microRNA-150-5p: A Novel Blood-Based Biomarker for Alzheimer's Dementia with Good Correlation to Cognition, Cerebrospinal Fluid Amyloid- β , and Cerebral Atrophy

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Research

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Abstract

Background: There is an urgent need for non-invasive, cost-effective biomarkers for Alzheimer's disease (AD), such as blood-based biomarkers. It is not only to support clinical diagnosis of dementia, but also to allow for timely pharmacological and non-pharmacological interventions evaluation. The aim of this study is to identify and validate a novel blood-based microRNA (miRNA) biomarker for dementia of Alzheimer's disease type (DAT). The miRNA correlations with AD pathology and AD clinical-radiological imaging were conducted.

Methods: We conducted miRNA-sequencing (miRNA-Seq) using peripheral blood mononuclear cells (PBMCs) isolated from a discovery cohort comprising DAT, mild cognitive impairment (MCI), and healthy subject (HS). Identified miRNA was validated in an independent cohort. Correlation analysis evaluated the relationships between miRNA expression and DAT clinical measures, including Mini-Mental State Examination (MMSE) and Montreal Cognitive Assessment (MoCA) scores, CSF A β ₁₋₄₂ and tau levels, and AD pattern cerebral atrophy. Furthermore, we conducted bioinformatics analysis and cell-based assay to identify miRNA target genes.

Results: MiRNA-seq identified a distinct miRNA (miR-328-3p, miR-7706, and miR-150-5p) expression signature differentiating DAT from MCI and HS. qPCR analysis reveals that miR-150-5p was consistent with the miRNA-seq data and was further validated. Specifically, we found that miR-150-5p expression was significantly upregulated in DAT compared to MCI and HS, and discriminated DAT from MCI and HS with a high accuracy with AUC of 0.86 and 0.86, respectively. We further found that higher miR-150-5p levels correlated with clinical measures of DAT, including lower global cognitive scores of MMSE and MoCA, lower CSF A β ₁₋₄₂, and higher CSF tau. Interestingly, we observed that higher miR-150-5p levels is associated with the lower grey matter volumes in the medial temporal lobe, posterior cingulate cortex and precuneus. These regions implicated default mood network and executive control network regions that are important for AD brain atrophy. Furthermore, pathway analysis identified the targets of miR-150-5p to be enriched in the Wnt signalling pathway, including programmed cell death 4 (*PDCD4*). We further found that *PDCD4* was downregulated in DAT blood and was downregulated by miR-150-5p at both transcriptional and protein levels.

Conclusions: Our findings demonstrated that miR-150-5p is a reliable clinical blood-based biomarker for DAT.

Introduction

The advancement of biomarker research in Alzheimer's disease (AD) has shifted the diagnosis of AD from a clinical syndrome to a biological construct [1]. However, the current AD biomarkers such as CSF amyloid- β 1-42 (A β ₁₋₄₂) and phosphorylated tau, as well as amyloid and tau PET are limited by either invasiveness or cost and hence are available only in very few academic centres [2–4]. As such, it is

imperative to develop a non-invasive and inexpensive blood-based biomarker that is specific for AD pathophysiology for clinical application in detecting AD in wider populations.

MicroRNAs (miRNAs) are small, conserved non-coding RNAs that modulate post-transcription gene expression by suppressing specific target miRNAs [5, 6]. miRNAs play vital roles in the body's essential processes including cell proliferation, cell development and stress responses amongst others. In the nervous system, miRNAs are also widespread and regulate key functions such as neurite outgrowth, dendritic spine morphology, neuronal differentiation, and synaptic plasticity [7, 8]. There has been increasing research interest in the relationship between miRNAs and neurocognitive disorders following the discovery of miRNAs dysregulation in neurocognitive disorders. Specifically, miRNAs dysfunction has been shown in mild cognitive impairment (MCI) and Alzheimer's disease dementia although they lack correlation with clinical phenotypes [9–13]. It has been shown that dysregulation of miRNAs in AD affects $A\beta_{1-42}$ and phosphorylated tau production, increases oxidative stress and neuroinflammation [14, 15].

Circulating miRNAs in whole blood, plasma, serum, CSF, or peripheral blood mononuclear cells (PBMC) are increasingly being examined as candidates of blood-based AD biomarkers [16–18]. A recent systematic review included 20 studies reported the potential for 102 dysregulated miRNAs in peripheral blood of AD patients as potential biomarker for AD [19]. With respect to miRNA dysregulation in the human brain, the same review reported the identification of 250 miRNAs from 27 studies. When cross-referencing the miRNAs from peripheral blood and the brain, 47 common miRNAs were found, of which 30 miRNAs were dysregulated in the brain tissue with known Braak Stages. Among these 30 miRNAs, 10 of them were observed to be dysregulated at Braak Stage III, and their expression levels in peripheral blood and the brain correlated with each other (miR-30e, miR-34c, miR-107, miR-200c, miR-210, miR-485) while others had opposing levels (miR-26b, miR-34a, miR-125b, miR-146a). Taken together, miRNAs do have the potential and show promise to be incorporated in clinical practice for diagnosis of AD, and for monitoring patients receiving preventive and therapeutic interventions. However, more studies need to be devoted to identifying correlations with current validated AD biomarkers to establish whether or not a particular miRNA can be considered a specific biomarker of AD. However, reliable miRNA-based tests to complement and support the clinical diagnosis of Alzheimer's disease is lacking. Therefore, studies focusing on the correlations of miRNAs with current Alzheimer's disease biomarkers are paramount.

