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Dicer and microRNA expression in Multiple Sclerosis and response to interferon therapy

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Abstract

Dysregulation of microRNA expression has been shown in multiple sclerosis (MS); however, the mechanisms underlying these changes, their response to therapy and the impact of microRNA changes in MS are not completely understood. Dicer mediates the cleavage of precursor microRNAs to mature microRNAs and is dysregulated in multiple pathologies. Having shown that interferons regulate Dicer in vitro, we hypothesized that MS patient IFN β 1a treatment could potentially alter Dicer expression. Dicer mRNA and protein levels, as well as microRNA expression, were determined in MS patient and healthy control PBL. Acute responses to IFN β 1a were assessed in 50 patients. We found that Dicer protein but not mRNA levels decrease in MS patients while both are selectively induced in patients responding well to IFN β 1a. Potential microRNA biomarkers for relapsing remitting multiple sclerosis (RRMS), secondary-progressive

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://>.

multiple sclerosis (SPMS) and IFN β 1a response are described. Contrasts in Dicer and microRNA expression levels between patient populations may offer insight into mechanisms underlying disease courses and responses to IFN β 1a therapy. This work identifies Dicer regulation as both a potential mediator of MS pathology and a therapeutic target.

Keywords

Multiple sclerosis; interferon; microRNA; Dicer; therapy; biomarkers

1. Introduction

Multiple sclerosis (MS) is a chronic autoimmune/inflammatory disease with a neurodegenerative component affecting the central nervous system. Several reports have suggested microRNA (miRNA or miR) dysregulation in MS patients and attempted to identify miRNAs as biomarkers (Otaegui et al., 2009; Junker et al., 2009; Keller et al., 2009; De Santis et al., 2010; Lindberg et al., 2010; Guerau-de-Arellano et al., 2011; Waschbisch et al., 2011; Guerau-de-Arellano et al., 2012; Martinelli-Boneschi et al., 2012; Angerstein et al., 2012; Sievers et al., 2012; Jernas et al., 2013; Fenoglio et al., 2013; Hecker et al., 2013; Sondergaard et al., 2013; Gandhi et al., 2013).

MicroRNA are now recognized as an important component of epigenetic regulation and miRNA expression profiles have been found to be disrupted in many pathologies (Esteller, 2011; Ha, 2011; Rottiers and Naar, 2012; Di Leva and Croce, 2013). Most miRNA are synthesized from RNA Pol II transcripts (primary miRNA or pri-miRs), processed in the nucleus by a type III RNase (Drosha/DGCR8) into shorter hairpin “pre-miRs” which are transported into the cytoplasm by Exportin5. In the cytoplasm, pre-miRs are recognized by another type III RNase, Dicer, and cleaved to their mature ~22nt form. Mature miRNA are then incorporated into the miRISC complex of proteins, which includes Dicer, Ago2, TRBP, PACT and other proteins. The miRNA guides the silencing complex to target mRNAs with sufficiently complementary sequences for either degradation or translational repression (Koscianska et al., 2011).

Currently, 2588 mature miRNA have been identified in humans and their expression profiles have been shown to be tissue, cell type and developmental stage specific. MiRNA are reported to contribute to the regulation of approximately half of the human genome (Koscianska et al., 2011; Di Leva and Croce, 2013). Alterations in miRNA expression profiles have been found in many pathologies (Esteller, 2011; Ha, 2011; Rottiers and Naar, 2012; Di Leva and Croce, 2013), particularly those associated with inflammation and immune function (Cobb et al., 2006; Asirvatham et al., 2008; Baltimore et al., 2008; O’Connell et al., 2012; Singh et al., 2013). It has been suggested that miRNA profiles may serve as diagnostic and/or prognostic biomarkers (Weber et al., 2010; Kosaka et al., 2010; Zhang et al., 2012; Chavali et al., 2013). Furthermore, miRNA are currently under investigation as targets for intervention – with the proposed use of synthetic miRNA mimics or antagonists (antagomiRs) as therapeutic agents (Krutzfeldt et al., 2005; Brown and Naldini, 2009; Castanotto et al., 2009; Rupaimoole et al., 2011; Chavali et al., 2013 30-34).

Aberrant expression of several components of the miRNA production pathway has been described in various diseases (Karube et al., 2005; Divekar et al., 2011; Oak et al., 2011; Mockenhaupt et al., 2011; Gascon and Gao, 2012; Sharma et al., 2013). For example, decreased Dicer expression in lung cancer is associated with diminished miRNA expression and poor prognosis (Karube et al., 2005). In MS, overexpression of Dicer mRNA in PBL has been reported (Jafari et al., 2015) while others have shown decreased Dicer protein in B cells from MS patients (Aung and Balashov, 2014). To our knowledge, no studies have analyzed Dicer mRNA and protein expression in the context of complete miRNA profiling. Furthermore, there have not been studies of Dicer expression in MS progression or treatment.

Dicer expression is required for normal development of oligodendrocytes and Schwann Cells (Shin et al., 2009; Bremer et al., 2010). Dicer expression has been shown to be subject to acute regulation in vitro as well as developmental regulation in vivo (Asada et al., 2008; Gonzalez-Gonzalez et al., 2008; Wiesen and Tomasi, 2009; Garcia-Lopez and del Mazo, 2012). Moreover, inflammatory mediators, including interferons and TLR ligands, have been found to cause acute regulation of Dicer in vitro and, interestingly, IFN α and IFN γ may have opposite effects on Dicer protein levels (Wiesen and Tomasi, 2009). Acute regulation of Dicer has not been shown in a therapeutic context and altered expression levels of Dicer in MS has not, to our knowledge, been reported in diagnostic or clinical response groups. Therefore, in this study, we established a population of 100 patients including healthy controls, untreated MS patients and both good and poor responders to weekly intramuscular (IM) IFN β 1a (Avonex[®], Biogen Idec, Boston, MA). In these patients' PBL, we determined Dicer mRNA and protein expression together with miRNA profiles in order to support high confidence determination of miRNA expression differences between our study groups.

2. Materials and Methods

2.1. Ethics statement

The Institutional Review Boards for both Roswell Park Cancer Institute and SUNY University at Buffalo approved this study and all participants signed approved consent forms.

