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Blood serum miRNA: Non-invasive biomarkers for Alzheimer's disease

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Abstract

There is an urgent need to identify non-invasive biomarkers for the detection of sporadic Alzheimer's disease (AD). We previously studied microRNAs (miRNAs) in AD autopsy brain samples and reported a connection between miR-137, -181c, -9, -29a/b and AD, through the regulation of ceramides. In this study, the potential role of these miRNAs as diagnostic markers for AD was investigated. We identified that these miRNAs were down-regulated in the blood serum of probable AD patients. The levels of these miRNAs were also reduced in the serum of AD risk factor models. Although the ability of these miRNAs to conclusively diagnose for AD is currently unknown, our findings suggest a potential use for circulating miRNAs, along with other markers, as non-invasive and relatively inexpensive biomarkers for the early diagnosis of AD, however, with further research and validation.

Keywords

Alzheimer's disease; microRNA; blood serum

Introduction

Alzheimer's disease (AD) is the most common cause of dementia with a worldwide population of over 24 million, a number expected to double in the next 15 years (Ferri, et al., 2005). Early and accurate diagnosis of AD is crucial, enabling early treatments to slow or delay the progression of the disease and provide prognostic information. Current methods of neuro-imaging biomarkers include magnetic resonance imaging (MRI) structural analyses (Barber, Mungas, et al., 2002). However, the sensitivity of these imaging techniques does

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not afford sufficient demarcations in the medical temporal atrophy between AD and non-AD dementia to provide clear and decisive diagnosis (Scheltens, et al., 2002, Wahlund, et al., 2000). Functional neuro-imaging techniques have been developed for probable diagnosis (Ballard, et al.), but again with insufficient accuracy in discriminating between AD and control individuals (Dougall, et al., 2004). Even though positron emission tomography (PET) imaging has been able to distinguish cases with probable AD from normal and non-AD cases, wide variations in sensitivity and specificity have been reported (Patwardhan, et al., 2004). Amyloid beta (A β) (Sjogren, et al., 2002) and hyperphosphorylated tau (de Souza, et al.) levels in the cerebrospinal fluid (CSF) have also been suggested as diagnostic markers for AD and show great promise. However, the ability to identify biomarkers through less invasive procedures, such as in a blood test, would be significant. Attempts to measure A β levels in blood, thus far, have led to inconsistent results (Ballard, et al., Blennow, et al., Hampel, et al., Hansson, et al., Irizarry, 2004).

In addition to proteins, blood serum contains circulating miRNAs, endogenous small RNAs of 21–25-nucleotides that post-transcriptionally regulate gene expressions (Lagos-Quintana, et al., 2001). MiRNAs have been reported to be transported in blood in liposomes (Kosaka, et al.), high density lipoproteins (Vickers, et al.), Argonaute2 (Arroyo, et al., Turchinovich, et al.), and other proteins (Wang, et al.), protecting them from being degraded. Recently, circulating miRNA levels have been proposed as potential diagnostic tools for a number of diseases (Gilad, et al., 2008, Wang, et al., Zeng, et al.). MiRNAs have been shown to be differentially expressed in AD patients (Cogswell, et al., 2008, Hebert, et al., 2008, Lukiw, 2007, Wang, et al., 2008) and altered in response to A β (Schonrock, et al.). In a recent study we showed that miR-137, -181c, -9 and -29a/b are involved in AD by modulating the ceramide levels (Geekiyanage and Chan). We showed that ceramides, a sphingolipid, are increased in the brain cortices of a subgroup of sporadic AD patients along with serine palmitoyltransferase (SPT) levels, the rate limiting enzyme in the *de novo* synthesis of ceramide. We demonstrated that SPT long chain 1 (SPTLC1) and SPT long chain 2 (SPTLC2), two subunits of SPT, are post-transcriptionally regulated by miR-137/-181c; and miR-9-29a/b, respectively. We observed significant correlations between SPT, their corresponding miRNAs (miR-137, -181c, -9 and -29a/b), and A β in the autopsy AD brain samples, as well as a direct involvement of SPT and miR-137/-181c in A β production through transfection studies. Hebert et al. (Hebert, et al., 2008) had previously demonstrated the involvement of miR-29a/b in A β production. In addition, we showed negative relationships between SPT and their respective miRNAs in AD risk factor models, where miRNA levels were observed to decrease in mice fed a high fat diet and in female mice, thereby suggesting a potential therapeutic value (Geekiyanage and Chan). Here, we investigated the expression levels of these miRNAs in the blood sera of a subgroup of mild and severe sporadic AD patients and mouse risk factor models to assess the potential of using these miRNAs as early diagnostic markers.

