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microRNA-146a as a biomarker for transmissible spongiform encephalopathy

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Abstract

The pro-inflammatory, innate-immune system ribonucleic acid mediator microRNA-146a, constitutively expressed in the brain and central nervous system (CNS) of both the mouse and the human, is pathologically up-regulated in multiple transmissible spongiform encephalopathies (TSEs) to several times its basal level. miRNA-146a: (i) exists as a ~22-ribonucleotide (nt) single-stranded non-coding RNA (sncRNA) whose sequence is unique and highly selected over evolution; (ii) is brain-, CNS- and lymphoid-tissue enriched and exhibits a 100% RNA sequence homology between the mouse and the human; (iii) has been repeatedly shown to play critical immunological and pro-inflammatory roles in the onset and propagation of several human CNS disorders including progressive, incapacitating, and lethal neurological syndromes that include prion disease (PrD) and Alzheimer's disease (AD); (iv) is a fascinating molecular entity because it is representative of the smallest class of soluble, information-carrying, amphipathic sncRNA yet described; (v) has capability to be induced by cellular stressors and the pro-inflammatory transcription factor NF-κB (p50/p65); (vi) has capability to post-transcriptionally regulate multiple mRNAs and cellular processes in neurological health and disease; (vii) is upregulated in human host cells after viral invasion by single-stranded RNA (ssRNA) or double-stranded DNA (dsDNA) neurotropic viruses; and (viii) has an immense potential in neuro-degenerative disease therapeutics via anti-NF-κB and/or anti-miRNA-146a treatment strategies. In this short communication we provide for the first time evidence that miRNA-146a is a prominent sncRNA species in experimental murine prion disease, progressively increasing in the pre-symptomatic stages in C57BL/6J, SJL/J or Swiss Albino murine scrapie prion models. The highest miRNA-146a levels were quantified in these three different murine scrapie models exhibiting full symptomology of prion infection. The results suggest that miRNA-146a levels in the brain may be useful as an accessory diagnostic, prognostic or response-to-treatment biomarker to monitor the onset and development of PrD in experimental murine models that may also be extrapolated to be a relevant adjunct biomarker in human TSEs.

Key words: *aging, microRNA-146a, prion disease, Alzheimer's-disease, bovine spongiform encephalopathy, Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker syndrome, NF-κB-(p50/p65), scrapie.*

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Introduction

Derived from the term '*proteinaceous infectious particle*', prion diseases (PrD) represent a group of transmissible spongiform encephalopathies (TSEs) that constitute a family of rapid onset, progressive, incurable and consistently lethal neurodegenerative disorders affecting both animals and humans [4,6,7,15,16,31,39]. Human prion diseases are not known to spread by social contact, but there is evidence that prion transmission occurs during invasive medical procedures, via contaminated neurosurgical instruments, by exposure to infected human cadaveric-derived pituitary or other hormones, *via* corneal and dural grafts and organ transplantation [15,16,26]. The incidence of PrD is relatively rare – currently in the USA about 300 cases of PrD are reported each year with a wide spectrum of clinical presentation, with most forms progressing slowly over decades, beginning with dysautonomia and neuropathy progressing to an insidious dementia with rapid cognitive decline and death. Largely because of their novel nature of containing no nucleic acids and unusual biophysical properties, prions, PrDs and TSEs have been intensively studied since their discovery in 1957 [8,10,15,16,26,39]. PrDs are known to be caused by a misfolded isoform of a highly conserved, ubiquitous brain-, central nervous system (CNS)-, peripheral nervous system (PNS)- and lymphoid tissue-enriched cellular prion sialoglycoprotein known as PrPc (prion protein – cellular). PrPc monomer is a 209 amino acid (MW ~200 kDa), predominantly α-helical glycosylated cell surface polypeptide, and the constitutively expressed PrPc appears to be involved in cell-cell adhesion, cell-cell interaction, intercellular signaling and communication, neuritogenesis and neuronal homeostasis, and may provide a protective role against induced physiological stress [4,7,9,10,19,26,46]. The misfolded, abnormal isoform of PrPc known as PrPsc self-associates into a pro-inflammatory, protease-resistant aggregate highly insoluble in most detergents [7,10,16,17,46]. The mechanisms of PrPsc neurotoxicity that drive the initiation, development and progression of PrD are highly complex and like Alzheimer's disease (AD), increased oxidative stress and chronic inflammation appear to be critically involved in the initiation and progression of PrD [19,21]. Typically, activated microglia accumulate within the immediate vicinity of abnormal PrPsc aggregates and release cytokines that play important roles in the inflammatory pathogenesis of PrDs [14,21,23-25]. These include the up-regulation of genes that promote pro-inflammatory pathological signaling and innate-immune system deficits that impact synaptic integrity [8,11,19]. As aggregates of PrPsc progressively accumulate they further induce inevitably fatal neuro-degenerative disease conditions including neuroinflammation typically discernable by massive microglial activation, and proliferation and the subsequent and self-reinforced up-regulation of cytokines such as interleukin 1α (IL-1α) and 1β (IL-1β), glial fibrillary acidic protein (GFAP), tumor necrosis factor α (TNF-α) and astrogliosis accompanied by multiple additional pathological changes in the neuronal transcriptome many of which target synaptic structure and function [15,19,23-25,39].

