

MicroRNA Expression in Alzheimer Blood Mononuclear Cells

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Abstract: Various coding genes representing multiple functional categories are downregulated in blood mononuclear cells (BMC) of patients with sporadic Alzheimer disease (AD). Noncoding microRNAs (miRNA) regulate gene expression by degrading messages or inhibiting translation. Using BMC as a paradigm for the study of systemic alterations in AD, we investigated whether peripheral miRNA expression is altered in this condition. MicroRNA levels were assessed using the microRNA microarray (MMChip) containing 462 human miRNA, and the results validated by real time PCR. Sixteen AD patients and sixteen normal elderly controls (NEC) were matched for ethnicity, age, gender and education. The expression of several BMC miRNAs was found to increase in AD relative to NEC levels, and may differ between AD subjects bearing one or two APOE4 alleles. As compared to NEC, miRNAs significantly upregulated in AD subjects and confirmed by qPCR were miR-34a and 181b. Predicted target genes downregulated in Alzheimer BMC that correlated with the upregulated miRNAs were largely represented in the functional categories of Transcription/Translation and Synaptic Activity. Several miRNAs targeting the same genes were within the functional category of Injury response/Redox homeostasis. Taken together, induction of microRNA expression in BMC may contribute to the aberrant systemic decline in mRNA levels in sporadic AD.

Keywords: Alzheimer disease, gene expression, microarray, neurodegeneration, noncoding small RNA

Introduction

MicroRNA are noncoding small RNA that bind target sites either in the 3' untranslated region (UTR) of mRNA to inhibit translation or at the coding regions to degrade the messages (Lee et al. 1993; Ruvkun et al. 2004; Ambros, 2004; Lim et al. 2005). Mature microRNAs (miRNA) are approximately 22 nucleotides in length and are expressed under the control of an RNA polymerase II promoter. The catalogue of human miRNA has expanded substantially in the last few years and the number of miRNA species is predicted to be close to one thousand (Hammond, 2006). Loss-of-function mutations have greatly facilitated elucidation of the biological functions of miRNA (Ambros, 2004) including their roles in development, cell differentiation (Ambros, 2004; Ouellet et al. 2006), lifespan (Boehm and Slack, 2005) and in diseases such as cancer (Hammond, 2006) and neurodegeneration (Bilen et al. 2006a). In mammals, neural tissues are believed to manifest the most complex and specific miRNA expression patterns relative to other organs (Babak et al. 2004; Strauss et al. 2006).

Alzheimer disease (AD) is a dementing illness characterized by progressive neuronal degeneration, gliosis, and the accumulation of intracellular inclusions (neurofibrillary tangles) and extracellular deposits of amyloid (senile plaques) in discrete regions of the basal forebrain, hippocampus, and association cortices (Chertkow et al. 2001; Selkoe, 1991). The etiology of sporadic AD is likely multifactorial, with carriage of the apolipoprotein E ϵ 4 (APOE4) allele constituting a strong risk factor for the development of this condition (Kamboh, 2004).

Gene expression studies in AD have shown substantial downregulation of various mRNA species in brain (Pasinetti, 2001), peripheral blood mononuclear cells (BMC) (Maes et al. 2006) and lymphocytes (Scherzer et al. 2004) relative to non-demented control values. Furthermore, numerous investigations have implicated impairment of protein synthesis in AD tissues (Keller, 2006) and diminished concentrations of specific proteins in the cerebrospinal fluid (CSF) of these patients (Puchades et al. 2003). In a small study of 13 miRNAs, levels of miR-9, miR-125b, and miR-128 were found to be increased in human AD

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hippocampus relative to control values (Lukiw, 2007). In light of the above, we hypothesized that augmented miRNA expression may be responsible for the suppression of specific mRNA species both in AD brain and peripheral tissues. To test this hypothesis in the latter, we screened 462 human miRNA (from let-7 family to miR-663) in BMC derived from well-characterized cases of mild sporadic AD and age-matched normal elderly control subjects and, based on predicted miRNA targets, ascertained whether the accruing data may account for the patterns of mRNA downregulation in Alzheimer BMC previously reported by our laboratories (Maes et al. 2006).

Materials and Methods

Subjects

This study was approved by the Research Ethics Committee of the Sir Mortimer B. Davis Jewish General Hospital (JGH). Written informed consent was obtained from all patients or their primary caregivers. Recruited patients with sporadic AD were assessed by a neurologist or geriatrician at the JGH-McGill University Memory Clinic, a tertiary care facility for the evaluation of memory loss in Montreal. All AD subjects underwent formal neuropsychological testing as previously described (Chertkow et al. 2001; McKhann et al. 1984). Normal elderly controls (NEC) were recruited from Family Practice Clinics at the JGH. The latter scored within one SD of age- and education-standardized normal values on a series of memory and attention tests. The Mini-Mental State Examination (MMSE) (Folstein et al. 1975) was administered to all subjects. Subjects with chronic metabolic and inflammatory conditions or acute illness were excluded from the study. Student's unpaired t-test was performed to assess statistical differences in age, years of formal education and MMSE scores between groups. Apolipoprotein E genotyping was performed as previously described (Maes et al. 2006). APOE4-positive persons were defined as individuals bearing one or two APOE4 alleles.

