Journal of Neuroimmunology xxx (2011) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Review article

miRNA profiling for biomarker discovery in multiple sclerosis: From microarray to deep sequencing

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ARTICLE INFO

Article history:
Received 9 September 2011
Received in revised form 14 October 2011
Accepted 17 October 2011
Available online xxxx

Keywords:
miRNA
Profiling
Biomarker
Multiple sclerosis
Neuroimmunology
Autoimmunity

ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level. miRNAs are highly expressed in cells of the immune and nervous system, attesting to their importance in Neuroimmunology. Besides their involvement in modulation of physiological and pathological processes, miRNAs hold high promise as disease biomarkers, therapeutic agents and/or drug targets. Several studies have recently explored the involvement of miRNAs in Multiple Sclerosis (MS) using a variety of miRNA profiling techniques. In this review, we discuss basic miRNA biology and nomenclature, the techniques available for miRNA profiling research and recent miRNA profiling studies in Multiple Sclerosis.

Contents

1. Introduction

MicroRNAs (miRNAs) are small, usually 19–24 nucleotides in length, non-coding RNAs that regulate gene expression at the post-

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doi:10.1016/j.jneuroim.2011.10.006

transcriptional level. The first microRNA (miRNA) was identified in 1993 by the laboratories of Victor Ambros and Gury Ruvkun (Wightman et al., 1993; Lee et al., 1993). Since then, miRNAs have captured great interest due to their involvement in physiological and pathological processes and their promise as disease biomarkers, therapeutic agents and/or drug targets. Several studies have recently explored the involvement of miRNAs in Multiple Sclerosis (MS) using a variety of miRNA profiling techniques. In this review, we discuss basic miRNA biology and nomenclature, the techniques available for miRNA profiling research and recent miRNA profiling studies in MS.

2. miRNA biogenesis

The biogenesis of miRNAs is a multi-step process that culminates in miRNA binding to the regulated target gene mRNA. Most miRNAs are independently encoded in intergenic regions or in antisense orientation to other genes (Lee et al., 2004), while others are encoded within introns of other genes (Eis et al., 2005). These different types of genomic encoding determine whether miRNAs will be independently transcribed or co-transcribed with a "host" gene. For example, since miR-155 is encoded within an intron of the BIC gene, miR-155 expression trails that of the BIC gene (Eis et al., 2005). miRNA genes are RNA transcribed by RNA polymerase II, giving rise to an approximately 100 nucleotide-long primary miRNA (pri-miRNA) characterized by a hairpin structure and overhangs (Y. Lee et al., 2004). Still in the nucleus, these overhangs are cleaved off by the enzymatic Drosha/DGRC8 complex to produce a 70 nucleotide-long precursor (pre)-miRNA that is transported into the cytoplasm by Exportin 5 (Lee et al., 2003; Yi et al., 2003). In the cytoplasm, pre-miRNAs are further processed by Dicer (Hutvágner et al., 2001; Ketting et al., 2001; Kim, 2005) and the resulting double-stranded (ds) RNA molecules are loaded onto the RNA-induced silencing complex (RISC). The RISC complex unwinds the ds pre-miRNA into single stranded mature miRNAs (Hammond et al., 2000; Martinez et al., 2002). Typically, one of the two strands is preferentially selected by the RISC complex to bind the 3′untranslated (3′UTR) region of target genes, while the other one is preferentially degraded. Mammalian miRNAs typically bind their target genes' 3′UTR through imperfect base pairing (Gregory et al., 2005). In most cases, the consequence of miRNA binding to its target gene is mRNA degradation and/or protein translation inhibition (Zeng et al., 2003), although there have been reports of miRNAs that stabilize their target transcripts (Place et al., 2008).

3. miRNA nomenclature

miRNA sequence information is publicly available in the miRBase repository (http://www.mirbase.org/), which is also responsible for miRNA nomenclature. miRNA names, such as hsa-miR-18b, contain information about the miRNA they represent. The first part of the name (e.g. hsa) indicates that **H**omo **sa**piens is the species of origin. A lowercase or uppercase "r" in hsa-mir versus hsa-miR indicates precursor versus mature miRNA, respectively. This is followed by a number which increases as more miRNAs are discovered. Therefore, one can infer that hsa-miR-18b was discovered earlier than hsa-miR-200. In addition, some miRNAs have a letter, such as a or b, after the number, differentiating highly similar sequences that are separately encoded. Finally, miRNA nomenclature allows to distinguish the two single stranded mature products that originate from the two strands present in the ds precursor miRNA. For example, hsa-miR-18b and hsa-miR-18b* indicate the major and minor, respectively, products found in the cell. The minor star strand was thought to be non-functional, but it is becoming clear that minor strands can be functional (Yang et al., 2011). Since the relative amount of the non-star and star products can vary in different cell types, the star nomenclature will be retired in the near future. In the new nomenclature, hsa-miR-18b-5p and hsa-miR-18b-3p will be used to indicate the 5′ or 3′ strand origin of the mature miRNA.

