Microarray and miRNA analyses of brain lesions in African-American and Caucasians with multiple sclerosis

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Citation

Abstract
Objective: To study if differences in gene expression in brain tissue among African-Americans (AA) and Caucasian Americans (CA) with multiple sclerosis (MS) exist. Understanding any genetic differences is critical for better understanding of MS and its outcomes.

Methods: Microarray and microRNA methods were used in chronic brain lesions of AA and CA patients with MS.

Results:
We found marked downregulation in GM2A (5.2 vs. 2.07), GALC (4.48 vs. 2.66), EIF1AY (4.54 vs. 1.57) and carboxypeptidase D (3.72 vs. 1.79), genes among chronic lesions taken from AA and CA brains and validated using real-time qPCR techniques. A total of 1108 genes were downregulated, compared to 467 genes that were upregulated in chronic MS lesions, compared to normal appearing brain matter (ratio of 2:1); a similar comparison between AA and CA brains revealed a total of 611 downregulated vs. 192 upregulated genes (ratio of 3:1).

Interpretation: Significant downregulation of GM2A, GALC, EIF1AY and carboxypeptidase D in the AA lesions as compared to CA cohort could have implications for MS.

INTRODUCTION

Multiple Sclerosis (MS) is a genetically complex immune mediated, demyelinating disease of the central nervous system. To date, no genetic variants have been unambiguously linked to disease severity until recently, when MGAT5, a gene coding for a glycosylation enzyme, was found to be significantly associated with outcome (Brynedal et al., Jan 29). However, no specific genes or gene products have been studied in the African-American populations that have a link to disease severity or poor outcomes have been unearthed.

Studies (Bailey, ; Alter, Aug; Oh & Calhoun, Sep; Kurtzke et al.) have shown that African Americans (AAs) develop multiple sclerosis (MS) less frequently than Caucasian Americans (CAs) and MS appears to be extremely rare in black Africans (Foster & Harries, Sep 12; Ames & Louw, Aug; Ames & Bowen, Aug; Kioy, Feb; Modi et al., Apr). The increased prevalence of MS in AAs compared to black Africans is probably due to genetic admixture of a resistant African population with a susceptible Caucasian population or other undetected factors. The presence of the DRB1*1501 allele in AA suggests that admixture with Northern European chromosomes contributes to MS susceptibility. However, the finding of a second MS susceptibility allele (DRB*1503), uniquely African in origin, indicates that MS susceptibility in this population is not entirely due to admixture with Northern European chromosomes. In addition, Cree et al (Cree et al., Dec 14; Cree et al., Nov) found that the human leukocyte antigen (HLA) allele DRB1*1501, long known to be associated with MS in the CA population, was also associated with AA MS, as was another closely related African HLA gene termed DRB1*1503. The benefits of disease-modifying therapies may also differ between AA and CA populations. In an exploratory post-hoc analysis of the EVIDENCE study, Cree et al (Cree et al., Nov) showed that AA subjects developed more new MS lesions on T2-weighted brain magnetic resonance imaging at 48 weeks and appeared less responsive to treatment than CA subjects on outcome measures, reaching significance only for T2-weighted lesion count at 48 weeks. Additionally, AA MS patients do not respond to interferon ß-1a as well as their CA counterparts.

It appears that African ethnicity (Cree et al., Dec 14) is a highly significant predictor of requiring a cane to ambulate after adjusting for age at onset, gender, and types and durations of medications commonly used to treat MS. The age at onset seems to be a strong predictive factor for disability and suggests that AA patients are at higher risk for
disability in part because of their older age at onset. Given the above data, it is worthwhile to note that no studies have explored the possibility that genetic markers or putative genes could play a role in increased disability, disease burden/presentation or treatment outcomes in the AA population.

Published literature suggests that large-scale analysis of transcripts in MS lesions can potentially unearth novel targets for therapy and recent transcription-profiling studies have found distinct gene expression patterns in diseased tissues, supporting such a hypothesis. It is possible that studies done at different stages of the disease may further uncover the mechanisms of MS pathogenesis. Hence, we studied brain microRNA (miRNA) and microarray analyses data in brains of AA and CA patients with MS patients to compile a database that could be used as a template for future research in this area.

