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Role of mi-RNAs in Diagnosing *APP* and *BACE-1* Targeted Cognitive Deficits of Alzheimer's Disease

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KEYWORDS Alzheimer's Disease. Amyloid Deposition. Amyloid Precursor Protein. Circulating miRNAs. Cognitive Health. Neuroinflammation

ABSTRACT miRNAs play vital pro-survival roles in regulating anti-inflammatory and memory based signalling cascades of the central nervous system. Amongst which, selective miRNAs including miR-17, miR-16, miR-101, miR-132, miR-31 and many more are studied as the post-transcriptional regulators of several conditions like Alzheimer's disease (AD). In the present study a total of 32 subjects comprising of 16 AD cohorts and 16 age adjusted control subjects were chosen from south Indian region (Coimbatore), screened for the *APOE* isoform incidence and the hippocampal amyloid deposition cascade associated relative expression of two major circulating miRNAs on *APP* and *BACE-1* genes. In addition, cognitive health of individual subjects was also calculated based on the MMSE tool. The outcome strongly portrayed that there exists a positive correlation between the relative up regulation and down regulation of miRNAs miR-132 and miR-31 on *APP* and *BACE-1* targeted cognitive deficits seen in the elderly aged AD women.

INTRODUCTION

miRNAs are small non-coding nucleic acid sequences that range from 15-20 base pair lengths and are mostly studied for their ability to regulate the post transcriptional mechanism of a target gene achieved by binding to the 3' untranslated region (3' UTR) region of target's complementary sequence (Angelucci et al. 2019). Recent studies report that altering miRNA expressions influences the epigenetic modifications that lead to varying effects on cognition. miRNAs play critical roles as both disease biomarkers (Lekka and Hall 2018), and molecular targets for disease therapeutics (Deverman et al. 2018).

The up-regulation and down-regulation of several mi-RNAs in *in-vivo* tissue and extracellular fluids have alternate effects on various signalling pathways that relay cognition and health (Song and Lee 2015; Maffioletti et al. 2020). Most relevant studies recently reported that altered expressions of miRNAs including miR-127-3p down-regulation in CSF, miR-93 and miR 501-3p down-regulation in serum, miR-342-3p up-regulation seen in the blood of AD patients have strong associations with cellular amyloid beta (Aβ) toxoid protein clearance. This in turn leads to severe decline in cognition and is stimulated through the activation of pro-inflammatory transcription factor (NF-κB), which is under the transcriptional control of several miRNAs including miR-9, miR-34a, miR-125b, miR-146a and miR-155 that are up-regulated (Zhao et al. 2014).

The most abundantly found miRNA in brain tissues, initially reported in nerve tissue of mice models was miR-132 (Lagos-Quintana et al. 2002). This mi-RNA sequence of 22bp length is believed to play a major role in inducing tau aggregation and impaired cognitive skills upon suppression (Hansen et al. 2016). In addition, numerous pathological reports have shown that the miR-132 is one of the most commonly seen miRNA down-regulated in post-mortem brains of AD subjects (Pichler et al. 2017). Similarly, a concomitantly expressing miRNA whose expression abolishes Aβ pathology and regulates the neuronal development and cognition function

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in Alzheimer's disease patients is miR-31 (Barros-Viegas et al. 2020). In the present study, the researchers have studied the effects of miR-132 and miR-31 expression in late onset AD (LOAD) patients with declined cognitive health and its effect on *APP* and *BACE-1* specific mutation occurrence seen in AD cohorts of Coimbatore region, Tamil Nadu, a south Indian state.

MATERIAL AND METHODS

Subject Recruitment and Sample Collection

A total of sixteen AD samples (n=16) along with sixteen age adjusted healthy controls (n=16) were chosen for the study. The AD cohorts were in-patients of Rahul's Elder Care Centre, Coimbatore, and were screened and confirmed for the disease pathology. From each of the chosen individuals aged above 55 years, approximately 5 ml of blood was collected in BD vacutainer Rapid Serum Tubes (2 ml of blood) and EDTA tubes (3 ml of blood) after receiving self-filled informed consent forms and MMSE questionnaires, respectively. In case of the AD patients, consent and MMSE assessments were carried out through the caregivers' access. All the sample collection procedures were carried out after obtaining Institutional Human Ethical Committee (IHEC) clearance and approval from Bharathiar University, Coimbatore, Tamil Nadu, India (BUHEC-009/2018; 10.08.2018).

