RESEARCH PAPER

Circulating miRNAs in myasthenia gravis: miR-150-5p as a new potential biomarker

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Abstract

Objective: Myasthenia gravis (MG) is a chronic autoimmune disorder where autoantibodies target the nicotinic acetylcholine receptors (AChR+) in about 85% of cases, in which the thymus is considered to play a pathogenic role. As there are no reliable biomarkers to monitor disease status in MG, we analyzed circulating miRNAs in sera of MG patients to find disease-specific miRNAs. **Methods:** Overall, 168 miRNAs were analyzed in serum samples from four AChR+ MG patients and four healthy controls using Exiqon Focus miRNA polymerase chain reaction (PCR) Panel I + II. Specific accumulation pattern of 13 miRNAs from the discovery set was subsequently investigated in the sera of 16 AChR+ MG patients and 16 healthy controls. All patients were without immunosuppressive treatment. Selected specific miRNAs were further analyzed in the serum of nine MG patients before and after thymectomy to assess the effect of thymus removal on the accumulation of the candidate miRNAs in patient sera. **Results:** Three miRNAs were specifically dysregulated in AChR+ MG patient sera samples. Hsa-miR150-5p, which induces T-cell differentiation, as well as hsa-miR21-5p, a regulator of Th1 versus Th2 cell responses, were specifically elevated in MG sera. Additionally, hsa-miR27a-3p, involved in natural killer (NK) cell cytotoxicity, was decreased in MG. Hsa-miR150-5p levels had the highest association with MG and were significantly reduced after thymus removal in correlation with disease improvement. **Interpretation:** We propose that the validated miRNAs: hsa-miR150-5p, hsa-miR21-5p, and hsa-miR27a-3p can serve as novel serum biomarkers in AChR+ MG. Hsa-miR-150-5p could be a helpful marker to monitor disease severity.

Introduction

Myasthenia gravis (MG) is a chronic T-cell mediated autoimmune disorder, where the attack of autoantibodies (abs) results in failure of neuromuscular transmission and fatigable weakness of skeletal muscles.[1,2] The most common immunological subtype of MG includes abs against the nicotinic acetylcholine receptors (AChRs) at the muscle membrane. Approximately 85% of patients are AChR ab seropositive (AChR+).[3] A substantial proportion of predominantly female AChR+ MG patients (~60%) has

thymic hyperplasia characterized by ectopic germinal center development, and hence AChR+ patients are recommended to undergo thymectomy to halt disease progress.[4] The production of anti-AChR abs depend upon CD4+ T cells[5] and characteristics of chronic autoimmune activation include long-term expression of markers for activation on T cells, lymphocyte trafficking as well as B cell-activating ability. Thus, these data provide evidence for persistent clonally expanded B helper CD4+ T cell populations in the blood of MG patients.[6] In the periphery, total Ig production is higher in the blood of MG

patients than in age-matched healthy control (HC) individuals,[7] indicating an active inflammatory state. The detection of MG-specific abs helps the diagnosis, along with clinical fatigue tests and neurophysiological assessment of neuromuscular transmission failure. However, in general, the ab titer does not predict the degree of muscle weakness or the response to therapy.[8,9] Consequently, there is an obvious need for reliable biomarkers, which correlate with even subtle fluctuations in disease severity for individual treatment regimen and clinical trials.

Mammalian microRNAs (miRNAs) have gained a lot of attention as powerful small non-coding RNA species inhibiting gene expression by degrading and/or blocking translation of their target messenger RNAs (mRNAs).[10] Mature miRNA regulated gene expression contributes to essential cellular processes such as differentiation, apoptosis and proliferation.[11] Quantitative detection of cellular miRNAs has been suggested to define disease status, as abnormal presence of certain miRNAs correlates with the pathogenesis of diseases such as cancer and diabetes.[12,13] Importantly, miRNAs can also be detected in the extracellular environment. This fraction of miRNAs is regarded as cell-free circulating molecules residing in various extracellular vesicles such as microvesicles, exosomes, and microparticles.[14] A growing list of reports indicates that these circulating miRNAs can be detected and quantitatively analyzed in biofluids, including serum, plasma, urine, and saliva.[15] Thus, the detection of circulating miRNAs in patient biofluids has been considered a novel method of detecting the progression of cardiovascular diseases and malignant growth.[14,15] A recent study has also identified circulating miRNAs as readily accessible blood biomarkers to monitor disease state in multiple sclerosis (MS), a T-cell mediated disorder of the central nervous system.[16] The aim of this study was to analyze the circulating miRNA profile in the serum of female patients with AChR+ generalized MG and to assess whether the level of any specific miRNA could serve as a new biomarker for MG patients.