Blood samples and extraction

Whole blood was collected and BMC were isolated as previously described (Maes et al. 2006). The cell pellet was lysed in Trizol (Invitrogen, Canada) and immediately stored at -80°C until further

processing. Extraction of RNA was performed as described by Lacelle et al. 2002 (Lacelle et al. 2002). Briefly, for 1 mL of Trizol, 0.2 mL of chloroform was added and mixed for 15 seconds. After 3 minutes incubation at room temperature (RT), the samples were centrifuged at $12,000\text{ g}$ (4°C) for 15 min. The upper aqueous layer containing RNA was transferred to another microcentrifuge tube for RNA extraction. Total RNA was solubilized in DEPC-treated water and purified using RNeasy Mini columns (Qiagen, Canada) according to manufacturer instructions.

Small RNA enrichment was performed according to Park et al. 2002 (Park et al. 2002). Total RNA samples were adjusted to $400\text{ }\mu\text{L}$ with RNase-free water and then $50\text{ }\mu\text{L}$ of NaCl (5M) and $50\text{ }\mu\text{L}$ PEG 8000 (v/v 50%) were added. The sample was incubated on ice for 2 hours, and centrifuged for 10 minutes at $13\text{ }000\text{ rpm}$ (4°C). Supernatant containing small RNA was transferred to a microcentrifuge tube and $50\text{ }\mu\text{L}$ of sodium acetate (3M, pH 4.6) and 1 mL of 100% ethanol were added. The samples were mixed and incubated at -20°C for 2 hours and centrifuged for 10 minutes at $12\text{ }000\text{ g}$ (4°C). The supernatant was discarded, and the pellet was washed with 1 mL of cold 75% ethanol and, centrifuged for 10 minutes at $12\text{ }000\text{ g}$ (4°C). The pellet was dried and dissolved in $12\text{ }\mu\text{L}$ of RNase-free water at 60°C for 10 minutes. Nucleic acid concentrations were determined at 260 nm by spectrophotometry and samples were stored at -80°C .

MicroRNA profiling

We applied rigorous selection criteria on samples to control for ethnicity, health status, age and education. Only samples with good yields in total RNA and small RNA enrichment were used (Supplementary Table 1). Small RNA samples were labeled with digoxigenin (DIG) at the 3' end using the DIG Oligonucleotide Tailing Kit, 2nd Generation (Roche Diagnostics, U.S.A.). One μg of small RNA was labeled in a total volume of $20\text{ }\mu\text{L}$ as described by Wang et al. (Wang et al. 2002). The human microRNA microarray (MMChip) consisted of 462 human anti-sense DNA sequences of microRNAs obtained from miRBase (<http://microrna.sanger.ac.uk/>) and spotted on nitrocellulose membrane as described (Wang et al. 2002). Microarray chips were pre-hybridized in 1 mL of DIG Easy Hyb solution (Roche Diagnostics, U.S.A.) at 42°C for

Table 1. Upregulated microRNA in Alzheimer BMC.

| MicroRNA | Fold change | Score (d) | q-value (%) | BMC targets (%) ^a |
|--------------|-------------|-----------|-------------|------------------------------|
| hsa-miR-34a | 1.2 | 1.3 | 0.0 | 21.7 |
| hsa-miR-579 | 1.2 | 1.2 | 0.0 | 12.3 |
| hsa-miR-181b | 1.4 | 1.1 | 0.0 | 31.3 |
| hsa-miR-520h | 1.1 | 1.0 | 0.0 | - |
| hsa-miR-155 | 1.2 | 0.9 | 5.1 | - |
| hsa-miR-517* | 1.1 | 0.9 | 5.1 | 11.3 |
| hsa-let-7f | 1.1 | 0.8 | 5.1 | 16.0 |
| hsa-miR-200a | 1.1 | 0.7 | 5.1 | 7.5 |
| hsa-miR-371 | 1.1 | 0.7 | 5.1 | - |

False discovery rate (q-values) was 5.1% of 16 significant miRNAs.

^aPredicted targets were determined for miRNAs having scores (d) higher than 1.0 or 0% q-values in both Tables 1 and 2.

1 hr. DIG-labeled small RNAs from one human subject were added to the membrane and hybridization was performed at 42 °C for 16 hrs. The membrane was washed twice (5 min) in solution 1 (2 × SSC, 0.1% SDS) at RT, incubated for 20 minutes at 37 °C in solution 2 (0.5 × SSC, 0.1% SDS), and 5 minutes in 1× maleic acid solution (100 mM maleic acid, 150 mM NaCl, pH 7.5) at RT. The membrane was blocked in 1.5% Blocking reagent (w/v; Roche Diagnostics, USA). The membrane was incubated for 30 minutes with 1:1500 Anti-Digoxigenin-AP Fab fragments (Roche Diagnostics, U.S.A.). The miRNAs hybridized on the MMchip were revealed by the alkaline phosphatase chromogenic reaction of NBT/BCIP dye as per manufacturer's instructions (Roche Diagnostics, U.S.A.).

Only MMchips with excellent hybridization intensities were analyzed. Hybridization intensities were measured using an Expression 1680 scanner (Epson, U.S.A.) and data acquired using Array-Pro Analyzer 4.5 software (Media Cybernetics, MD, USA). Net intensity was derived from whole cell area measurement and corrected using mean intensity of ring background of surrounding spots. Microarray data analyses were performed with SAM software, version 3.02 (Significance Analysis of Microarrays, Stanford University, CA, U.S.A.). The variable "Block" was used to account for the experimental batches (block effect). Kolmogorov—Smirnov statistics were generated by Gene Set Enrichment Analysis (GSEA) software (Subramanian et al. 2005).

Hierarchical clustering analysis was performed using GenePattern software (www.broad.mit.edu/cancer/software/genepattern/; Broad Institute, MA, U.S.A.) Functional attribution of correlated gene targets was made according to the SOURCE database

(<http://source.stanford.edu>) and Gene Ontology Tree Machine (<http://bioinfo.vanderbilt.edu/gotm>).