2.2. Study population

A total of 100 patients and age, sex matched healthy control subjects were recruited to this IRB approved study. Subjects were enrolled in one of four subgroups: 25 healthy controls (HC), 24 MS on no therapy (NT), 50 patients receiving weekly intramuscular IFN β 1a – 25 defined as good responders (GR) and 25 as poor responders (PR) (Table 1). These 50 patients averaged 18.2 years since diagnosis and had been treated with IFN β 1a for an average of 10.26 years each (Table 1). The poor responders were defined as patients presenting with clinical (relapses and/or increased disability) and/or MRI activity despite IFN therapy while GR included stable patients (no MRI or clinical activity for at least 2 years on IFN). While the majority of our enrolled patients were diagnosed as relapsing remitting multiple sclerosis (RRMS), 40% of our Poor Responders were secondary

progressive multiple sclerosis (SPMS) patients who continued to receive IFN β 1a due to ongoing relapses (Lublin and Reingold, 1996). Our untreated MS patient population was 80% RRMS, 20% SPMS (Table 1). All patients were evaluated clinically within one month of their study participation and assessed using the Expanded Disability Status Scale (EDSS) to quantify disability. EDSS scores can range from 0.0 - 10.0 with ambulatory MS patient scores 1.0 - 4.5 and scores 5.0 - 6.5 indicating degrees of impaired ambulation. Patients with scores ≥ 7 are not ambulatory.

The 50 subjects on IFN β 1a therapy had blood drawn in EDTA tubes immediately prior to an Avonex injection and again 48hrs later. Healthy controls and patients not receiving IFN β 1a had blood drawn at 0 and 48 hrs. Samples were coded so that their diagnosis, treatment and study group remained unknown until data acquisition was complete.

2.3. Sample preparation

Whole blood samples, collected in EDTA vacutainers, were separated over Ficoll (Ficoll-Plaque PLUS, GE Healthcare) and recovered leukocytes were lysed for total RNA (miRvana kit, Life Technologies, Grand Island, NY) or total protein (RIPA lysis buffer [Sigma, St. Louis, MO] with protease and phosphatase inhibitors [Thermo Scientific, Rockford, IL]). Plasma samples were also preserved.

2.4. Western blotting

Discontinuous SDS-PAGE (12/7/4%) gels were loaded with 40ug of total protein lysates, run and transferred to PVDF membranes by standard methods. Westerns were probed with antibodies to Dicer (Cell Signaling Technology, Danvers, MA), β actin (Abcam, Cambridge, MA) and goat anti-rabbit-HRP (Promega, Madison, WI) and visualized on a BioRad ChemiDoc XRS CCR camera with FemtoGlow substrate (Michigan Diagnostics, Royal Oak, MI). Quantity One software (BioRad, Hercules, CA) was used to normalize the quantified Dicer and actin bands. Our data are presented as actin normalized Dicer protein levels (Dicer/actin protein level). A healthy control separate from our study population donated a larger volume of blood which provided a common protein sample included in every blot for further normalization.

2.5. RNA analyses

DICER1 and GAPDH mRNA were assayed by real time RT-qPCR (primer sequences can be found in Wiesen and Tomasi, 2009). Reverse transcription used Superscript II (Life Technologies) and real time PCR used Sybr Green master mix (Roche Diagnostics, Indianapolis, IN). All assays were carried out on an ABI 7900 HT (Life Technologies) and analyzed with SDS software. Relative expression levels were determined by the $\Delta\Delta C_t$ method (Magner et al., 2000). GAPDH was used as a reference gene and both intra- and inter-assay CV (%) were $<15\%$. In addition, ACTB, B2M, STAT1, MxA and TRAIL mRNA absolute expression levels were determined by real time RT-qPCR standard curve method (assay specifics and references to primers can be found in Weinstock-Guttman et al., 2008).

For sequencing, all patient RNA samples were subjected to quality control analysis by Nanodrop spectrophotometer (Wilmington, DE) and Bioanalyzer 2100 nanochip (Agilent, Santa Clara, CA). TrueSeq small RNA libraries (Illumina, San Diego, CA) were prepared (RPCI Genomics core facility) and submitted for 50 cycle sequencing on an Illumina HiSeq 2000 (SUNY Univ. at Buffalo Sequencing facility). Sequence data were trimmed in CLC Workbench (CLCbio, Cambridge, MA). In order to profile known miRNAs in our small RNA-seq datasets, we used miRanalyzer version 0.2 (Hackenberg et al., 2009), which identifies miRNAs annotated in miRBase v20 (Kozomara and Griffiths-Jones, 2011) with the default parameter settings. miRanalyzer aligned reads by using bowtie 0.12.8. Subsequently, the expressed miRNAs and their copy numbers, obtained from miRanalyzer, were analyzed using EdgeR version 2.12 (Robinson et al., 2010) in order to find miRNAs that were differentially expressed between the groups (i.e. NT vs HC, pre- vs post-treatment for Good Responders and Poor Responders). The miRNAs that had more than 10 copies expressed were retained in the analysis of differential expression.

MiRNA target predictions used the miRwalk website (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/micronapredictedtarget.html>) and predictive algorithms (DIANA-microT, miRanda, miRDB, PicTar5, miRwalk, PITA, RNA22, RNAhybrid and TargetScan/TargetScanS) (Dweep et al., 2011). miRNA naming conventions were used as specified in miRbase (<http://www.mirbase.org/>). Gene Ontology analyses were carried out with DAVID Bioinformatics Resources 6.7 (<http://david.abcc.ncifcrf.gov/home.jsp>) (Huang et al., 2009). Analysis of overlaps among gene and GO term lists used GeneSifter Intersector (<http://public.genesifter.net/intersector/>).

2.6. Statistics

Statistical differences in Dicer protein and mRNA levels in our study populations were determined with SigmaPlot and Excel analyses using ANOVA for comparison of multiple groups of data. Student t test and Mann-Whitney Rank Sum test were used in pairwise comparisons of expression data.

Analysis of differential expression within our miRNA sequence data was carried out with edgeR; we applied two different approaches. For differential expression between a pair of study groups (e.g. MS vs HC), we ran the function exactTest. For the pairs of pre- vs post-treatment of Good and Poor Responders, we ran the functions glmFit and glmLRT to detect miRNAs that are differentially expressed between the two groups. To provide statistical confidence, we used p value and false discovery rate (FDR) thresholds of 0.05 to define differentially expressed miRNA.