Material and Methods

Subjects

In order to obtain as homogenous group of MG patients as possible, our selection criteria were (1) female patients with early-onset MG (onset <50 years of age) with objective clinical muscle fatigue along with disturbed neuromuscular transmission on repetitive nerve stimulation and/or single-fiber electromyography, (2) positive serum AChR abs (≥0.2 nmol/L), (3) mild generalized disease (Myasthenia Gravis Foundation of America [MGFA] class 2), (4) no immunosuppressive treatment for at least 6 months, and

(5) no thymoma. Symptomatic treatment with acetylcholinesterase inhibitors (AChEIs) was allowed. Serum samples were obtained from MG patients and from age-matched healthy female blood donors after informed consent. These studies on blood samples were approved by local Ethics Committees (Uppsala-Sweden [Dnr 2010/446] and Paris-France [CPP no.29-10 – authorization number ID RCB 2010-A00250-39]). Demographics of the discovery and validation sets are shown in Table 1.

The discovery set (N = 8) included four MG patients from France, who had not undergone thymectomy at the time of serum sampling. All MG patients had an MGFA class of 2A and a hyperplastic thymus was identified in all cases later upon thymectomy. The discovery set also included four age-matched female French blood donors as HCs.

The validation set (N = 32) consisted of 16 Swedish MG patients with MGFA class 2A as well as 16 age-matched healthy female Swedish blood donors (Table 1). All of the patients had undergone thymectomy and underwent a clinical examination at the same date as the blood samples were collected, revealing an MG Composite score[17] ranging from 1 to 7.

Furthermore, in order to assess the effect of thymectomy, nine French MG patients who had serum samples collected both before and after thymectomy were included. These patients had an MGFA class of 2A–2B at thymectomy time (Table 4).

Table 1. Demographic data of subjects in the discovery set and validation set.

	Healthy controls	MG patients
Discovery set		
N	4	4
Age	33.2 ± 8.2	32.7 ± 7.1
Disease duration (months)	n.a.	8.0 ± 2.8
AChEI (N)	n.a.	4
Thymectomy	n.a.	No
Validation set		
N	16	13 (16)[1]
Age	40.1 ± 9.9	42.2 ± 7.7
Disease duration (years)	N.A	19.2 ± 10.2
AChEI (N)	N.A	13
Post-thymectomy (years)	N.A	15.9 ± 7.4

Clinical characteristics of the patients included in the discovery set (French) as well as the validation set (Swedish). The results are, where applicable, presented as mean ± SD. All patients and controls in the validation set were female and age matched in each individual case. N, number of patients; AChEIs, acetylcholinesterase inhibitors; N.A, not applicable. None of the analyzed patients had current immunosuppressive treatment. All data are displayed as mean ± SD.
[1]Sixteen patients were included in the validation cohort, although due to hemolyzed samples 13 samples were used.

RNA isolation and miRNA expression analysis

Blood samples were collected in tubes without any additives and then centrifuged, after at least 20 min storage in room temperature, at 1900 *g* and 20°C for 5 min in Sweden and at 1800 *g* and 4°C for 20 min in France. The serum samples were subsequently stored at −80°C until use. RNA isolation was performed using a miRCURY RNA Isolation Kit (Exiqon, Vedbaek, Denmark) according to the manufacturer's instructions. Isolated RNA samples were used for cDNA synthesis with Universal cDNA Synthesis Kit II (Exiqon).

miRNA analysis using PCR arrays

Initial miRNA detection experiments were performed on Serum/Plasma Focus microRNA polymerase chain reaction (PCR) Panel I + II (V1.M) by using SYBR® Green master mix (Exiqon) as recommended by the manufacturer. This panel covers the analysis of 168 human miRNAs and was used for profiling the discovery set of four patients and four HCs. Analysis of the raw qRT-PCR amplification data was performed according to the Exiqon's recommendations. The qRT-PCR data from Serum/Plasma Focus microRNA PCR Panels were examined with GenEx software (Exiqon). In this process the inter-plate calibration, approved quality controls (RNA-spike-in), and hemolysis test (hsa-miRNA23a-3p – hsa-miRNA451a) were included. Hereafter, we use the miRBase (www.mir-base.org) recommended naming of the miRNAs, whereby the hsa-miRNA indicates human mature miRNA, for the miRNAs analyzed in this study. Reference miRNAs, hsa-miR93-5p, hsa-miR-191-5p, hsa-miR-423-4p, and hsa-miR-103a, were chosen by analyzing the suggested candidate genes in the applications "NormFinder" and "GeNorm" available in the GenEx software (Fig. S1).