QRT-PCR validation

For real time PCR validation, 0.1 µg of small RNA were quantified using the NCode qRT-PCR kit (Invitrogen, U.S.A.). Mature DNA sense sequences of tested miRNAs were used as forward PCR primers. 5S rRNA served as reference gene, and was probed using an internal forward primer (CAGGGTCGGCCTGGTTAGTACTTG).

For the miRNA let-7f, the qRT-PCR was performed using the TaqMan MicroRNA Reverse Transcription kit, TaqMan MicroArray assay (hsa-let-7f, RT 382) and TaqMan Fast Universal PCR (No AmpErase UNG; Applied Biosystems, U.S.A.) with 1 ng of small RNA. The TaqMan specific primer U24 small nucleolar RNA (RNU24, RT 1001) was used as reference gene.

All real time PCR reactions were performed on a 7500 Fast System Real Time PCR cyclor (Applied Biosystems, U.S.A.) according to manufacturer's instructions. MicroRNA fold changes between diagnostic groups or genders were calculated by the delta Ct method.

Results

MicroRNA expression in Alzheimer BMC

Mean ages between the NEC (76 ± 6 years) and AD (78 ± 5 years) groups were not significantly different (p = 0.26; Supplemental Table 1). Subjects in the AD group had fewer years of formal education (12.6 years

as compared to 15 years for NEC, $p = 0.05$) and scored significantly lower on the MMSE (23/30 as compared to 29/30 for NEC, $p < 0.0001$). Yields in small RNA enrichment did not significantly differ between NEC and AD samples ($p = 0.95$).

The study consisted of 16 NEC and 16 AD miRNA expression profiles, analyzed in four independent experimental blocks with equal numbers of women and men. Only upregulated miRNA expression was found to be significantly altered in Alzheimer BMC by T-statistics (Table 1). The relative increases in miRNA in Alzheimer BMC were modest, in the range of 1.1 to 1.4-fold. Significant microarray data analyzed by SAM (Table 1) was subsequently ranked by Kolmogorov—Smirnov statistics using the Gene Set Enrichment Analysis (GSEA) software (Subramanian et al. 2005). The enrichments of the significant upregulated miRNA were confirmed for miR-34a and 181b. (Fig. 1A). Although miR-155 was ranked in second position, the fold increase in AD was not validated by quantitative PCR (see below).

We observed slightly different levels of miRNA expression between male and female BMC as illustrated by the heat map generated by GSEA (Fig. 1A), but the differences analyzed by SAM were not significant (data not shown). Next, we stratified NEC and AD miRNA signatures according to APOE4 status (Table 2). Similarly to the entire cohort, we found common miRNA upregulated in AD, with tendencies for over-representation of certain miRNA in the APOE4-negative (i.e. miR-34a, 517*, let-7f, 200a), and possibly the APOE4-positive (i.e. miR-371; miR-181b) strata.

We next performed a higher order analysis of the cohort's miRNA expression signatures by hierarchical clustering using Pearson correlations (Fig. 1B). An apparent classification of NEC and AD was obtained, although three female NEC signatures subgrouped with AD miRNA profiles. The complexity of the different levels of clusters may be related to the inherent experiment block effect existing between the MMchips. Sub-grouping for gender or using the significantly upregulated miRNAs of Table 1 did not improve the classifications (data not shown).

Real time PCR validation

The above comparison for the whole cohort was validated by real time PCR. Levels of selected miRNAs in 10 samples (NEC and AD) were determined by qRT-PCR, and the average Ct values were

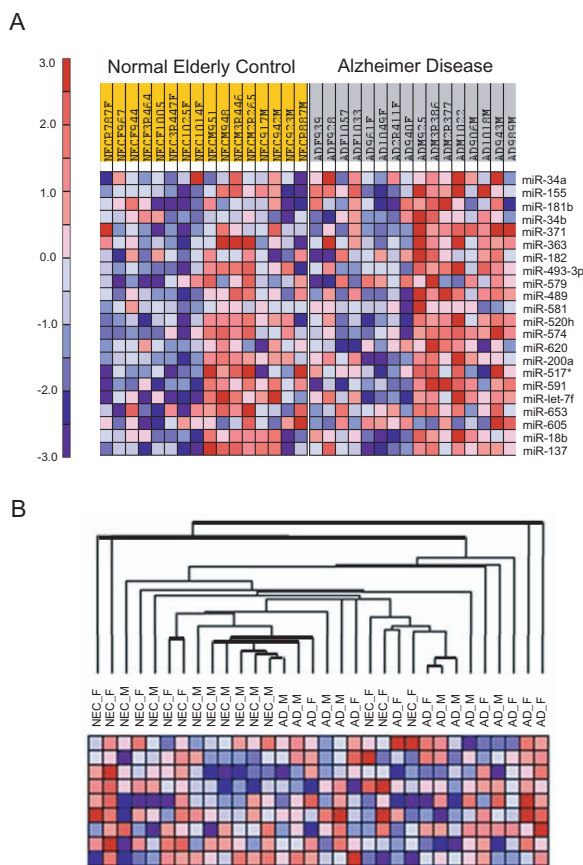


Figure 1. Gene set enrichment analysis and hierarchical clustering of microRNA expression in Alzheimer BMC. (A) The significantly upregulated miRNA in AD are ranked in order of significance from top to bottom. (B) The hierarchical clustering of subjects was determined using Pearson correlation.

used to estimate the difference in expression levels between diagnostic groups (Fig. 2).