In this study, we evaluated PBMC as a source of miRNA as 1) it's a major cellular component of the human immunity; 2) stable levels in bloodstream; 3) can be obtained using minimally invasive procedure; 4) low cost and 5) low risk of being contaminated when compared to miRNAs extracted from other body fluids [20]. Here, in a discovery and an independent cohort of individuals with dementia of Alzheimer's disease type (DAT), mild cognitive impairment (MCI) and healthy individuals (HS), we profiled the expression of PBMC-derived miRNAs using high throughput miRNA-seq. We aimed to identify and validate miRNA that is specific for DAT. We further correlated the miRNA with AD clinical biomarkers and studied its target gene in biological pathways.

Methods

Study participants

Participants with mild DAT and MCI were recruited from a tertiary neurology center (National Neuroscience Institute, Singapore) while HS were recruited from the community between 2015–2018. The diagnoses of DAT and MCI were made using the NIA-AA criteria [2, 16] and supported by a Clinical Dementia Rating [21] (CDR) score of 1 and 0.5 respectively. DAT participants were in the mild stage of dementia as reflected by the CDR score of 1 and Mini-Mental State Examination (MMSE) [22], as well as Montreal Cognitive Assessment (MoCA) [23] scores being in the mild impairment range. All individuals received a consensus diagnosis from a panel comprising cognitive neurologists, psychologists, and specialist nurses. Key exclusion criteria included serious neurological or psychiatric comorbidities, diagnosis of dementia with Lewy bodies (DLB), vascular dementia (VAD) and frontotemporal dementia (FTD) as well as a history of alcohol or drug abuse.

The medical history and demographic characteristics of the study participants were collected via a standardized interview. Study participants underwent a standardized assessment of their global cognition using the MMSE [22] and MoCA [23], blood test, lumbar puncture for CSF analysis [24] and a 3T-MRI scan within six months of clinical and neuropsychological evaluations.

MiRNA extraction and sequencing

Extraction of PBMC from whole blood was performed as described by Maes OC et al., [25] with slight modification. Following extraction, PBMC were resuspended in QIAzol Lysis Reagent (Qiagen) and stored at -80°C until further use. Total RNAs including miRNA was extracted from PBMC using miRNeasy Mini Kit (Qiagen) following manufacturer's instructions. Concentration and Quality of extracted RNAs were measured using NanoDrop1500 (Thermo Fisher Scientific). RNA integrity number (RIN) of RNAs was assessed by Bioanalyser, where RNA samples with $RIN \geq 8.0$ and RNA ratio [28s/18s]: ≥ 1.5 were used for library preparation and miRNA-seq.

The small RNA libraries were prepared by Beijing Genomics Institute (BGI) using the TruSeq small RNA kit (Illumina). During this analysis, all the runs were considered as independent. The sequences were assessed for quality using FASTQC. The Primer, adapter and contaminant sequences were removed using Cutadapt/TRIMMOMATIC. Read lengths shorter than 15 nucleotides were removed. The cleaned sequences were collapsed using the FastX-toolKit (fastx collapser) and reduced to unique reads. The subsequent file was processed using miRDEEP2 pipeline. The reads were mapped to the human genome (hg19) and precursor miRNA sequences for miRbase version 21. The final output of the count file summarizing the mapped miRNAs for each sample was used for further analysis.

Validation of microRNA by qPCR

Differentially expressed miRNAs were validated using Taqman MicroRNA Assays (Applied Biosystem). Each Assay contains miRNA-specific Taqman minor groove binder (MGB) probe and PCR primers for hsa-

miR-150-5p, hsa-miR-150-3p, hsa-miR-328-3p, hsa-miR-7706 and along with U6 small nuclear RNA (U6) which served as a reference control for miRNA expression normalization. MiRNA expression changes were calculated relative to U6 using the $2^{-\Delta\Delta C_t}$ method.

MRI image acquisition and pre-processing

High resolution T1-weighted MPRAGE (Magnetization Prepared Rapid Gradient Echo) sequences performed on a 3T Prisma Fit System (Siemens, Erlangen, Germany) were obtained as follow: 192 continuous sagittal slices, TR/TE/TI = 2300/2.28/900 ms, flip angle = 8°, FOV = 256 × 240 mm², matrix = 256 × 240, isotropic voxel size = 1.0 × 1.0 × 1.0 mm³, bandwidth = 200 Hz/pixel. We used the Computational Anatomy Toolbox (<http://dbm.neuro.uni-jena.de/cat12/>) protocol in Statistical Parametric Mapping (SPM12) (<http://www.fil.ion.ucl.ac.uk/spm/>), to process the T1 images for voxel-based morphometry (VBM) analysis. Specifically, all 3D T1-weighted MRI scans were normalized using an affine transformation followed by non-linear registration, corrected for bias field inhomogeneities. Images were then segmented to derive subject-level GM, WM, and CSF components [26]. The Diffeomorphic Anatomic Registration Through Exponentiated Lie algebra algorithm (DARTEL) was used to normalize the segmented scans into the standard MNI space which provides better precision in spatial normalization to the template [27]. All obtained segmented, modulated, and normalized GM and WM images were then smoothed using an 8-mm full-width-half-maximum isotropic Gaussian smoothing kernel.