Screening of the individual miRNAs

Screening of the individual miRNAs was performed with specific microRNA LNA™ PCR primers (Exiqon) by using ExiLENT SYBR® Green master mix (Exiqon). All qRT-PCR reactions were carried out on 384-well plates in the presence of ROX Reference Dye (Life Technologies, Stockholm, Sweden) and analyzed with the Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies). The sera were analyzed for potential cellular miRNA contamination due to hemolysis during the sera samples preparation. The ΔCT value of established hemolysis markers ($\Delta CT_{(hemolysis)} = CT_{(hsa-miR-23a-3p)} - CT_{(hsa-miR-451a)}$), have been recommended as the qualitative measurement of the hemolysis in serum samples (Exiqon). A $\Delta CT > 7$ in serum samples indicate a high risk of hemolysis and therefore these samples were not used for further analysis.

Normalization of individual miRNA levels on Serum/Plasma Focus microRNA PCR Panel was done to the above-mentioned reference genes according to GenEx manual guidelines (Exiqon). Relative quantities were calculated by using the healthy blood donors as a control group. Quantification of relative miRNA expression on the validation set was performed with the comparative CT method using the formula $2^{\wedge-\Delta\Delta CT}$, where $\Delta CT = [(CT$ gene of interest$-CT$ reference gene$)$ sample $A-(CT$ gene of interest$-CT$ reference gene$)$ sample $B]$ by using hsa-miR-93-5p as the reference miRNA.[18]

Statistical analysis

Log conversion of the data in the discovery set was done in order to obtain data more similar to a normal distribution for the statistical tests. Unpaired two-tailed *T*-test of independent samples as well as one-way analysis of variance (ANOVA) analysis was performed, comparing MG and control groups with the null hypothesis that the mean values of the different miRNAs were the same across MG and control categories. In the discovery set, candidate miRNAs were selected if they were found to be significantly different expressed in the MG versus control group. Exclusively miRNAs that were found to be up-or downregulated in each individual patient were selected for the validation set analysis. Pearson correlation was performed in order to determine the correlation coefficient between disease duration, AChR ab titer, age and differentially expressed miRNAs. In order to establish a possible effect on validated miRNAs after thymectomy, one-sample (paired) two-tailed *t*-test was performed based on within-patient change before and after surgery with the null hypothesis that the mean change was zero. As 13 markers were analyzed for a final assessment of MG relation, a multiplicity method (Bonferroni-Holm adjustment) was applied to strengthen the statistical conclusion. Statistical significance was defined as $P < 0.05$.

Results

Significantly elevated or reduced miRNAs in sera of MG patients compared to HCs in the discovery set (*N* = 8)

The first step was to evaluate the miRNA profile in sera of female patients with AChR+ generalized MG. For this purpose, we first analyzed discovery set samples of four AChR+ MG patients and four age-matched HCs.

Sera were tested using microRNA PCR arrays containing 168 human miRNA detection primer pairs. None of the

Table 2. Differentially expressed miRNAs in the discovery set.

| miRNA | P-value | Change (fold) | |
		Elevated	Decreased
hsa-miR-150-5p	0.0042	13.2	
hsa-miR-296-5p	0.010	11.8	
hsa-miR106b-3p	0.0045	9.9	
hsa-miR-130b-3p	0.016	9.8	
hsa-miR-210	0.0067	8.3	
hsa-miR-363-3p	0.050	7.4	
hsa-let-7c	n.s.	7.3	
hsa-miR-34a-5p	0.0093	4.0	
hsa-miR-21-5p	0.046	3.3	
hsa-miR-421	0.0027	3.1	
hsa-miR-424-5p	0.0025	2.8	
hsa-miR-30e-5p	0.042		1.5
hsa-miR-27a-3p	n.s.		5.8

Selection of miRNA of interest among the 116 miRNA validated from the discovery set of 168 miRNAs. Comparing four MG patients versus four healthy controls, miRNA were selected when a P-value <0.05 was found either with a t-test or a one-way ANOVA test. Hsa-let-7c and hsa-miR-27a-3p were also added because of a clearly elevated or reduced level in the MG patients, although not significant (n.s., $P > 0.05$).

discovery set samples contained hemolysis ($\Delta CT < 6.5$). Normalization was done to all of the recommended reference miRNAs (hsa-miR93-5p, hsa-miR-191-5p, hsa-miR-423-4p, and hsa-miR-103a). Out of all 168 miRNAs, 116

miRNAs showed amplification in more than 60% of the samples and were thus further assessed. Thirteen miRNAs were found to be strongly elevated or reduced in the MG patients (Table 2). Out of these candidates, 11 miRNAs had *P*-values <0.05 after statistical analysis with *t*-test or ANOVA, visualized in the Volcano plot (Fig. 1). These data were supported by the principal component analysis, indicating different expressions of the 11 miRNAs between MG patients and HCs (data not shown) and a heat map analysis (Fig. S2). A summary of all analysis steps of the candidate miRNAs is shown in Figure S3.