In addition to the informatics described for miRNA sequence analysis, Excel (Microsoft, Redmond, WA) and SigmaStat/SigmaPlot (Systat, Chicago, IL) were used for Pearson correlation and Regression analyses. A Type I error level of 0.05 was used.

3. Results

3.1. Patient demographics

We recruited 74 MS patients and 25 healthy controls for this study (Table 1). Most patients were diagnosed as RRMS but a small population of SPMS patients was also included (Table 1). All assays were carried out on two blood samples (taken 48hrs apart) from each subject. In the two groups receiving IFN β 1a, the first blood draw was immediately prior to a scheduled dose and the second draw was 48hrs later. Similarly, healthy controls and untreated patients had blood drawn twice with the samples 48hrs apart. Treated patients also had an intermediate blood draw 4hrs after IFN β 1a injection to monitor interferon responsive gene induction. Statistical analyses addressed all MS patients in their study groups and also segregated subgroups according to diagnosis.

Type I Interferon (IFN β) is a front-line treatment for MS, however, neutralizing antibodies against IFN β have been identified in some MS patients who respond poorly to IFN β therapy (Vosslamber et al., 2009; Serana et al., 2014). Neutralizing antibodies are not, however, unambiguous markers of non-responsive patients (Vosslamber et al., 2009; Serana et al., 2014). A panel of 4 IFN β -responsive mRNA (MxA, B2m, STAT1, TRAIL) was monitored at baseline (time 0), 4 and 48hrs after IFN β 1a injection to characterize patient responses (Weinstock-Guttman et al., 2008). Treated groups all showed induction of STAT1, MxA and TRAIL mRNA 4 and 48hrs after receiving IFN β 1a. When analyzed as groups, the clinically defined Poor Responders and Good Responders did not show statistically different responses (Supplementary Figure 1) suggesting that these patients' poor clinical responses were not due to a lack of IFN β 1a bioactivity.

3.2. Dicer protein expression levels

To determine whether Dicer expression is altered in MS patients, we quantified Dicer protein in PBL from untreated MS patients and healthy control volunteers. Sample analyses were carried out fully blinded to patient groups. The initial 'unblinding' identified patient study group assignments (HC, NT) and yielded the data shown in Figure 1A and Supplementary Figure 2. Untreated MS patient PBL Dicer protein levels were 40% lower than the levels found in age and sex matched HC ($p=0.0493$) (Figure 1A). Further analysis by diagnosis suggested that Dicer protein levels decreased with advancing disease (Figure 1B). These two groups (HC & NT) did not receive any treatments so their samples served as biological replicates (providing sample numbers $n=46, 38, 10$ for HC, RRMS, SPMS respectively). Based on these analyses, we hypothesized that the decreased Dicer protein expression associated with MS was most likely a function of disease progression.

3.3. Dicer correlation with disease

To determine whether the changes seen in untreated RRMS patient Dicer protein expression levels were associated with clinical status, potential correlations between Dicer protein levels and EDSS were examined. Pearson correlation and Regression analyses showed a negative correlation between Dicer protein expression levels and the first three EDSS quartiles for RRMS NT patients ($r = -0.59, p = 0.025$) (Figure 1C). The inverse correlation between Dicer and EDSS was apparent and statistically significant through all quartiles;

however, Dicer expression appeared to plateau between the third and fourth quartile which weakened the correlation over the full range ($r = -0.45$, $p = 0.0083$). These analyses suggest that the reduction in Dicer protein expression seen in RRMS patients (Figure 1B), while not significant as a group, did demonstrate a statistically significant change when examined in the context of disease status. (Figure 1C). Also, Pearson correlation and Regression analyses did not reveal statistically significant correlations between Dicer protein level and age or time since diagnosis (data not shown). Separation of male and female subject data from our four main study groups (HC, NT, GR, PR) did not identify statistically significant differences associated with gender. We conclude, therefore, that Dicer protein expression is negatively correlated with disability in active MS patients.

3.4. Dicer protein response to IFN β 1a treatment

Dicer protein expression was analyzed in PBL from Good and Poor Responders before and 48hrs after Avonex. Changes in Dicer protein expression are shown here as fold change in which actin normalized Dicer protein levels 48hrs after IFN β 1a treatment are compared to the expression level before treatment (Figure 2A). IFN β 1a given as weekly IM injections did not alter Dicer expression in the PR patient group. However, GR Dicer levels were enhanced by 46% post-IFN β 1a (Figure 2A). The HC and NT groups did not receive treatment so their samples were biological replicates with average values ~ 1.0 (1.04 for HC, 1.14 for RRMS NT). In response to IFN β 1a treatment, GR Dicer levels trended toward but did not achieve a statistically significant difference relative to either HC or NT. As a group, PR Dicer protein levels did not appear to respond to IFN β 1a treatment and did not differ significantly from HC or NT. However, comparison between the RRMS GR and PR subpopulations demonstrated a differential response in good responders not present in poor responders and the difference did achieve statistical significance ($p=0.046$, Mann-Whitney Rank Sum test) (Figure 2A).

3.5. Dicer mRNA expression in MS

To determine whether the apparent induction of Dicer in response to IFN β 1a treatment was transcriptionally driven, *DICER1* mRNA was quantified from the same PBL samples described above. Despite the significant changes in Dicer protein expression levels, there were no statistically significant differences in *DICER1* mRNA levels between the HC and NT populations. As above, untreated MS patients and healthy control samples were essentially biological replicates and the ratios between these samples did not show substantial change (Figure 2B). However, the fold change in *DICER1* mRNA calculated from the pre and post-treatment samples demonstrated a 2.3-fold increase in *DICER1* mRNA after IFN β 1a treatment of good responders ($p=0.0263$ vs NT, $p=0.0013$ vs HC) (Figure 2B). Despite induction of STAT1, MxA and TRAIL mRNA (data not shown), IFN β 1a treatment did not significantly alter *DICER1* mRNA levels in the PR group (Figure 2B). Thus, an acute response to IFN β 1a treatment in good responders is seen in both *DICER1* mRNA and Dicer protein expression.