Of the five selected miRNAs tested by qPCR, only one miRNA (miR-155) was not validated. Importantly, the delta Ct fold differences estimated in AD for miR-34a, 181b, 200a and let-7f corresponded to their order of significance reported in Table 1. The qPCR data suggest that the increase in miRNA levels in AD may be underestimated in the microarray platform (Table 1).

Target predictions

Target predictions for the significantly upregulated miRNA in Alzheimer BMC ascertained from the miRBase (Sanger Institute, <http://microrna.sanger.ac.uk/targets/v4/>) were compared with the down-regulated mRNAs previously reported in Alzheimer BMC (Maes et al. 2006). Of the predicted hundreds of targets for any specific miRNA, a range of 10 to 30 downregulated transcripts in Alzheimer

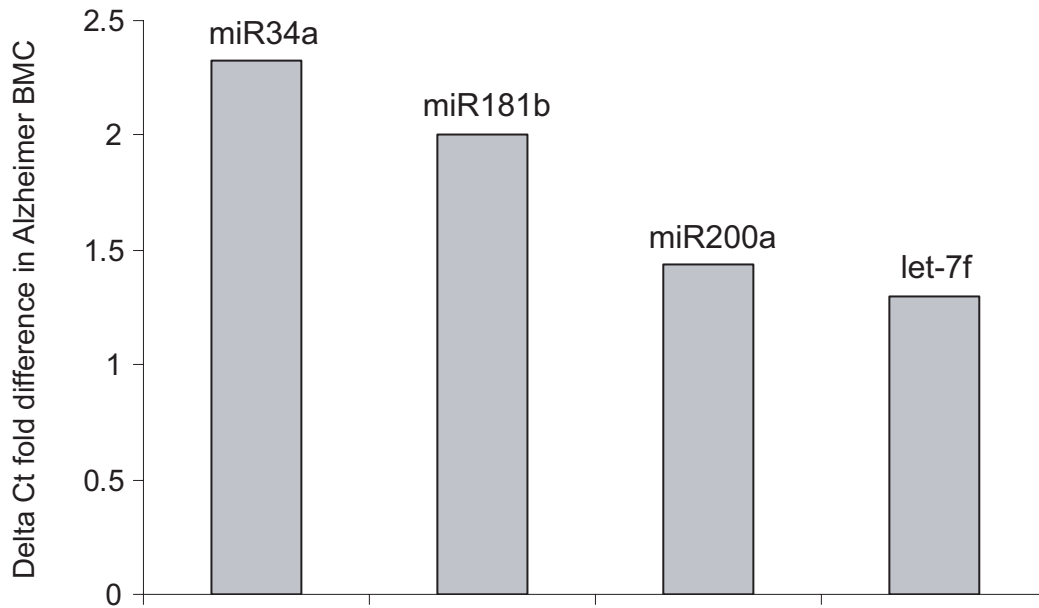


Figure 2. Quantitative real time PCR of miRNA levels in AD. Fold differences in miRNA levels between NEC and AD were estimated by the delta Ct method.

BMC were correlated, however only transcripts with significantly lower levels in AD are reported in Table 3. The percentage of targets per miRNA is reported in Table 1. Interestingly, miR-181b, which exhibited the greatest fold-increase expression in Alzheimer BMC, targeted the highest proportion of downregulated genes in AD BMC (31.1%), followed by miR-34a (21.7%). In contrast, targets of miR-200a correlated least with the genomic data (7.5%). Target genes with significantly downregulated transcript levels in AD frequently correlated with miR-181b (NDUFS3, HSF2), let-7f (HERPUD1, TBPL1) and miR-34a (HNRPR, BTF3).

The functional categories for these putative targets are summarized in Figure 3. Interestingly,

genes with multifunctional roles in Synapse Activity represented the most extensively targeted category. The enriched GO categories specified by GOTREE for the targeted downregulated genes were: Transcription ($P = 0.001$), Protein Transport ($P = 0.006$), and the Peroxisome ($P = 0.005$). The highest number of individual miRNAs targeting a common gene within the same functional category corresponded to Injury Response/Redox Homeostasis. MicroRNAs let-7f, 34a 181b and 200a shared common targets in several functional categories (Table 3).

The enriched GO categories for the downregulated targets identified for some of the individual upregulated miRNAs were as follows: Cell Homeostasis and Peroxisome for miR-34a;

Table 2. Altered miRNA expression in APOE4-negative and -positive strata

| APOE4 status | MicroRNA | Fold change | Score (d) | q-value (%) |
|--------------|--------------|-------------|-----------|-------------|
| negative | hsa-miR-34a | 1.4 | 1.8 | 0.0 |
| negative | hsa-miR-517* | 1.2 | 1.7 | 0.0 |
| negative | hsa-let-7f | 1.2 | 1.5 | 0.0 |
| negative | hsa-miR-200a | 1.2 | 1.4 | 0.0 |
| positive | hsa-miR-371 | 1.4 | 1.1 | 22.2 |
| positive | hsa-miR-181b | 1.7 | 1.0 | 22.2 |

False discovery rates (q-values) were 0% in the negative stratum and 22.2% in the positive stratum of 4 and 4 significant miRNAs respectively.

