Can Peripheral MicroRNA Expression Data Serve as Epigenomic (Upstream) Biomarkers of Alzheimer's Disease?

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common form of dementia. However, biomarkers that require testing in the brain tissue pose a formidable practical barrier to AD diagnostic innovation. MicroRNAs (miRNAs) are responsible for control of gene expression at the posttranscriptional level and are essential for the function of neuronal networks and neuronal survival. miRNA expression can impact the regulation of *APP* (amyloid beta A4 precursor protein), *PSEN1* (presenilin 1), *PSEN2* (presenilin 2), and *BACE1* (beta-secretase 1) genes in the brain that were previously implicated in AD pathophysiology. Little is known, however, on the extent to which peripheral tissue (e.g., whole blood) miRNA variation might offer clinical predictive value for AD. Moreover, few studies have examined multiple peripheral miRNA expression data at the same time. We report here, to the best of our knowledge, the first whole-blood-based and parallel study of seven miRNAs (*hsa-miR-9-5p*, *hsa-miR-29a-3p*, *hsa-miR-106a-5p*, *hsa-miR-106b-5p*, *hsa-miR-107*, *hsa-miR-125a-3p*, and *hsa-miR-125b-5p*) in relation to AD susceptibility. Notably, these miRNAs are situated ''upstream'' to the genes implicated in AD. We measured the whole-blood miRNA expression by a real-time polymerase chain reaction in a large study sample ($n = 281$), comprising patients with AD ($n = 172$) and healthy controls ($n = 109$). A reduction in whole-blood expression of *hsa-miR-9-5p*, *hsa-miR-106a-5p*, *hsa-miR-106b-5p*, and *hsa-miR-107* was significantly associated with an increased risk of AD ($p < 0.05$). Notably, after receiver operating characteristics curve analyses, *hsa-miR-106a-5p* displayed, as a predictor variable, 93% specificity and 68% sensitivity. On the other hand, the expression of *hsa-miR-29a-3p*, *hsa-miR-125a-3p*, and *hsa-miR-125b-5p* was not significantly different between patients and controls ($p > 0.05$). In conclusion, these observations warrant replication in larger samples while making a contribution to translational research, precision medicine, and biomarker literatures, by expanding the current efforts for AD diagnostic innovation to the realm of epigenomic pathways such as miRNA expression variation among patients.

Introduction

ALZHEIMER'S DISEASE (AD) is a progressive neurodegenerative disorder and the most common form of dementia. The current diagnostic approaches rely on measurement of the attendant memory loss and cognitive decline but offer only modest diagnostic insights. Innovative and robust biomarkers of AD are, therefore, sorely needed (Van Giau and An, 2016). In this context, for decades, the neurobiology of AD and putative biomarkers of this complex disease have received intensive research attention in numerous neuroscience laboratories and international scientific consortia. However, the biomarker candidates identified in the brain tissue warrant further ''translation'' in peripheral tissues (e.g., whole blood) that are amenable to clinical diagnostics development (Fig. 1). Biomarkers that require testing in the brain tissue pose a formidable barrier to progress in AD diagnostics discovery and development owing to obvious practical limitations of brain biopsy in patients.

MicroRNAs (miRNA) are a group of endogenous, small, noncoding RNA molecules consisting of nearly 22 nucleotides and are responsible for control of gene expression at the posttranscriptional level by binding to the 3′ untranslated region of target mRNAs (Ambros, 2004). miRNAs are essential for the function of neuronal networks and neuronal survival (Esteller, 2011). For example, miRNA expression can impact the regulation of *APP* (amyloid beta A4 precursor protein), *PSEN1* (presenilin 1), *PSEN2* (presenilin 2), and