3.6. MicroRNA expression

To characterize specific miRNA profiles for our study groups and to determine whether the changes in Dicer expression were reflected in miRNA profiles, we carried out next generation sequencing analysis of all small RNA expressed in subject PBL. After quality assessment of all patient RNA samples, 76 patients (19 subjects in each study group) were found to have paired samples of adequate quality for small RNA library preparation with 71 patients yielding high quality data for analysis. Analysis of all expressed miRNA, after filtering and matching, demonstrated that PR profiles were closest to untreated MS patients and GR were closest to Healthy Controls. These correlations suggest that miRNA profiles are associated with clinical status. The average number of miRNA identified in each study population did not vary significantly among the groups; however, the specific miRNA expressed and their levels showed significant differences between subgroups (Tables 2-5, Supplementary Table 1).

The expression levels of specific Dicer-dependent miRNAs may contribute to the initiation and/or progression of MS. To examine associations between miRNA expression and disease progression, Pearson correlation coefficients were calculated for all pairings between EDSS scores and the normalized miR expression for each miR in each patient. In untreated RRMS patients, two miRs (miR-146b, -874) were negatively correlated with EDSS and two others (miR-107, -3614) were positively correlated (Figure 3A-D). Interestingly, miR-107, positively correlated with EDSS, has been shown to target Dicer (Asirvatham et al., 2008; Martello et al., 2010). Future studies will be needed to determine whether induction of miR-107, and potentially other Dicer targeting miRNAs, are integral to Dicer suppression in MS.

Analysis of differentially expressed miRNA revealed miR profiles specific to untreated RRMS or SPMS, good responders and poor responders (Tables 2-5, Supplementary Table 1). Figure 4A displays contrasting levels of miRNA expression between RRMS NT patients and HC volunteers for several of the most significantly affected miRs (see Table 2 for statistics). The enhanced expression of miR-9 shown here in RRMS PBL is consistent with the findings of Junker et al in MS brain lesions (Junker et al., 2009). Also, four independent publications have cited miR-145 as a potentially significant diagnostic marker for RRMS (Junker et al., 2009; Keller et al., 2009; Sondergaard et al., 2013; Gandhi et al., 2013). In agreement with these reports, miR-145 was among the miRs most strongly differentially expressed in our RRMS patients (Figure 4A&B) and its differential expression in our study was nearly identical to that previously reported by Keller et al (Keller et al., 2009). In addition, our observations extend the biomarker potential of miR-145 by showing that its levels are normalized following IFN β 1a treatment (Figure 4B). Gene expression changes in response to IFN β 1a have been characterized as acute (differentially expressed in hours or days post-injection but returning to basal levels in less than 1 week) or chronic (differentially expressed in treated individuals but stable between injections) (Weinstock-Guttman et al., 2008). Interestingly, the differential expression of miR-145 suggests a chronic IFN β 1a response in both Good and Poor Responders. The other miRs shown in Figure 4A have not previously been shown to be dysregulated in MS.

While our study focused on RRMS patients, we had a small cohort of SPMS patients and identified miRNA dysregulated in SPMS relative to RRMS. Due to the low number of patients analyzed in the SPMS NT subgroup, our differentially expressed miRNAs ($p < 0.05$) showed high false discovery rates (FDR) (Supplementary Table 1). Therefore, these miRs should be considered candidates for further characterization (Supplementary Figure 3). However, there were sufficient SPMS patients in the PR study group to generate reliable statistics ($p < 0.05$ and $FDR < 0.05$) (Table 5). Most of these miRs have not previously been associated with MS; however, altered expression of miR-494 and miR-323 were reported in RRMS but not investigated in SPMS (Jernas et al., 2013; Fenoglio et al., 2013) (Figure 4C). The identification of miRs differentially expressed in MS diagnostic groups such as these offers potential insight into mechanistic differences in disease progression which could have clinical significance.

In response to IFN β 1a treatment, we identified 5 chronically altered miRNA common to both Good and Poor Responders as well as 34 miRs chronically regulated only in GR and 2 restricted to PR groups ($p < 0.01$, $FDR < 0.05$). Our data also identified several acutely regulated miRNA -- 2 miRNA acutely altered in the GR (Table 6) and one (miR-3614-5p) in the PR groups. MiRNAs whose IFN β 1a-mediated alterations are restricted to specific responder groups offer potential as prognostic biomarkers as well as a means to investigate mechanistic differences in the response to treatment options. Further analysis of the regulated miRNAs showed that miR-3614 was acutely upregulated in all of the IFN β 1a treated patient groups ($p < 0.0004$) but that the SPMS PR response was significantly weaker than the RRMS GR response ($p = 0.0419$) (Figure 4D). The acute regulation of this restricted subset of miRNA suggests that these miRNA may have relatively short half-lives. It will be important, in future work, to determine the specific target genes regulated by these miRs.

The changes in miRNA expression in disease and in response to various treatments are mechanistically relevant in the context of their target mRNAs. Therefore, as an initial foray into how these miR profiles may direct future mechanistic studies, we compiled the predicted mRNA targets for all of the differentially expressed miRNAs calculated to have $p < 0.05$ and $FDR < 0.05$ – a total of 64 unique miRNAs. We used miRwalk (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/micronapredictedtarget.html>) to aggregate the predictions from multiple algorithms (DIANA-mT, miRanda, miRDB, miRWalk, RNAhybrid, PICTAR5, PITA, RNA22, and TargetScan) (Friedman et al., 2009; Dweep et al., 2011). In this analysis, we accepted target genes predicted by at least 7 algorithms (Asirvatham et al., 2008). After assembling all the predicted targets for each differentially expressed miR in our MS study group comparisons, GeneSifter Intersector software (<http://public.genesifter.net/intersector/>) was used to further strengthen our predictions by identifying those targets common to at least three of the paired study populations. For instance, the 1070 genes that were identified as likely targets for miRNAs differentially expressed in RRMS NT vs HC, RRMS GR vs HC and RRMS GR vs NT pairings showed 10 genes as likely targets of all three pairings (Supplementary Figure 4 and Supplementary Table 2). These genes were then taken as high confidence targets for continued analysis. Further studies will be necessary to validate the genes targeted by these differentially expressed miRNA and to determine their response to IFN β and potential roles in multiple sclerosis.