4. miRNAs in health and disease

The importance of miRNAs in biological processes is evidenced by their high conservation across species (Lim et al., 2003). miRNAs have been estimated to approximately target 33% of human genes (Lewis et al., 2005), attesting to their importance in gene regulation. Most cellular processes, from differentiation and proliferation to apoptosis, can be regulated by miRNAs (Ambros, 2004; Bartel, 2004; Ambros, 2003). In addition, miRNAs are involved in disease processes. Changes in miRNA gene expression have been associated with the development and progression of malignancies (Calin et al., 2004) and other diseases (Tili et al., 2007). The role of miRNAs in autoimmunity is just beginning to be explored, but there is ample evidence that miRNAs play a crucial role. miRNAs are highly expressed in immune cells (C.-Z. Chen et al., 2004). Interestingly, miRNA deficiency in T cells results in excessive pro-inflammatory responses or autoimmunity (Muljo et al., 2005; Zhou et al., 2008) and miR-181a (Li et al., 2007) and the miR-17–92 cluster (Xiao et al., 2008) have been shown to modulate T cell activation. Overall, it is becoming clear that miRNA dysregulation contributes to human autoimmune disease, including MS. miRNAs are of interest in MS because they are highly expressed in both the immune cells that mediate the disease (Chen et al., 2004) and in the central nervous system that constitutes the target organ (Krichevsky et al., 2003).

5. miRNAs as MS biomarkers

Several studies have recently explored miRNAs as potential MS biomarkers (Du et al., 2009; Junker et al., 2009; Keller et al., 2009; Otaegui et al., 2009; Cox et al., 2010; De Santis et al., 2010; Lindberg et al., 2010; Guerau-de-Arellano et al., in press). Some of the features desired in a biomarker include: biological rationale, clinical relevance, practicality and correlation with disease activity, disability/prognosis and treatment effect (Bielekova and Martin, 2004). miRNAs are exceptional biomarker candidates. There is evidence that miRNAs are involved in diverse physiological processes (Ambros, 2003; Bartel, 2004), potentially providing a biological rationale for their use. They have been proven to be clinically relevant, correlating with disease activity and prognosis in several human diseases, particularly malignancies. In addition, they are extremely practical, with many advantages over other proposed biomarkers such as mRNA and proteins. First, while there are more than 30,000 genes coding for protein and mRNA, about 1500 miRNAs have been identified in humans as of miRbase v17 (Kozomara and Griffiths-Jones, 2011). Therefore, miRNA analysis is more manageable and requires fewer resources than microarray or proteomics analysis, particularly in the clinic. Another advantage is durability. Since miRNAs are short RNA molecules, they are more resistant to ribonucleases than mRNA and have been detected in archived formalin-fixed samples (Nuovo, 2010). Lastly, miRNAs can be detected in a large number of samples, including tissue biopsies, whole blood, blood cells, serum, plasma and urine. Several miRNA profiling platforms, from microarray to deep sequencing, are currently available to prospectively identify miRNAs (Table 1).

6. miRNA profiling techniques

6.1. Classical microarray

miRNA identification through classical microarray is based on the hybridization of glass-printed probes to miRNAs (Babak et al., 2004; Calin et al., 2004). The arrays containing probes are typically available through commercial sources or institutional array facilities. Prior to hybridization to the array, miRNAs are labeled by attaching

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Table 1
miRNA profiling methods.

	Hybridization microarray	Taqman-based assays	Bead-based arrays	Nanostring nCounter	Deep sequencing
Method	Probes printed on slide array for hybridization to fluorescent dye-labeled miRNAs.	miRNAs reverse transcribed to cDNA and miRNAs amplified by stem-loop multiplex Taqman Real-Time PCR on a microfluidic card (TLDA), OpenArray or integrated fluidic circuits (Biomark)	Color beads coated with miRNA-specific probes and bound to biotynilated miRNA sample for flow cytometry detection.	miRNAs ligated to a specific miRTag sequence that allows hybridization to a color probe	miRNAs ligated to 5′ and 3′ adaptors to allow reverse transcription and PCR amplification of all miRNAS prior to sequencing and quantification
RNA requirements[a]	30 ng–5 μg	1–350 ng with preamp. 0.35–1 μg w/o preamp.	250–500 ng	100 ng	1-5 μg
Advantages	Use of LNA probes increases Tm.	High sensitivity for low RNA samples. OpenArray and Fluidigm are highly customizable.	Adaptable to custom-made multiplex miRNA assays	Digital direct counting of miRNA molecules	All (even unknown) miRNAs are potentially identifiable
Commercial Suppliers[b]	Exiqon (LNA arrays), Agilent, Affymetrix, Invitrogen, LCSciences	ABI (TLDA cards and OpenArray), Fluidigm (BioMark)	Luminex	Nanostring	Illumina, ABI (SOLiD) and Roche (454)