MRI regions of interest derivation

We also applied a multiple seed-based approaches to test the association between GMV and miRNA levels specifically in regions of interest (ROIs) belonging to the Default mode network (DMN) and executive control network (ECN). We selected nine ROIs covering the DMN and ECN based on a prior study [28]. The DMN ROIs included the left and right parahippocampal cortex (L and R HIP), medial prefrontal cortex (mPFC), posterior cingulate cortex (PCC) and precuneus (PCN) and the ECN ROIs included the left and right dorsolateral prefrontal cortex (L and R DLPFC) and the left and right posterior parietal cortex (L and R PPC) in standard space. Average GMV from these network ROIs were derived using the MarsBar toolbox in SPM12. Following derivation of miRNA levels, a median split analysis was carried out for each of the miRNAs. A binary assignment was formulated with participants less than the median level of miRNA levels were assigned as “0” and those with greater than median levels of miRNAs were assigned “1”.

CSF processing for A β ₁₋₄₂ and tau measurement

CSF was collected via lumbar puncture then centrifuged at 2000 g for 10 min at 4 °C. The resulting supernatant was aliquoted and stored at -80 °C until further analysis. CSF levels of A β ₁₋₄₂ and tau were measured using the INNOTEST® (Fujirebio Europe NV) according to the manufacturer's instructions [29]. A standard protocol for collection of CSF and measurement of A β ₁₋₄₂ and tau was used for all participants.

Plasmid Constructs

pmirGLO- PDCD4 -3'UTR: A 1,236 bp DNA fragment was amplified from human *PDCD4* cDNA (NCBI reference number: NM_014456.5) 3'UTR region containing four miR-150-5p predictive binding sites using primer set: 5'-CTAGCTAGCATGTTGGCTGCTGCTGTTGAGATAC-3' and 5'-ACGGTCGACTGCCTCCCAGGTTCAAGCAATTCT-3'. The purified DNA fragment was then digested with *NheI* and *SaI* restriction enzymes and inserted into the pmirGLO vector (Promega) [12, 13]. The sequence of formed plasmid was confirmed by Sanger sequencing.

Luciferase assay

Human embryonic kidney (HEK)293T cells were cultured in 6-well plates prior transfection. Cells were cotransfected with miR-150-5p mimic and empty vector or *PDCD4* 3'UTR plasmid using lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Cells were harvested 48 hours post transfection and subjected to measure respective luciferase activity with the Dual Luciferase Reporter Assay Kit (Promega) by using a GloMAX 20/20 luminometer (Promega).

mRNA Reverse transcription and qPCR

Total mRNAs were reverse-transcribed by iScript™ cDNA Synthesis kit (Bio-rad) and generated cDNA was subjected to qPCR using All in One™ qPCR Mix (Genecopoeia). β -actin was used as a reference gene for mRNA expression normalization. Relative expression of the genes was calculated using the $2^{-\Delta\Delta CT}$ method relative to values of β -actin.

Western blot analysis

Proteins were extracted from cells using RIPA lysis buffer supplemented with protease inhibitor cocktail (MedChemExpress) and phosphatase inhibitor cocktail (MedChemExpress). Protein concentrations were measured using RC-DC protein assay kit II (Bio-Rad). 20 μ g of protein lysates were separated by SDS-PAGE and then transferred to polyvinylidene fluoride membrane (PVDF membrane, Millipore). The membranes were blocked with 5% milk in tris buffered saline with tween 20 (TBST) for 1 hour at room temperature and incubated with a 1:1000 dilution of primary antibodies (rabbit anti-*PDCD4*, Merck; mouse anti- β -actin, Santa Cruz) at 4°C overnight. The membranes were then washed with TBST, followed by incubation of horseradish peroxidase (HRP)-conjugated secondary antibody (1:3000, GE Healthcare). Protein bands were visualized by Pierce ECL Western Blotting Substrate (Thermofisher Scientific). Band intensity was quantified using ImageJ software.

Statistical analysis

Statistical analysis for evaluation and correlation

Evaluation of miRNA, mRNA and protein expression levels, ROC curves, and Pearson correlation were analysed with GraphPad Prism 6.0. One-way ANOVA with Tukey's multiple comparisons test was used for evaluation of expression levels and values were expressed as mean $\pm$ SEM. Differences between the study participants were considered statistically significant at $p < 0.05$.

Voxel-based morphometry and region of interest analyses

Group differences in grey matter volume (GMV): To examine group differences, we built a voxel-wise Analysis of Covariates (ANCOVA) general linear model with GMV as the dependent variable, binary group membership as either below or above median miRNA levels with age and gender as nuisance covariates. The GM regions which showed significant group differences based on miRNA median split were examined using a threshold of Family-wise error correction of $p < 0.05$ or an uncorrected $p < 0.001$ and a minimum cluster size of 100 voxels [30, 31]. Significant clusters in the GM were anatomically identified using the Automated Anatomical Labelling atlas labels.