As an initial step to identify biological processes, cellular components and molecular functions associated with the differentially expressed miRNAs, the target gene lists were assessed to define their associated Gene Ontology (GO) terms (DAVID Bioinformatics Resources 6.7 <http://david.abcc.ncifcrf.gov/>). The resulting lists of GO terms associated with these predicted gene targets ($p < 0.05$, Benjamini < 0.05) were then handled as described above to determine GO terms common to multiple differentially expressed miR target lists. Frequently identified terms are related to regulation of transcription, chromatin, cell adhesion, signaling and anterior/posterior pattern formation. Interestingly, even though our analysis used PBL, several of the differentially expressed miR targets are associated with neurological functions such as *neuron differentiation*, *neuron projection* and *regulation of neurogenesis*. It is important to remember that analysis of PBL miRNA will include miRNA expressed in each circulating cell population as well as miRNA shed from tissues, often in exosomes, that become associated with PBL (Junker, 2011; Best et al., 2015; Mushtaq et al., 2015).

Examination of GO terms associated with individual population comparisons led us to the observation that good responder associated terms commonly included neurological categories, such as *neuron projection*, *regulation of neurogenesis* and *cell morphogenesis*, whereas poor responder terms, while including *neuron differentiation*, were more commonly associated with chromatin and organelles. This type of analysis, using predicted targets and their associated GO terms for distinct sets of differentially expressed miRNA may be instructive in the design of future mechanistic studies.

4. Discussion

MicroRNA mediated regulation of gene expression is now well-established with 40-60% of all genes directly targeted by miRNA (Selbach et al., 2008; Friedman et al., 2009; Tan and Marquez, 2014). Expression of Dicer, the RNase III enzyme required for the final maturation step of most miRNAs, is critical for normal cellular development and differentiation and has been found to be dysregulated in cancer and several other pathologies (Ha, 2011; Rottiers and Naar, 2012; Di Leva and Croce, 2013; Emde et al., 2015; Kim et al., 2015). Conditional Dicer knockouts have shown that Dicer is required for murine brain development and organism survival (Hebert et al., 2010; Gascon and Gao, 2012; Pang et al., 2014). Loss of Dicer, or specific miRNA, expression has been shown to disrupt oligodendrocyte and Schwann cell differentiation, prevent myelination and promote neuron degeneration (Shin et al., 2009; Bremer et al., 2010; Dugas et al., 2010; Gokey et al., 2012; Li and Yao, 2012). We previously showed that multiple inflammatory mediators (TLR ligands, interferons, ROS) can acutely regulate Dicer expression in vitro (Wiesen and Tomasi, 2009). Interestingly, we also found that $IFN\alpha$ and $IFN\gamma$ both regulate Dicer expression but have opposite effects on expression levels (Wiesen and Tomasi, 2009). Thus the ability to acutely regulate Dicer may offer a novel therapeutic approach to many pathologies.

Multiple studies over the past six years have shown that miRNA expression profiles are disrupted in MS (Otaegui et al., 2009; Junker et al., 2009; Keller et al., 2009; De Santis et al., 2010; Lindberg et al., 2010; Guerau-de-Arellano et al., 2011; Waschbisch et al., 2011;

Guerau-de-Arellano et al., 2012; Martinelli-Boneschi et al., 2012; Angerstein et al., 2012; Sievers et al., 2012; Jernas et al., 2013; Fenoglio et al., 2013; Hecker et al., 2013; Sondergaard et al., 2013; Gandhi et al., 2013). MicroRNA profiling is useful in the identification of potential biomarkers for diagnosis, prognosis and assessment of response to therapy as well as in mechanistic studies of gene expression alterations in disease or therapy. To determine whether Dicer is regulated in vivo, we studied MS patients receiving IFN β 1a (Avonex) therapy. Our studies were designed to address differences between untreated MS patients and healthy controls but also to distinguish differences among patients receiving IFN β 1a (Avonex) therapy and clinically defined as good or poor/partial responders. We also sought to enroll sufficient patients in each study group to provide good statistical power for analysis.

We found that PBL from MS patients express, on average, ~40% less Dicer protein than comparable healthy controls. When our untreated MS patients were segregated by diagnosis, we observed that patients with more progressive disease (SPMS) expressed significantly less Dicer protein than did RRMS patients. To further dissect the relationship between Dicer expression and disease progression, we considered each patient's disability status (EDSS). Consideration of these patient data demonstrated that Dicer protein levels decreased with disease progression. This is seen in the strong inverse correlation between untreated RRMS Dicer protein level and EDSS in patients with EDSS <4. In patients with higher EDSS, Dicer protein levels were low and did not decrease further with progression.

The apparent reduction of Dicer protein expression in MS patient PBL raises the important question of whether MS therapy can reverse the low Dicer levels. In the group of MS patients clinically defined as good responders to IFN β therapy, we found 46% higher Dicer protein expression 48 hrs after IFN β 1a administration. In contrast, the clinical poor responder group did not show significant alterations in Dicer protein levels. To our knowledge, this is the first report of Dicer levels responding to treatment in patients. Thus, Dicer protein levels correlate with disability, progression, response to therapy and, likely, clinical course. Since the poor responders induced MxA and other interferon responsive genes after treatment, their poor response to therapy is not likely due to neutralizing antibodies or other upstream signaling defects. Rather, we propose that these patients' failure to induce Dicer may be linked to their poor clinical response and their expression of specific miRNAs that may disrupt/prevent a productive response. The findings of reduced Dicer expression in MS patients, the correlation of Dicer protein with EDSS and the restoration of Dicer protein restricted to good clinical responses to IFN β 1a treatment suggest that Dicer may play a mechanistic role in MS. Further studies with animal models in which Dicer levels can be directly manipulated should address Dicer's role in MS development, progression and response to therapy. It will also be important, in future studies, to determine Dicer activity, its correlation with Dicer mRNA and protein expression as well as the factors which may regulate Dicer activity such as associated proteins and post-translational modifications.