TLDA: Taqman Low Density Array; LNA: Locked Nucleic Acid; Tm: melting temperature; ABI: Applied Biosystems; w/o: without.
[a] Guideline total RNA amount and concentration required to perform assay.
[b] Examples of miRNA profiling technique suppliers. Additional core facility support is generally required to perform the profiling. Readers are advised to further investigate all the breadth of companies and core services providing these services.

a fluorescent dye. The fluorescence associated with the area where specific probes have been spotted is measured to provide a raw expression signal. One limitation of this method is that, since miRNAs are only 19–24 nucleotides long, the Tm of the probes has a wide range that may diminish specificity and/or sensitivity for low GC content miRNAs. This issue can be addressed through the use of Locked-Nucleic Acid technology (LNA) probes (Castoldi et al., 2006). LNA probes contain chemically modified nucleotides that fix the oligonucleotide conformation and increase its Tm value. As a result, miRNA detection has improved considerably in this and other platforms. The main advantage of this method is that it can simultaneously detect a large number of miRNAs. In addition, microarrays are highly flexible and can be adapted to the needs of the experiment, either assaying most known miRNAs or a group of selected miRNAs. Another advantage of microarrays is their ability to relatively quickly incorporate probes to detect the miRNAs most recently described in miRBase.

6.2. Taqman-based arrays

Taqman-based arrays are very commonly used in miRNA profiling. Prior to Real-Time PCR amplification, miRNAs are reverse transcribed to cDNA using stem-loop primers (Mestdagh et al., 2008). Stem-loop primers extend the length of the miRNA sequence to make it more easily detectable by Real-Time PCR. In addition, these primers reduce the likelihood of detecting genomic DNA or precursor miRNA and enhance the stability of miRNA-primer complexes, thus improving efficiency and assay sensitivity. As a consequence, a high reverse transcription efficiency is achieved, highly similar miRNA species can be differentiated and mature miRNA can be better discriminated from precursor miRNA species (Chen et al., 2005). After reverse transcription, an optional pre-amplification step of up to 14 cycles can be introduced, which allow one to analyze samples of as little 1 ng of total RNA. The Taqman Real-Time PCR reaction can then be performed on various platforms. In Taqman Low-Density Arrays (TLDA, Applied Biosystems), the Real-Time PCR reaction is performed on a micro fluidic card that permits simultaneous amplification of 384 small RNAs. Typically, two cards are used to cover the human miRNAs described as of miRbase v14. The new OpenArray platform (Applied Biosystems), simultaneously performs 754 Taqman Real-Time PCRs for 3 samples on a single OpenArray surface, with considerable cost reduction. In addition, OpenArrays can be custom-made to analyze fewer selected miRNAs on a larger number of samples. Fluidigm offers an alternative platform of microfluidics chips, called Integrated Fluidic Circuits, that simplifies workflow, lowers costs and increases throughput. The Fluidigm platform supports many commercially available Taqman Real-Time PCR assays. Overall, Taqman-based techniques have high specificity and sensitivity and a wide dynamic range (Mestdagh et al., 2008).

6.3. Bead-based methods

The leading bead-based method for miRNA detection, from Luminex, uses magnetic microspheres tagged with unique DNA tag sequences that allow to identify the bead and that specifically bind to a chimeric probe that in turn recruits a specific miRNA to the complex. To avoid spurious binding, tag sequences have been selected for lack of hybridization to other known DNA/RNA sequences, as well as to other tag sequences that may be present in the assay. The miRNA specificity is provided by the specific biotinylated chimeric probes, which have 100% complementarity to both the miRNA sequence and one of the unique tag sequences. After hybridization of the miRNA to the probe, the miRNA-probe hybrid binds the specific tags on microspheres. The biotin in the chimeric probe allows fluorescent labeling of the complex. The microspheres can then be detected on flow instruments that decode each bead and read the amount of fluorescence. This method is able to detect up to 500 custom-chosen miRNAs in a single reaction. While this technique has not been as widely used as microarray or TLDA so far, we may see an increase in the near future, since variations of bead-based miRNA capturing and enrichment followed by varied detection techniques could conceivably improve miRNA detection in certain specimens such as blood or plasma.