METHODS

Brain tissue was obtained at autopsy less than 3 hours after postmortem. For our study, lesions were dissected from brain tissue obtained from the human brain and spinal fluid resource center in Los Angeles, CA, 90073; its website is http://www.loni.ucla.edu/uclabrainbank. This resource center is supported by the National Multiple Sclerosis Society and National Institute of Neurological Disorders and Stroke, among others. Informed consent is obtained by the center prior to collection of all its tissue and fluid samples from patients and control subjects.

A total of 5 lesions from the AA brain (3 were chronic lesions and 2 from normal appearing tissue) and 8 lesions from the CA brains (6 chronic lesions and 2 normal appearing tissue) were studied. The age of the AA patient was 76, and the disease duration was 11 years; the age range of the CA was from 50-71. None of the patients were on immune-modulating agents for several months prior to death (Table 1).

Gene expression profiles were compared in AA and CA brains between chronic lesions, normal appearing white or gray matter (NAWM or NAGM) which are clinically ‘silent’ and have minimal or no inflammation. The lesions were taken from paraffin sections in all cases, stained with H&E and histologically examined for evidence of pathology. These were used as the basis for characterization of demyelination, presence of activated microglia/macrophages and infiltration/ perivascular cuffing of lymphocytic cells in and around the MS lesions, and in adjacent NAWM and NAGM. Special staining with Luxol Fast Blue was used to demonstrate demyelination and CD68 immunohistochemistry was used to localize the activated macrophage/microglia in and around the MS lesions. (see Figure 1)

RNA ISOLATION

Total RNA was isolated from frozen brain sections using trireagent (Molecular Research Center) and further cleaned up on a Qiagen RNEasy column using standard protocols that included an on-column Dnase treatment. The quality of the RNA was verified using an Agilent 2100 BioAnalyzer and a
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nanodrop spectrophotometer. Samples were deemed of good quality if the 260/280 and 260/230 ratios as were between 1.8-2.0 and if the 28S/18S ribosomal ratio was >1.8.

**MICROARRAY METHODS**

Total RNA (2 ug) was converted to labeled cRNA using standard Affymetrix protocols as described elsewhere (Chittur et al.). The fragmented cRNA was hybridized to Human Genome U133 plus 2.0 GeneChips and scanned on a GeneChip scanner using standard protocols.

**STATISTICAL ANALYSIS**

The raw data (CEL files) were imported into GeneSpring GX software (v9) for further analysis. The data was quantile normalized using the GCRMA algorithm. Selection of statistically significant genes from each expression profile was done using a p-value cut off of ≤ 0.05 with the cross gene error model (CGEM) combined with Welch t-test. The multiple testing correction (Benjamini and Hochberg false discovery rate, p-value <0.05) was integrated within each test. A fold change cut-off at 1.5 fold was implemented to generate the final list of differentially expressed genes. Hierarchical clustering was used to organize the data in discrete expression profiles.

**QUANTITATIVE REAL-TIME PCR**

To verify the data obtained from microarrays, 5 µg of total RNA was taken from the same pool of RNA as used for the microarray experiments. The RNA was reverse transcribed to cDNA which served as the template for quantitative PCR (qPCR). Real-time relative qPCR (SYBR Green; Applied Biosystems) was performed in triplicate using a HT7900 sequence detection system according to the manufacturer’s instructions. Primers were specifically designed using Primer express software (Applied Biosystems). 1 µg of cDNA was amplified in 1x SYBR green buffer. PCR conditions were: 10 min at 95°C for AmpliTaq Gold DNA polymerase activation, 45 thermal cycles of 15 sec at 95°C to denature and 1 min at 60°C to anneal and extend. Relative expression levels were analyzed using the $2^{-\Delta\Delta C_T}$ method using the GAPDH expression level as a control (For primer sequences see Table 2).