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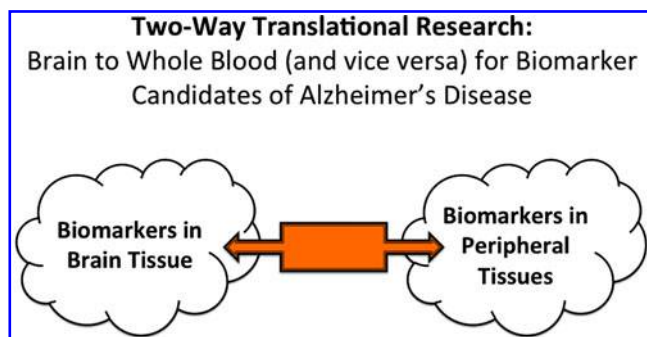
FIG. 1. Underscoring the need for ''bi-directional translational research'' so as to move brain-derived and peripheral tissue-based biomarker candidates toward robust clinical diagnostic applications.

BACE1 (beta-secretase 1) genes in the brain that were previously implicated in AD pathophysiology (De Strooper and Christen, 2010). Little is known, however, on the extent to which whole-blood miRNA variation targeting such AD-relevant candidate genes might offer clinical predictive value.

We report here, to the best of our knowledge, the first whole-blood-based and parallel study of seven miRNAs (*hsa-miR-9-5p, hsa-miR-29a-3p, hsa-miR-106a-5p, hsa-miR-106b-5p, hsa-miR-107, hsa-miR-125a-3p,* and *hsa-miR-125b-5p*) in the same study sample, in relation to AD susceptibility. Notably, these miRNAs are situated ''upstream'' to the genes implicated in AD (Table 1).

Materials and Methods

Study sample

The participants were recruited from the Neurology Department of the Medical Faculty of Mersin University in Turkey. The total study sample ($n = 281$) comprised AD patients ($n = 172$) and healthy controls ($n = 109$). Both study groups had a Turkish descent and were aged 60 years and older. The patients with AD were diagnosed according to the

TABLE 1. CANDIDATE MICRORNA BIOMARKERS FOR ALZHEIMER'S DISEASE, THEIR TARGET GENES, AND THE BRAIN REGIONS WHERE THE MICRORNAS ARE EXPRESSED

miRNAs	Target gene	Evidence of expression in the brain tissue	Reference
hsa-miR-9	BACE1	Frontal cortex Hippocampus	Ludwig et al. (2016) Taguchi (2013) Lukiw (2007)
hsa-miR-29a	BACE1	Frontal cortex	Hébert et al. (2008)
hsa-miR-106a	APP	Temporal cortex Cerebral cortex	Patel et al. (2008)
hsa-miR-106b	APP	Temporal cortex Cerebral cortex	Hébert et al. (2008) Hébert et al. (2009)
hsa-miR-107	PSEN1	Neocortex	Wang et al. (2008)
hsa-miR-125a	PSEN2	Temporal cortex	Wang et al. (2011)
hsa-miR-125b	PSEN1	Frontal cortex Hippocampus	Lukiw (2007)

miRNA, MicroRNAs.

DSM-IV-TR (American Psychiatric Association, 2000) and the ICD-10 Classification of Mental and Behavioral Disorders (World Health Organization, 1992). The patients were late-onset AD type (except four individuals). The healthy control subjects had no history of AD, were evaluated with the DSM-IV-TR and ICD-10, and were to be ascertained as control subjects. Importantly, co-morbid complex disorders such as hypertension, diabetes, and vascular disease were neither among the cases nor among the controls, as determined by detailed medical history, thus lending confidence for the present AD peripheral biomarker association study. The data on age and gender were recorded and accounted for in multivariate statistical analyses between the cases and the controls. The present study sample comprised, by and large, the late-onset type of AD, except four patients. The study was approved by the Institutional Research Ethics Committee of the Faculty of Medicine at Mersin University. A written informed consent was obtained from all subjects. A 10 mL whole-blood sample was drawn from each subject into a coded and heparinized tube for miRNA expression analysis.