6.4. NanoString nCounter technology

The novel NanoString nCounter technology has recently joined the miRNA market, after the successful implementation of this technology for multiplexed direct digital counting of other RNA molecules (Geiss et al., 2008). The basis of this technology is the use of single stranded DNA molecules hybridized to various synthetic RNA segments labeled with different fluorochromes, which create a molecular barcode through permutations in fluorochrome order. The reporter probes can then bind RNAs and allow their measurement. This technology has been successfully used for mRNA profiling and several adaptations have been implemented to adapt it to the detection of miRNAs. miRNA detection is possible through the use of miRTags, single-stranded DNA sequences that are ligated to the 3′ end of a miRNA. A bridge sequence with complementarity to both miRNA and miRTag is used to provide specificity during the ligation step. After removal of the bridge, the miRNA–miRtag adduct can be bound by a color-coded reporter probe and a biotynilated capture probe. The capture probe allows immobilization to a streptavidin-

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coated slide and extension of the entire length of the DNA backbone for accurate measurement of its color code. 100 ng of total RNA (in a 3 μl volume) allow accurate detection of less than 1 RNA copy/cell. A major advantage of this technique is bypassing the need for amplification and reverse transcription. This eliminates enzymatic bias and simplifies the nCounter workflow to consist of hybridization, post-hybridization processing and digital data acquisition. Another advantage is that, since it directly counts miRNA molecules providing a digital readout, statistical bioinformatics analysis is simpler than for TLDA, albeit several normalization procedures are performed to ensure reproducibility. Currently, about 750 human and human-associated viral miRNAs can be simultaneously detected with the Nanostring platform.

6.5. Deep sequencing

The next wave of miRNA profiling uses deep sequencing techniques to allow detection of virtually all miRNAs expressed in the sample, regardless of whether they have been previously described or whether they are of human or other (e.g., an unknown infectious organism present in the sample) origin. Next generation sequencing techniques include Genome Analyzer sequencing (Illumina), 454 sequencing (454/Roche) and SOLiD system sequencing (ABI) (Rothberg and Leamon, 2008).

The first step in all of these techniques is the generation of a small RNA library. For this purpose, 5′ and 3′ RNA adaptors are ligated to either end of the miRNAs. The 3′ adaptors are synthesized so that they specifically target mature miRNAs or other small RNAs carrying the 3′ hydroxyl group generated by Dicer's cleavage. The RNA is then reverse transcribed and PCR amplified. In 454 and SOLiD, the library generation step is performed with the template attached to beads enclosed within droplets of water-in-oil emulsion (Rothberg and Leamon, 2008). In contrast, in Illumina sequencing, the amplification occurs in a classical liquid phase (Morin et al., 2010) and one of the two primers used for PCR amplifications contains an “index” sequence that tags cDNAs originating from different samples. The 48 different indices available later allow simultaneous sequencing of up to 48 samples on the same lane of a flow cell.

Both Illumina and 454 sequence by synthesis, but there are important differences. Illumina uses sequencing by synthesis using fluorescently labeled nucleotides. The nucleotides carry a reversible terminator signal that allows addition of a single nucleotide at each step. At each nucleotide addition, the color is read and recorded. The terminator sequence is then removed to allow a subsequent nucleotide addition and reading (Shendure et al., 2004). In contrast, 454 technology sequences by pyrosequencing, a method in which nucleotide addition releases inorganic pirophosphate (PPi) that activates luciferase activity and produces light (Rothberg and Leamon, 2008). This technique does not require nucleotide removal at each step, resulting in increased nucleotide sequencing speed. As opposed to sequencing by synthesis, the SOLiD system sequences by ligation: fluorescently labeled probes, when complementary to the template, are ligated to the sequencing primer. These probes allow identification of two template nucleotides at a time and each template nucleotide is read twice, providing high sequencing accuracy (Rothberg and Leamon, 2008). Perhaps the most challenging aspect of deep sequencing is the bioinformatics analysis required to select the small RNA sequences that are actually miRNAs versus other small RNAs or degradation products. A number of bioinformatics tools and expertise are now available to aid with these analyses.

6.6. General considerations

Best results in any of these techniques are achieved with the highest amount, purity and quality of RNA samples. Even though small RNAs are not degraded as easily as mRNA, care must be taken to obtain high quality RNA. The best small RNA recovery is achieved through the use of column-based methods adapted to small RNA molecules, such as Ambion's miRVana, Qiagen's miRNeasy or Exiqon's miRCURY RNA isolation kits, among others. It is preferable that total, and not just small RNAs, are isolated. Additional RNA species improve small RNA isolation by acting as carriers. An additional advantage of total RNA is that it can be later assayed for expression of predicted miRNA targets to identify potential miRNA-target gene correlations. RNA integrity can be assessed with manual or automated gel electrophoresis, such as with Agilent's Nano chips run on the Bioanalyzer. Automated gel electrophoresis provides several readouts of DNA or protein contaminants in the sample, as well as an RNA integrity number (RIN) representative of the large RNA species relative to small (or degraded) RNA species. Typically, RIN values of 8 or higher indicate that most of the RNA is not degraded. Deep sequencing requires the high amounts of starting RNA (a minimum 1 μg/run, preferably at high concentration) with a minimum RNA Integrity Number (RIN) of 8. Other techniques, such as TLDA and classical microarray, may allow miRNA detection under less optimal circumstances. TLDA cards can be used to detect miRNAs in samples with 1 ng total RNA using a preamplification step. Microarrays have a wide range of starting RNA sample (30 ng–5 μg) depending on the supplier platform used and whether or not an amplification step is introduced prior to RNA labeling.