**MICRONORNA ISOLATION AND MICROARRAY METHODS**

Total RNA (25ug) was isolated from the tissue samples using trizol extraction and the miRNA component was enriched using the mirVana miRNA isolation kit (Ambion). The presence of miRNA was validated using the small RNA assay on the Agilent Bioanlyzer. 5 ug of this was then spiked with the kit-supplied positive controls following which a 3'-amine modified tail was added using the manufacturers recommended protocol. The tailed miRNA was labeled with amine-reactive Cy5, hybridized to the mirVana miRNA Bioarrays and scanned on a Genepix 4000B scanner.

The scanned images were analyzed using GeneSpring GX. The miRNA probes were filtered to exclude those with poor signal in all samples. The remaining probes were subjected to a Mann-Whitney test comparing NAWM samples to plaques (p-value <0.05) followed by a filter on probes showing 1.5 fold differential expression between these two conditions. The list was further culled to include 471 probes corresponding to only human miRNA. This list was then used as input for the TargetScan 5.1 module in GeneSpring to identify putative known miRNA targets in the human genome. The resulting target list was then compared with the list of differentially expressed genes from the HU133 plus 2.0 microarray analysis to identify common entities.

**RESULTS**

A total of 1108 genes were down regulated, compared to 467 genes that were upregulated, between MS brain lesionosal vs. non-lesional areas (NAWM/NAGM), an approximate ratio of 2:1. A comparison of the lesional vs non-lesional gene expression profiles (set at a cut-off of 1.5 fold) within AA and CA brains revealed a total of 611 down regulated vs. 192 upregulated genes (3:1 ratio, approximately). Additionally 637 genes to be further differentially expressed in only the CA samples and a further 629 genes were
differentially expressed only in AA Samples. (see table 3 and supplementary table 1)

**Figure 4**
Table 3: Representative differentially expressed genes

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Fold Change (AA/CA)</th>
<th>Fold Change (CA/AA)</th>
<th>Gene Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>201613245.5</td>
<td>-2.07</td>
<td>0.30</td>
<td>CPD</td>
<td>Carboplatin D</td>
</tr>
<tr>
<td>20161335.5</td>
<td>-1.72</td>
<td>1.79</td>
<td>GM2A</td>
<td>Ganglioside 2 activator</td>
</tr>
<tr>
<td>20161357.5</td>
<td>-1.79</td>
<td>1.79</td>
<td>GM2A</td>
<td>Ganglioside 2 activator</td>
</tr>
<tr>
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<td>-1.72</td>
<td>1.79</td>
<td>CPD</td>
<td>Carboplatin D</td>
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<tr>
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<td>-1.72</td>
<td>1.79</td>
<td>GM2A</td>
<td>Ganglioside 2 activator</td>
</tr>
</tbody>
</table>

Supplementary Table 1

While GM2A was down regulated in both AA (5.20) and CA (2.07) lesions, this difference in was less profound when MS brains (AA and CA) were compared against normal brain (3.01). Other genes that were down regulated were GALC in AA (4.48) vs. CA (2.06) and carboxypeptidase D, in AA (3.72) vs. CA (1.79), respectively. Furthermore, EIF1AY gene was down regulated in CA (4.54) vs. AA (1.57). Myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) were both down regulated in AA (2.2) and CA (1.7) lesions, with no significant differences. Expression levels of these genes as validated by qPCR data for up and down regulated genes is shown in figure 2. All corresponding expression data for miRNA targeting these genes (GM2A, GALC, CPD, EIF1AY) are shown in table 4.

