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Original Article

Tumour necrosis factor-α (TNF-α) and miRNA expression in frontal and temporal neocortex in Alzheimer's disease and the effect of TNF-α on miRNA expression in vitro

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Abstract: Micro-RNAs (miRNAs) are short non-coding RNAs capable of regulating gene expression at the translational level. A number of studies have suggested that the expression of several miRNAs is changed in AD. The pro-inflammatory cytokine tumour necrosis factor-α (TNF-α) is increased in serum and CSF in AD. We measured the expression of *TNFA* and several AD candidate gene-associated miRNAs (*let7a/b, miR-128a/b, miR-27a/b, miR-155*) in frontal and temporal neocortex from AD and control brains. The expression of these miRNAs was also measured after incubating non-differentiated (NDC) and retinoic acid -differentiated (DC) SH-SY5Y neuroblastoma cells with TNF-a. *TNFA* expression was similar in AD and control brains but *miR-128a/b* levels were significantly reduced in the temporal cortex and *miR-128b* in the frontal cortex in AD. MiRNA levels did not correlate with *TNFA* expression in brain tissue but exposure of NDC and DC SH-SY5Y cells to TNF-α caused a variable dose-dependent response in the level of some of the miRNAs studied. Our brain tissue findings argue against a role for TNF-a in influencing the expression of these miRNAs in AD.

Keywords: Alzheimer's disease, Tumour necrosis factor-α, microRNA, SH-SY5Y cells

Introduction

Micro-RNAs (miRNAs) are highly conserved, short non-coding single-stranded RNAs involved in translational regulation of messenger RNA (mRNA). They recognise and bind generally but not exclusively to the 3'-untranslated (3'UTR) of specific mRNAs and in doing so generally cause gene silencing [1,2]. Some miRNAs are reported to be brain-specific or brain-enriched [3-5]. The number of miRNAs expressed in human brain is higher than in other organs, most likely reflecting the greater diversity of cell types and subtypes [6,7].

Recently, changes in miRNA expression were identified in Alzheimer's disease (AD) that were thought to contribute to its development [8-11]. One study found altered expression of several miRNAs in brain tissue and cerebrospinal fluid (CSF) of early- and late-stage AD patients compared with controls [12]. Other studies suggested that certain miRNAs up-regulate APP and BACE therefore potentially contributing to the overproduction of Aβ in AD [13-15].

The role of the immune system in the clearance of Aβ from the brain has been the subject of a number of recent studies [16-18]. Multiple cytokines are produced in response to Aβ-mediated activation of microglia and elevated levels of these cytokines have been found in serum and CSF from AD patients [16,17,19]. TNF-α is considered to be one of the most important pro-inflammatory cytokines produced by activated microglia and of major importance in AD. Elevated levels of TNF-α, encoded for by TNFA (OMIM:191160), appear to correlate with disease progression [20,21] and with the onset of cognitive deficits [22,23].

We measured miRNAs let-7a, let-7b, miR-128a, miR-128b, miR-27a, miR-27b, miR-155 as well as TNFA in brain tissue from patients with AD

and age-matched controls. These miRNAs were selected for their reported abundant expression in brain, involvement in inflammation and microglial activation [24-26], implicated in cell cycle regulation [27,28] and apoptosis [29]. In addition, other predicted targets of miR-27a/b and miR-128a/b as identified by the online software 'TargetScanHuman release: 5.1' include ACE, ECE1 and ECE2, which influence cerebral blood flow and are also capable of degrading Aβ. We also investigated if TNF-α influences the expression of any of these miRNAs in non-differentiated (NDC) and retinoic acid - differentiated (DC) SH-SY5Y neuroblastoma cells.