MMSE

A total of sixteen (n=16) English for India version of the Mini-Mental State Examination (MMSE) Test Forms acquired from the Psychological Assessment Resources (PAR) Inc., United States and was used to assess the cognitive health of all AD patients (PAR customer No: CU-10009460). The MMSE scores were calculated based on the PAR guidelines.

DNA/RNA Extraction

The genomic DNA from collected blood samples was isolated using modified Miller et al. (1988) method. Finally the DNA pellets were pooled out using ice cold absolute alcohol, suspended in 100µl of 1X TE buffer and checked for purity using Nanodrop (NanoDrop™ 2000/2000c Spectrophotometer-Thermo Scientific™) and was further stored at 4°C until use. In addition, the miRNeasy Serum/Plasma Kit (QIAGEN, Hilden, Germany) was used to isolate the total Cell-free RNA from 200µl of serum separated out from the collected blood samples.

APOE Genotyping

APOE genotyping was performed by digesting the isolated DNA products using *HhaI* restriction enzyme (NEB Inc. England) for 18 hours at 37°C set water bath, and assessing the RFLP patterns cumulatively on 3 percent Agarose gel electrophoresis or 12 percent polyacrylamide agarose gel electrophoresis system (Mohtasebi et al. 2021).

qRT-PCR

The total RNA from the collected blood samples was isolated using the RDP-Trio Reagent RNA Isolation protocol (HiMedia TM India), yielding an approximate RNA product of 100 ng concentration with RIN~3.95 based cut-off threshold on optical density at 260/280 nm wavelength between the range of 1.6-2.2. All the RNA samples were reverse transcribed to obtain concomitant cDNA sequence using the Hi-cDNA synthesis kit (HiMedia TM India) protocol. The genes *APP* and *BACE-1* spanning for a sequence length of 134bp and 189bp was screened for relative quantification on QuantStudio™ 5 Dx Real-Time PCR System- Thermal cycler (Thermo fisher Scientific™ - Applied Biosystems), where each of the reaction mixture consisted of 1µl (25 nanomoles) of each corresponding gene-specific primers (*APP:* F-52 -GGCTCTGGAGGTACCCACTGAT', R-5'-GGTTG-GCTTCCACCACGTTT'; *BACE-1*: F-52 -TG-GAGGGCTTCTACGTTGTC and R-52 -CAGAGTG-GCAGCAGCATGAAGAG), 2µl of cDNA synthesised and 6 µl using Maxima SYBR Green/Fluorescein qPCR Master Mix (2X) (Thermofisher Scientific, India) made up to a volume of 12 µl with DEPC treated water (Caykara et al. 2019). Normalisation against a reference was carried out using qPCR Human Reference Total RNA (Takara Bio Inc. India) of β-actin gene. A negative control (nuclease-free

water) was also included in each run set in the thermal cycler. The hot start PCR was set with the reaction comprised of an initial denaturation step of 95°C for 2 minutes, followed with 30 cycles of 95°C denaturation step for 5 seconds and an annealing step at 60°C for 10 seconds with an extension step of 10 seconds at 52°C.

miRNA Expression

Candidate miRNAs from serum were validated using TaqMan Micro RNA Assays (Thermo Fisher Scientific) by qRT-PCR. Where 10µl of serum RNA was reverse transcribed to cDNA using the Hi-cDNA synthesis kit. 10µl of Real Time PCR product was pre-amplified using the TaqMan PreAmp Master Mix for 20 cycles -where, the pre-amplification primer pool containing two target miRNA primers (hsa-miR-132 [Hs04231496_s1], and hsa-miR-31 [Hs04231431_s1]) along with one normalisation miRNA primer (cel-miR-39-3p) were matched. The qRT-PCR was set up with a final reaction volume of 25µl in triplicate copies. The final primer concentrations were set to 0.03X in each reaction volumes. Relative expression of the individual miRNAs was calculated using the Levak's method by calculating the $2^{\tilde{}C}_{T}$ values with reference to the standard spike-in (cel-miR-39-3p) miRNA expression for normalisation (Souza et al. 2020).