Selection of miRNA genes for expression

We selected the miRNAs targeting the *APP*, *BACE1*, *PSEN1*, and *PSEN2* genes that had been previously implicated in relation to AD-related phenotypes. These miRNAs were predicted using MicroCosm Targets, TargetScan, miRNAMap, microInspector, PicTar, miRWalk, mirbase, mirdb, Patrocles, Diana lab, and Human MiRNAs & Diseases databases. Subsequently, we specified and corroborated the normal blood tissue expression of the candidate miRNAs that were identified earlier using Ferrolab (Ferrolab, the Bioinformatics and Data Mining Group, 2010) and Miracle (Zotos et al., 2012) databases. Finally, to demonstrate the efficacy values (matching scores) of the identified miRNAs' match with their target genes, we used the microRNA database (Wang, 2008).

Synthesis of primers and probes

We used the pdC and fluorogenic Zip Nucleic Acids (ZNA™) probe technologies for synthesis of the probes. Substitution of C-5 propynyl-dC (pdC) for dC is an effective strategy to enhance base pairing. Using these base substitutions, duplex stability and melting temperatures are raised by C-5 propynyl-C 2.8° per substitution. ZNA probes provide broad flexibility in assay design and represent an effective alternative to minor groove binder and locked nucleic acid-containing oligonucleotides (Paris et al., 2010). The specific stem–loop primers, reverse transcriptase–polymerase chain reaction (RT-PCR) primers, and probes for the miRNAs were designed using Primer Express 3.0 software provided by Applied Biosystems (Table 2) (Yılmaz, 2013).

Reverse-transcriptase PCR

RNA was extracted from whole blood using the method of acide guanidinium–phenol chloroform (Chomczynski and Sacchi, 1987). Reverse-transcriptase reactions contained 10 ng total RNA, 50 nM stem–loop reverse transcriptase (RT) primer (Metabion International AG), 5×RT buffer, 10 mM dNTPs, 0,25 mM MultiScribe Reverse Transcriptase (Thermo Scientific), 0,1 mM RiboLock RNase inhibitor (Thermo

Table 2. List of the MicroRNAs Characterized, the Reverse Transcriptase–Polymerase Chain Reaction, and Real-Time Polymerase Chain Reaction Primer-Probe Sequences Utilized in the Experiments

miRNA	miRNA ID[a]	miRNA sequence ID[b]	Primer and probe sequences
hsa-miR-26b-5p	407017	NR_029500.1	5′-RT-GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGCA TACGACACCTAT-3′
			5′-F-GCCGCTTCAAGTAATTCAGG-3′
			5′-PR-FAM-TG(pdC)ATA(pdC)GA(pdC)A(pdC)CTATCC-ZNA4-BHQ-1-3′
hsa-miR-9-5p	407046	NR_029691.1	5′-RT-GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGCA TACGACTCATAC-3′
			5′-F-GCCGCTCTTTGGTTATCTAGCT-3′
			5′-PR-FAM- TG(pdC)ATA(pdC)GA(pdC)T(pdC)ATA(pdC)AG-ZNA4-BHQ1-3′
hsa-miR-29a-3p	407021	NR_029503.1	5′-RT-GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGCA TACGACTAACCG-3′
			5′-F-GCCGCTAGCACCATCTGAAAT-3′
			5′-PR-FAM- TG(pdC)ATA(pdC)GA(pdC)TAA(pdC)CGAT-ZNA4-BHQ1-3′
hsa-miR-106a-5p	406899	NR_029523.1	5′-RT-GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGCA TACGACCTACCT-3′
			5′-F-GCCGCAAAAGTGCTTACAGTGC-3′
			5′-PR-FAM- TG(pdC)ATA(pdC)GA(pdC)CTACCTGC-ZNA4-BHQ1-3′
hsa-miR-106b-5p	406900	NR_029831.1	5′-RT-GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGCA TACGACATCTGC-3′
			5′-F-GCCGCTAAAGTGCTGACAGT-3′
			5′-PR-FAM- TG(pdC)ATA(pdC)GA(pdC)ATCTGCAC-ZNA4-BHQ1-3′
hsa-miR-107	406901	NR_029524.1	5′-RT-GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGCA TACGACTGATAG-3′
			5′-F-GCCGCAGCAGCATTGTACAGGG-3′
			5′-PR-FAM- TG(pdC)ATA(pdC)GA(pdC)TGATAG(pdC)C-ZNA4-BHQ-1-3′
hsa-miR-125a-3p	406910	NR_029693.1	5′-RT-GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGCA TACGACGGCTCC-3′
			5′-F-GCCGCACAGGTGAGGTTCTTG-3′
			5′-PR-FAM-TGCATACGACGGCTCCCA-ZNA4-BHQ1-3′
hsa-miR-125b-5p	406911	NR_029671.1	5′-RT-GTCGTATGCAGTGCAGGGTCCGAGGTATTCGCACTGCA TACGACTCACAA-3′
			5′-F-GCCGCTCCCTGAGACCCTAAC-3′
			5′-PR-FAM- TG(pdC)ATA(pdC)GA(pdC)T(pdC)A(pdC)AAGT-ZNA4-BHQ1-3′
miR-universal R primer			5′-R-GTGCAGGGTCCGAGGTAT-3′