Associations between default mode network and executive control network regions of interest GMV and miRNA levels: Pearson's correlation analysis was used to assess the association between ROI GMV and miRNA levels. Partial Pearson's correlation analysis was used to assess the association between ROI GMV and miRNA levels after controlling for age at visit as a covariate. Multiple comparisons correction across the nine ROIs was conducted using FWE-correction at $p < 0.0055$.

Statistical analysis for bioinformatics

All the miRNAs with reading counts < 50 summed up for all samples for each group (HS/MCI/DAT individuals) were removed. The read counts were normalized using Quantile Normalization or the raw file was analyzed for differentially regulated miRNA using DESEQ2 pipeline (TMM Normalization) or using SAMSEQ pipeline (Quantile Normalization) or Quantile normalized data. P -values were calculated using Wilcoxon-Mann-Whitney (WMW) test. The adjusted p values (FDR) were calculated using Benjamini-Hochberg approach. For hierarchical classification and K means clustering approach e1701 and caret packages were used in R.

Results

MiRNA-sequencing reveals an expression signature that distinct DAT from MCI and HS

We performed Hiseq2000 microRNA-sequencing (miRNA-seq) in the PBMCs of age matched discovery cohort of 19 HS, 16 MCI, and 19 DAT individuals (Table 1). A total of 956 mature miRNAs originating from 739 unique precursors met the expression cut-off (normalized read counts > 50). These samples showed similar read count distributions for individual genes and were virtually identical after Quantile Normalization, confirming successful normalization. To determine distinctive miRNA signatures among HS, MCI, and DAT subjects, we performed sample clustering analysis using the hierarchical clustering approach. Four distinct clusters of miRNA expressions were observed, with DAT being enriched in one of them but none in MCI and HS (Fig. 1a). This indicates that there is a distinct miRNA expression signature that differentiates DAT from MCI subjects, and DAT from HS subjects.

Table 1
Characteristics of discovery cohort.

Discovery cohort	Healthy Subjects (HS)	Mild cognitive impairment (MCI)	Alzheimer's dementia (DAT)	P-value
Sample numbers	19	16	19	
Gender	10 (53%)	5 (31%)	9 (47%)	0.425
Male	9 (47%)	11 (69%)	10 (53%)	
Female				
Age, years	65.6±6.5	64.4±7.3	67.7±6.3	0.226
Education, years	12.1±2.2	11.6±3.1	8.6±3.1	< 0.001***†
MMSE score	29.1±1.2	27.4±1.9	23.1±4.4	< 0.001***†#
MoCA score	27.5±1.9	25.1±2.	20.5±5.0	< 0.001***†
Values are represented as mean±SD or number (%). Demographic characteristics were compared between disease group using the χ^2 test for gender, and Kruskal-Wallis with Dunn's test for numerical variables. *** $p < 0.001$ for DAT vs HS subjects; † $p < 0.05$ for DAT vs MCI subjects; # $p < 0.05$ for MCI vs HS subjects.				

Volcano plot further revealed that there is a distinct miRNA expression signature between DAT and HS (Fig. 1b), DAT and MCI (Fig. 1c); while the miRNA expression was indistinguishable between HS and MCI (Fig. 1d). Specifically, when comparing DAT with HS, miR-328-3p (Fig. 1e) and miR-7706 (Fig. 1f) were found to be downregulated in DAT, whereas miR-150-5p were found to be highly upregulated in DAT (Fig. 1g). Our findings suggested a distinct miRNA expression signature differentiating DAT from MCI and HS. miR-150-5p was selected for further evaluation in another independent cohort because miR-150-5p have been found to be upregulated in the postmortem brain tissue of AD patients by next generation sequencing[32]. Further investigation will warrant the role of miR-150-5p on AD

MiR-150-5p expression is upregulated in DAT

We validated the miR-150-5p in an independent cohort comprising 16 HS, 27 MCI, and 21 DAT (Table 2) and found that the expression of miR-150-5p was significantly upregulated by 76.9% in DAT compared to HS, and by 73.3% compared to MCI individuals (Fig. 2a). This finding was consistent with earlier miRNA-seq data that miR-150-5p was highly expressed in DAT (Fig. 1g). Moreover, findings from the AUC analysis showed that miR-150-5p levels discriminated DAT from MCI and HS individuals with a good accuracy of AUC 0.86 (95% CI 0.76-0.96) and AUC 0.86 (95% CI 0.73-0.99) respectively (Fig. 2b). However, miR-150-5p levels did not discriminate MCI from HS individuals with an AUC of 0.53 (95% CI 0.33-0.72).

Table 2
Characteristics of an independent cohort.