Material and Methods

Patient information

AD (n=7) and control (n=7) blood serum samples were from the University of Kentucky (UK) Alzheimer's disease center tissue bank (ADC). The samples have been clinically diagnosed by neurologists, neuropsychologists, and other staff members in the ADC clinic. A complete description of the samples including age of the patient, gender, MMSE scores and the clinical diagnoses is provided in Table 1. The standard MMSE test in the UDS battery was used with no correction factors. The discrimination of AD, MCI and control cases were performed using current consensus-based methodologies which are previously well- described in a clinical-pathological consensus conference (Jicha, et al.). Based on this study, we placed individuals with MMSE scores of 29 and 30 (n=7) in the “control” group

and individuals with MMSE scores of 10–20 (n=6) and 1 subject with MMSE score of 8 in the “probable AD” group. Finally, based on Jicha, et al. we placed subjects with MMSE scores of 23–28 in the “MCI/probable Early AD” group. The MCI subjects included in this study only contains patients exhibiting amnesic MCI (indicative of prodromal AD) and not indicative of other type of MCI (e.g., vascular changes on MRI, or Parkinsonism). The patient dietary information is not available. Blood samples were obtained from living research subjects with appropriate IRB approval. Blood sera were separated by centrifugation at 3000 rpm for 5 mins.

Mice blood serum collection

Wild type C57/BL on a hybrid background, C3H/He (Charles River) x C57BL/6 (Jackson laboratories) were used in the diet and gender specific studies. Blood was collected from mice under anesthesia through puncturing of the aorta into venous collection tubes coated with clot activator and silicone. Blood serum was separated by centrifugation at 1600 g for 15 mins following 30 mins of clotting at room temperature. All procedures conducted were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Quantitative RT-PCR (qRT-PCR)

Total miRNAs were extracted using miRNeasy Mini Kit (Qiagen) and RNeasy MinElute Cleanup Kit (Qiagen) and total RNA was quantified using ND-1000 nanodrop spectrophotometer. Quality control was performed by assessing the OD ratio of 260/280 nm. In addition the PCR products were run on agarose gels. qRT-PCR was conducted using miScript SYBR Green PCR Kit (Qiagen) and MyiQ real time PCR detection system following reverse transcription using miScript Reverse Transcription Kit (Qiagen) according to manufacturer’s instructions. All miRNA primers were purchased from Qiagen and the relative expressions were calculated using the comparative CT method with spiked cel-miR-39 (Kroh, et al.), internal miR-22, internal miR-191 and internal miR-126 as the normalizing controls for human sera and internal miR-22 as the normalizing control for mouse sera.

Statistical analysis

Statistical significances were determined using both 2 tailed *t* tests and Mann-Whitney tests for the human sera samples and 2 tailed *t* tests were used on mice sera samples.

Results

MiRNAs are down-regulated in blood serum of probable AD patients

The expression levels of the miRNAs, that were previously shown to regulate SPT and A β , and were down-regulated in the brain cortices of a subgroup of sporadic AD patients (Geekiyana and Chan), were quantified in blood sera of 7 control (MMSE scores 29 and 30), 7 amnesic MCI/ probable early AD (MMSE scores 23–28) and 7 probable sporadic AD (MMSE scores 8–19) subjects (see Table 1 for patient information). Currently there is no generally agreed upon normalizing RNA with respect to blood serum or plasma. The generally used normalizing ribosomal RNA (RNU6B etc.) in miRNA analysis is typically not present in the blood. Therefore, the human blood sera from patients were spiked with *C. elegans* miRNA-39, cel-miR-39 (Kroh, et al.), prior to miRNA extraction. Cel-miR-39 was selected as it demonstrates no sequence homology to any known human, mouse, or rat miRNA. In addition miR-22 and miR-191 are abundantly expressed in the blood serum (Qiagen, 2011) and have not shown to be differentially expressed in the literature with respect to AD and therefore were also used for normalization. Further, we have observed that miR-126 expression levels remained unchanged in the brains of AD patients. Therefore,