[a]www.ncbi.nlm.nih.gov/gene
[b]www.ncbi.nlm.nih.gov/SNP
F, forward; PR, probe; R, reverse; RT, reverse transcriptase.

Scientific), and nuclease-free water to a total reaction volume of 15 μL. The reaction was performed on an automated Thermal Cycler (Techne Flexigene). RT-PCR was performed for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C; then, cDNA samples were held at −20°C.

Quantitative-comparative CT (ΔΔCT) real-time PCR

Quantitative-comparative C_T ($\Delta\Delta C_T$) real-time PCR was performed in an ABI Prism 7500 Real-Time PCR System (Applied Biosystems) using the SDS 2.0.6 software. The hsa-miR-26b-5p (Applied Biosystems application note cms 044972) was used as an endogenous control. We used a reference RNA sample (AM7155; Life Technologies). The 25 μL PCR included 5 μL RT-PCR product, 2×TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM of each primer (Primer F and Universal Primer R; Metabion

International AG), and 200 nM TaqMan® probe. The reactions were incubated in a 96-well plate of preincubation at 52°C for 2 min, and at 94°C for 10 min, followed by 50 cycles at 94°C for 15 sec, and at 60°C for 90 sec. All reactions were run in triplicate. $\Delta\Delta C_T$ values were used and calculated of $2^{-\Delta\Delta C_T}$ values (Livak and Schmittgen, 2001).

Statistical analyses

The data were processed and analyzed using the statistical package IBM SPSS-V22.0 (IBM Corp.) for Windows. The normality assumption of the $2^{-\Delta\Delta C_T}$ values was checked by the Shapiro–Wilk test. Since the assumption of normality was not met, the $2^{-\Delta\Delta C_T}$ values were expressed as median (50th percentile), first quartile (25th percentile), and third quartile (75th percentile), and the comparison between groups was performed using the Mann–Whitney U test. Univariate and

multivariate logistic regression analyses were used to obtain odds ratio of variables that were significantly related with disease. The chi-squared test was used for categorical variables (gender), and the *t*-test was used for continuous variables (age) between AD patients and controls. Differences (two-tailed *p*) less than 0.05 were regarded as significant. A receiver operating characteristics (ROC) curve was performed, and the area under the curve was assessed for the specificity and sensitivity of AD prediction for the whole-blood miRNAs.

Results

The study sample comprised 281 individuals: 172 patients with AD (111 men and 61 women) and 109 (56 men and 53 women) controls. The age distribution for the patients and controls was 73.92 ± 1.06 and 69.42 ± 1.10, respectively. Age and gender were statistically controlled in univariate and multivariate analyses, as noted earlier.

We found that a decrease in whole-blood expression of *hsa-miR-9-5p*, *hsa-miR-106a-5p*, *hsa-miR-106b-5p*, and *hsa-miR-107* was significantly associated with an increased risk of AD ($p < 0.05$) (Tables 3 and 4). In contrast, the expression of *hsa-miR-29a-3p*, *hsa-miR-125a-3p*, and *hsa-miR-125b-5p* was not significantly different between patients and controls ($p > 0.05$).