Independent Cohort	Healthy Subjects (HS)	Mild cognitive impairment (MCI)	Alzheimer's dementia (DAT)	P-value
Samples numbers	16	27	21	
Gender	10 (62%)	19 (70%)	9 (43%)	0.15
Male	6 (38%)	8 (30%)	12 (57%)	
Female				
Age, years	57.8± 5.6	57.8± 6.7	58.7± 4.8	0.834
Education, years	n=16 13±2.7	n=25 13.4±3.8	n=19 8.9±4.4	< 0.001**+++
MMSE score	28.3±1.3	27.3±1.8	20.5±6.7	< 0.001***+++
MoCA score	26.6±3.2	26.1±2.5	17.1±7.1	< 0.001***+++
CSF Aβ ₁₋₄₂ , pg/ml	n=5 1173.2±274.2	n=13 872.1±380.5	n=17 701.5±396.6	< 0.05*
CSF tau, pg/ml	n=5 302.0±41.0	n=13 326.8±222.6	n=17 543.4±416.1	0.1368
Values are represented as mean±SD or number (%). Values are represented as mean (SD) or n (%). Demographic characteristics were compared between disease group using the χ^2 test for gender, and Kruskal-Wallis with Dunn's test for numerical variables. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for DAT vs HS subjects; +++ $p < 0.001$ for DAT vs MCI subjects.				

To confirm the specificity of miR-150-5p in detecting DAT, we evaluated the expression pattern of miR-150-3p, a mature form of miR-150 but is not differentially expressed by miRNA-seq analysis, to serve as a negative control. No significant differences were observed among the three cohorts with regards to the expression pattern (Fig. 2c) and with AUC less than 0.60 (Fig. 2d).

Higher miR-150-5p expression correlates with lower cognitive performance, lower CSF Aβ₁₋₄₂ and higher CSF tau

To further confirm if miR-150-5p is a reliable biomarker for DAT, we studied the relationship between the elevated levels of miR-150-5p and the clinical cognitive performance outcome of the patients. MMSE and MoCA are commonly used clinical assessment for dementia[22, 23]. In both MMSE and MoCA, the lower

the score, the more severe the cognitive impairment of patients are. Correlation analysis showed that expression levels of miR-150-5p correlated inversely with both MMSE ($r = -0.3968$; $p = 0.001$, Fig. 3a) and MoCA scores ($r = -0.4091$; $p = 0.001$, Fig. 3b), that a higher miR-150-5p expression levels in PBMC was associated with lower MMSE and MoCA scores. Our results indicate that the higher miR-150-5p expression levels in PBMC is associated with poor cognitive outcome of the patient, which is consistent with the clinical diagnosis.

Next, we conducted correlation study between miRNA expression with AD pathology diagnosis. CSF $A\beta_{1-42}$ levels have been previously demonstrated to be lower in patients with pathologically confirmed DAT, compared to HS [33]. We found that there is a negative correlation between miR-150-5p expression levels and CSF $A\beta_{1-42}$ levels ($r = -0.3516$, $p = 0.0355$, Fig. 3c). The increased miRNA expression associated with decreased $A\beta_{1-42}$ is consistent with the higher expression of miR-150-5p in DAT cohort (Fig. 2a). In addition, we found a positive correlation between miRNA expression and CSF tau levels, that the increased miR-150-5p is associated with increased CSF tau, ($r = 0.3317$, $p = 0.0553$, Fig. 3d), indicating that higher miR-150-5p expression levels is associated with greater neurodegeneration.

Higher miR-150-5p levels are associated with lower voxel-wise grey matter volume in AD specific brain regions

To study the correlation between miR-150-5p and AD brain atrophy patterns, we examined the influence of miR-150-5p expression levels on *in vivo* MRI-based brain structural measures involving whole-brain grey matter volume. We carried out whole-brain voxel-based morphometry analyses to assess voxel-wise grey matter differences between individuals with low and high miR-150-5p. We found that participants with higher than median levels of miR-150-5p showed lower voxel-wise grey matter volume primarily in parietal and temporal regions involving the medial temporal lobe, posterior cingulate cortex, and precuneus (FWE corrected $p < 0.05$, Fig. 4a) when compared to participants with lower than median levels of miR-150-5p. On the other hand, participants with lower than median miR-150-5p levels did not show reduced voxel-wise grey matter volume compared to participants with higher than median miR-150-5p levels. To further investigate miR-150-5p influence on grey matter volume, we carried out a Pearson's correlation analyses between miR-150-5p expression levels and grey matter volume from specific regions-of-interest comprising the default mode network and executive control networks, two major networks targeted in AD. We found that higher miR-150-5p levels are associated with lower grey matter volume in the default mode network regions comprising the left hippocampus (Fig. 4b), right hippocampus (Fig. 4c), medial prefrontal cortex, posterior cingulate cortex (FWE corrected $p < 0.0055$) and precuneus (uncorrected $p = 0.007$). Additionally, higher miR-150-5p levels are associated with lower grey matter volume in the executive control network regions comprising the left dorsolateral prefrontal cortex, right dorsolateral prefrontal cortex, left posterior cingulate cortex (Fig. 4d) and right posterior parietal cortex (Fig. 4e) at the FWE-corrected $p < 0.0055$ threshold. Our result demonstrated that miR-150-5p is able to detect the cerebral atrophy in AD specific regions.