Statistical Analysis

Student t-test for independent samples was used to study the comparisons between groups of normally distributed continuous variables and $\chi 2$ test was used preferentially to compare the frequencies between cognitive levels. Comparison of circulating miRNA levels (non-normally distributed variables) was performed by the Wilcoxon (Mann-Whitney U) test.

RESULTS

After choosing 35 in-patients from the elder care centre, samples (of whole blood) could only be obtained from 16 female patients scoring an MMSE record of 3±1.94 (Mean±SD) with an average age of 72.93±6.78 (Mean±SD). Amongst which 4 individual subjects showed severe symptoms of stroke and diabetes, with the majority of them being diagnosed with type two diabetes mellitus (T2D). The confirmed AD patients (n=2) exhibited hippocampal depositions of senile plaques and also were witnessed with occurrences of recurrent seizures. These subjects also showed severe decline in cognitive health having MMSE scores < 2. The clinical characteristics of chosen subjects are given in Table 1, and the cumulative *APOE* genotype patterns (Fig. 1) predicted in AD subjects showed ε2=ε4>ε3/4>ε3=ε2/3>ε2/4, with ε2 and ε4 allele being the most frequently occurring allelic variation. In the case of control subjects the observed genotype pattern was ε2/3>ε3/3>ε2/2. The gene expression studied for all the subjects showed a significant increase of *APP* and *BACE-1* gene expressions in AD subjects depicting

Table 1: Demographic and clinical backgrounds of the subject groups

	Control (n=16) *Mean or Percentile*	*AD (n=16)* *Mean or Percentile*	*p value*
Age	72± 5.5	71.875± 4.787	0.943
Systolic pressure	87± 12.75mm Hg	162±13.16mm Hg	0.667
FPG	167± 1.78mg/dL	210± 2.754mg/dL	0.875
MMSE score	0.000	3/30± 1.94	0.000
APOE Genotype			
ε2	25	0.000	0.000
ε3	12.5	37.5	1.678
ε4	25	0	0.000
ε2/ε3	12.5	50	1.235
ε3/ε4	18.75	12.5	1.075
ε2/ε4	6.25	0.000	0.000
Use of			
Donepezil	0.000	5± 2.75mg/day	0.000
Rivastigmine	0.000	1.5± 1.32mg/day	0.000
Memantine	0.000	10± 1.23mg/day	0.000

FPG: Fasting plasma glucose; MMSE: Mini mental state examination; APOE: apolipoprotein E