A naturally occurring TSE of herbivorous mammals of the orders *Artiodactyla* and *Ruminantia* have been considered the essential '*prototype*' of PrD – these include scrapie of sheep and goats (both of the family *Bovidae*; subfamily *Caprinae*), and bovine spongiform encephalopathy (BSE; or "mad cow disease") of cattle (family *Bovidae*, subfamily *Bovinae*). The animal PrDs of the family *Bovidae* are closely related in their genetic and molecular neurobiology, neuropathology and clinical presentation to the human neurological disorders Creutzfeldt-Jakob disease (CJD) and Gerstmann-Straussler-Scheinker syndrome (GSS) [7,14-16,19,29; https://www.nhs.uk/conditions/creutzfeldt-jakob-disease-cjd/; last accessed 5 December 2021]. Importantly, TSEs can be experimentally studied *via* the inoculation of PrPsc and/or prion brain extracts into laboratory animals such as mice, voles, gerbils and hamsters. This causes a recapitulation of the PrD and TSE in sensitive animals which can be further studied, analyzed, monitored and investigated in a biohazard safety level 2 or 3 (BSL-2 or BSL-3) laboratory [8,9,23-25, 33,39,40; www.ehs.msu.edu/lab-clinic/bio/handling-prions.html; last accessed 5 December 2021].

Behavioral and neuropathological processes accompanying TSEs in humans include: (i) a rapidly developing disorientation and dementia; (ii) motor disturbances including progressive difficulty in walking, changes in gait and muscle stiffness; (iii) confusion, delirium and hallucinations; (iv) exhaustion, fatigue and lethargy; and (v) profound difficulty in cognition, speaking and understanding [4,9,11,15,17; www.hopkinsmedicine.org/health/conditions-

and-diseases/prion-diseases; last accessed 5 December 2021]. A definitive diagnosis of TSE and PrD can be difficult largely because these symptoms are common to several neurological diseases including AD. At the molecular-genetic level, TSEs are in part characterized: (i) by decreased activity of cholinergic and γ-amino butyric acid (GABA) pathway-related enzymes while adrenergic pathways are relatively spared; (ii) by distinctive spongiform changes in the cortex and/or neocortex of brain tissues; (iii) by association with long prodromal and incubation periods; (iv) by synaptic and dendritic damage and dysfunctional connectivity in the limbic system of the brain; (v) by progressive neuronal atrophy and loss; (vi) by gliosis and the unusually rapid 'spongiform' proliferation of neuroglial cells; (vii) by an atypical inflammatory response in neural tissues which further stimulates spongiform change; and (viii) by the induction of sncRNAs including miRNA-146a [7-9, 14-16,26,40].

To date, there is no treatment or vaccine currently available for any PrD or TSE and anti-viral, antibiotic and/or anti-microbial pharmaceuticals or vaccines have no effect on the transmission or propagation of PrD [6,7,39,43,47]. Unfortunately, all human PrD clinical trials have failed to show any significant survival benefit [43,47]. As mentioned earlier, PrDs appear to involve neuropathological pro-inflammatory changes that accompany alterations in neuronal and synaptic function from initial asymptomatic stages and a considerable prodromal period before the sudden onset of rapid cognitive decline, inflammatory neurodegeneration, insidious global CNS dysfunction, functional collapse and death [11,23,29,39]. Reliable biomarkers enabling early PrD diagnosis, prognosis and the gauging of the effectiveness of potential PrD treatments are therefore needed to increase the time window for remedial therapeutic intervention and clinical management of TSEs. The pro-inflammatory, innate-immune system microRNA mediator *Homo sapiens* microRNA-146a (hsa-miRNA-146a-5p) has been shown to be pathologically up-regulated in various brain regions of all TSEs so far examined including those of susceptible animals and humans. Currently miRNA-146a is widely considered as an NF-kB (p50/p65)-regulated 'pro-inflammatory microRNA' and neuro-inflammatory regulator over-expressed in multiple forms of human neurological and neurodegenerative disease [1,3,14,18,21,24,29,36,48].