MiR-150-5p targets are involved in Wnt signaling pathway that is linked to AD pathobiology

To capture the molecular mechanisms that underlie the dysregulation of miR-150-5p in AD, we first, identified potential targets of miR-150-5p using three widely used miRNA target prediction algorithms (miRDB, Insect Genome and TargetScan). 120 common targets of miR-150-5p were revealed and were then subjected to Gene Ontology (GO) categories analysis to identify the functional role of miRNA targets. GO enrichment analysis showed that miRNAs regulate its targets negatively and is required for protein binding, as well as were enriched in nucleocytoplasm regions (Fig. 5a). In addition, we mapped 120 predicted miRNA targets in KEGG pathway using DAVID 6.8 to further identify miRNA targets in biological pathways (Fig. 5b). Next, we further narrowed down 11 candidate miR-150-5p target genes based on the overlapping genes between enriched GO categories and KEGG pathways with FDR < 0.06 and found that these genes were related to AD and/or Wnt signaling pathway (Fig. 5c). Among these 11 genes, we focus on programmed cell death 4 (*PDCD4*) because it is a multifunctional tumor suppressor which regulates cell apoptosis, cell growth, protein translation and signal transduction, and impairs PI3K/AKT signaling pathway in cellular AD models[34, 35]. qPCR revealed that *PDCD4* was significantly downregulated in DAT compared to MCI, which is opposite to that of miR-150-5p (Fig. 5d). In addition, we found that there are miR-150-5p putative binding sites (TTGGGAT) located at the 3'UTR region of its mRNA (Fig. 5e). To further confirm the *PDCD4* is the target of miR-150-5p, we conduct the cell-based analysis. Co-expression of miR-150-5p with 3'UTR of *PDCD4* in HEK 293T cells significantly downregulates the *PDCD4* at the transcription level by luciferase assay (Fig. 5f and g). We further showed that expression of miR-150-5p in SH-SY5Y cells reduced *PDCD4* at the mRNA level by qPCR assay (Fig. 5h), and at protein levels by western blot analysis (Fig. 5i). Therefore, *PDCD4* is a potential downstream target of miR-150-5p, which may be related to miR-150-5p-associated pathogenesis in AD.

Discussion

In the present study, we systematically identified a potential PBMC-based miRNA biomarker for DAT. Our initial miRNA profiling allowed the identification of miRNAs with high differential expression in DAT and MCI individuals compared to HS individuals which was consistently upregulated in the discovery cohort and independent cohorts. Expression levels of miR-150-5p was upregulated in PBMC of patients with DAT and was closely correlated to cognitive performance, CSF A β_{1-42} and tau levels, as well as MRI AD pattern of atrophy. Our results demonstrated that higher levels of miR-150-5p was associated with derogatory effects on brain structure, especially in regions of the brain belonging to the default mode and executive control networks, key brain networks implicated in AD. Furthermore, miR-150-5p was subjected to miRNA targets prediction, functional enrichment analysis, and verification of miRNA targets expression. Our bioinformatics analysis revealed that selected targets of miR-150-5p were markedly associated with Wnt signaling pathway which has been implicated in AD [36]. We further showed that target genes *PDCD4* expression levels were downregulated in DAT. These results provide evidence that

upregulation of miR-150-5p suppresses its downstream target genes. Therefore, our findings suggest that miR-150-5p is a potential as a biomarker for the diagnosis of DAT.

A previous study showed that PBMC is involved in the pathogenesis of AD due to its central role in inflammation during the early phase of AD development [37]. Lymphocytes and monocytes, which represent the majority of immune cells in PBMC, can infiltrate blood brain barrier (BBB) and lead to inflammatory cascade in the brain of AD [38]. Furthermore, it is also reported that dysfunction of BBB in AD enables exchange of miRNAs between the brain and peripheral blood [39]. A recent review reported that a higher correlation of transcriptomic changes (pattern of gene expression) and epigenomic changes (pattern of DNA methylation) in the CNS and blood [40]. Taken together, it is hypothesized that that changes in the expression of miRNAs in the PBMC could reflect the neuropathogenic changes in the CNS and PBMC may serve as a potential source to find miRNAs signatures that could help to discriminate cognitively healthy subjects from diseased patients. To explore the role of PBMC derived miRNAs for DAT, we unbiasedly profiled the miRNAs from PBMC of 54 study participants. (Fig. 1a). Additionally, we validated the aberrantly expressed miRNA in an independent cohort of 64 study participants. As verified by qPCR, miR-150-5p was upregulated in DAT versus HS individuals and DAT versus MCI individuals with an AUC of 0.86 and 0.86, respectively (Fig. 2a **and b**).

In addition to differential expression of miRNA in HS, MCI, and DAT individuals, another important consideration is the reliability of the miRNA with regards to cognitive, neuroimaging, and CSF biomarkers for AD. In this regard, we observed miR-150-5p expression levels to be inversely correlated to cognitive tests including MMSE and MoCA, demonstrating that higher miR-150-5p levels was associated with lower cognitive scores (higher disease severity) (Fig. 3a **and b**). Our findings also demonstrate that miR-150-5p expression levels were negatively correlated with CSF $A\beta_{1-42}$ levels and positively correlated with CSF tau levels (Fig. 3c **and d**). In addition, subjects with DAT also had lower CSF $A\beta_{1-42}$ and higher CSF tau compared to HS and MCI subjects. As expected, subjects with MCI had intermediate levels of both markers (Table 2). These findings further support the concept that CSF is the gold standard of AD diagnosis where cognitive impaired AD patients with a low concentration of CSF $A\beta_{1-42}$ are usually accompanied by a higher concentration of CSF tau levels [41]. Furthermore, the concentration of CSF $A\beta_{1-42}$ in DAT was significantly higher compared to MCI subjects but was comparable to MCI subjects. Similar finding was found in the expression of miR-150-5p among three groups. Thus, not only miR-150-5p expression differentiates DAT from HS, but it also correlates with cognitive performance and CSF $A\beta_{1-42}$ as well as CSF tau levels and thus has wide clinical implications.

