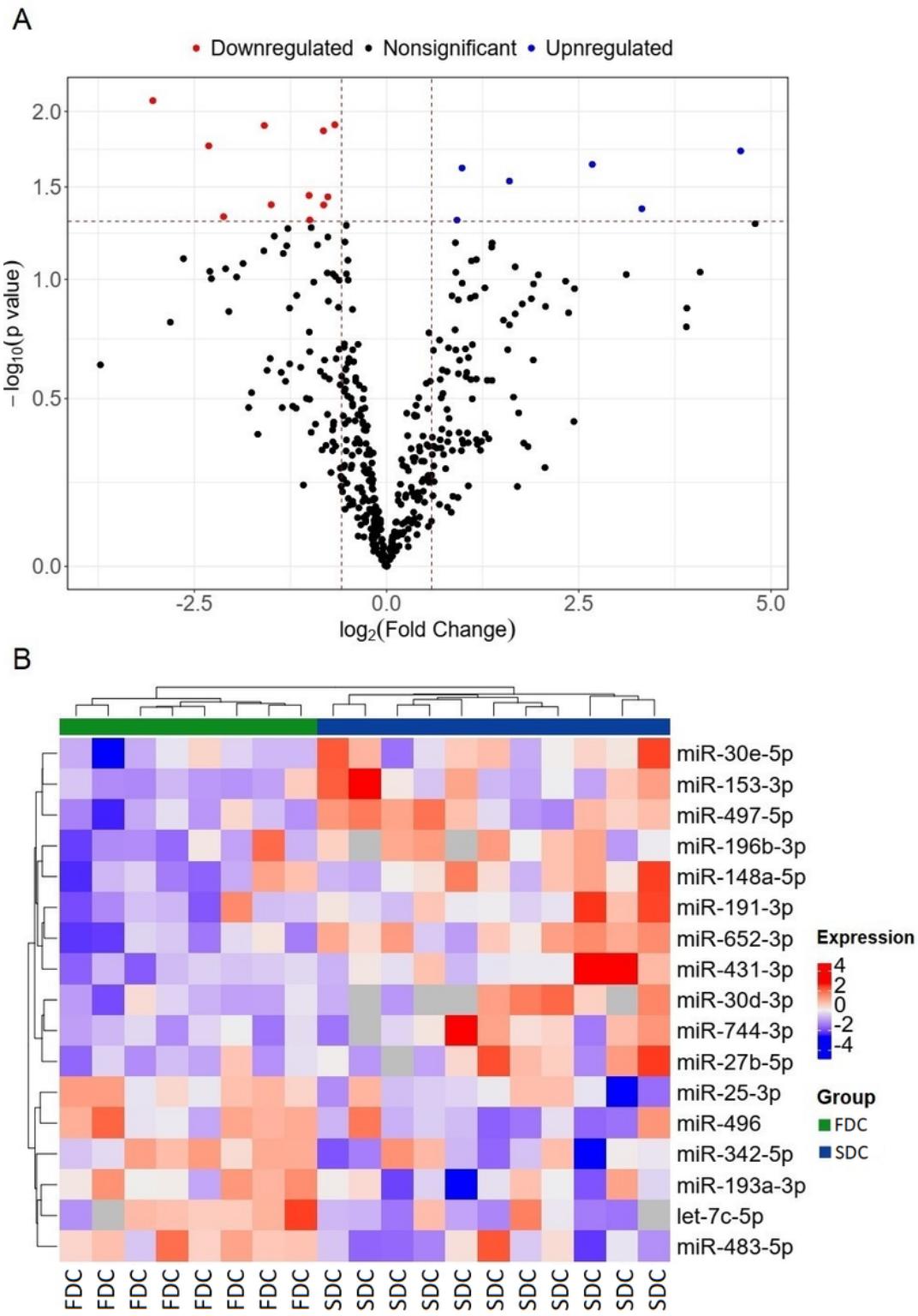

Figure 1

A: Volcano plot of the distribution of 17 differentially expressed miRNAs between patients with FDC and SDC in the discovery study, mapping six upregulated miRNAs (blue dots) and 11 downregulated miRNAs (red dots) in the FDC group; B: Heatmap of all differentially expressed miRNAs between AD patients with FDC or SDC.