To further define the molecular and genetic mechanism of TSE infection the aim of this study was to quantify miRNA-146a abundance along a 0- to 320-day time course in the brain cortex and cerebrospinal fluid (CSF) of 3 different murine models of prion disease spanning the asymptomatic to the symptomatic stages. Interestingly microRNA-146a was found to significantly increase in both the brain and CSF during this ~46 week time course, and may be useful as an accessory diagnostic, prognostic and/or clinical biomarker for the development of human PrDs and the therapeutic interventions designed to treat them.

Experimental methods

Control and scrapie-affected mice

Pre-symptomatic and symptomatic scrapie injected C57BL/6J female mice were studied at 0, 80, 160, 240 and 320 days of age post-injection as previously described in detail [22,29,33]. Briefly, 5 µl of a 1% brain homogenate prepared from mice clinically affected with the 139A scrapie prion strain was microinjected stereotaxically into the murine cortex at 6 weeks of age; microinjections were carried out under general anesthesia (sodium pentobarbital 70 mg/kg i.p.) using a stereotaxic instrument (Stoelting, Wood Dale IL; the stereotaxic coordinates used for the murine cortex were A+1.0, 0+2·0, H+1·5) [22]. Since no cerebral dominance for scrapie infection has been shown, injections were made in the right hemisphere [22,33]. All control and scrapie-infected mice were maintained with a 12 hr on –12 hr off light cycle and were given food and water *ad libitum* [22,33]. Control C57BL/6J and SJL/J mice were injected with 1% homogenate of normal mouse (C57BL/6J) brain. For the experiments using Swiss Albino mice, the scrapie strain designated SSBP/I Chandler strain, which had been passed serially through 18 sheep, 8 goats and two A.R.C. Swiss mouse passages prior to use. Control Swiss Albino mice were injected with 1% normal mouse brain homogenate; all experimental work was undertaken under biohazard safety level 2 or 3 (BSL-2 or BSL-3) containment. Beginning at 70 days post-injection, all mice were examined every 7 days for clinical symptoms; mice were sacrificed within 1 week of the end of the incubation period; additional experimental details and procedures have been previously reported in detail [22,23,29,33,40].

Mouse and human brain total RNA

Murine control and scrapie-affected brain cortex and cerebrospinal fluid (CSF) samples used in these studies were selected from multiple total RNA extract sources (see Acknowledgements section for brain tissue and/or CSF sources). All tissue samples and extracts were used in accordance with the institutional review board and biosafety guidelines at the donor institutions [12,28]. Archived total RNA isolated from the short incubation model of murine scrapie (strain 139A in Compton White [CW] mice) used here have been previously analyzed for chromatin structural aberrations during prion infection [33]. The current study focused on mouse brain cortex and CSF miRNA-146a abundance in 3 murine prion models; miRNA-146a is a human and murine brain-enriched miRNA known to be associated with the regulation of gliosis, glial cell proliferation, and the innate-immune and inflammatory response [12,18,28,35; see below].

Small non-coding RNA (sncRNA) isolation and Northern dot blot analysis

A guanidine isothiocyanate- and silica gel-based membrane total RNA purification system and miRNA isolation kit (PureLink, Invitrogen, Carlsbad, CA) were used to isolate total small non-coding RNA (sncRNA; that includes microRNA), and the concentration and quality of these total small RNA were determined using RNA 6000 Nano LabChips and a 2100 Bioanalyzer (Caliper Technologies, Mountain View, CA; Agilent Technologies, Palo Alto, CA). Both human and mouse brain tissues typically yield about 1 µg of total RNA per mg wet weight of tissue; there were no significant differences in total RNA yield or RNA spectral quality between any murine control or prion-affected brain samples [12,13,24,28,29,35,41].