Validation of the miRNA expression (*N* = 32)

The 11 miRNAs from Table 2 that were found to significantly differentiate between MG patients and HCs in the discovery set were further analyzed by qRT-PCR in sera from a cohort of Swedish MG patients and healthy blood donors. Additionally, let-7c and miR-27a-3p were added as these miRNAs were markedly elevated or decreased, although not significantly, in the MG samples compared to controls, respectively. The hemolysis quote (hsa-miR23a-hsa-miR451) was <7 in all control samples and in 13 patient samples, thus the three patient samples with hemolysis were excluded from further analysis. Out of the selected 13 miRNAs, five miRNAs (hsa-miR210, hsa-miR296-5p, hsa-miR106-3p, hsa-miR34a-5p, and
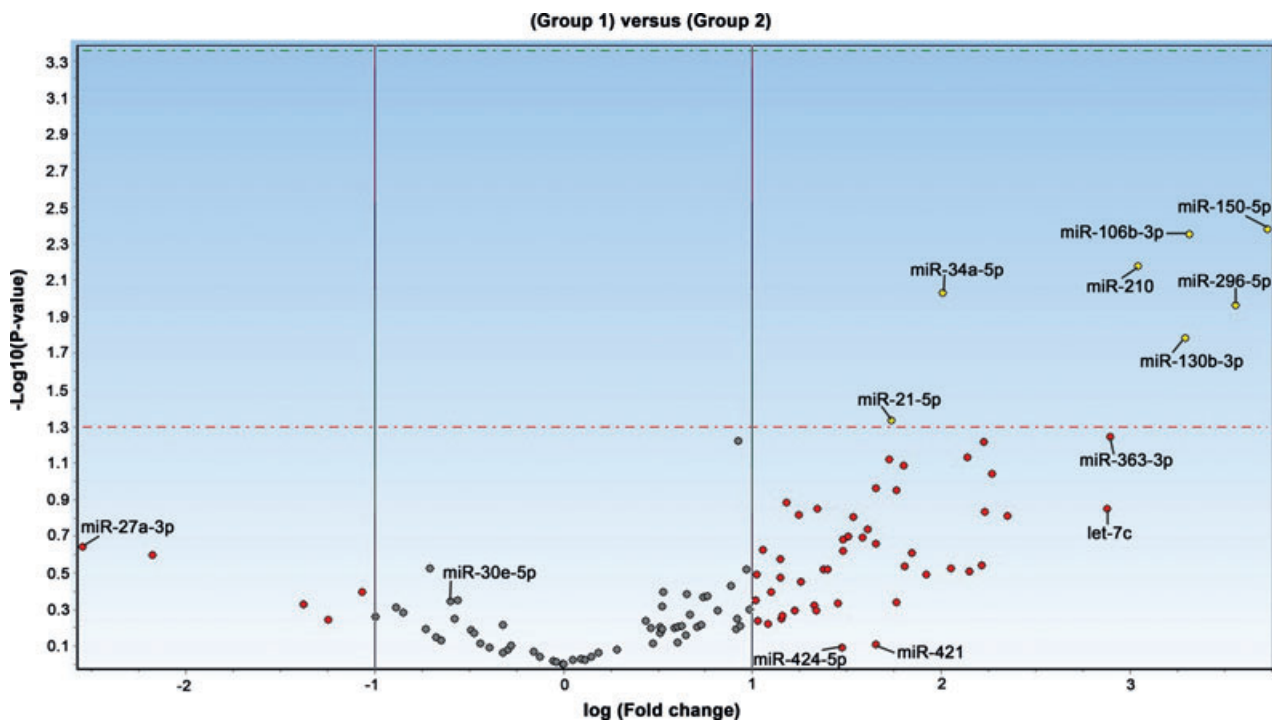

Figure 1. Volcano plot of the 116 expressed miRNAs in the discovery set. Group 1 represents the MG patients (*N* = 4) and group 2 refers to the healthy controls (*N* = 4). The differently expressed miRNAs (Table 2) that were further analyzed in the validation set are named in the plot.

hsa-miR130b) showed amplification on qRT-PCR in <20% of the samples and were hence excluded from further analysis. Of the reference genes, hsa-miR93-5p was shown to have the most stable amplification in all of the individual samples with good expression (*CT* values ≤30). For the remaining eight miRNAs of interest, hsa-mir-93-5p was thus used to calculate ΔCT and further relative expression levels. On the basis of these results, we were able to validate three miRNAs that were differentially expressed in MG patients and HCs: hsa-miR150-5p (*P* = 0.00046; Fig. 2A and B) and hsa-miR21-5p (*P* = 0.004; Fig. 2C and D) that were significantly elevated and hsa-miR27a-3p (*P* = 0.046; Fig. 2E and F), which was significantly reduced. The data obtained from the validation set are displayed in Table 3, which shows the odds ratio, *P*-values and area under the ROC curve (AUC) (Fig. 3). The strongest association with MG in the validation set was observed for hsa-miR150-5p, with an AUC value of 0.841 (*P* = 0.002; Fig. 3). Disease