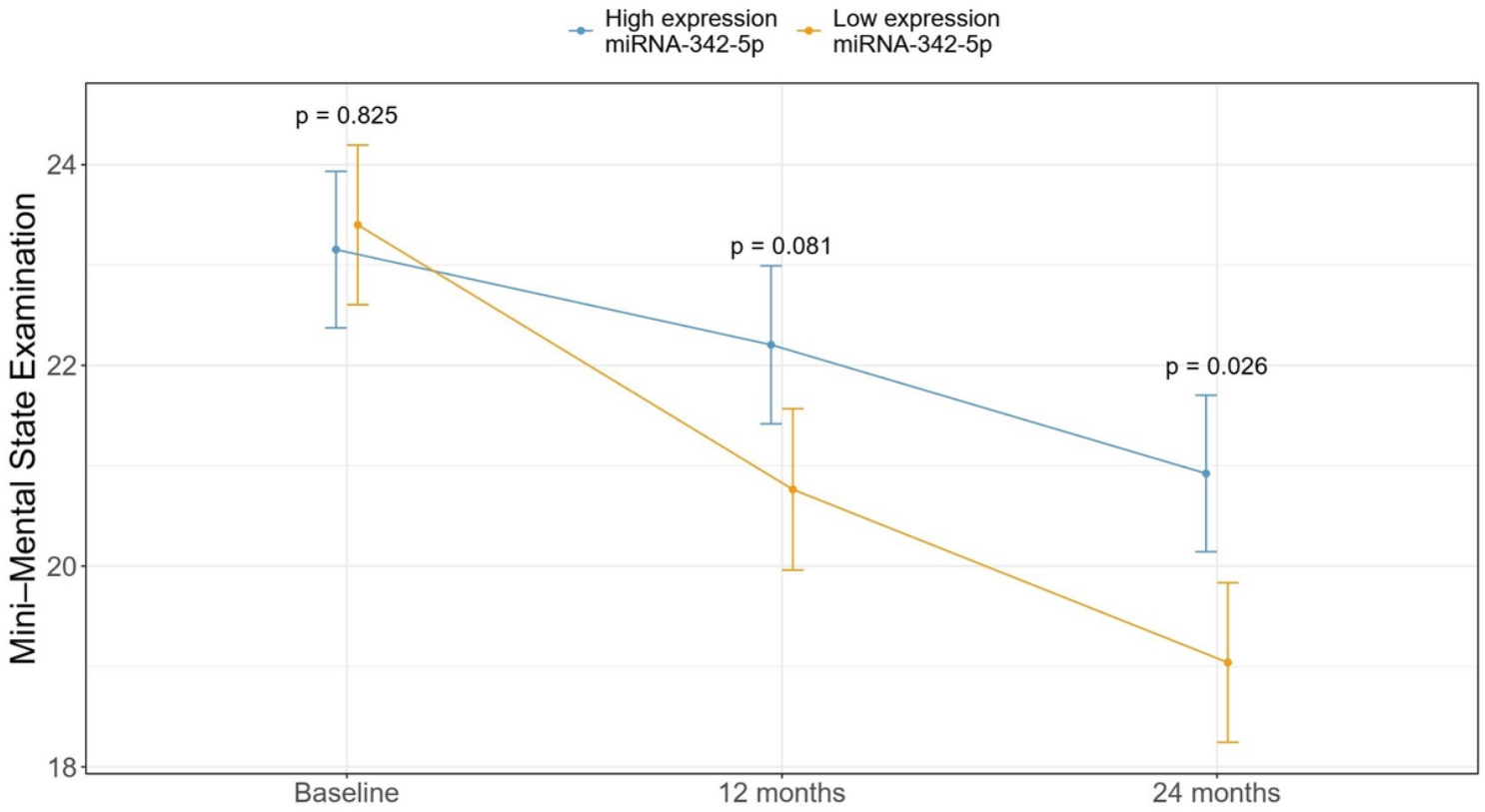

Figure 2

The association between the mean MMSE score in patients with high and low expression of miR-342-5p at baseline and after one year and two years of follow-up.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.xlsx
- Supplementarydata.docx