For miRNA-146a quantitation using Northern dot blot arrays, total radiolabeled sncRNA from control or prion-associated brain tissues and/or CSF was probed against miRNA-146a dot-blot panels spotted onto an 8 × 12 array pattern and containing fixed amounts of synthetic human miRNA-146a targets as previously described [11-14,29]. Using a vacuum dot blot apparatus synthesized miRNA-146a (Integrated DNA Technologies; Coralville, Iowa, USA; 1-800-328-2661; LC Sciences, Houston TX) was spotted onto GeneScreen Plus nylon membranes using a Biomek 2000 laboratory automated workstation (Beckman, Fullerton, CA). Blots were cross-linked, baked, hybridized, and probed using the radiolabeled probes according to the manufacturer's protocol (NEN Research Products, Boston, MA, USA). Murine cortex or CSF sncRNA species were end-labeled using [γ-^{32}P]-δATP (6000 Ci/mmol; Amersham-GE Healthcare) according to the manufacturer's protocols (Invitrogen-Thermo Fisher Scientific; Waltham, MA, USA). miRNA-146a showing the strongest hybridization signals were studied further; miRNA-146a Northern dots hybridized in an 8 × 12 position array pattern were next excised from the grid and counted in liquid scintillation fluid [28,29]. The [γ-^{32}P]-δATP radiolabel-based miRNA analytical assay was found to be at least as sensitive as fluorometrically based miRNA detection as previously described in detail and considerably more sensitive than any PCR-based technique used in our laboratory [1,12,18,29,41].

Behavioral assessment

As ataxia is the major biomarker for scrapie symptomology in experimental murine models, an assessment of balance, walking and coordination for each control or scrapie animal was made beginning at 10 weeks post-injection. All mice were examined weekly for clinical symptoms that included significant disturbances in ataxia. The clinical test consisted of a monitor of motor coordination (MMC) combined with visual inspection (VI) of experimental animals placed on a grid apparatus containing a series of level parallel bars of 3 mm diameter, placed 7 mm apart from each other with a total length of 18 inches. Controls (clinically normal animals) could easily traverse the full length of the apparatus without any problem; an animal was scored as positive when it failed to walk along the grid without foot slippage between bars [22]. In positively symptomatic mice this indicated locomotor difficulties; the reliability of the test was supported by the fact that it was rare that a scrapie-positive assignment was reversed on subsequent evaluation [22,33]. In parallel with MMC analysis disturbances in ataxia were also verified in parallel by visual inspection of each experimental animal under study (VI; Table I).

Data analysis and interpretation

In Northern dot blots, sncRNA signals including those for miRNA-146a were acquired using data-acquisition software provided with a GS250 molecular

Table I. Behavioral Assessment of ataxia – real time monitor of motor coordination (MMC) and visual inspection (VI) of all experimental animals

	C57BL/6J					SJL/J					SWISS ALBINO				
Day	0	80	160	240	320	0	80	160	240	320	0	80	160	240	320
MMC	–	–	+	++	+++	–	–	+	++	+++	–	–	+	++	+++
VI	–	–	+	++	+++	–	–	+	++	+++	–	–	+	++	+++
Controls	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

MMC – animals who scored negative (–) displayed no symptoms of ataxia and were able to traverse the full length of the grid apparatus; complete details of the grid apparatus are described by Kim et al. [22]; animals who scored positive (+) displayed symptoms of ataxia and were unable to traverse the full length of the grid apparatus; number of '+' symbols indicates the degree of difficulty; '+' designates some difficulty; '++' indicates moderate difficulty; '+++' indicates moderate-to-severe difficulty (see manuscript text); control (untreated) animals showed no level of difficulty (no symptoms of ataxia) at any time-point; time-points 0-320 are days post-infection (see Figs. 1 and 2); VI – during MMC testing animals were closely observed in parallel using visual inspection (VI) regarding their ability to traverse the entire length of the grid apparatus; scoring scheme is identical to that used for MMC evaluation (see manuscript text) [22].

imager (Bio-Rad, Hercules, CA). miRNA ribonucleotide sequence analysis was performed using visual inspection, miR-BASE (http://microrna.sanger.ac.uk/sequences; last accessed 5 December 2021), the DBD transcription factor database search (Medical Research Council, Cambridge, UK), and/or GeneSpring algorithms (GeneSpring, Redwood City, CA). Statistical significance was analyzed using a two-way factorial analysis of variance (p, ANOVA; SAS Institute, Cary, NC); a $p < 0.05$ was deemed as being statistically significant.