Materials and methods

Human brain tissue

This retrospective case-control study used brain tissue that had been donated to the Human Tissue Authority (HTA) licensed South West Dementia Brain Bank (SWDBB), Bristol. Sections of post mortem brain tissue were collected in RNAlater (Ambion, UK) and frozen at -80°C until RNA extraction. mRNA and miRNA from frontal and temporal neocortex (Brodmann areas 6 and 22) was isolated from 6 control (73–94 y, mean 87 y; 5 male, 1 female) and 12 AD (67–97 y, mean 81 y; 5 male, 7 female) . The AD cases had 'definite AD' according to CERAD criteria [30]. The study had local Research Ethics Committee approval.

Cell culture and TNF-α exposure

SH-SY5Y human neuroblastoma cells were cultured in Dulbecco's modified Eagles medium (DMEM) (Sigma) (2 mM glutamine (Sigma), 1% essential amino acids (Sigma), 15% fetal bovine calf serum (FCS) (Autogene Bioclear). Cells were differentiated by addition of 10 μM retinoic acid (RA) (Sigma-Aldrich, Dorset, UK) for 5 days and 50 ng/ml BDNF for 2 days in DMEM. DC and NDC were incubated in serum-free medium for 12-14 h before exposure to 1 ng/ml, 10 ng/ml or 50 ng/ml TNF-α (R&D systems, UK) for 15 min and 3 h. All cells were discarded after 15 passages as the sensitivity of SH-SY5Y cells to TNF-α was shown to decrease with increased passages [31]. All experiments were repeated 3 times, and on each occasion the mean values were determined for triplicate samples.

mRNA/miRNA extraction and real-time PCR (RT-PCR)

mRNA/miRNA was extracted using the mirVANA extraction kit (Ambion) according to the manufacturer's instructions. cDNA was produced from the mRNA and miRNA by use of the High Capacity c-DNA Archive Kit (Applied Biosystems). RT-PCR was performed using the ABI 7000 sequence detection system (Applied Biosystems) with Assay-on-Demand Gene Expression Products for TNFA, GAPDH and TaqMan Universal PCR Master Mix. RT-PCR of miRNAs was performed using TaqMan miRNA assays, detecting the mature miRNAs, (hsa-let-7a/7b, hsa-miR-155, has-miR-128a/128b, has-miR27a/27b) (Applied Biosystems) as described by the manufacturer. Expression of TNFA relative to GAPDH (calibrator gene) mRNA and of the individual miRNAs relative to RNUB6 (internal normalizing control) was calculated by the $2^{-\Delta\Delta Ct}$ method [32]. The results were expressed as the fold difference in gene expression between AD and controls.

Statistical analysis

Statistical tests were performed using GraphPad Prism v5 for windows. Mann-Whitney test was used for the comparison of expression levels across the subject groups. Differences with a $p < 0.05$ were considered significant. Since mRNA and miRNA levels were expressed as an exponential function (the fold-change relative to the appropriate control measurements) the values from repeat analyses were presented as the geometric means and interquartile ranges.

Results

Expression of TNF-α and miRNAs in AD and control cases brain tissue

Expression of TNFA, let-7a/b, miR-128a/b, miR-27a/b and miR-155 was detected in temporal and frontal neocortex from all brain samples studied although expression varied considerably between cases in all diagnostic groups. TNFA levels were particularly variable (**Table 1**) but did not differ significantly between the AD and controls in either temporal or frontal neocortex.

Expression of miR-128a/b was significantly decreased in the temporal neocortex in AD (miR-

Table 1. Expression of *TNFA* and *miRNA*s in AD and control brain tissue

AD	Frontal neocortex		Temporal neocortex	
Control=1	$2^{-\Delta\Delta Ct}$ Geometric mean (IQR)	p-value	$2^{-\Delta\Delta Ct}$ Geometric mean (IQR)	p-value
TNFA relative to *GAPDH*	0.72 (0.9)	NS	0.83 (1)	NS
Let7a	0.9 (0.59)	NS	0.9 (0.77)	NS
Let 7b	0.95 (0.45)	NS	0.54 (0.46)	NS
miR-128a	0.92 (0.27)	NS	0.68 (0.24)	0.006
miR-128b	0.69 (0.11)	0.006	0.77 (0.28)	0.044
miR-155	0.95 (0.38)	NS	0.96 (0.98)	NS
miR-27a	0.7 (0.75)	NS	0.80 (0.47)	NS
miR-27b	0.66 (0.59)	NS	0.62 (0.37)	NS