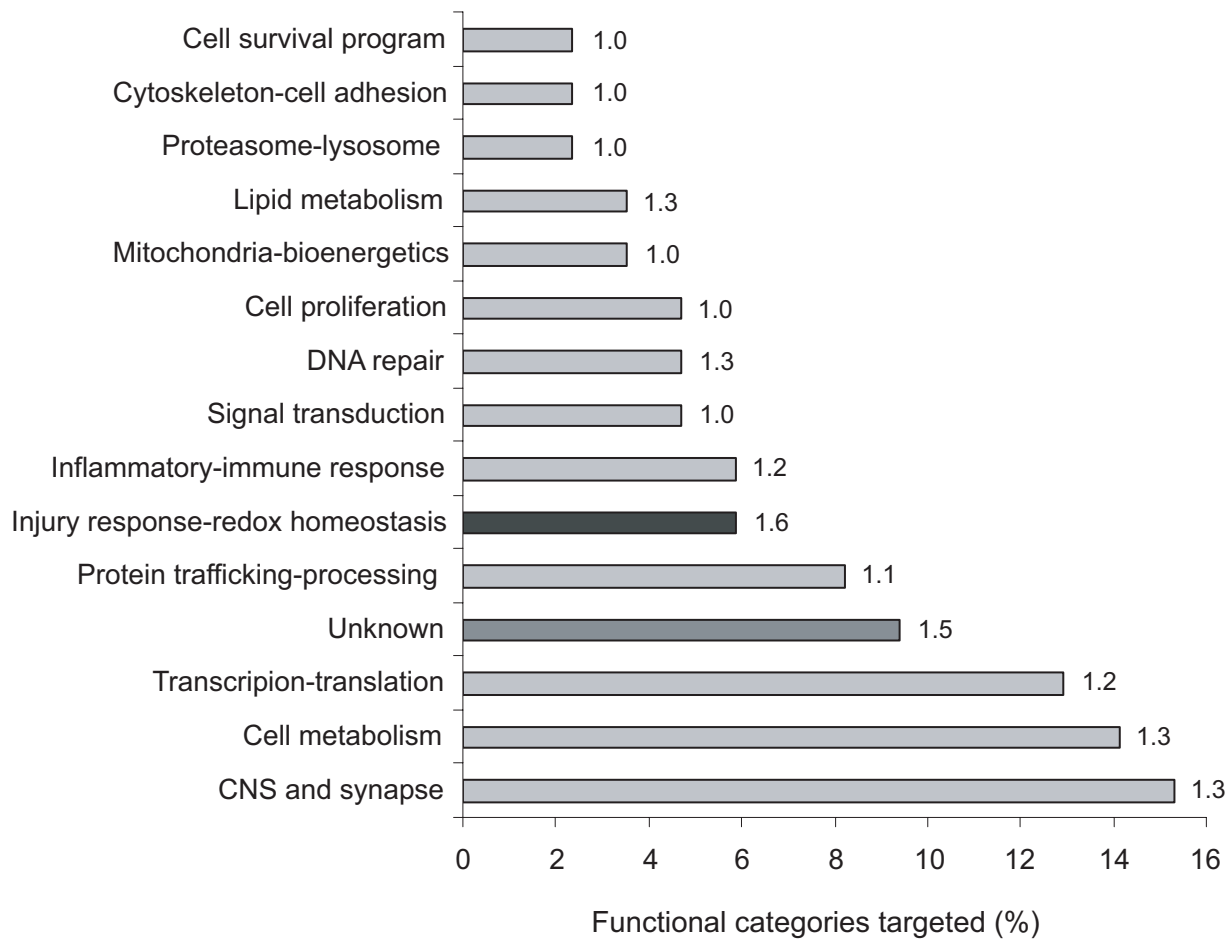


Figure 3. Functional categories of predicted microRNA targets downregulated in Alzheimer BMC. Targets of upregulated BMC miRNA were compared to previous downregulated mRNA data in Alzheimer BMC (Maes et al. 2006), and summarized according to percentage of targets per functional category. The average number of miRNAs targeting the same gene within each category is reported.

Cell Cycle and DNA Damage for miR-181b; and Vesicle Processes for miR-517*. Represented GO categories were also associated with miRNAs, such as: Cell Cycle with miR-200a and DNA Damage with miR-517*.

Discussion

Our study was based on the same population described in a previous transcriptome analysis (Maes et al. 2006) in order to compare microRNA action and their target genes at the message level. We made a stringent sample selection based on: (1) well-ascertained cases and controls matched for ethnicity, gender and age and (2) excellent total RNA quality and yield of purified microRNA for the screening of 462 currently-known human microRNA species. We found that human BMC expressed a broad range of miRNAs, representing 20% of the 462 miRNA spotted on nitrocellulose membranes in the array format (Supplemental Table 2).

MicroRNA expression profiles have shown greater accuracy in classifying cancers than have gene expression profiles (Lu et al. 2005). Although our classification of miRNA expression signatures may have been affected by experimental variations, we obtained relatively good discrimination of NEC vs. AD cases. However, the complexity and variability of miRNA expression observed between subjects could have also arisen from the diversity of cell types comprising the BMC fraction (Jison et al. 2004). It still remains to be determined whether the miRNA signature of a specific blood mononuclear cell type would be more effective in the classification of neurodegenerative diseases.