We also observed that the reduced basal level of Dicer protein expression in untreated MS patients was not reflected in their *DICER1* mRNA levels; suggesting post-transcriptional regulation (Asirvatham et al., 2008; Wiesen and Tomasi, 2009; Jakymiw et al., 2010;

Martello et al., 2010; Gibbings et al., 2012). However, MS patients who respond well to IFN β 1a showed a 2.3-fold increase in *DICER1* mRNA suggesting a transcriptional response of Dicer to therapy. The lack of correlation between *DICER1* mRNA and Dicer protein was not entirely surprising since previous work showed that Dicer protein levels can change significantly in the absence of Dicer mRNA alteration (Wiesen and Tomasi, 2009; Jakymiw et al., 2010; Gibbings et al., 2012). Also important is that Dicer has been found to have alternative promoters and polyadenylation sites as well as splice variants and feedback regulation by several Dicer-dependent miRs (Singh et al., 2005; Irvin-Wilson and Chaudhuri, 2005; Asirvatham et al., 2008; Jakymiw et al., 2010; Martello et al., 2010; Hamaya et al., 2012). The potential for feedback regulation of Dicer by miRs (esp. miR-107) suggests the importance of future studies into the potential use of miRs or their antagonists in treatment options.

MS patients demonstrate two distinct patterns of Dicer regulation – in untreated MS, Dicer protein decreases without concomitant changes in mRNA while, in good responders, IFN β 1a administration induces both Dicer mRNA and protein. Thus, Dicer protein expression levels have potential as a biomarker for the presence of disease and both Dicer mRNA and protein responses may serve as biomarkers for response to IFN β 1a therapy. However, whether a decrease in Dicer is specific to MS or more generally found in inflammatory/autoimmune disease is uncertain (Divekar et al., 2011). A recent article (Jafari et al., 2015) reported increased levels of *DICER1* mRNA in RRMS patient PBL but did not examine Dicer protein. In contrast, another recent report found decreased Dicer protein and mRNA expression in RRMS patient B cells (Aung and Balashov, 2014). The disagreement between Jafari et al and the data we report here may be due to patient populations (especially EDSS scores), the cells examined and/or cell recovery methods. Furthermore, the data from the broad range of patients analyzed here suggests potential differences in Dicer regulation as MS advances and offers a perspective for the differences reported in various patient populations.

Using next generation sequencing, we identified 52 unique miRNAs differentially expressed among our study groups. Of these, 13 (25%) had previously been associated with MS thus providing additional support for an MS miR signature. Most previous studies used limited approaches (arrays or RT-qPCR) to identify differentially expressed miRs (Keller et al., 2014). Sequencing of all small RNAs, as reported here, provides greater breadth of coverage and is likely to identify more differentially expressed miRNAs. The distinction between good and poor IFN β 1a responders and our assessment of expression before and 48hrs after an IFN β 1a dose has allowed us to define more precise patterns as well as acute responses to treatment within these populations. For example, we have identified miR-3614 as acutely responsive to IFN β 1a. In contrast, other miR (e.g. miR-369, miR-100) expression levels change with therapy (i.e. treated patients vs untreated) but appear to be chronically regulated since they are stable between the pre and post-treatment samples. This acute/chronic distinction may have mechanistic implications and/or may reflect miRs with shorter or longer half-lives. Our data also demonstrate that, while some miRs (e.g. miR-145) may be good biomarkers to distinguish MS patients from healthy controls, they may not correlate well with response to therapy or progression of disease. For example, miR-145 has been

identified, by our results and others (Junker et al., 2009; Keller et al., 2009; Sondergaard et al., 2013; Gandhi et al., 2013), as a biomarker for discrimination between MS patients and healthy controls. However, the data reported here show that healthy levels of miR-145 expression are restored in IFN β 1a treated patients regardless of whether or not they demonstrate a clinical response to therapy. Additionally, our study design identified 34 miRNAs that are significantly altered in response to IFN β 1a only in those patients who show good clinical responses. This provides a distinction between miRs that respond to IFN β 1a independent of clinical response (e.g. miR-145) and those that may be prognostic of patient responses to therapy (e.g. miR-494 or 100-5p). Further study of specific miRNAs will be required to validate their utility as biomarkers. Natalizumab and Glatiramer Acetate treatments have also been reported to alter MS patient miRNA expression (Waschbisch et al., 2011; Sievers et al., 2012); it will be important, in future studies, to address Dicer expression levels and activity in these and other treatments. These miRNAs' expression and responses to treatment should also be evaluated in other neurological and autoimmune diseases especially in view of several studies that have implicated miR-125 in inflammatory weakening of the blood brain barrier and miR-494 as a key regulator in Parkinson's Disease (Le et al., 2009; Lattanzi et al., 2013; Reijerkerk et al., 2013; Stappert et al., 2013; Xiong et al., 2014).

The observation of increased expression of several miRNAs (see Figure 4 & Table 2) despite a significant reduction in Dicer levels appears contradictory; however, it is important to recall that miRs are processed from pri-miR transcripts whose regulation may be altered in disease independently of Dicer. Also, there is evidence of preferential pre-miR processing by Dicer (Martin et al., 2014 and W.J.M. & T.B.T., unpublished data) and that specific miRs have distinct half-lives which vary over a large range (minutes to weeks) (Gantier et al., 2011; Winter and Diederichs, 2011). These factors may play a role in altered miR profiles especially when Dicer expression is reduced.

Several studies (Asirvatham et al., 2008; Tokumaru et al., 2008; Jakymiw et al., 2010; Martello et al., 2010) have addressed the regulation of Dicer by certain miRNAs (let-7, miR-103/107). We find here that miR-107, known to target Dicer (Martello et al., 2010), is increased, especially in SPMS, and directly correlates with EDSS. The enhanced miR-107 expression may play a role in post-transcriptional inhibition of Dicer protein expression. Post-transcriptional inhibition of Dicer expression could contribute to blocking Dicer's response to IFN β 1a treatment. The demonstration of Dicer modulation in MS and in response to IFN β suggests opportunities for other therapeutic approaches. For example, Dicer regulation as shown here might also be found in MS patients, or others, treated with corticosteroids. High dose corticosteroids are standard therapy for MS relapses (Krieger et al., 2014). Our recent in vitro studies have shown Dicer induction by hydrocortisone (Wiesen and Tomasi, manuscript submitted) suggesting that enhanced Dicer expression may play a role in acute therapy for MS relapses.