Results

Compared to age- and gender-matched control animals, scrapie-infected prion mouse models in all 3 murine strains – including C57BL/6J, SJL/J or Swiss Albino mice murine models – exhibited: (i) a basal level of miRNA-146a in asymptomatic control murine brain cortex (Fig. 1); (ii) an increasing level of miRNA-146a in asymptomatic murine cortex up to day 240 (Fig. 1); (iii) significantly higher miRNA-146a levels at day 160 and 240 in both cortex and CSF in asymptomatic animals (Figs. 1 and 2); and (iv) a significant increase in miRNA-146a in the brain and CSF of C57BL/6J, SJL/J or Swiss Albino mice symptomatic for prion infection at day 320 (Figs. 1 and 2). Scrapie-infected animals at 240 days post-infection exhibited occasional and sporadic symptoms of scrapie while 100% of all animals at 320 days were fully symptomatic. Interestingly, miRNA-146a levels were quantified to be just over twice as abundant in murine cortical regions of the brain when compared to CSF in the same animals. The transition from the asymptomatic (at 160 days) to fully symptomatic stage (at 320 days) was accompanied by a significant 2.5-, 2.1- and 2.0-fold increase of miRNA-146a in the brains of C57BL/6J, SJL/J and/or Swiss Albino mice, respectively. All 3 scrapie murine models studied exhibited a significant increase in cortical and CSF miRNA-146a abundance between the asymptomatic (160 day), and the partially symptomatic (240 day) and fully symptomatic stage (at 320 days). The abundance of miRNA-146a in CSF ranged between 2.3- and 2.7-fold between asymptomatic age- and gender-matched controls and fully symptomatic scrapie (at 320 days) infected murine scrapie models (Fig. 2). Interestingly, in prion-infected C57BL/6J mice significant increases in miRNA-146a in CSF were already increasing at 160 days post-infection in pre-symptomatic animals to ~1.6-fold compared to age- and gender-matched controls. Similar patterns of miRNA-146a increase were found in the prion-infected SJL/J and Swiss Albino murine strains. As further discussed below: (i) the observed increase in miRNA-146a lies in parallel with the onset of the symptoms of experimental scrapie in 0- to 320-day animals; and (ii) this increasing miRNA-146a abundance in pre-symptomatic animals may be useful diagnostically for PrD as it occurs well before fully symptomatic scrapie has appeared in any of the 3 strains of prion-infected mice at day 320 (Fig. 2).

In the behavioral assessments at 240 days post-injection, about 35% of scrapie-injected mice were unable to complete the MMC behavioral assessment task (as described above) while at 320 days post injection, 90-100% of scrapie-injected mice were unable to complete this behavioral assessment task. All control animals were clinically normal and all were able to complete this behavioral assessment task. The results indicate parallel increases in miRNA-146a abundance with the development of fully symptomatic scrapie in experimental animals, which is especially apparent over the time course of 240 days to 320 days post-injection.