In order to validate miRNAs as biomarkers of AD, there is a need to understand their relationship with *in vivo* markers of brain structure and AD neuropathology such as amyloid- β and tau. However, there is currently limited evidence illustrating the association between miRNA levels and brain structure in AD. Thus, how miRNA levels influence and relate to *in vivo* measures of brain structure and function in AD remain a key gap. In this regard, our findings illustrate a derogatory influence of miR-150-5p levels on both voxel-wise and regional grey matter volume in DAT (Fig. 4). Prior studies suggest that effects of

miRNA levels on neuropathological markers of AD including amyloid- β and phosphorylated tau could be related to such changes in brain structure. For instance, one study illustrated high accuracy in the diagnosis of prodromal AD using PBMC miRNA markers in accordance with amyloid- β deposition as observed on PET imaging [42]. Evidence also indicates that miRNAs regulate genes that are responsible for amyloid- β and phosphorylated tau up-regulation with increasing evidence suggesting that miRNAs influence amyloid- β production [15]. Additionally, prior studies show alterations of miRNA levels primarily in the grey matter such as those involving the temporal lobes of the brain which are also key regions of amyloid- β and phosphorylated tau deposition in AD [43–45]. Studies in post-mortem human tissue illustrate miRNA expression correlates with the presence of early AD-related pathology in grey matter, specifically with the density of diffuse amyloid- β plaques [45]. Such findings support the notion that patterns of miRNA expression in the cortical grey matter may contribute to, and explain underlying AD pathogenesis [45, 46].

The presence of amyloid- β plaques likely further initiate or potentiate downstream changes that culminate in neurofibrillary tau pathology, cell death, synapse elimination, and eventually cognitive impairment in individuals with altered miRNA levels [45]. Expression of various miRNAs can thus form important candidates in understanding disease progression and modulation in AD. Such events as well as miRNA influence on synaptic loss and synaptic damage are possible mechanisms underlying grey matter loss especially within the hippocampus as well as cognitive decline related to AD. While our group is one of the first to show associations between brain changes and miRNA levels, nonetheless, much remains to be explored regarding the relationship of miRNA levels and brain structure in regions known to be affected in AD.

A single miRNA is able to regulate multiple target mRNAs, and likewise a single target mRNA can be regulated by multiple miRNAs [47]. Using both target prediction and pathway enrichment approaches, we found out some miR-150-5p targets are enriched in the Wnt signaling, PI3K-AKT and thyroid hormone signaling pathways (Fig. 5b). Interestingly, these signaling pathways has been implicated in neurodevelopment and pathogenesis of AD [36, 48, 49]. In addition, miR-150-5p targets were found enriched in the nucleus, nucleoplasm, nuclear membrane, cytosol, cytoplasmic mRNA processing body, and cytoplasm (Fig. 5c), which further demonstrated that the likelihood of miRNA targets shuttles between cytoplasm and nucleus.

It is noteworthy that miRNA negatively regulates the expression of its targets [5, 6]. Consistently, miR-150-5p targets, *PDCD4* were found to be downregulated in DAT compared to MCI and HS (Fig. 5d), which reflected the most enriched category for GO biological process that miR-150-5p is a negative regulator (Fig. 5a). *PDCD4* (programmed cell death 4), has been demonstrated to be an important tumour suppressor in various cancers by inducing cell apoptosis. Until recently, *PDCD4* was found to be regulated by miR-21 via PI3K/AKT signaling pathway in *in vitro* model of AD [34]. In the treatment of SH-SY5Y cells with A β_{1-42} , *PDCD4* expression were repressed by miR-21. In this study, our results validated that *PDCD4* was remarkably reduced in DAT individuals. Through luciferase assay, we identified that miR-150-5p

suppressed expression of *PDCD4* at the transcription level. So far, little is known about the biological role of *PDCD4* in miR-150-5p-based regulatory mechanisms of AD. Further investigation is warranted.

Conclusions

Our findings identified miR-150-5p as a potential candidate biomarker for DAT. miR-150-5p was well correlated with CSF A β _{1–42} and tau, AD pattern of cerebral atrophy and cognitive performance. To the best of our knowledge, this study is the first to demonstrate the role of miR-150-5p as a potential biomarker of DAT coupled with clinical measures (Fig. 6). While miR-150-5p has the potential of being developed as a clinical biomarker for DAT, future longitudinal studies with larger cohorts are needed to further validate the utility of miR-150-5p in DAT.