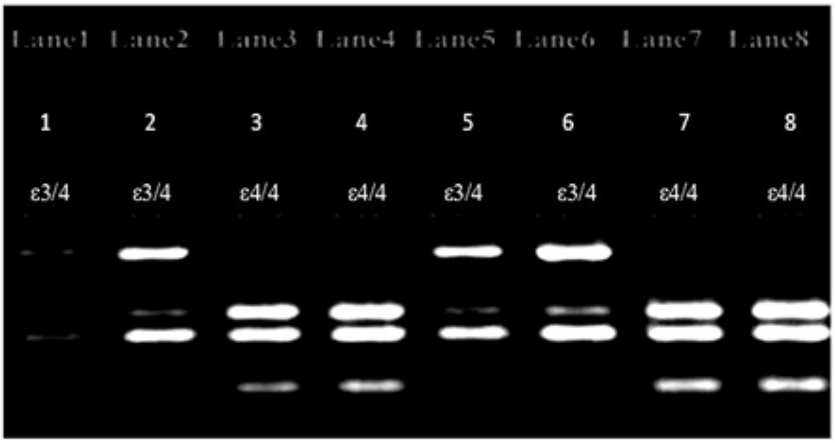

Fig.1. The image shows gel picture retained from the Gel documentation system. The lanes 1 to 8 represent each of the individual samples 1 to 8 after the RELP reation on 3% agarose gel. The variation seen in band lengths represent various of ApoE genorype having specific base pair size as follows: ε2/2 (104bp and 91bp): ε3/3 (91bp, 53bp and 32bp): ε4/4 (72bp, 53bp and 32 bp) for homozygous genotypes and ε2/3 (104bp, 91bp, 53bp and 32bp): ε3/4 (91bp, 53bp and 32bp), ε2/4 (104bp, 53bp and 32bp) respectively

nearly 3 and 3.5 folds higher transcriptional activity when compared to that of the expression seen in control subjects. Although not achieving the significance, comparison between circulating miRNAs and relative gene expressions of the hippocampal cascade genes show a tendency of relevant difference for miR-132 and miR-31 against miR-39-3P (OR = 1.025; 95% confidence interval; p=0.035@ p≤0.05) as predicted in the graphical representation shown in Figure 2.

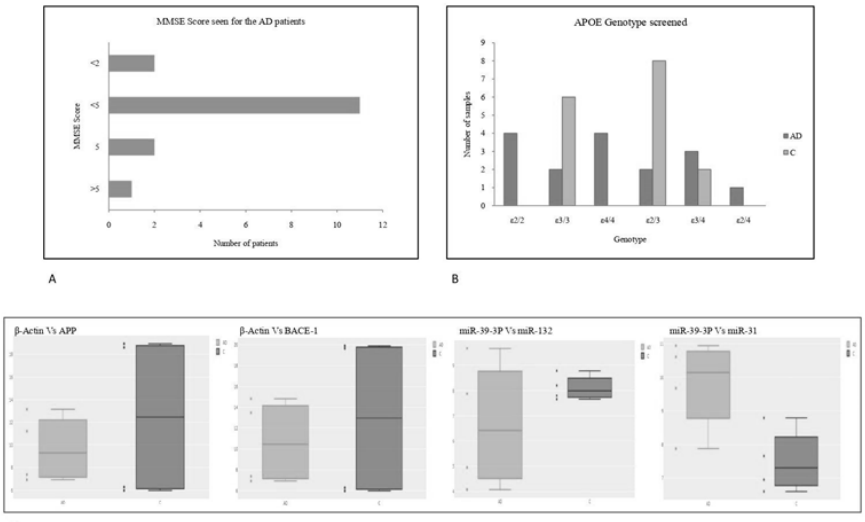

Fig. 2. A) The graphical representtion of MMSE scroes obtained by the D individuals, approximately more than 69% of the total (n=16) individuals scored below 5, B) The genotype screening results for both the AD and Control subjects represented in a graphical outcome, showing higher prevalence rates of ε2/3 in conrol and ε2/2=ε4/4 in AD subjects. C) The expression profiles observed for the genes APP and BACE-1 screened against β-actin and miR-132, mi-31 observed during the real time quantitative analysis

DISCUSSION

miRNAs have played critical roles in the pathophysiology of AD (Tan et al. 2014; Kumar and Reddy 2016; Kumar et al. 2017; Cosín-Tomás et al. 2017; Fransquet and Ryan 2018; Hampel et al. 2018). The neuropathological regulator miR-132 is involved in several mechanisms including neuroinflammation, synaptic plasticity and neuronal cell development. It is studied to be a negative regulator of inflammatory response in PC12 (Zhang et al. 2019). The TNF receptor associated factor 6 (TRAF6) is a direct target of miR-132 in promoting cellular inflammation (Ji et al. 2018). Several animal model studies have reported that the down-regulation of miR-132 in AD mouse model brains have resulted in elevated phosphorylation of Tau proteins correlating the increased levels of hippocampal amyloid β depositions (Salta et al. 2016; Riancho et al. 2017). The miR-132/-212 double knockout mouse model exhibits cognitive deficit behaviours (Hansen et al. 2016).