6.7. Bioinformatics analysis

All of the miRNA profiling techniques discussed require normalization to standardize the data and identify biologically relevant changes. Normalization of TaqMan TLDA and NanoString cards are simpler and quite straight forward compared to the other techniques. This is mostly because of the data size. TaqMan assays quantify expression in terms of cycle threshold (Ct) and normalization to the predefined invariant endogenous control or average miRNA expression (Mestdagh et al., 2009) is performed by simply subtracting the Ct values (DeltaCt). Molecule counts reported by NanoString are first normalized to the spike-in positive controls and then housekeeping genes. The most commonly used normalization methods for microarray data are robust multiple-array average (RMA), quantile and Loess normalizations (Meyer et al., 2010). Standard procedure to normalize miRNA sequencing experiments is to scale reads to the total number of sequence reads aligning to the genome (Marques et al., 2010). Recently, trimmed mean of M values (TMM), quantile and Loess normalization methods are also suggested to eliminate technical and biological biases in miRNA-sequencing (Meyer et al., 2010). After normalization statistically significant changes in miRNA expression can be identified using *t*-test, ANOVA, z-score, Mann–Whitney test, Bayesian method etc. Then, p-values can be adjusted for multiple comparisons using Bonferroni correction. Finally, functional annotation and pathway analysis tools, such as DAVID (http://david.abcc.ncifcrf.gov) and ToppGene (http://toppgene.cchmc.org/), can be used to evaluate differentially expressed miRNAs.

6.8. Future directions in miRNA profiling

In the near future, we expect to see new or adapted platforms that address newly recognized challenges in miRNA detection.

One new challenge in miRNA biology is the existence of miRNA isomers. Isomers vary from the canonical sequence described on miRBase by nucleotide additions. Non-template 3′ additions of adenines, uridines or cytidines are quite common and are introduced by enzymatic activity. These non-template additions occur in physiological settings and the type and extent of nucleotide addition is highly specific to each miRNA (Newman et al., 2011; Wyman et al., 2011). The effects of nucleotide additions are still incompletely understood but it appears it can affect miRNA stability or repression capability

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(Jones et al., 2009; Katoh et al., 2009). It is important to note that most miRNA profiling techniques have been specifically designed to detect canonical miRNA sequences and may not properly detect isomers. Deep sequencing is by far the least biased technique in that regard and should be able to deliver information on all isomers. Other techniques, such as Nanostring, have been shown to be adaptable to detect and quantify specific isomers (Wyman et al., 2011), although they are still not commercially available.

Another challenge in miRNA studies is the detection of miRNA in certain types of specimens where miRNA is present in low amounts (such as plasma or serum) or where miRNA may be difficult to extract (such as in formalin-fixed tissues). We may soon see additional miRNA profiling techniques that combine the techniques described here with various miRNA capture methods to eliminate the need of RNA extraction and ease miRNA detection. HTG Molecular Diagnostics has recently released the quantitative nuclease protection (qNPA) technology system. In this technique, cells are lysed to release RNA and DNA oligonucleotides complementary to the miRNAs hybridize to their targets. The sample is then treated with S1 nuclease, which removes non-protected RNA and excess oligonucleotides. The RNA from the RNA-DNA duplexes is removed, yielding a specific oligonucleotide library. The oligonucleotides in the library are then bound to specific oligonucleotide linkers on an array surface and are detected with a horseradish peroxidase probe that produces measurable luminescence in the presence of substrate. This technique has been successfully used to detect miRNAs in formalin-fixed tissues. These and other improvements should further facilitate the use of miRNA analysis at clinical sites.

7. miRNA studies in MS

The availability of commercial profiling platforms has greatly facilitated prospective biomarker studies in MS and other autoimmune diseases. miRNAs have been suspected to play an important role in MS, based on their high expression in immune cells that mediate disease, as well as in target organ central nervous system cells. Moreover, miRNAs had been shown to play an important role in autoimmune processes in general (Xiao et al., 2008; Zhou et al., 2008) and other autoimmune diseases (Pauley et al., 2009).

Since 2009, a number of MS miRNA profiling studies have been reported (summarized in Table 2). The first study, by Otaegui et al. (2009) investigated miRNA expression differences in relapsing-remitting MS (RRMS) patients and controls. Peripheral blood mononuclear cells (PBMC) were profiled for 364 miRNAs with TLDA cards. The study included RRMS patients during relapses and remissions and the most statistically robust differences were obtained in the relapse to control comparison, with three up-regulated miRNAs: miR-18b, miR-599 and miR-493. In addition, miR-148a, miR-184, miR-193 and miR-96 were considered not significant, but promising candidates, differentially expressed in RRMS during remission as compared to controls.