Despite the heterogeneity of BMC, we observed a significant increase in the expression of miR-34a and 181b in mild sporadic AD as compared to age-matched normal controls. A higher upregulation of miR-181b in Alzheimer BMC may also occur in APOE4-positive AD subjects. However, the higher FDR of 22% and low number of APOE4-positive

Table 3. Predicted miRNA targets downregulated in Alzheimer BMC.

| Upregulated microRNA | Target ^a | Target Gene Name | Fold change | Score (d) | Function |
|------------------------------------------|---------------------|-----------------------------------------------------|-------------|-----------|--------------------------------|
| Cell development and metabolism | | | | | |
| 181b | AHCY | S-adenosylhomocysteine hydrolase | 0.46 | -1.14 | methylation/homocysteine |
| 34a | BCKDK | branched chain alpha-ketoacid dehydrogenase kinase | 0.56 | -1.91 | amino acid pathway |
| let-7f; 34a | FXYD2 | FXYD domain containing ion transport regulator 2 | 0.66 | -1.61 | ion transport |
| 34a | SHMT1 | serine hydroxymethyltransferase 1 | 0.75 | -1.59 | serine catabolism |
| 181b | <u>GPI</u> | glucose phosphate isomerase | 0.75 | -1.52 | glycolysis |
| 34a | AMPD2 | adenosine monophosphate deaminase 2 | 0.50 | -1.47 | energy metabolism (brain) |
| 181b; 517* | HMBS | hydroxymethylbilane synthase | 0.35 | -1.24 | heme biosynthesis |
| 34a | LTF | lactotransferrin | 0.15 | -0.93 | iron homeostasis |
| 34a | CD151 | CD151 antigen | 0.59 | -2.66 | cell adhesion (sensory neuron) |
| 181b | <u>NDUFS3</u> | NADH dehydrogenase Fe-S protein 3 | 0.08 | -1.19 | mitochondrial complex I |
| Cell survival program | | | | | |
| 579 | PSAP | prosaposin | 0.79 | -1.61 | anti-apoptotic |
| 34a | BIK | BCL2-interacting killer | 0.54 | -1.01 | apoptosis |
| CNS and synapse | | | | | |
| let-7f; 34a | CRB3 | crumbs homolog 3 | 0.39 | -2.19 | tight junction |
| 34a | P2RY2 | purinergic receptor P2Y, G-protein coupled, 2 | 0.51 | -1.84 | APP processing |
| 579 | RXRG | retinoid X receptor, gamma | 0.49 | -1.74 | synaptic plasticity |
| 34a | GFAP | glial fibrillary acidic protein | 0.47 | -1.65 | myelination |
| 181b; 517* | ACTG1 | actin, gamma 1 | 0.77 | -1.63 | axon growth |
| 34a | <u>HNRPR</u> | heterogeneous nuclear ribonucleoprotein R | 0.45 | -1.49 | axon motor neuron |
| DNA repair | | | | | |
| 181b; 200a | <u>MCM3</u> | minichromosome maintenance deficient 3 | 0.70 | -1.68 | DNA damage response |
| 181b | MMS19L | MMS19-like (MET18 homolog) | 0.59 | -1.39 | DNA repair |
| Inflammatory-immune response | | | | | |
| 579 | <u>DDT</u> | D-dopachrome tautomerase | 0.76 | -2.68 | inflammation |
| let-7f; 34a | CHST12 | carbohydrate sulfotransferase 12 | 0.79 | -1.85 | immune response |
| 517* | LAMP2 | lysosomal-associated membrane protein 2 | 0.50 | -1.73 | immune response |
| 181b | SLA | Src-like-adaptor | 0.53 | -1.41 | immune response |
| Injury response-redox homeostasis | | | | | |
| let-7f; 200a | HERPUD1 | homocysteine-inducible, ubiquitin-likedomainmember1 | 0.41 | -2.26 | stress response |
| 517* | HMG2 | high-mobility group nucleosomal binding domain 2 | 0.71 | -1.71 | oxidative stress |
| let-7f; 181b | <u>TBPL1</u> | TBP-like 1 | 0.41 | -1.69 | stress response |
| 181b | <u>HSF2</u> | heat shock transcription factor 2 | 0.34 | -1.64 | stress response |
| Lipid metabolism | | | | | |
| let-7f | ELA3B | elastase 3B, pancreatic | 0.53 | -2.87 | cholesterol biosynthesis |
| 181b | FDXR | ferredoxin reductase | 0.51 | -1.85 | cholesterol biosynthesis |

(Continued)

Table 3. (Continued)

| Upregulated microRNA | Target ^a | Target Gene Name | Fold change | Score (d) | Function |
|-------------------------------|---------------------|------------------------------------------------------------|-------------|-----------|-------------------------------|
| let-7f; 34a | MECR | mitochondrial trans-2-enoyl-CoA reductase | 0.50 | -1.17 | fatty acid metabolism |
| Proteasome-lysosome-transport | | | | | |
| 579 | <u>UBE2M</u> | ubiquitin-conjugating enzyme E2M | 0.50 | -2.13 | ubiquitin cycle |
| 181b | <u>DNAJC7</u> | DnaJ (Hsp40) homolog, subfamily C, member 7 | 0.76 | -1.82 | protein folding |
| 181b | NDRG2 | NDRG family member 2 | 0.43 | -1.76 | protein chaperone (misfolded) |
| 579 | SNX2 | sorting nexin 2 | 0.34 | -1.68 | protein sorting |
| Signal transduction | | | | | |
| 181b | AGGF1 | Angiogenic factor with G patch and FHA domains 1 | 0.54 | -1.44 | signal transduction |
| 181b | MAP3K6 | mitogen-activated protein kinase kinase kinase 6 | 0.58 | -0.93 | signal transduction |
| Transcription-translation | | | | | |
| 34a | BTF3 | basic transcription factor 3 | 0.45 | -3.31 | transcription |
| 200a | <u>DDX5</u> | DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 5 | 0.73 | -2.34 | spliceosome |
| 34a | <u>IRF1</u> | interferon regulatory factor 1 | 0.59 | -1.46 | transcription |
| let-7f; 181b | TAF1A | TATA box binding protein- assoc. factor, RNAPol I, A | 0.58 | -1.35 | transcription (rRNA) |
| Unknown | | | | | |
| 517* | <u>MLF2</u> | myeloid leukemia factor 2 | 0.71 | -2.04 | unknown |
| 181b; 579 | <u>BEX2</u> | Brain expressed X-linked 2 | 0.33 | -1.89 | unknown (brain) |
| let7f; 181b; 579 | SSX2 | synovial sarcoma, X breakpoint 2 | 0.46 | -1.75 | unknown |
| 517* | ZNF691 | zinc finger protein 691 | 0.14 | -1.60 | unknown |
| 181b | RBMXL1 | RNA binding motif protein, X-linked-like 1 | 0.42 | -1.27 | unknown |
| let-7f, 517* | OBFC1 | oligonucleotide/oligosaccharide- bindingfoldcontaining1 | 0.55 | -1.11 | unknown |