duration among the Swedish MG patients ranged from 4 to 38 years (mean 19.1 ± 10.2 years). We did not find any correlation between disease duration and the differentially expressed miRNAs; the following Pearson correlation coefficients were found: hsa-miR27a-3p = 0.33 (*P* = 0.27); hsa-miR150-5p = 0.25 (*P* = 0.40) and hsa-miR21-5p = −0.31 (*P* = 0.91). There was also no correlation between current age (mean: 42.4 ± 7.7) or AChR ab titer and the differentially expressed miRNAs. Applying the Bonferroni-Holm adjustment, a *P*-value of 0.00598 was obtained for hsa-miR150-5p and an adjusted *P*-value of 0.048 for hsa-miR21-5p.

Effect of thymectomy on miRNA expression in MG patients

In order to establish whether thymectomy affects the circulating miRNA profile in serum of MG patients, we
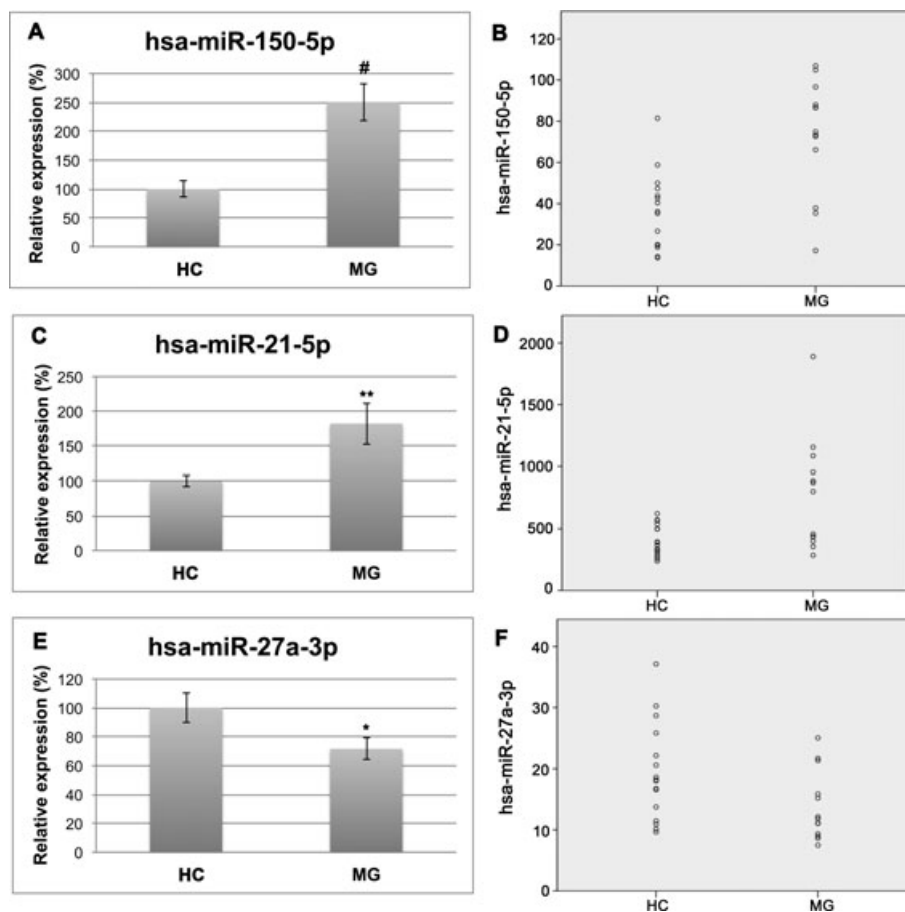

Figure 2. Significantly dysregulated miRNAs in MG patients (*N* = 13) compared to age-matched healthy controls (HC; *N* = 16). Relative expression as well as distribution in individual samples is shown for the significantly elevated hsa-miR-150-5p (A and B); hsa-miR-21-5p (C and D) and significantly reduced hsa-miR-27a-3p (E and F) after normalization to the reference gene hsa-miR-93-5p. In the left lane, results expressed as % ±SEM where the control samples were set to 100%. In the right lane, results are expressed according to the formula $2^{(-\Delta\Delta CT)} \times 100$ in order to have numbers in a comparable range. *$P \leq 0.05$; **$P \leq 0.01$; #$P \leq 0.001$.

included nine French female patients who had serum samples drawn just before thymectomy (mean age 30.2 ± 6.5 years) and 7.6 ± 3.7 years after thymectomy.