Abbreviations

A β = amyloid- β ; PBMC = peripheral blood mononuclear cells; DAT = dementia of Alzheimer's disease type; MMSE = Mini-Mental State Examination; MoCA = Montreal Cognitive Assessment; MCI = mild cognitive impairment

Declarations

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Author's Contributions

S.Y.C., A.V., K.P.N., A.S.B., H.T., B.Z.W., Y.J.T., and F. Z. performed the study and acquired, analysed, interpreted the data, draft and edited the manuscript. A.S.N., S.C.L., K.O., and E.K.T analysed and interpreted the data. N.K. and L.Z conceived and designed the study, acquired, analysed, interpreted the data and drafted the manuscript with input from the co-authors.

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Availability of data and materials

All data associated with this study are available upon request.

Ethics approval and consent to participate

The study was approved by the SingHealth Centralized Review Board and adhered to the tenets of the Declaration of Helsinki and local clinical research regulations. A signed informed consent was also

obtained from each patient.

Consent for publication

Not applicable.

Competing Interests

Authors declare that they have no competing interests.

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Figures

Figure 1

Figure 1

MiRNA-sequencing data analysis reveals Alzheimer's dementia is distinct from MCI and healthy subjects. (a) Hierarchical clustering analysis. HS, MCI and DAT individuals from discovery cohort were labeled with blue, grey and red letters. The MC replicates sequenced on different lanes are marked by black rectangles, suggesting that the technical error was small. The dendrogram showed four clusters: blue, yellow, grey and red lines. DAT were enriched in the group shaded by the red rectangle. Volcano plots were drawn by

plotting $-\log_{10}$ (FDR) against $\log_2$ (fold change) of each miRNA in comparison between (b) DAT and HS, (c) DAT and MCI, and (d) MCI and HS subjects. Wilcoxon-Mann-Whitney (WMW) p-values with Benjamini-Hochberg adjustment were used as FDR. Differentially expressed genes at $< 15, 30\%$ cutoffs were colored by blue and green. Expression patterns of differentially expressed (e) miR-328-3p, (f) -7706 and (g) -150-5p. Dots represented individual expression values. Data shown as means $\pm$ SD.

Figure 2

Figure 2

MiR-150-5p expression is upregulated in dementia of Alzheimer’s disease type individuals. Expression levels (a) and receiving operating curve (ROC) analysis (b) of miR-150-5p in PBMC of an independent cohort. Expression levels (c) and ROC analysis (d) of miR-150-3p in PBMC of an independent cohort. Data are presented as the mean $\pm$ SEM. One-way ANOVA with Tukey post hoc test was used to compare the differences between each disease group for all data sets. The statistical significance levels were set at *** p < 0.001, ns = no significant.

Figure 3

Figure 3

Association between expression levels of miR-150-5p and cognitive assessment, CSF A β 1-42 and tau levels. Correlations of miR-150-5p expression levels with (a) MMSE score and (b) MoCA score, (c) CSF A β 1-42 levels, and (d) CSF tau levels are shown. Lower scores indicate cognitive decline. Lower CSF A β 1-42 and higher CSF tau levels indicate higher risk of associated with dementia of the Alzheimer's type. Data were analyzed by a Spearman correlation test. The statistical significances were set at ** $p < 0.01$ and *** $p < 0.001$.

Figure 4

Figure 4

High miR-150-5p levels are associated with lower voxel-wise grey matter volume in AD specific regions. (a) Higher than median miRNA levels are associated with lower voxel-wise grey matter volume in temporal and parietal regions. Individuals with higher than median level of miR-150-5p showed reduced grey matter volume in parietal and temporal regions. (b-c) Higher miR-150-5p levels are associated with lower grey matter volume in the default mode network. Increasing levels of miR-150-5p were related to

lower grey matter volume in the left and right hippocampal regions of interest comprising the default mode network. (d-e) Higher miR-150-5p levels are associated with lower grey matter volume in the executive control network. Increasing levels of miR-150-5p were related to lower grey matter volume in the left and right posterior parietal cortex regions of interest comprising the executive control network.

Figure 5

Figure 5

PDCD4 is the downstream target of miR-150-5p. (a) Enriched GO terms for biological process, molecular function and cellular components [50]. (b) Enriched KEGG pathway analysis. Significantly enriched terms were presented with cut-off $p < 0.01$. (c) Top 11 potential miR-150-5p targets which were implicated to AD pathology and/or Wnt signaling pathway. (d) Expression levels of PDCD4 in PBMC of an independent cohort. (e) One of the four putative miR-150-5p binding sites (AACCCUC-red), position 2081-2096 at the 3'UTR of PDCD4 shown. (f) Overexpression of miR-150-5p by miR-150-5p mimic. (g) Luciferase assay of PDCD4 upon miR-150-5p overexpression. (h-i) Overexpressed miR-150-5p downregulates PDCD4 (h) mRNA by qPCR and (i) protein level by western blot. The statistical significances were set at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ by student's t test.

Figure 6

Schematic workflow for identification of miR-150-5p as a biomarker in PBMC of DAT. The schematic diagram describes the miRNA expression profile using miRNA-seq, followed by validation of predictive

miR-150-5p in PBMC of an independent cohort. Subsequently, miR-150-5p was identified as a potential DAT biomarker by using clinical and experimental approaches.