**Table 4: Representative differential expression in miRNA**

<table>
<thead>
<tr>
<th>Name</th>
<th>FC (AA)</th>
<th>FC (CA)</th>
<th>Gene Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa_let-7a</td>
<td>3.17</td>
<td>2.15</td>
<td>MBP</td>
</tr>
<tr>
<td>hsa_let-7b</td>
<td>2.15</td>
<td>1.54</td>
<td>MBP</td>
</tr>
<tr>
<td>hsa_let-7c</td>
<td>2.08</td>
<td>1.54</td>
<td>MBP</td>
</tr>
<tr>
<td>hsa_miR-105</td>
<td>1.54</td>
<td>1.05</td>
<td>MBP</td>
</tr>
<tr>
<td>hsa_miR-186</td>
<td>1.54</td>
<td>1.05</td>
<td>MBP</td>
</tr>
</tbody>
</table>
A comparison of miRNA expression (see supplementary table 2) between NAWM and plaques showed 97 human miRNAs (37 up and 60 down in MS) to be differentially expressed at a fold change of 1.5 or greater. A similar comparison within the races showed 150 miRNAs (64 up and 86 down) in AA plaques vs AA NAWM and 125 miRNAs (58 up and 67 down in CA plaques) vs CA NAWM tissue. Moreover 116 miRNAs (54 up and 62 down in CA plaques) and 197 (84 up and 113 down in CA NAWM) were differentially expressed as compared to their corresponding tissue from AA samples.

Supplementary Table 2

DISCUSSION

The paucity of AA brain tissue with MS in brain banks makes it impossible to evaluate and reproduce results across multiple subjects particularly when CA brain with MS is more readily available. This is one of the pitfalls of this study. Although we evaluated data from multiple lesions, our data are severely limited owing to lack of AA brain tissue as starting material and hence, definitive conclusions are hard to make. Our study is the first of its kind and hence, a starting point.

In MS, longitudinal monitoring of plaque activity allows for identification of temporal patterns and underlying cellular events that drive tissue destruction and could provide clues to disease progression and evolution. Study of plaque activity (chronic vs. acute) in different subsets of patients and samples obtained at different time points in the evolution of a plaque could help in understanding the disease mechanisms better.

When lesions in the AA and CA brains were compared, we found 604 down regulated vs. 193 upregulated genes in our whole genome analysis with an arbitrary cut-off set at 1.5. We found that the differentially expressed down regulated gene cohort in AA brain had the most significant correlation to disease pathology. We found significant quantitative differences in specific gene expression in AA brain lesions compared to CA brains that may be relevant to MS pathology.

GM2A downregulation was seen both in AA (5.20) and CA (2.07) lesions; the difference in downregulation was less profound when MS brains (AA and CA) were compared against normal brain (3.01 vs. 1.51) but present. The gene for GM2A encodes a glycolipid transport protein that acts as a substrate-specific co-factor for the lysosomal enzyme beta-hexosaminidase A, which together with GM2A catalyzes the degradation of the ganglioside GM2 and other molecules containing terminal N-acetyl hexosamines. GM2A gene mutations can result in GM2-gangliosidosis type AB or the AB variant of Tay-Sachs disease. A related study in a mouse model (Liu et al., Jul 22) confirmed that the GM2A deficiency, caused by mutations in the GM2A gene, resulted in GM2 ganglioside accumulation in the cerebellum with resultant deficits in balance and coordination. The possibility that GM2A downregulation could impact its interaction with beta-hexosaminidase A raises an intriguing possibility that relative GM2 accumulation between AA and CA brains could offer one clue into the pathological basis of the disease in these diverse populations.

Secondly, GALC was down regulated in AA (4.48) vs. CA (2.66) brain lesions. GALC encodes a lysosomal enzyme (galactosylceramidase) which hydrolyzes the galactose ester bonds of galactosylceramide, galactosylsphingosine, lactosylceramide, and monogalactosyldiglyceride. A deficiency in this gene has been associated with Krabbe’s disease. The downregulation of GALC in MS lesions (AA and CA combined) was 2.94 vs. 1.23 (normal brain). Taken together, the downregulation of GM2A and GALC may be particularly relevant given that MS in the AA population is...
more aggressive and responds poorly to treatment compared to MS in CA. It is noteworthy that both genes are involved in the pathogenesis of Tay-Sachs and Krabbe’s diseases. A rare form of Tay-Sachs’ disease, known as Late Onset Tay-Sachs disease (LOTS), occurs in patients in their 20s and early 30s and is frequently misdiagnosed. Symptoms of LOTS may present in adolescence or early adulthood, include speech and swallowing difficulties, unsteadiness of gait, spasticity, cognitive decline, and psychiatric illnesses, symptoms that can mimic MS.