Table 3. Association between miRNAs and groups in the validation cohort.

| miRNA | MG patients versus HC | | | |
	Dysregulation (mean fold)	AUC	Standard error	*P*-value
hsa-miR-150-5p	2.5 (up)	0.841	0.081	0.002
hsa-miR-21-5p	1.8 (up)	0.779	0.089	0.011
hsa-miR-27a-3p	1.4 (down)	0.279	0.097	0.044

Differentially expressed miRNAs in the validation set of 13 miRNAs from the discovery set in the cohort of Swedish female MG patients (*N* = 13) and Swedish age-matched female healthy controls (HC; *N* = 16). The standard error is under the nonparametric assumption. The null hypothesis indicates a true area of 0.5.
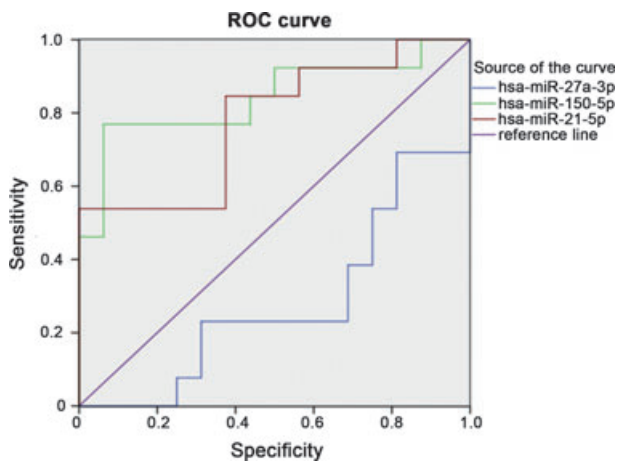

Figure 3. ROC curve of all dysregulated miRNAs, indicating sensitivity of each miRNA for MG. The null hypothesis indicates a true area of 0.5 (reference line).

Their epidemiological data and age at sampling are shown in Table 4. AChR abs were analyzed for all these patients at thymectomy time with titers ranging from 0 (for one patient, who subsequently became seropositive for AChR+ abs) to 970 nmol/L. As we aimed at identifying a miRNA that could be a suitable and powerful marker to follow all patients (with and without thymus), we selected the three miRNA that were found to differentiate between both non-thymectomized and thymectomized patients versus HCs. The change in miRNA levels in serum samples before and after thymectomy was analyzed on log-transformed data. The estimated difference in levels was then reverse transformed prior to presentation by taking 10^difference. The result is the ratio of the values after thymectomy divided by values before, or more specifically the ratio of their geometric means. The only statistically significant result among the three miRNA candidates was found for hsa-miR150-5p (*P* = 0.0242, Table 5), which supports the findings during the prior validation process. The estimated ratio for this variable was 0.27, which can be interpreted as that the values after thymectomy are 27% (95% CI: 9–80) of the values taken before surgery. Looking at miR-150-5p in individual patients (Fig. 4), seven out of the nine patients had a decrease in serum sample levels and two patients had increased levels. Intriguingly, the reduced level of miR150-5p after thymectomy in seven patients was also accompanied by either significant improvement in clinical status or clinical remission (Table 6), whereas the two patients with increased miR150-5p post thymectomy were found to suffer from clinical fluctuations.

Discussion

In the present report, we have analyzed the presence and abundance of circulating miRNAs in MG patient sera. The circulating miRNA with the highest association with MG identified in this study was hsa-miR-150. miR-150

Table 4. MG patients were sampled before and years after thymectomy.

Patient #	Age at thymectomy	Germinal centers of the thymus	AChRab titer before thymectomy (nmol/L)	AChRab titer after thymectomy (nmol/L)	Follow-up after thymectomy (years)
1	39	None	0.96	0.72	10
2	21	Few	18.1	5.0–50 (variable)	10
3	29	Few	4.4	4.8	10
4	35	Few	18.4	43–93 (variable)	10
5	23	Few	0.94	1.3	3
6	35	None	0	0.5	6
7	29	Few	970	152	7
8	37	Few	18.7	–	2
9	25	None	0.5	0	15

The initial sera samples were collected just before thymectomy and the age as well as acetylcholine receptor antibody (AChR ab) titer refers to this time point. –, missing data.

has been identified as a miRNA selectively expressed in mature, resting B and T cells and this miRNA is strongly upregulated as maturation/differentiation of T cells progresses.[19,20] In mice with transgenic expression of miR-150, the number of pre-B cells decrease in the bone

Table 5. Change in miRNA candidates after thymectomy.