Table 2. miRNA expression in NDC SH-SY5Y cells after exposure to TNF-α

15mins	*miR-128a* $2^{-\Delta\Delta Ct}$ geometric mean	*miR-128b* $2^{-\Delta\Delta Ct}$ geometric mean	*miR-27a* $2^{-\Delta\Delta Ct}$ geometric mean	*miR-27b* $2^{-\Delta\Delta Ct}$ geometric mean	*let-7a* $2^{-\Delta\Delta Ct}$ geometric mean	*let-7b* $2^{-\Delta\Delta Ct}$ geometric mean	*miR-155* $2^{-\Delta\Delta Ct}$ geometric mean
1 ngTNF-α	1.32	1.4	1.04	1.36	0.8	0.72	0.97
10ngTNF-α	1.12	1.66	1.2	0.77	1.43	0.87	0.74
50ngTNF-α	0.75	1.11	0.91	0.61	2	1.51	1.21
3 h							
1 ngTNF-α	2.2	0.59	0.96	1.05	0.91	1.55	0.95
10ngTNF-α	2.09	1.38	0.98	1.28	0.92	1.89	1.56
50ngTNF-α	1.33	1.33	1.08	1.14	1.58	1.62	1.15

128a p=0.006, miR-128b p=0.044) and miR-128b (p=0.006) in the frontal cortex compared to controls. (**Table1**). There was no association between the expression of TNFA and any of the miRNAs studied in the frontal or temporal cortex in either of the cohorts.

miRNA expression in DC and NDC SH-SY5Y cells after TNF-α exposure

The effect of TNF-α exposure on miRNA gene expression in vitro is summarised in Tables 2 and 3. In NDCs, let-7a/b expression increased and miR-128a and miR-27b decreased in a dose-dependent manner after 15 min exposure to TNF-α. After 3 h incubation only let-7a/b and miR-128a showed a response to TNF-α (**Table 2**).

The expression of miR-155 in control DCs was significantly lower than in control NDCs (2^{-DDCt} = 0.339) and after exposure to TNF-α reduced to the minimum level of detection (**Table 3**). MiR-128b expression was 2-fold higher ($2^{-\Delta\Delta Ct}$ = 2.21) in control DCs compared to control NDCs and miR-128b expression was consistently reduced in DCs after 3h TNF-a.

Discussion

Some studies have reported that miRNA expression profiles are altered in AD which may have an aetiological basis in AD. TNFA expression in brain was generally low in all individuals studied irrespective of their diagnostic groups and there was considerable variation between individuals. The generally low levels of TNFA expression in

Table 3. miRNA expression in DC SH-SY5Y cells after exposure to TNF-α

15mins	*miR-128a* $2^{-\Delta\Delta Ct}$ geometric mean	*miR-128b* $2^{-\Delta\Delta Ct}$ geometric mean	*miR-27a* $2^{-\Delta\Delta Ct}$ geometric mean	*miR-27b* $2^{-\Delta\Delta Ct}$ geometric mean	*let-7a* $2^{-\Delta\Delta Ct}$ geometric mean	*let-7b* $2^{-\Delta\Delta Ct}$ geometric mean	*miR-155* $2^{-\Delta\Delta Ct}$ geometric mean
1 ngTNF-α	0.53	1.13	1.11	1.07	0.03	0.44	0.07
10ngTNF-α	1.3	0.88	1.52	1.5	0.42	0.4	0.03
50ngTNF-α	2.0	1.08	1.81	2.39	1.32	0.78	0.04
3 h							
1 ngTNF-α	1.18	0.37	1.19	1.19	0.76	1.02	0.16
10ngTNF-α	1.2	0.38	0.74	0.85	0.57	1.31	0.11
50ngTNF-α	1	0.54	1.05	1.15	1.45	1.35	0.32

AD and control tissue may reflect a short half-life of TNF-α mRNA in brain tissue and the absence of factors which normally regulate TNFA mRNA stability [33,34]. There was no difference between mean TNFA expression levels in tissue from AD when compared to control subjects, confirming results of a previous study [35]. There was also no evidence of a correlation between TNFA and miRNA gene expression in brain tissue.