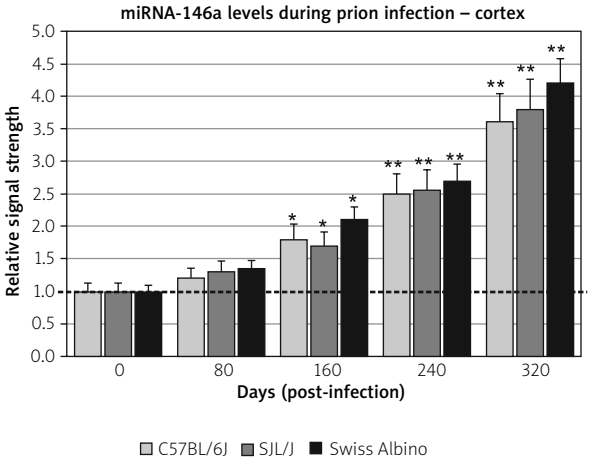

Fig. 1. Mean plus one standard deviation (x + 1 SD) abundance levels of miRNA-146a in the brain cortex of 3 murine strains infected with scrapie prion agent 139a; control abundance levels for each strain was set to 1.0 and miRNA-146a levels in prion-infected C57BL/6J, SJL/J or NIH-Swiss Albino mice were expressed as 'relative signal strength' (fold-change) increases; C57BL/6J, SJL/J and/or Swiss Albino mice have been extensively utilized in transmissible spongiform encephalopathy (TSE) studies and their utilization has been extensively documented [6,22,23,33]; a horizontal dashed line at 1.0 is shown for ease of comparison; murine TSE models at 0, 80, and 160 days were pre-symptomatic; at 240 days were partially symptomatic (in about 35% of all animals); only mice at 320 days exhibited full symptomology of prion infection; these symptoms included prominently ataxia, aggression, behavioral changes, hyperactivity, pruritus (severe itching of the skin), rubbing and biting of limbs, difficulty walking and gait, progressing tremor, sensitivity to noise and movement, lethargy and withdrawal from littermates (see text); number of animals per time-point $N = 3$; N = 3-5 assays per animal per time-point; $^{*}p < 0.05$, $^{**}p < 0.01$ (ANOVA).

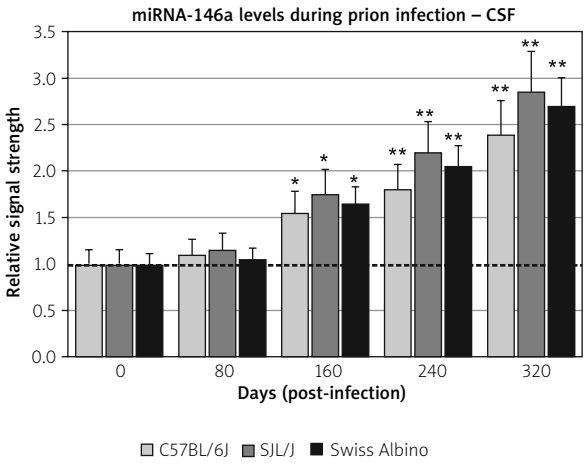

Fig. 2. Mean plus one standard deviation (x + 1 SD) abundance levels of miRNA-146a in the CSF of 3 murine strains infected with scrapie prion agent 139a; control abundance levels for each strain were set to 1.0 and miRNA-146a levels in C57BL/6J, SJL/J or Swiss Albino mice were expressed as 'relative signal strength' (fold-change); a dashed line at 1.0 is shown for ease of comparison; again murine TSE models at 0, 80 and 160 were pre-symptomatic; at 240 days were partially symptomatic; only mice at 320 days exhibited full symptomology of prion infection (see text); a dashed horizontal line at 1.0 is included for ease of comparison and interpretation. The transition from asymptomatic scrapie to symptomatic scrapie was accompanied by a significant 2.3- to 2.7-fold increase in CSF miRNA-146a (see text); number of animals per time-point $N = 3$; N = 3-5 assays per animal per time-point; $^{*}p < 0.05$, $^{**}p < 0.01$ (ANOVA).

Discussion

miRNA-146a and inflammatory neurodegeneration in TSEs

Previous studies have shown that a specific group of miRNAs, sncRNAs, important in shaping the transcriptome of the cell, are known to be involved in a protracted cytokine storm, inflammatory neuropathology and neurodegeneration associated with progressive age-related neurological diseases that include PrD, AD, neurotropic viral infection, age-related macular degeneration (AMD; a progressive inflammatory neurodegeneration of the central region of the retina of the human eye) and related human neurodegenerative diseases [11-14, 21,27,31,34-37,41,44,48]. This group includes, prominently, an intensively studied miRNA-146a expressed in murine and human brain and lymphoid tissues that is known to be under transcriptional control by a triplet of upstream binding sites for the pro-inflammatory transcription factor NF-κB (p50/p65), activated by both naturally occurring and artificial cellular stressors [13,35,36,44]. To cite just one