Soon after, Keller et al. (2009) reported differential miRNA expression in whole blood samples of RRMS patients using microarray technology. The relapse or remission status at the time of blood draw was not reported. Out of the 866 miRNAs profiled, 165 were significantly different in MS. The top ten most significant dysregulated miRNAs were miR-145, miR-186, miR-664, miR-20b, miR-422a, miR-142-3p, mir-584, miR-223, miR-1275 and miR-491-5p. With the exception of miR-20b, all these miRNAs were up-regulated in MS. miR-145, their top biomarker candidate, was up-regulated 3 fold in MS over controls and was capable of differentiating MS from controls in the profiling study population with 89.5% specificity, 90% sensitivity and 89.7% accuracy.

Du et al. (2009) reported over-expression of miR-326 in PBMC and CD4+ T cells of MS patients undergoing relapses, but not during remission. Although this miRNA was not identified in a profiling study, a handful of other miRNAs were unchanged. In addition, they identified a mechanism by which miR-326 may contribute to disease through negative regulation of the transcription factor ets-1 and enhanced pathogenic Th17 cell development.

Next, Junker et al. (2009) looked at miRNA expression in active and inactive brain lesions from MS patients and compared it to normal white matter from healthy individuals. TLDA cards were used to profile 365 miRNAs from laser-capture microdissected tissue. Among the deregulated miRNAs, they found overexpression of miR-155, a miRNA associated with immune activation, in astrocytes. Together with also up-regulated miR-326 and miR-34a, miR-155 was capable of targeting CD47, a negative regulator of macrophage activity.

In contrast to prior studies that have profiled complex tissues or mixed cell populations, Lindberg et al. (2010) investigated miRNA expression in purified B cells and CD4+ and CD8+ T cells using TLDA cards. 10 differentially expressed miRNAs were identified in CD4+ T cells from RRMS patients. miR-17-92 was upregulated two-fold in MS patients samples and was also upregulated upon polyclonal T cell activation. Since miR-17–92 overexpression in transgenic mice causes enhanced T cell activation and autoimmunity, this increase may reflect the reported increased T cell activation of MS T cells. Lindberg et al. also identified several predicted targets of the miR-17–92 cluster in the PI3K signaling cascade, including PI3 kinase regulatory subunit 1 (PI3KR1) and phosphatase and tensin homolog (PTEN). These two predicted targets negatively regulate the Pi3K activation cascade. Transfection with miR-17–92 inhibitors resulted in an increase in PI3KR1 and PTEN while the expression of PI3K cascade downstream molecules was decreased.

Last year, De Santis et al. (2010) reported microarray profiling of 723 miRNAs in the regulatory T cell population of MS patients and controls. This study investigated whether miRNA expression differences in the regulatory T (Treg) cell population of MS patients may underlie the reduced function of this population observed in MS patients. 23 miRNAs were differentially expressed, with a predominance of up-regulated genes. Since the analyzed population corresponded to CD4+CD25high T cells that may also include activated T cells, a confirmatory Real-Time PCR was performed using $CD4^+CD25^{high}CD127^{-/low}$ Treg cells. A trend towards increased expression of miR-106b, miR-19a, miR-19b and miR-25 was observed. Since these miRNAs are involved in regulation of the TGFbeta pathway by silencing CDKN1A/p21 and BCL2L11/Bim (Petrocca et al., 2008), they may reduce Treg development or activity. In addition, miR-17–92, also found increased in MS $CD4^+CD25^+$ T cells, has been proposed to contribute to TGFbeta pathway inhibition (Petrocca et al., 2008). In summary, miRNA dysregulation may mediate Treg function defects in MS.

The largest study performed so far included 59 MS (24 RRMS, 17 SPMS and 18 PPMS) and 34 Healthy donor controls (Cox et al., 2010). Whole blood was profiled for 733 miRNAs, using Illumina's proprietary sentrix array matrix (SAM) surface coupled to hybridization microarray. 23 miRNAs were differentially expressed in MS with mild fold changes ranging from −1.59 to 1.36. In contrast to previous studies, there was a predominance of down-regulated miRNAs with only a single miRNA up-regulated. miR-17 and miR-20a, the top candidate biomarkers, were consistently decreased in MS patients regardless of presentation. Gene expression analysis in Jurkat cells transfected with miRNA mimics or inhibitors was used to identify genes regulated by these miRNAs. Several pathways potentially affected by these miRNAs were identified, including translation regulation, Activin A signaling regulation, Vitamin B7 metabolism and immune response pathways.