We observed a statistically significant decrease in miR-128a/b in temporal neocortex from AD compared to control brains and reduced miR-128b in the frontal cortex of AD brains. In contrast to these findings, miR-128 was previously shown to be up-regulated in the hippocampus in AD [36]. Diverse expression of miRNAs in distinct areas of the brain has been previously been noted, including substantial variation in relative miRNA levels across different regions [9]. Recently miRNA deregulation in response to Aβ exposure in hippocampal neurons and in the hippocampus of Aβ42-depositing APP23 mice at the onset of Aβ plaque formation was reported. It was suggested this may be an important factor contributing to the cascade of events leading to AD [37].

Several other reports have indicated the importance of miR-128 in neurogenesis and AD: it plays an important role in the differentiation of neuronal stem cells (NSCs) into neurons or astrocytes, regulates tau degradation and adenosine 2B receptor expression [38-40]. However, further investigation is needed to identify if the decreased levels observed for miR-128a/b in AD causes translational changes affecting genes for which interactions have been validated or other potential target genes. This is a challenging task since each miRNA can bind multiple targets and many miRNAs can bind the same target [4,9].

It has been suggested that miRNAs are normally relatively stable [41] but that their expression profile may change rapidly, as demonstrated previously in primary hippocampal neurons in response to Aβ treatment [37] and as we observed in this study in neuroblastoma cells in response to TNF-α.

We noticed that RA-induced differentiation of SH-SY5Y neuroblastoma cells alone reduced the expression of miR-155 and increased the expression of miR-128b. The increased expression of miR-128 in RA-differentiated neuroblastoma cells was reported previously to impair cell growth [42]. The expression of several other miRNAs was reported to be altered in cells exposed to RA [43]. Although miR-155 was not mentioned in that report, our findings indicate that it can be included in the list of miRNAs sensitive to RA-induced cell differentiation.

We found that miR-155 expression in differentiated neurons was severely reduced by TNF-α as has been reported previously in immune cells [44]. MiR-155 is a multifunctional miRNA, involved in numerous biological processes including haematopoiesis, inflammation and immunity. Deregulation of miR-155 has been associated with cancer, cardiovascular disease and viral infections [25]. It also affects the expression of type 1 angiotensin II receptor (AT1R) and PICALM (phosphatidylinositol binding clathrin assembly protein) [45], molecules thought to be involved in AD [46,47].

miRNAs are generally regarded as negative regulators of mRNA translation and repression of miRNAs would be expected to enhance

mRNA translation of target genes. However, under certain stress conditions miRNAs increase translation of their targets [48]. Previous work showed that members of the let-7 family of miRNAs bound to the 3'UTR of TNFA mRNA and by doing so stimulated translation in cells that were deprived of serum and therefore in a particular phase of cell cycle arrest [48].

It is possible that the initial up-regulation of these miRNAs targets genes involved in the TNF-R signaling pathways leading to neuronal cell survival or cell death. Over-expression of a miRNA complex including miR-27a sensitized HEK293T cells to TNF-α cytotoxicity and miR-27a negatively regulated the expression of FADD (Fas Associated protein with Death Domain), a protein involved in cell apoptosis [29]. It seems likely that the high serum and CSF levels of TNF-α observed in AD will influence miRNAs in this disease in vivo. However, further work is needed to establish the full range of miRNAs that are affected and the biological relevance of their targets to AD.

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