important example cytokine IL-1β, Aβ42 peptide and/or hydrogen peroxide-induced reactive oxygen species (ROS; stressors known to be overly abundant in AD brain) were found to specifically upregulate miRNA-146a in transgenic murine models for AD (TgAD) [35]; miRNA-146a is known to selectively target the 3'-untranslated region (3'-UTR) of the messenger RNA (mRNA) that encodes complement factor H (CFH) and in doing so down-regulates the expression of this large, soluble complement control glycoprotein [28,35]. Decreased CFH contributes to loss of complement and innate-immune system control and increased pro-inflammatory signaling in the brain and CNS and contributes directly to progressive neurodegeneration [11,14,21,24,25]. In this report we quantified the abundance of miRNA-146a in the pre-symptomatic and symptomatic phases of experimental scrapie in the brain and CSF of 3 strains of prion-infected mice and found a graded progression of increased miRNA-146a abundance. These results indicate an increased gradation of miRNA-146a abundance in both the murine cortex and CSF over the time-points of 0 to 320 days post-infection across which these experimental murine models were studied (Figs. 1 and 2). These findings suggest an early and highly interactive role for miRNA-146a in experimental PrD and that this inducible microRNA appears to be an early sncRNA biomarker for TSEs in murine animal models of prion infection.

Pre-symptomatic vs. symptomatic experimental TSE in murine models

Experimental PrDs in murine models are associated with an extensive prodromal (pre-symptomatic) period. The pre-symptomatic period in mice consists of behavioral changes including gait ataxia, aggression, hyperactivity, sleep abnormalities, difficulty walking, altered gait and randomized twitching, and withdrawal from littermates followed by the symptomatic phase in which animals exhibit advanced ataxia in gait (especially in the lower limbs), tremor (especially of the head and neck), motor and movement disturbances, and locomotor incoordination which rapidly progresses to recumbency and death [4,6,7,9,11]. Symptomatic physiological signs of PrD may also include fever and increased mean body temperature, loss of balance and coordination, dysphagia, abnormal jerking movements and highly asymmetrical rapid-onset motor presentation, loss of bladder and bowel control, neurological signs including insomnia, progressive loss of brain function and mobility, and decreased levels of consciousness [7,24,25,33].

At the molecular level, studies suggest that increased oxidative stress is one of the important early factors that initiate conversion of PrPc to PrPsc which drives the initiation and development of PrD and the transition from the pre-symptomatic to the fully symptomatic phase in experimental PrD. Redox-labile metals copper, iron and environmentally abundant neurotoxins such as aluminum bound with PrPsc may promote neurodegeneration *via* increased Fenton cycling leading to excessive production of free ROS with glial cell-mediated inflammatory responses [30,38]. From mouse studies there is evidence: (i) that over time phagocytes may aid in the dissemination of PrPsc of exogenous origin by facilitating their entry into lymphoid tissues where they propagate; and (ii) are subsequently shuttled into the circulation and across the blood-brain barrier into the CNS, a translocation that may be mediated in part by extracellular vesicular trafficking [2,32]. In contrast, other types of phagocytes may remove infective PrPsc peptides *via* phagocytosis and this may be a useful therapeutic strategy in clinical treatment strategies for PrD that may modulate the transition from prodromal (pre-symptomatic) to the symptomatic phase.

miRNA-146a as a diagnostic biomarker for PrD

microRNAs have emerged as promising, cost-effective and non-invasive biomarkers for inflammatory neurodegenerative disorders such as AD, and miRNA abundance patterns have been readily detected and analyzed in different biofluids, including the extracellular fluid (ECF), blood serum and CSF as well as in micro-vesicles [1,3,21,32,42,50]. Accumulating evidence further indicates that pro-inflammatory miRNAs are centrally involved in neurodegenerative disease pathology including the regulation and maintenance of synaptic homeostasis and plasticity processes, suggesting that they may be useful in monitoring the prodromal period for synaptic impairment and ensuing motor and/or cognitive dysfunction [20,45,50]. Taken together these studies highlight the relevance of specific miRNAs such as miRNA-146a as potential biomarkers for neurobiological and/or neuropathological events associated