Out of the seven miRNAs whose expression was analyzed in the whole blood, four turned out as being significantly associated with AD. Hence, we conducted further joint analysis of these four miRNA variables together as composite predictors of AD (Table 5). Additionally, after ROC curve analyses, *hsa-miR-106a-5p* displayed, as a predictor variable, 93% specificity and 68% sensitivity (Table 6).

TABLE 3. MICRORNA EXPRESSION VALUES IN PATIENTS WITH ALZHEIMER'S DISEASE AND HEALTHY CONTROLS

miRNA name	Patients/ controls (n)	Median expression values in patients/ controls	25%	75%
hsa-miR-9-5p	165/94	0.0556	0.0032	0.3064
		0.2746	0.0311	1.1623
hsa-miR-29a-3p	150/98	0.1096	0.0133	0.9599
		0.2025	0.0253	1.0583
hsa-miR-106a-5p	148/84	0.0041	0.0004	0.5763
		1.5704	0.2565	10.254
hsa-miR-106b-5p	162/85	0.1086	0.0182	0.7135
		0.3557	0.1092	1.1317
hsa-miR-107	165/85	0.0078	0.0005	0.3019
		0.3590	0.0173	1.3872
hsa-miR-125a-3p	158/85	0.1611	0.0168	1.5207
		0.2951	0.0287	1.0873
hsa-miR-125b-5p	155/80	0.0760	0.0087	0.4437
		0.0847	0.0072	0.5696

Median (50th percentile), first quartile (25th percentile), and third quartile (75th percentile) are reported here. n = Numbers of individuals included in the statistical analyses for the patient and the control groups; the numbers noted for each miRNA association analysis are the numbers of patients and controls who could be characterized technically for the given miRNA. Note that in general, miRNA expression levels were lower in patients than in controls.

TABLE 4. LOGISTIC REGRESSION ASSOCIATION ANALYSES OF INDIVIDUAL MICRORNA EXPRESSION DATA WITH ALZHEIMER'S DISEASE

Logistic regression variable	p	Odds ratio	95% CI
hsa-miR-9-5p	0.001*	0.797	0.696–0.912
hsa-miR-29a-3p	0.194	0.994	0.984–1.003
hsa-miR-106a-5p	0.001*	0.814	0.717–0.923
hsa-miR-106b-5p	0.008*	0.817	0.703–0.949
hsa-miR-107	0.001*	0.736	0.625–0.867
hsa-miR-125a-3p	0.098	1.149	0.974–1.355
hsa-miR-125b-5p	0.608	1.039	0.897–1.204

*$p < 0.05$ is considered statistically significant, and after adjustment for age and gender. Note that in the case of miRNAs that were significantly associated with AD, they appear to offer a protective role against AD, in that their higher expression confers a lower risk for AD.

AD, Alzheimer's disease.

Discussion

AD is a common form of dementia, with a paucity of robust clinical diagnostics (Hallock and Thomas, 2012; Yılmaz et al., 2016). Moreover, it is essential that future diagnostics also consider the feasibility of practical applications in the clinic, not to mention in both developed and developing countries (Evans et al., 2015; Reddy et al., 2015; Wonkam and Hurst, 2014). Blood contains circulating miRNAs and could serve as a source of novel noninvasive biomarkers for the diagnosis of common complex neurodegenerative disorders such as AD (Kumar and Reddy, 2016). With the availability of biotechnologies targeted for biomarker discovery, much research has been conducted on AD and its putative diagnostics in the brain tissue and peripheral samples such as the whole blood. For these two sets of biomarker literature to be meaningful, we need to examine the emerging findings from both the brain biomarker research and peripheral diagnostics research in a context of AD pathophysiology. Only through such two-pronged approaches and insights, we will be able to decipher and translate biomarker data between the brain and the periphery in a context of AD (Fig. 1).