the expressions of the respective miRNAs in the blood serum were also normalized to miR-126. The expression levels of miR-137, miR-181c, miR-9, miR-29a and miR-29b (Figure 1) were significantly (both $P < 0.05$, student's t test and Mann-Whitney test) down-regulated in the blood serum of probable AD patients when normalized to spiked cel-miR-39 (Figure 1A), internal miR-22 (Figure 1B), internal miR-191 (Figure 1C) or internal miR-126 (Figure 1D). The expression levels of the respective miRNAs (Figure 1) were significantly (both $P < 0.05$, student's t test and Mann-Whitney test) down-regulated in the blood serum of amnesic MCI/ probable early AD patients when normalized to spiked cel-miR-39 (Figure 1A), internal miR-22 (Figure 1B), internal miR-191 (Figure 1C) or internal miR-126 (Figure 1D) with the exception of miR-137, where the down regulation was not statistically significant when normalized to spiked cel-miR-39 and internal miR-191. However, a statistical significance may be achieved with the exclusion of the case exhibiting an MMSE score of 28, from the amnesic MCI/ probable early AD group.

Decreased miRNA expression levels in the blood serum of high fat diet fed mice

High dietary fat intake is identified as a potential risk factor for AD (Baker, et al., Bayer-Carter, et al., Julien, et al., Oksman, et al., 2006, Refolo, et al., 2000). We previously showed that the expression levels of miR-137, miR-181c and miR-9 were down-regulated in the brain cortices of high fat diet fed mice (Geekiyana and Chan). The miRNA expression levels were measured in the blood serum of male wild-type mice fed a 60% kcal high fat diet for a period of 5 months (starting at 4 months of age). In accordance with the expression levels in the brain, the expression levels of miR-137 ($P = 0.045$, student's t test), miR-181c ($P = 0.046$) and miR-9 ($P = 0.03$) (Figure 2) expression levels were down-regulated in the blood serum of mice fed a high diet. As the expression levels of miR-22 were stable across the human sera samples, miR-22 was used for normalization of miRNA expressions in the sera of mice fed a high fat diet.

miRNA are differentially expressed in blood serum according to gender

Research suggests that AD pathology may be more prevalent in females than in males (Alberca, et al., 2002, Bachman, et al., 1992, Brookmeyer, et al., 1998, Burns and Zaudig, 2002, Henderson and Buckwalter, 1994, McPherson, et al., 1999, Ripich, et al., 1995). We previously reported that the miR-137, miR-181c and miR-29a/b-1 expression levels are down-regulated in the cerebral cortices of female wild-type mice compared to males (Geekiyana and Chan). Here we demonstrate that the expression levels of miR-137 ($P = 0.01$), miR-181c ($P = 0.02$) and miR-29b-1 ($P = 0.046$) expression levels are down-regulated (Figure 3) in the blood serum of female mice (9 months of age) vs. male mice. However, the expression levels of miR-29a did not differ between the groups. As the expression levels of miR-22 were stable across the human sera samples, miR-22 was used for normalization of miRNA expressions in female vs. male mice sera.

Discussion

MiRNA levels were down-regulated in the sera of patients with probable AD (MMSE scores 8–19) and amnesic MCI/probable early AD (MMSE score 23–28) compared to normal patients (MMSE scores 29 and 30). This suggests a potential role for these miRNAs as early diagnostic markers. Moreover, screening for miRNAs in the sera as biomarkers, that i) directly affect a fundamental feature of AD neuropathology, ii) are diagnostically sensitive enough to detect, iii) can detect AD early in the course of the disease progression, and iv) are non-invasive, simple to perform and inexpensive, make them potentially good diagnostic biomarkers in accordance with the criteria described by the National Institute on Aging (1998).