	Ratio	Lower 95% CI	Upper 95% CI	P-value
hsa-miR150-5p	0.27	0.09	0.80	0.0242
hsa-miR27a-3p	0.35	0.11	1.07	0.0614
hsa-miR21-5p	0.40	0.12	1.31	0.1126

Back-transformed data on the French female MG patients (N = 9) for the three significantly altered miRNAs. Estimate with 95% confidence interval of the ratio between samples after and before thymectomy with a P-value of a hypothesis of ratio = 1 or equivalently the difference in log scale = 0.
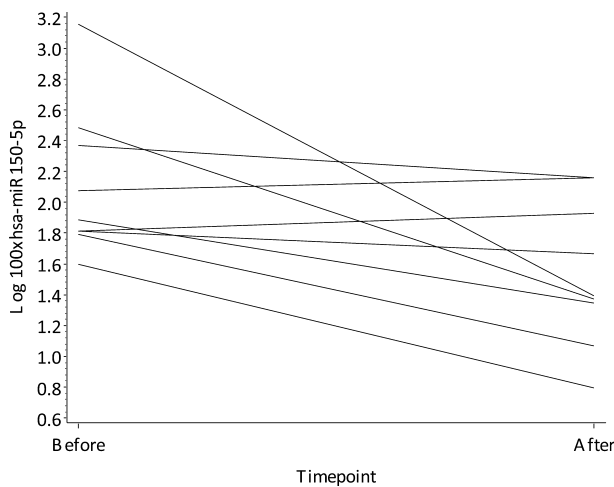

Figure 4. Serum levels of 100*hsa-miR150-5p in Log scale for the nine French female MG patients of the validation dataset is displayed where each patient is represented by a line that connects the serum level before and after thymectomy.

marrow and over time long-lived mature B cells accumulate.[21] Another important role has been shown for miR-150 in directing the development and maturation of natural killer (NK) cells,[22] that may be involved in the onset of autoimmunity and accumulate in the target organs of certain autoimmune diseases.[23–25] Furthermore, overexpression of miR-150 results in a substantial reduction in invariant NK T (iNKT) cells in the thymus and in the peripheral lymphoid organs.[22] Hence, one could speculate that there is a connection between high levels of miR-150 and the immune defect involved in the persistent inflammatory response, specifically of the thymus, in MG. In patients with early-onset AChR+ MG there is a distinct involvement of the thymus, with thymic hyperplasia of lympho-proliferative origin (reviewed in[26]). More specifically, thymic epithelial cells are believed to play a central role in the inflammatory environment through upregulating the autoimmune response. Although no prospective study has yet been able to show a beneficial effect of thymectomy alone, AChR+ MG patients are believed to benefit more from the removal of the thymus than patients with MuSK abs or patients without any detectable abs against AChR or MuSK.[27]

Our patient material in the pre-and post thymectomy cohort did not have any immunosuppressive treatments before or after thymectomy. This makes disease course evaluation of the surgery unbiased by other immunomodulatory medications. Intriguingly, the observed elevated expression levels of hsa-miR-150 in our patient sera were found to significantly decrease after thymectomy, which correlated well with an accompanying clinical improvement. However, as hsa-miR150-5p was still significantly upregulated in the validation set of Swedish patients who all had undergone thymectomy, the levels did not completely normalize after thymus removal. We hypothesize that this could be due to the fact that miR-150-5p expression is high not only in the thymus but also in the lymph nodes and spleen,[28] and thus the activation of miR150-5p

Table 6. Change in hsa-miR-150-5p, clinical status, and AChR ab titers after thymectomy.

Patient	% Change in hsa-miR150-5p after thymectomy	Clinical change after thymectomy	Follow-up of AChR ab titer after thymectomy
1	−37.7	Improvement/almost remission	Slightly decreased
2	+30.2	No real improvement/fluctuations	Very variable
3	−98.2	Improvement	Stable
4	−92.3	Remission	Increased
5	−81.0	Remission	Stable
6	+21.8	Slight improvement/fluctuating symptoms	Slightly increased
7	−29.0	Remission	Decreased
8	−71.1	Slight improvement	–
9	−33.3	Improvement	Slightly decreased

Individual changes in hsa-miR150-5p levels after thymectomy as well as evolution of clinical MG status and global change in acetylcholine receptor antibody (AChR ab) titer at follow-up sampling after thymectomy. (–) not known.

in the lymph nodes and spleen would not be affected after thymus removal. The AChR ab titers varied substantially among the patients and there was often no clear reduction in ab titer after thymectomy. In this sense, hsa-miR150-5p levels in the sera seem more responsive to thymectomy and change in clinical status.