An *in-vivo* study on the 3xTg-AD mouse model depicts that miR-31 expression inhibits AD neuropathology by downregulating the expression levels of APP and BACE1 proteins targeting the amyloidogenic pathway. Similarly, the present study shows that there exists a critical role played between the circulating peripheral miRNAs (miR-132 and miR-31) in *APP* and *BACE-1* targeted hippocampal amyloid deposition and cognitive deficits seen in the elderly population chosen. The gene expression studies of pathological genes and respective miRNAs noted strong correlations for the down-regulation of miR-132 having been implying strong associations in increased expressions of *BACE-1* gene thereby causing delayed amyloid clearance pathway. This can be eventually understood by the correlated increase seen in the expression levels of *APP* and *BACE-1* genes with reference to the miR-132 down-regulation and up-regulation of miR-31 investigated against the miR-39-3P levels in AD subjects. The concomitant up-regulation and down-regulation of the miRNAs are accountably higher for the ε4/4 genotype carrying cognitively preserved older women.

CONCLUSION

Plenty of findings in animal and human post-mortem samples have been reported with implication that loss of function or down-regulation in miR-132 directly affects the bimodal amplification of Tau pathology and Aβ depositions in AD pathology. This puts forward putatively the targets of miR-132 in AD based therapeutic approaches. Though the effects of miR-31 are reported very mildly during recent years, the relative up- and down-regulation of miR-132 in reciprocating expressions of miR-31 play crucial roles in abrogation of cognitive health in elderly aged groups. The present study is one of the novel approaches put forth in studying the relative expressions of circulating miRNAs in blood serum samples collected from the chosen south Indian population. The results strongly suggest that there is a positive correlation between the relative co-reciprocating expressions of miR-132 and miR-31 in delivering alternative relay of *APP* and *BACE-1* transcriptional theories that repeal cognitive functions seen in elderly aged AD witnessed women. The loss of circulating miR-132 thereby exacerbates the deposition of Aβ depositions in the hippocampal regions of the AD brains, and this leads to the elevated transcriptional activities of *APP* and *BACE1* genes targeting the inositol-1,4,5-trisphosphate 3-kinase B pathway that up-regulates the tau phosphorylation and neuronal damage witnessed in AD patients. A strong recommendation is also put forth to the scientific community to predominantly indulge in identifying tissue specific miRNAs, which serve as the key diagnostic biomarkers in AD pathology.

RECOMMENDATIONS

Tissue based miRNAs as biomarkers in the field of therapeutics are more advanced in *in-vivo* models and their evidence is being successfully used in several diagnostics in the near future. However, the role of circulating peripheral miRNAs as a biomarker tool in predictable diagnostics for neurological diseases such AD has to be carried forward. These findings will target stage specific therapeutics and risk predictions for mild cognitive impairments to AD during the disease progression.

AUTHORS CONTRIBUTIONS

(SS: Performed work, PB: Designed and generated idea and prepared manuscript; PVM: vali-

dation and approval of idea; BK and DI: formatting and literature collection)

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CONFLICTS OF INTEREST

The authors of this manuscript show no conflicts of interest

ABBREVIATIONS

AD	:	Alzheimer's Disease
APOE	:	Apolipoprotein E
APP	:	Amyloid Precursor Protein
Aβ	:	Amyloid Beta
BACE-1	:	beta-site Amyloid Precursor Protein Cleaving Enzyme
CSF	:	Cerebrospinal Fluid
LOAD	:	Late Onset AD
MMSE	:	Mini Mental State Examination
mRNA	:	messenger RNA
PAR	:	Psychological Assessment Resources
RFLP	:	Restriction Fragment Length Polymorphism
T2D	:	Type Two Diabetes Mellitus
TE	:	Tris EDTA
UTR	:	Untranslated Region
EDTA	:	Ethylenediaminetetraacetic Acid
ITPKB	:	inositol-1,4,5-trisphosphate 3-kinase B
TRAF6	:	TNF Receptor Associated Factor 6

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