In a previous study (Geekiyana and Chan) we identified that a subgroup of AD patients display increased levels of ceramide along with increased SPTLC1/2 protein levels in neocortices. However, the SPTLC1/2 mRNA levels did not differ from their control counterparts. We observed negative correlations between the expression levels of miR-137/-181c and SPTLC1, and between miR-9/-29a/b and SPTLC2 protein expressions, in these autopsy brain samples. These results in combination with cell culture studies suggested that SPTLC1/2 are post-transcriptionally regulated by their respective miRNAs. Similar negative relationships were identified between SPTLC1/2 and the corresponding miRNAs in AD risk factor models, i.e. high fat diet and gender specific. In addition, positive correlations between SPTLC1/2 and A β , and negative correlations between the respective miRNAs and A β were observed in these brain samples. Cell culture studies with “target protectors” and over-expression assays showed a direct effect of miRNAs on SPT and in turn on A β protein expression. These results together suggested that these miRNAs and SPT are involved in AD and represents a potential therapeutic target. In this current study, we suggest a prospective use for the circulating miRNAs as diagnostic markers.

We also observed that the same miRNAs were down-regulated in the blood serum of high fat diet and gender specific models. Expression of miR-137 is negatively regulated, epigenetically and transcriptionally by MeCP2 and Sox2 (Szulwach, et al.). Additionally, Sox2 genetic polymorphisms are associated with diabetic neuropathy in female patients but not in males (Gu, et al., 2009), providing a possible explanation for the dysregulation of miR-137 in female mice. Research performed on the treatment for Rett syndrome, a disease caused by mutations in MeCP2, suggests an increase in MeCP2 levels with the consumption of a high fat diet (Haas, et al., 1986, Liebhaber, et al., 2003). This may provide a potential explanation for the down-regulated miR-137 levels observed in mice fed a high fat diet. Further, miR-181c is positively and transcriptionally regulated by Akt1 (Androulidaki, et al., 2009). Akt activity has been observed to be decreased in response to high fat diet (Tremblay, et al., 2001), providing a possible explanation for the reduced miR-181c levels observed in mice fed a high fat diet. Interestingly, Akt1 expression levels have been shown to decrease considerably with age in the myocardial tissue of women (Camper-Kirby, et al., 2001) while MeCP2 expression levels increases with age in frontal cortices of males and females (Samaco, et al., 2004). The reduction in Akt expression in these elderly women may provide an explanation for the gender specific reduction of miR-181c observed with the mice in this study. However, further analysis is needed including age matched male samples to conclude the gender specific regulation of miR-181c by Akt. MiR-9 is negatively regulated by RE1-silencing transcription factor (REST) while it is positively regulated by cAMP-response element binding protein (CREB) (Laneve, et al.). In contrast, NF B, c-Myc and hedgehog signaling transcriptionally repress miR-29a/b (Mott, et al.). High dietary fat intake has been shown to activate cortical NF B in rats (Zhang, et al., 2005); however we did not observe miR-29a/b-1 to be down-regulated in mice fed a high fat diet. Differential expression patterns of hepatic miR-29 family have been observed between different mouse strains fed with methyl-deficient diets (Pogribny, et al.), suggesting species and strain variations may contribute to the unchanged miR-29a/b expression level observed in this study. In support of the gender difference observed in the miRNA expressions in this study, estrogen is known to protect neurons against inflammation by suppressing the activation of NF B (Wen, et al., 2004). However, this protection may be reduced in post-menopausal elderly women making them more vulnerable to NF B mediated suppression of miR-29a/b.

It must be noted that the blood serum samples used in this study were only clinically diagnosed and further studies are necessary to assess their ability to discriminate them from other forms of dementia. In addition, independent validation of these miRNAs as biomarkers will also be required. We note that the circulating miR-137, -181c, -9 and 29a/b levels are

low in the blood serum. Nevertheless, the qRT-PCR are able to distinguish specifically between normal and AD patients.