The other miRNA that was found to be significantly elevated in MG patients was hsa-miR-21-5p. miR-21 is known to act as an oncomir, that is, a miRNA that is associated with different cancer forms, in B cell lymphoma and chronic lymphocytic leukemia.[29] In addition, several studies indicate a functional role of miR-21 in various immune cells, such as B- and T cells as well as dendritic cells.[30–32] For example, miR-21 is a major regulator of Th1 versus Th2 cell responses, defining a new mechanism for regulating polarized immune-inflammatory responses through altering the balance of different cytokines. Novel animal experiments indicate that miR-21 limits the immune response-mediated activation of the IL-12/IFNγ pathway as well as Th1-polarization.[32] Increased expression of miR-21 was previously found in peripheral mononuclear cells (PBMC) of MS patients[33] as well as in diabetes.[34]

The only miRNA that was significantly reduced in MG patient sera was hsa-miR27a-3p, which may reflect NK cell cytotoxicity in MG. From mice with AChR+ experimental autoimmune MG, NK cells are required for the development of MG-specific auto reactive T and AChR ab producing B cells.[35] Intriguingly, miR-27a downregulates NK cell cytotoxicity both in resting and activated NK cells,[36] suggesting that low levels of this particular miRNA in MG patients could reflect increased NK cell activation. Furthermore, clinical studies of AChR+ MG patients have shown that double-filtration plasmapheresis reduces NK cell cytotoxicity and AChR ab titer in patients who respond clinically.[37]

Double-stranded RNA (dsRNA)-signaling activation has been proposed to contribute to the etiology of MG.[38] As dsRNA can be produced during various virus infections,[39] this recent study poses the hypothesis that MG could develop after a viral infection.[38] In this context, it is quite exciting that cytomegalovirus infection can trigger the rapid degradation of two mature cellular miRNAs, miR-27a, and miR-27b, in various cell types.[40] Thus, the downregulation of hsa-miR27 in MG patients might be theoretically due to underlying persistent virus infection.

A recent report indicated miRNA profile alterations in PBMCs derived from MG patients.[41] More specifically, Cheng et al. (2013) observed a correlation between decreased levels of miR-320a and increased proinflammatory cytokines such as IL-2 and IL-17 in these cells.[41] Nevertheless, we did not observe altered levels of hsa-

miR-320a in the sera of our MG patients compared to HCs. Another study by the same group also identified reduced levels of the let-7 miRNA family in MG patient-derived PBMCs.[42] Although we did see increased, although not significantly, levels of let-7c in our discovery set; altered let-7c levels could not be confirmed in the validation set. Another potential MG-specific miRNA would be miR-146a, which very recently was proven to be elevated in MG patients PBMCs,[43] nevertheless, we did not detect any increase in the sera of our MG patient cohort. These divergent results are probably due to detection of distinct miRNA populations in different studies. In our experiments, we analyzed the presence of circulating extracellular miRNAs in MG patient sera samples, whereas in the above-mentioned reports the cellular miRNAs were profiled. Cellular miRNAs accumulate inside of the cells whereas circulating miRNAs originate from cells but are released into the extracellular environment. Thus, not all the cellular miRNAs can be identified as circulating miRNAs in the biofluids.

MG is a rare disease, hence the number of patients included is low. However, when evaluating the result of each marker the whole process should be taken into account. The alternative to multiplicity adjustment is replication, which, especially in medical applications, is often considered as the preferred approach. The marker hsa-miR150-5p was statistically significant in three independent populations, which provides strong evidence of a true relation. Under an assumption (null hypothesis) that none of the originally 168 markers are related to MG the probability to see at least one marker achieve this result is only 2.1%. Additionally, the disease duration differed between the patients in the discovery cohort and validation cohort. Nevertheless, we found no correlation between miRNA profile and disease duration.

In summary, this extensive analysis defined three human miRNAs that were linked to generalized AChR+ MG disease in female patients. The identified miRNAs: hsa-miR150-5p, hsa-miR21-5p, and hsa-miR27a-3p all could serve as potential biomarkers for disease stages in MG and we suggest validation of these biomarkers in a larger international cohort for diagnostic and therapeutic purposes. Among these three miRNAs hsa-miR-150-5p is of specific interest as its decrease after thymectomy correlated well with improvement in MG disease status. Additionally, these miRNAs could be of interest as targets of therapy in other autoimmune disorders as well.[44]

Acknowledgment

Author Contributions

Conception and design of the experiments: T. P., R. P., S. B. A., A. R. P. Performed the experiments: T. P. Analysis of data: R. P., A. R. P. Statistical analysis: M. A. Contribution of reagents/materials/analysis tools: R. P., F. T., S. B. A., M. A., A. R. P. Manuscript contribution: T. P., R. P., M. A., S. B. A., A. R. P.

Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Most stably expressed reference genes in the control samples, according to the application GeNorm in Exiqon software.

Figure S2. Heat map plots of the expression of selected 42 miRNAs in discovery group samples. Color key is given for association of different miRNAs with either MG patients ($N = 4$) or healthy controls ($N = 4$).

Figure S3. Summary of the steps covered by the manuscript. Three separate populations were analyzed: A: four MG patients + four healthy controls, B: 16 MG patients + 16 healthy controls, C: nine MG patients where marker evaluation is performed before and after thymus operation. The markers analyzed in subsequent steps are the statistically significant markers from the prior step. *Although this *P*-value was not significant in the first step, this miRNA was selected due to the considerately altered accumulation pattern in all patients.