The differentially expressed miRs reported here may help define other targets which offer potential insights into MS pathology and treatment. For instance, Reijerkerk et al (Reijerkerk et al., 2013) characterized miRNA associated with diminished blood brain barrier (BBB) integrity in MS. They identified miR-125a-5p as a critical regulator of BBB

function and showed that inflammation reduced miR-125a expression. Several authors have linked miR-125 to neuronal differentiation and we have found reduced miR-125a expression in SPMS. Identification of miR-125 targets and transcriptional regulators may therefore offer new therapeutic approaches.

Xiong et al (Xiong et al., 2014) showed that miR-494 is elevated in Parkinson's Disease and identified DJ-1, a putative sensor of oxidative stress, as its target. Elevated miR-494 reduced DJ-1 expression and exacerbated neurodegeneration in dopaminergic neurons (Xiong et al., 2014). We found that miR-494 is elevated in RRMS and further elevated in SPMS but was restored to healthy control levels in patients responding well to IFN β 1a (Figure 4C). This pattern suggests that a process similar to that described in Parkinson's Disease may be functioning in MS thus identifying a potential target and mechanism for future investigations.

Given the underlying heterogeneity of multiple sclerosis (Weinstock-Guttman et al., 2008; Keller et al., 2009; Otaegui et al., 2009) and partial efficacy of the available treatments (Weinstock-Guttman et al., 2008; Keller et al., 2009; Sievers et al., 2012; Serana et al., 2014), biomarkers, specifically including miRNA and Dicer, could be helpful tools in diagnosis, prognosis and the identification of the most suitable therapeutic treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PBL	peripheral blood leukocytes
miRNA or miR	microRNA
EDSS	expanded disability status scale
logFC	log fold change
FDR	false discovery rate
GO	gene ontology

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Highlights

Dicer protein expression is reduced in MS patient PBL relative to healthy controls

Decreasing Dicer expression correlates well with advancing disability (EDSS)

Dicer mRNA and protein expression are enhanced after IFN β therapy

Specific microRNA levels correlate with diagnosis, EDSS and response to IFN β therapy

Dicer is a potential therapeutic target for MS therapy as well as other pathologies

Figure 1. Dicer protein expression correlation with diagnosis and EDSS

A, Dicer protein expression in PBL is displayed here as average actin normalized Dicer protein level. The healthy control group included 46 samples analyzed while the untreated group included 48 samples. *, $p < 0.05$. B, Dicer protein expression was quantified from patient/volunteer PBL and is shown here stratified by diagnostic group (RRMS NT, untreated relapsing remitting MS [n=38]; SPMS NT, untreated secondary progressive MS [n=10]). *, $p < 0.05$. C, Pearson correlation analysis demonstrated a strong negative correlation between actin normalized Dicer protein levels and patients' EDSS scores ($r = -0.59$, $p = 0.025$) (n=38).

Figure 2. Restricted Dicer protein and mRNA responses to intramuscular IFNβ1a treatment
 A, Comparison of Dicer protein levels 48 hrs post-IFNβ1a to levels immediately before IFNβ1a treatment shows enhanced expression only in Good Responders (GR). Data shown represent paired samples from 23 (HC), 16 (RRMS NT), 25 (RRMS GR) and 15 (RRMS PR) patients/volunteers. B, Comparison of Dicer mRNA levels 48 hrs post-IFNβ1a to levels immediately before IFNβ1a treatment show enhanced expression only in Good Responders. Data shown represent paired samples from 23 (HC), 18 (NT), 21 (GR) and 15 (PR) RRMS patients/volunteers. Changes in Dicer protein (A) and mRNA (B) expression were quantified from patient/volunteer PBL and are shown here as average fold change between post-treatment (48hr) and pre-treatment (0hr) samples. Healthy Control and untreated RRMS (NT) volunteers did not receive any treatment between blood draws. RRMS patients clinically defined as Good after the 0hr blood draw. Error bars represent SEM; *, $p < 0.05$ vs HC and NT; **, $p < 0.05$ vs PR.

Figure 3. Expression levels of several miRNAs correlated with clinical status (EDSS)
Specific miRNA expression in untreated RRMS patients was found to correlate with EDSS (n=34). Pearson correlation coefficients for (A) miR-107, (B) miR-3614-5p, (C) miR-146b-5p, (D) miR-874 were $\rho=0.57$, 0.60 , -0.55 , -0.53 respectively.

Figure 4. Selected miRNA that demonstrate potential as biomarkers to distinguish untreated MS patients from healthy controls or to identify response to therapy

A, Multiple miRNA are differentially expressed between healthy controls (light bars)(n=36) and untreated RRMS patients (dark bars) (n=30) (see Table 2). Examples shown here demonstrate that miRNA expression may be up or down regulated in RRMS ($p < 0.002$). B, miR-145 expression in untreated RRMS (NT) (n=30), healthy controls (HC) (n=36), RRMS patients who respond well to IFN β 1a (GR) (n=38) and RRMS patients who respond poorly to IFN β 1a (PR) (n=24) (all differentially expressed relative to NT, $p < 0.01$). C, miR-323b (dark bars) and miR-494 (light bars) are differentially expressed in RRMS NT (n=30), SPMS NT (n=4) and in RRMS GR (n=38) response to IFN β 1a. RRMS & SPMS NT vs HC, $p < 0.01$; GR vs RRMS NT, $p < 0.001$. D, Sequencing and quantitation of miR-3614-5p expression was carried out in PBL samples taken from study volunteers immediately prior to and 48 hours after IFN β 1a injection. In RRMS PBL, miR-3614-5p was acutely enhanced by IFN β 1a treatment in each of our study populations (RRMS GR post vs HC, $p = 2.40 \times 10^{-6}$; RRMS PR post vs HC, $p = 8.50 \times 10^{-6}$; GR pre vs post, $p = 1.77 \times 10^{-8}$; PR pre vs post, $p = 0.00038$). SPMS PR samples appear to show acute

Table 1

Patient demographics.