Carboxypeptidase D downregulation in AA brain was 3.72, compared to 1.79, for CA brain. Our finding may be significant since carboxypeptidase D (CpD) is a 180-kDa type I membrane protein and downregulation of CpD leads to downmodulation of transforming growth factor-beta (TGF-beta), suggesting a role for CpD in a positive feedback loop. A similar mechanism has been shown in autoimmune diseases such as lupus (Hoff et al., Nov). JAM3 expression was significantly down regulated in the AA samples compared to CA lesions. The expression of adhesion molecules at tight junctions regulates cell-to-cell adhesion in epithelial or endothelial cell sheets. The protein encoded by this immunoglobulin superfamily gene member is localized in the tight junctions between endothelial cells. The encoded protein is a member of the junctional adhesion molecule protein family and acts as a receptor for another member of this family. Downregulation of JAM3 could facilitate blood-brain barrier transmigration, a parallel that can be drawn from studies in patients with ulcerative colitis wherein down-regulation of the key TJ transmembrane protein occludin occurs in regions that show active transmigration of polymorphonuclear cells. (Kucharczik et al., Dec).

An important finding in our earlier study in B cells was an 11.7-fold downregulation of eukaryotic translation initiation factor 1A, Y-linked (EIF1AY) gene. In this study, EIF1AY gene was down regulated in CA brain (4.54) significantly more than in AA brain (1.57). It is thought that EIF1AY encodes for minor histocompatibility antigen (mHA) and B-cell mediated antibody response to Y-chromosome-encoded histocompatibility antigens (H-Y antigens) associated with maintenance of disease remission in graft-vs-host disease (Miklos et al., Apr 1). It is possible that EIF1AY downregulation is more robust in the CA brain and perhaps linked to better disease remission.

SYNE1 levels in AA were twice as high as in CA lesions. This gene encodes a spectrin repeat containing protein expressed in skeletal and smooth muscle and peripheral blood lymphocytes that localizes to the nuclear membrane. SYNE1 is the first identified gene responsible for a recessively inherited pure cerebellar ataxia (Gros-Louis et al., Jan). Further studies are required to explore if SYNE1 gene is consistently overexpressed in the AA cohort and whether increased SYNE1 has a role in the ataxia in the AA patient population.

We found that IGF1 (somatomedin C) upregulation in AA brain is 2.95 vs. 1.66 in CA brain. It has been shown that IGF1 targets oligodendrocytes raising the possibility that apoptosis may be better prevented in the AA brain.

MicroRNAs (miRNAs) are small noncoding RNAs that repress gene expression by binding mRNA target transcripts, causing translational repression or mRNA degradation. They are predicted to target one-third of all genes in the genome, where each miRNA may target approximately 200 transcripts. Given the large number of miRNAs and potential targets, miRNAs have a key regulatory role in many biological processes.

Until recently, no data was available that explored the levels of miRNA expression in multiple sclerosis (Du et al., Dec; Keller et al., ; Otaegui et al.). Since no miRNA expression data is currently available for MS samples from the AA cohort, this study presents the first catalog of these post-transcriptional modulators. Regulation of gene expression is a complex phenomenon involving various mechanisms of which miRNA silencing is one such pathway. We hypothesized that specific miRNA levels for the genes that we studied could have influenced gene expression. We demonstrate that different sets of miRNA are differentially expressed in these samples. We subjected our lists of differentially expressed miRNA to Target Scan 5.1 to identify putative conserved and non-conserved target genes. These gene lists so obtained were cross-compared with genes differentially expressed in our data sets and found that they are concordant. Various miRNA such as mir-326 and mir-186 have been recently implicated in MS pathogenesis and indeed their expression levels in our study are consistent with these findings. Multiple miRNAs can target a single gene and multiple genes can be targeted by a single miRNA. The specificity of this relationship in each disease state is not yet known and remains to be further explored.

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