with the onset and/or propagation of inflammatory neurodegeneration. The role of miRNA-146a abundantly expressed in murine and human brain and biofluids has been expanded to other TSEs, suggesting it is an integral part of the innate-immune and/or inflammatory brain cell responses with wide neurophysiological action [3,11,40,42,50]. However, because miRNA-146a is significantly upregulated in multiple forms of prion-like disorders involving inflammatory neurodegeneration *increased miRNA-146a alone cannot in itself be completely diagnostic for any TSE*. What is essential in successful human TSE diagnosis is the integrated knowledge and assessment of multiple biomarkers and interrelated factors that include the patient's age, gender and lifestyle, family, medical, genetic and clinical history, cognitive, physical, behavioral and geriatric assessment, laboratory examination of multiple biofluids, especially within the systemic circulation (lymphatics, glymphatics and blood serum) and CSF, and multiple neuroimaging-modalities of the brain's cortex and/or limbic system compared to unaffected control regions within the same brain [45,50]. For example there is evidence that miRNA-146a is induced by at least 18 single-stranded RNA (ssRNA) or double-stranded DNA (dsDNA) neurotropic viruses including SARS-CoV-2, the causative agent of COVID-19 [37]. Clinical treatment of suspected TSE patients with broad spectrum antiviral and/or antimicrobial medications and/or vaccines might help exclude a diagnosis of viral involvement in suspected PrD. Additional validation studies in larger numbers and strains of experimental prion-infected animals and PrD in humans, including longitudinal studies will be needed to establish the role of miRNA-based biomarkers as reliable diagnostic, prognostic and disease-monitoring tools.

Conclusions

In the current experiments we monitored the abundance of the pro-inflammatory miRNA-146a in early, middle (pre-symptomatic), and late (symptomatic) stages of TSE in C57BL/6J, SJL/J or Swiss Albino murine models experimentally infected intra-cerebrally with scrapie agent or PrD extracts over a 0- to 320-day time course. In all three murine models the results show an increasing presence of miRNA-146a in both the brain cortex and CSF. Interestingly there is some evidence that overly abundant, amphipathic miRNAs may leak across biophysical and biological membrane barriers from cortical tissue into the ECF (the fluid surrounding neural cells) and then translocate into the CSF, perhaps *via* vesicle-mediated processes involving exosomes and/or extracellular micro-vesicles [1,32]. Examination and extrapolation of data suggests that this increase in the NF-κB-regulated miRNA-146a in CSF could be one useful indicator and biomarker not only in murine prion models but also for human forms of PrD.

As a critical component of the complement system and innate-immune related neurological dysfunction miRNA-146a is an inducible sncRNA of the brain and CNS that lies at a critical intersection of several important neurobiological adaptive immune-response processes. These include highly interactive associations with CFH, Toll-like receptor pathways, innate-immunity, inflammatory signaling, cytokine production, astrogliosis, apoptosis and neural cell decline [5,12-14,18,20,21,24,28,31,35,42,44,45,48,50]. Inflammatory neurodegeneration mediated by microglial activation and astrogliosis is now widely recognized as a hallmark of most types of both human and experimental neurodegeneration including those encountered in PrD [8,9,14,25,34,40]. Since neuroinflammation is a common neuropathological hallmark of many neuro-degenerative disorders, and PrD shares many molecular and clinical similarities with other more prevalent neurodegenerative diseases such as AD, we speculate the study of miRNA-mediated neuroinflammation in PrD could help uncover important insights into the role of pro-inflammatory and epigenetic neurobiology in the diagnosis, prognosis and clinical management of TSEs and other progressive neurodegenerative conditions [11,25,31]. Individual miRNA-146a and other microRNA molecular patterns and signatures have been found useful in distinguishing between prodromal AD and healthy age-matched controls [3,49]. Importantly, while increasing miRNA-146a in the CSF in pre-symptomatic, and peaking in fully symptomatic experimental prion disease in murine models is a very valuable and significant indicator, *it alone cannot be diagnostic for TSEs, and additional biomarkers including extensive clinical observation, family history and genetic testing and neuroimaging-modalities of the brain should contribute collectively to a more definitive diagnosis of PrD* [45,50].

Future investigation on the role of potentially pathogenic miRNAs such as miRNA-146a in the brain

and CSF of TSEs should open up new horizons for the application of miRNA-modulatory strategies useful to prevent, diagnose, monitor and/or treat the course of PrD infection.

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Ethics statement

The acquisition, handling, experimental and analytical procedures involving all animal tissues were carried out in an ethical manner in close accordance with the ethics review board policies at brain and tissue donor institutions and at the Louisiana State University (LSU) Health Sciences Center. The ethical use of all murine tissues and their analyses were also carried out in strict accordance with the Institutional Biosafety Committee and the Institutional Review Board Committee (IBC/IRBC) ethical guidelines IBC# 18059 and IRBC# 6774 at the LSU Health Sciences Center, New Orleans, LA 70112, USA.

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Disclosure

The authors report no conflict of interest.

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