Demographics	Health controls	No Therapy	Good Responders	Poor Responders
Sample size	25	24	25	25
Age, Mean $\pm$ SD	50 $\pm$ 9	52 $\pm$ 11	54 $\pm$ 7	51 $\pm$ 11
Females, n (%)	20 (80%)	22 (88%)	21 (84%)	20 (80%)
Caucasians, n (%)	25 (100%)	22 (88%)	24 (96%)	25 (100%)
Disease course:	--			
RR		19 (32%)	25 (42%)	15 (25%)
SP		5 (33%)	0 (0%)	10 (67%)
Disease duration, years	--	16.9 $\pm$ 11.5	18 $\pm$ 8.8	18.1 $\pm$ 11.1
EDSS, median (IQR)	--	3.5 (1.6)	2.3 (0.8)	4.4 (2.1)

Table 2

Differential miRNA expression in RRMS patients.

	logFC	p value	FDR
hsa-miR-451a	-1.71	0.00001	0.0019
hsa-miR-144-5p	-1.98	0.00001	0.0020
hsa-miR-484	1.34	0.00008	0.0062
hsa-miR-9-5p	1.43	0.00009	0.0062
hsa-miR-323b-3p	1.33	0.00010	0.0062
hsa-miR-101-3p	-0.46	0.00022	0.0113
hsa-miR-15b-5p	0.79	0.00035	0.0153
hsa-miR-145-5p	1.30	0.00050	0.0182
hsa-miR-337-5p	1.58	0.00053	0.0182
hsa-miR-485-5p	1.40	0.00076	0.0220
hsa-miR-654-3p	0.73	0.00078	0.0220
hsa-miR-377-3p	1.44	0.00140	0.0342
hsa-miR-493-5p	0.72	0.00143	0.0342
hsa-miR-301b	0.47	0.00193	0.0426
hsa-miR-409-3p	0.66	0.00224	0.0462

Differential miRNA expression between RRMS patients (untreated) (n=30, 15 subjects with 2 biological replicates each) and healthy controls (n=36) (p<0.01).

logFC = log fold change, p value calculated by edgeR, FDR=false discovery rate.

Table 3

Differential miRNA expression in Good Responders.

	logFC	p value	FDR
hsa-miR-654-3p	-0.88	1.40E-05	0.0036
hsa-miR-377-3p	-1.70	2.58E-05	0.0036
hsa-miR-136-5p	-1.96	5.07E-05	0.0036
hsa-miR-150-5p	-1.08	6.10E-05	0.0036
hsa-miR-223-5p	0.63	6.23E-05	0.0036
hsa-miR-411-5p	-0.81	1.08E-04	0.0052
hsa-miR-100-5p	1.59	0.0002	0.0071
hsa-miR-136-3p	-0.77	0.0002	0.0077
hsa-miR-410	-0.77	0.0002	0.0077
hsa-miR-409-3p	-0.74	0.0004	0.0113
hsa-miR-3614-5p	1.63	0.0005	0.0113
hsa-miR-21-3p	0.51	0.0005	0.0113
hsa-miR-381-3p	-0.82	0.0006	0.0113
hsa-miR-21-5p	0.36	0.0006	0.0113
hsa-miR-485-3p	-0.97	0.0008	0.0155
hsa-miR-493-3p	-1.20	0.0009	0.0155
hsa-miR-323b-3p	-0.93	0.0009	0.0157
hsa-miR-369-3p	-1.14	0.0010	0.0157
hsa-miR-889	-0.79	0.0011	0.0157
hsa-miR-494	-1.36	0.0011	0.0157
hsa-miR-199ab-3p	-0.45	0.0012	0.0160
hsa-miR-376c-3p	-0.98	0.0014	0.0179
hsa-miR-369-5p	-1.20	0.0015	0.0185
hsa-miR-411-3p	-1.37	0.0016	0.0185
hsa-miR-539-3p	-1.36	0.0018	0.0210
hsa-let-7i-5p	0.37	0.0019	0.0210
hsa-miR-758-3p	-1.29	0.0025	0.0266
hsa-miR-431-5p	-0.79	0.0026	0.0269
hsa-miR-487a	-1.23	0.0031	0.0307
hsa-miR-301b	-0.43	0.0042	0.0400
hsa-miR-485-5p	-1.16	0.0046	0.0424
hsa-miR-15b-5p	-0.59	0.0051	0.0455

Differential miRNA expression between relapsing-remitting MS patients who responded well to Avonex (GR) (n=38) and untreated RRMS patients (n=30) (p<0.01).

Table 4

Differential miRNA expression in Poor Responders.

	logFC	p value	FDR
hsa-miR-145-5p	-1.45	0.0001	0.0329
hsa-miR-654-3p	-0.81	0.0002	0.0333
hsa-miR-410	-0.82	0.0003	0.0333
hsa-miR-409-5p	-1.71	0.0007	0.0410
hsa-miR-889	-0.94	0.0008	0.0410
hsa-miR-15b-5p	-0.85	0.0008	0.0410

Differential miRNA expression between relapsing-remitting MS patients who responded poorly to IFN β 1a (PR) (n=24) and untreated RRMS patients (n=30) (p<0.01).

Table 5

Differential miRNA expression between SPMS and RRMS patients who respond poorly to IFN β 1a therapy.

	logFC	PValue	FDR
hsa-miR-874	-1.79	6.53E-05	0.0153
hsa-miR-99a-5p	-2.24	0.0001	0.0153
hsa-miR-141-3p	-1.10	0.0002	0.0153
hsa-miR-125b-5p	-1.99	0.0002	0.0153
hsa-miR-342-3p	-1.14	0.0006	0.0345

Differential miRNA expression between secondary progressive MS patients who responded poorly to IFN β 1a (SPMS PR) (n=10) and relapsing-remitting MS patients who responded poorly (RRMS PR) (n=24) (p<0.01).

Table 6

Acute miRNA response to IFN β 1a treatment.

	logFC	p value	FDR
hsa-miR-3614-5p	-3.30	1.77E-08	4.44E-06
hsa-miR-27a-3p	-0.40	9.37E-05	0.0117

Differential miRNA expression induced by acute response to Avonex treatment in relapsing-remitting MS patients who responded well to Avonex (GR) (n=19) (p<0.01). Patients were sampled immediately before and 48hrs after their scheduled IFN β 1a dose.