In the case of the seven miRNAs examined in the present study, they are reportedly all expressed in various brain regions (Table 1), and were associated in some studies with AD, when measured in the brain and/or peripheral tissues (Kumar and Reddy, 2016). On the other hand, and to the

TABLE 5. MULTIVARIATE LOGISTIC REGRESSION ANALYSES OF FOUR SIGNIFICANT MICRORNA ASSOCIATIONS WITH ALZHEIMER'S DISEASE

miRNAs analyzed together as composite predictors of AD	p	Odds ratio	95% CI
hsa-miR-9-5p	0.012	0.882	0.801–0.973
hsa-miR-106a-5p	0.003	0.856	0.774–0.947
hsa-miR-106b-5p	0.002	0.798	0.691–0.922
hsa-miR-107	0.001	0.711	0.620–0.815

*$p < 0.05$ is considered statistically significant, and after adjustment for age and gender. *p*-Values and the odds ratios are derived after composite analyses of the four miRNAs in statistical analysis.

Table 6. The Receiver Operating Characteristics Curve Analyses of the Four MicroRNAs That Were Significantly Associated with Alzheimer's Disease in the Present Sample

Compared groups	Test variables	AUC	95% CI	p	Specificity	Sensitivity	Cut-off
Patients versus controls	hsa-miR-9-5p	0.642	0.575–0.709	<0.005	0.585	0.594	≤0.107
	hsa-miR-106a-5p	0.786	0.728–0.845	<0.005	0.929	0.676	≤0.066
	hsa-miR-106b-5p	0.632	0.562–0.703	<0.005	0.682	0.605	≤0.196
	hsa-miR-107	0.692	0.627–0.757	<0.005	0.624	0.642	≤0.081

AUC, area under the curve; CI, confidence interval.

best of our knowledge, no previous study has examined these seven miRNAs in the same study sample, with a view to their potential for future clinical diagnostics applications. Moreover, no previous whole-blood-based study of *miR-106a-5p* and *miR-106b-5p* was reported in clinical association studies of AD.

We underscore that the miRNA expression levels were, by and large, lower in patients than in healthy controls. Additionally, *hsa-miR-9-5p*, *hsa-miR-106a-5p*, *hsa-miR-106b-5p*, and *hsa-miR-107*, which were significantly associated with AD in the present study (Tables 3 and 5), seem to confer protection against AD, since controls had displayed a higher expression of these miRNAs in the whole blood. From a biological plausibility standpoint, this makes sense, because the observed significantly lower expression of *hsa-miR-9-5p*, *hsa-miR-106a-5p*, *hsa-miR-106b-5p*, and *hsa-miR-107* in patients would mean that the corresponding target genes for these miRNAs (*BACE1*, *APP*, and *PSEN1*) would be expressed in higher abundance owing to the release of the traditionally, inhibitory effect of miRNAs on the target genes regulated by them.

Taken together, these observations bring about new possibilities for future clinical development of miRNAs, *hsa-miR-9-5p*, *hsa-miR-106a-5p*, *hsa-miR-106b-5p*, and *hsa-miR-107*, as potential peripheral diagnostics for AD. We wish to underscore that considering the ROC analyses reported in Table 6, *hsa-miR-106a-5p*, in particular, warrants future research attention as a putative regulatory or epigenomic biomarker of AD susceptibility.

Conclusions

In sum, we report here, to the best of our knowledge, the first whole-blood-based and parallel study of seven miRNAs (*hsa-miR-9-5p*, *hsa-miR-29a-3p*, *hsa-miR-106a-5p*, *hsa-miR-106b-5p*, *hsa-miR-107*, *hsa-miR-125a-3p*, and *hsa-miR-125b-5p*) situated ''upstream'' to the genes targeting AD. These observations require replication in larger samples while making a contribution to translational research, precision medicine, and biomarker literatures, by expanding the current efforts for AD diagnostics to the realm of epigenomic regulatory mechanisms such as miRNA expression variation among patients.

Acknowledgments

Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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Abbreviations Used

AD = Alzheimer's disease
miRNA = MicroRNA
ROC = receiver operating characteristics
RT-PCR = reverse transcriptase–polymerase chain reaction