The discrimination of the AD patients was performed using a current consensus-based methodology described in (Jicha, et al.). Nevertheless, it is acknowledged that comorbid pathologies can contribute to the development of cognitive impairment, and thereby confound the application of the Preclinical Alzheimer's disease Workgroup recommendations (Jicha, et al.). Therefore, we recognize the possibility that some subjects included in this study may contain non-AD pathologies. Consequently, all clinical diagnosis may not necessarily coincide directly with AD neuropathological features, i.e. some amnesic MCI/probable early AD and probable AD subjects included in this study may not develop AD neuropathologies and some normal subjects may develop AD. Finally, as with any biomarker, other diseases with similar risk factors, cerebrovascular disease, cardiovascular disease and diabetes (Beerli, et al., 2009, Ewers, et al., McClean, et al., Slevin and Krupinski, 2009), may have similar blood miRNA profiles. Although, these diseases play a significant role in AD as major risk factors (Carlsson, Caselli, et al., Ettorre, et al.) and thus would provide insight into the possible risk of developing AD, further studies are needed to determine whether these miRNAs can be used to distinguish AD from these other diseases. Therefore, whether these miRNA profiles could provide conclusive diagnosis is currently unknown, nonetheless, miRNA profiles along with other biomarkers and cognitive tests could potentially provide a more comprehensive and early diagnosis and prognosis of AD.

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Figure 1. Down-regulated miRNA expression levels in probable AD patients

The expression levels of miR-137, -181c, -9, -29a and -29b in blood serum of probable AD (n=7), amnestic MCI/Probable Early AD (n=7) and control (n=7) patients measured by qRT-PCR. Relative expressions shown are normalized to spiked cel-miR-39 (A), internal miR-22 (B), internal miR-191 (C) and internal miR-126 (D) and average control patient expressions. The statistical significance between control and AD sera were determined by 2-tailed student *t* tests and Mann-Whitney tests (*, $P < 0.05$).

Figure 2. Reduced miRNA expression in high fat diet fed mice

The expression levels of blood serum miR-137 (*, $P < 0.05$), -181c (*, $P < 0.05$), -9 (*, $P < 0.04$), -29a and -29b in mice fed a high fat diet were measured by qRT-PCR. The expression of miR-126 is shown as a control. Relative expressions shown are normalized to miR-22 and average chow control diet expressions. The statistical significance between control and AD sera were determined by 2-tailed student *t* tests.

Figure 3. Gender specific down-regulation of miRNA

The expression levels of blood serum miR-137 (*, $P < 0.02$), -181c (*, $P < 0.02$), -29a, -29b (*, $P < 0.05$), and -9 in male and female mice ($n=6$) were measured by qRT-PCR. The expression of miR-126 is shown as a control. Relative expressions shown are normalized to miR-22 and average male ($n=6$) expressions. The statistical significance between control and AD sera were determined by 2-tailed student *t* tests.

Table 1**Patient information**

Patient information includes MMSE scores, clinical diagnosis, age and gender. This information was provided by the University of Kentucky (UK) Alzheimer's disease center tissue bank (ADC). The discrimination of AD and control cases were performed using current consensus-based methodologies which are previously well-described in a clinical-pathological consensus conference (Jicha, et al.).

MMSE	Clinical diagnosis based on Jicha et al. 2011	Group	Age	Gender
30	Normal	Normal	90	Female
30	Normal	Normal	91	Male
29	Normal	Normal	84	Male
29	Normal	Normal	88	Male
30	Normal	Normal	82	Female
29	Normal	Normal	88	Female
30	Normal	Normal	85	Male
25	Early AD	Amnestic MCI/Probable Early AD	94	Female
25	MCI	Amnestic MCI/Probable Early AD	85	Female
28	MCI	Amnestic MCI/Probable Early AD	87	Male
27	MCI	Amnestic MCI/Probable Early AD	86	Female
23	Early AD	Amnestic MCI/Probable Early AD	87	Female
24	Early AD	Amnestic MCI/Probable Early AD	88	Female
25	Early MCI	Amnestic MCI/Probable Early AD	89	Female
17	AD	Probable AD	89	Female
16	AD	Probable AD	84	Female
19	AD	Probable AD	96	Female
17	AD	Probable AD	90	Male
8	AD	Probable AD	86	Female
15	AD	Probable AD	92	Female
13	AD	Probable AD	80	Male