^aUnderlined gene symbols indicate similar down-regulation in AD affected brains (Maes et al. 2006).

chips in the NEC group does not allow us to determine conclusively the impact of APOE4 status on miRNA expression. Nevertheless, the APOE4 allele is associated with an earlier onset of the disease (Hsiung et al. 2004), and higher expression of miRNAs could occur in these subjects as suggested by our results. If validated, induction of miRNA expression may provide a surrogate marker of AD progression.

In this regard, it is interesting to note that a reduction in dicer activity promotes tau toxicity in *Drosophila* (Bilen et al. 2006b). Thus, certain hallmark neuropathological features of AD may represent events of dysregulated miRNA processing. A reduction of miRNA processivity should

instead lead to a decrease in miRNA levels. In fact, increases in miRNAs levels were reported in human AD hippocampus (Lukiw, 2007), and this possible paradox in humans should be addressed in future investigations. However, the miRNAs observed in Alzheimer BMC differed from the few miRNAs screened in the latter study. In both studies, induction of specific miRNAs may contribute to the suppression of multiple mRNA species and thereby impact a host of cellular mechanisms (Lukiw, 2007). Moreover, concomitant induction of several miRNA species may act in an additive or synergistic manner to inhibit gene expression (Krek et al. 2005). In fact, studies have determined that individual microRNAs can downregulate

several mRNA species (Lim et al. 2005) and induce mRNA instability (Sood et al. 2006).

The 3' UTR of target genes and the cellular context strongly influence the action of miRNA (Didiano and Hobert, 2006), and our study provides insights leading to the identification of probable miRNA targets in human BMC. We discerned a prevalence of putative downregulated genes in the functional categories of Synapse Activity, Transcription, and Injury/Redox Homeostasis. Importantly, Cell Cycle and DNA Damage related GO categories are targeted by miR-181b, 200a, and 517*.

Taken together, these observations further support our model linking the development of AD pathology to systemic dysfunction in the cellular stress/antioxidant response and genomic maintenance (Maes et al. 2006). These data are commensurate with reports of augmented oxidative DNA and RNA damage and deficient transcription and translation in AD brain and peripheral tissues (Markesbery and Lovell, 2006; Shan and Lin, 2006). The latter impairments may, in turn, contribute to the cytoskeletal abnormalities and neurofibrillary degeneration characteristic of AD-affected neural tissues (Maes et al. 2006).

Caution must be exercised in extrapolating from BMC miRNA data sets to gene expression profiles in AD brain given the different 3' UTR regions inherent to neuronal mRNA relative to peripheral tissues (Sood et al. 2006). A wide array of miRNA species should be surveyed in AD-affected brain tissue to determine whether miRNA dysregulation exists therein comparable to that observed in the current Alzheimer BMC study.

The current study provides, to our knowledge, first evidence of augmented microRNA expression in Alzheimer BMC, as well as the framework for future miRNA-target experiments on altered cellular functions related to this disease. For example, miRNAs may account for the suppression of mRNA species implicated in the cellular stress response and DNA repair previously reported in Alzheimer BMC (Maes et al. 2006). Dysregulation of BMC miRNA in sporadic AD may shed new light on the pathogenesis of AD and possibly provide useful diagnostic/prognostic biomarkers of this common affliction.

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Supplemental Materials

Supplemental Table 1. Demographics of cohort and small RNA yields.