Our own group has performed miRNA profiling in naïve $CD4^+$ T cells of MS patients (Guerau-de-Arellano et al., in press). Naïve T cells represent T cells that have undergone thymic maturation but have not yet been activated by their cognate antigen. Therefore, assaying this population can reveal miRNAs that predispose MS

Please cite this article as: Guerau-de-Arellano, M., et al., miRNA profiling for biomarker discovery in multiple sclerosis: From microarray to deep sequencing, J. Neuroimmunol. (2011), doi:10.1016/j.jneuroim.2011.10.006

Table 2
miRNA profiling studies in Multiple Sclerosis.

	Tissue	Method	MS subtype	Disease status	Treatment status	Identified miRNAs[a]
Otaegui et al.	PBMC	TLDA	RRMS	Relapses and remissions	Unspecified (no acute steroids)	miR-18b, miR-493 and miR-599 (in relapse)
Keller et al.	Whole Blood	Hybridization microarray	RRMS	Unspecified	GA, IFNβ or none (no steroids in 3 months	miR-145, miR-186, miR-664, miR-20b, miR422a, miR-142-3p, mir-584, miR-223, miR-1275 and miR-491-5p (in RRMS)
Du et al.	PBMC, $CD4^+$ T cells	Real-Time PCR	RRMS	Relapsing or remitting	Treatment-naive	miR-326 (in relapse)
Junker et al.	MS brain lesions	TLDA	SPMS, RRMS, PPMS and Marburg variant	Unspecified	Unspecified	miR-650, miR-155, miR-326, miR-142-3p, miR-146a, miR-34a, miR-21, miR-23a and miR-656 (in active lesions)
Lindberg et al.	$CD4^+$ T cells	TLDA	RRMS	Unspecified	No treatment for 6 months	miR-485-3p, miR-376a, miR-1, miR-497, miR-193a, miR-200b, miR-126, miR-486, miR-17-5p and miR-34a (in CD4+ T cells)
De Santis et al.	$CD4^+CD25^+$	Hybridization microarray	RRMS	Stable	Treatment-naive	miR-29c, miR-107, miR-210, let-7i, miR-15a, miR-19a, miR-19b, miR-138-2*, miR-324-3p and miR-301a (in $CD4^+CD25^+$ T cells)
Cox et al.	Whole blood	Hybridization microarray	RRMS, SPMS, PPMS	Unspecified	No treatment for 3 months	miR-768-3p, let-7d, let-7f let-7g, let-7i, miR-106a, miR-126, miR-126* and miR-140-5p (in combined MS)
Guerau-de-Arellano et al.	Naïve $CD4^+$ T cells	TLDA	RRMS PPMS SPMS	Stable	Treatment-naive	miR-660, miR-518d-3p, miR-586, miR-128, miR-564, miR-708, miR-378, miR-346, miR-645 and miR-566 (in combined MS)

[a] Top 10 most significantly dysregulated miRNAs are listed. Additional miRNAs may be found in the original manuscript.

patients' T cells to exaggerated activation or pathogenic T cell differentiation upon antigen encounter. In fact, myelin-specific T cells exist in all individuals (Giegerich et al., 1992; Lovett-Racke et al., 1998), suggesting that it is not the mere presence of these cells but their tendency to develop into pathogenic phenotypes that results in MS. Furthermore, MS T cells have been shown to have reduced costimulatory requirements (Allegretta et al., 1990; Lovett-Racke et al., 1998) and a tendency to differentiate into pro-inflammatory phenotypes as compared to healthy donor cells (Pelfrey et al., 2000). We hypothesized that miRNA dysregulation in MS patients naïve CD4+ T cells may underlie these defects and performed TLDA analysis of 756 miRNAs. After p value and false discovery rate exclusion analysis, 85 miRNAs were found to be significantly and differentially expressed. Among the dysregulated miRNAs, miR-128 and miR-27b, were found to directly target BMI-1, a pro-Th2 factor that stabilizes GATA-3. These miRNAs contributed to driving Th cell differentiation away from Th2 pathway and towards pro-inflammatory Th1 responses and increased the severity of adoptive transfer experimental autoimmune encephalomyelitis. These studies indicate that miRNAs dysregulated in MS patients' naïve CD4+ T cells can indeed contribute to MS pathogenesis.

These studies highlight that miRNA changes are associated with MS. Although there has been little overlap between studies, several miRNAs stand out. For example, the miR-17–92 cluster has been found over-expressed in two studies using purified CD4+ T cells (Lindberg et al., 2010) and CD4+CD25+ T cells of RRMS patients (De Santis et al., 2010). In contrast, they were down-regulated in whole blood of MS patients encompassing the three major presentations (primary-progressive, RRMS and Secondary-progressive MS (Cox et al., 2010)). The reason for the discrepancy is unclear, but both differences in population choice (RRMS versus all 3 MS subtypes) and the tissue examined (CD4+ T cells versus whole blood) may very likely be the cause. Similarly, miR-326 was identified as over-expressed in CD4+ T cells of RRMS patients (Du et al., 2009) and was again found increased in active MS lesions (Junker et al., 2009). However, none of the purified T cell studies so far has reproduced these results. Overall, the differences in the miRNAs identified in the studies published so far are probably to be expected, due to the wide variation in the tissue or cell type assayed, disease subtype, treatment status and profiling techniques utilized.

The biological information that can be inferred from these profiling studies is starting to emerge (Du et al., 2009; De Santis et al., 2010; Lindberg et al., 2010; Guerau-de-Arellano et al., in press) miRNAs dysregulated in MS have been found to favor pro-inflammatory T cell phenotypes (Du et al., 2009; Guerau-de-Arellano et al., in press) or have been associated with potential regulation of T cell activation (Lindberg et al., 2010) or Treg function (De Santis et al., 2010). It is important to note that the biological consequence of miRNA changes is more difficult to assess in complex specimens, such as whole blood or PBMC, than in purified populations. miRNA composition varies depending on the cell type (Chen et al., 2004) and profiling of mixed cell populations may reflect changes in these populations. Although miRNAs identified from these samples can potentially be useful disease biomarkers, it is more difficult to causally associate them with modulation of a biological process. In order to address the role of miRNAs in MS pathogenesis, it is preferable to analyze purified cell populations. Once a miRNA has been identified as dysregulated, the usual workflow leading to identification of an altered pathway involves identification of putative targets through predictive algorithms, such as Targetscan and PicTar, miRanda and RNAhybrid, among others. Targetscan (www.targetscan.org) searches for conserved 7mer or 8mer matches of nucleotides 2–7, i.e. the seed region, of the miRNAs in the 3′UTR of genes (Lewis et al., 2005). An option to search for evolutionarily non-conserved or mammalian conserved targets also exists. Targetscan also allows one to identify targets with incomplete seed region match if it is compensated by conserved 3′ pairing (Friedman et al., 2009). Each match is given a context score that allows ranking sites from more likely (most negative values) to less likely biological targets (Grimson et al., 2007). PicTar (http://pictar.mdc-berlin.de) identifies targets based on alignment of evolutionarily conserved 3′ UTR sequences in various species providing the best predicted binding with a perfect 7-nucleotide seed match, but allowing some mismatches that do not affect free energy (Krek et al., 2005). These algorithms usually identify several hundred predicted targets. Therefore,

Please cite this article as: Guerau-de-Arellano, M., et al., miRNA profiling for biomarker discovery in multiple sclerosis: From microarray to deep sequencing, J. Neuroimmunol. (2011), doi:10.1016/j.jneuroim.2011.10.006

the choice of targets to further validate is usually aided by a hypothesis-driven process or through identification of potentially affected pathways with a pathway analysis bioinformatics tool. Another approach combines mRNA and miRNA expression analysis to identify those mRNA–miRNA pairs with an inverse relationship. Validation of targeting of a gene by the miRNA requires a luciferase assay, in which the 3′UTR of the predicted target gene is tagged to a luciferase gene to allow tracking of gene suppression when the miRNA of interest is present (Zeng and Cullen, 2003). In addition, experiments that assess the effect of miRNA overexpression or inhibition in the cells where the miRNA was originally identified as dysregulated allows one to more conclusively determine how the miRNA contributes to disease development.

Since the discovery of miRNAs in 1993 (Lee et al., 1993; Wightman et al., 1993) and the first report of miRNA association with human disease (Calin et al., 2004), there has been a tremendous effort to identify miRNAs associated with MS. The results of these studies are now becoming apparent. Several miRNAs have been associated with MS, MS relapses and/or MS pathogenesis. Although the studies published so far are very promising, a consensus regarding which miRNAS can be used as MS biomarkers needs to be reached. Future studies designed with higher numbers of patient and control samples and more defined patient populations (MS subtype, disease status and treatment status) will be instrumental to uncover biomarkers of widespread clinical applicability.

8. Conclusions

The discovery of MS biomarkers should greatly improve the diagnosis and management of MS. miRNAs offer several advantages over other types of biomarkers such as mRNA or protein, including increased stability and proven biological relevance in several diseases. Ultimately, miRNAs offer an opportunity to link a biomarker with an altered biological process and a therapy targeting this process and/or miRNA. The current broad spectrum of miRNA profiling methods available should help achieve this lofty goal.

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Please cite this article as: Guerau-de-Arellano, M., et al., miRNA profiling for biomarker discovery in multiple sclerosis: From microarray to deep sequencing, J. Neuroimmunol. (2011), doi:10.1016/j.jneuroim.2011.10.006