| Diagnosis | Subject ID | Gender | Age | Education | MMSE | APOE | Total RNA (µg) | Small RNA (µg) | Ratio ^a (%) |
|-----------|------------|--------|-----|-----------|------|------|----------------|----------------|------------------------|
| NEC | 967 | F | 80 | 11 | 29 | 2,3 | 41.7 | 1.2 | 2.9 |
| NEC | 944 | F | 68 | 18 | 30 | 3,3 | 80.4 | 1.7 | 2.1 |
| NEC | 1014 | F | 83 | 21 | 29 | 3,3 | 73.6 | 2.1 | 2.8 |
| NEC | 3R447 | F | 78 | 17 | 29 | 3,3 | 23.5 | 1.1 | 4.5 |
| NEC | 3R464 | F | 78 | 10 | 28 | 3,3 | 115.8 | 1.2 | 1.0 |
| NEC | R787 | F | 87 | 15 | 30 | 3,3 | 26.8 | 1.7 | 6.5 |
| NEC | 1005 | F | 68 | 15 | 30 | 3,4 | 97.0 | 1.2 | 1.3 |
| NEC | 1025 | F | 71 | 15 | 28 | 3,4 | 51.1 | 3.4 | 6.6 |
| NEC | 917 | M | 72 | 15 | 29 | 3,3 | 23.5 | 1.8 | 7.7 |
| NEC | 923 | M | 84 | 12 | 27 | 3,3 | 43.2 | 1.0 | 2.2 |
| NEC | 942 | M | 72 | 11 | 29 | 3,3 | 57.8 | 1.9 | 3.3 |
| NEC | 948 | M | 80 | 16 | 30 | 3,3 | 34.9 | 1.4 | 4.1 |
| NEC | R887 | M | 74 | 20 | 30 | 3,3 | 56.4 | 3.1 | 5.5 |
| NEC | 951 | M | 80 | 16 | 30 | 3,4 | 15.1 | 1.1 | 7.0 |
| NEC | R265 | M | 73 | 22 | 30 | 3,4 | 44.3 | 1.0 | 2.3 |
| NEC | 3R446 | M | 71 | 9 | 29 | 4,4 | 62.9 | 1.7 | 2.6 |
| AD | 940 | F | 75 | 13 | 21 | 3,4 | 52.9 | 1.1 | 2.1 |
| AD | 961 | F | 79 | 10 | 23 | 3,4 | 100.1 | 1.8 | 1.8 |
| AD | 939 | F | 78 | 6 | 27 | 3,3 | 76.9 | 1.1 | 1.4 |
| AD | 2R411 | F | 80 | 14 | 24 | 3,3 | 38.4 | 2.6 | 6.8 |
| AD | 1049 | F | 84 | 16 | 24 | 2,4 | 18.9 | 2.6 | 13.9 |
| AD | 1057 | F | 72 | 11 | 27 | 3,4 | 37.6 | 1.1 | 2.8 |
| AD | 928 | F | 81 | 13 | 13 | 2,2 | 39.4 | 1.1 | 2.9 |
| AD | 1033 | F | 88 | 12 | nd | 3,3 | 48.0 | 0.9 | 1.9 |
| AD | 906 | M | 73 | 14 | 25 | 3,3 | 28.1 | 1.3 | 4.7 |
| AD | 925 | M | 80 | 15 | 28 | 3,3 | 52.1 | 1.4 | 2.6 |
| AD | 943 | M | 75 | 20 | 17 | 3,3 | 77.8 | 1.6 | 2.0 |
| AD | 989 | M | 69 | 11 | 21 | 4,4 | 36.7 | 0.9 | 2.6 |
| AD | 1018 | M | 76 | 12 | 23 | 4,4 | 34.3 | 2.8 | 8.1 |
| AD | 1022 | M | 84 | 12 | 22 | 3,4 | 61.0 | 2.5 | 4.1 |
| AD | 2R377 | M | 81 | 15 | 26 | 3,4 | 117.0 | 1.3 | 1.1 |
| AD | 3R386 | M | 79 | 7 | 25 | 3,3 | 73.8 | 1.8 | 2.5 |

^aPercentage of small RNA over total RNA.

Supplemental Table 2. miRNA expression in elderly human blood mononuclear cells.

| High (intensity 20–8)^a. | Medium (intensity <7) | Low (intensity <3) |
|-------------------------------------------|---------------------------------|------------------------------|
| hsa-let-7c | hsa-let-7g | hsa-let-7i |
| hsa-let-7f | hsa-miR-34a | hsa-miR-10a |
| hsa-miR-10b | hsa-miR-34b | hsa-miR-18b |
| hsa-miR-18a | hsa-miR-92b | hsa-miR-23b |
| hsa-miR-27b | hsa-miR-125b | hsa-miR-26a |
| hsa-miR-95 | hsa-miR-136 | hsa-miR-93 |
| hsa-miR-137 | hsa-miR-181b | hsa-miR-107 |
| hsa-miR-188 | hsa-miR-182 | hsa-miR-146a |
| hsa-miR-200b | hsa-miR-195 | hsa-miR-148a |
| hsa-miR-373* | hsa-miR-200a | hsa-miR-152 |
| hsa-miR-376a* | hsa-miR-219 | hsa-miR-155 |
| hsa-miR-377 | hsa-miR-373 | hsa-miR-192 |
| hsa-miR-380-5p | hsa-miR-489 | hsa-miR-363 |
| hsa-miR-509 | hsa-miR-515-5p | hsa-miR-371 |
| hsa-miR-510 | hsa-miR-518a | hsa-miR-424 |
| hsa-miR-517* | hsa-miR-520b | hsa-miR-431 |
| hsa-miR-520h | hsa-miR-539 | hsa-miR-449b |
| hsa-miR-523 | hsa-miR-548b | hsa-miR-493-3p |
| hsa-miR-551a | hsa-miR-562 | hsa-miR-513 |
| hsa-miR-561 | hsa-miR-577 | hsa-miR-569 |
| hsa-miR-574 | hsa-miR-579 | hsa-miR-575 |
| hsa-miR-582 | hsa-miR-600 | hsa-miR-581 |
| hsa-miR-585 | hsa-miR-607 | hsa-miR-587 |
| hsa-miR-591 | hsa-miR-620 | hsa-miR-605 |
| hsa-miR-598 | hsa-miR-623 | hsa-miR-608 |
| hsa-miR-603 | hsa-miR-624 | hsa-miR-638 |
| hsa-miR-609 | hsa-miR-627 | hsa-miR-652 |
| hsa-miR-612 | hsa-miR-646 | |
| hsa-miR-621 | hsa-miR-647 | |
| hsa-miR-633 | hsa-miR-653 | |
| hsa-miR-641 | hsa-miR-661 | |
| hsa-miR-649 | | |
| hsa-miR-659 | | |
| hsa-miR-660 | | |

^aHybridization intensity after DIG immunodetection (see Methods section).