Circulating microRNA miR-21-5p, miR-150-5p and miR-30e-5p correlate with clinical status in late onset myasthenia gravis

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There are no biomarkers for late onset myasthenia gravis (LOMG; onset > 50 years). We evaluated circulating microRNA in a discovery cohort of 4 LOMG patients and 4 healthy controls and in a prospective diagnostic validation cohort of 73 LOMG patients (48 male) with longitudinal follow-up samples. In immunosuppression naive patients, levels of miRNAs miR-150-5p, miR-21-5p and miR-30e-5p decreased in parallel with clinical improvement after initiation of immunosuppression and their levels positively correlated with the clinical MG composite score. Levels of miR-150-5p and miR-21-5p were lower in patients with ocular compared to generalized LOMG. Circulating miR-150-5p, miR-21-5p and miR-30e-5p correlate with the clinical course in LOMG.

1. Introduction

Myasthenia gravis (MG) is a chronic autoimmune disease caused by autoantibody attack of the neuromuscular junction proteins (Meriggioli and Sanders, 2009). The MG diagnosis is based on the clinical picture of fluctuating muscle weakness and fatigability, neuromuscular transmission failure on electrophysiological studies and serum detection of antibodies against the acetylcholine receptor (AChR), muscle specific tyrosine kinase (MuSK) or low-density lipoprotein receptor related protein 4 (LRP4).

One of the difficulties in predicting clinical outcome during follow-up in MG is that the disease is largely heterogeneous and disease progression is different depending on the subgroup that the patient is classified into. To date, well-described MG subgroups include: age of onset [early onset MG (EOMG; onset ≤50 years) versus late onset MG (LOMG; onset > 50 years); presence of a thymoma [thymoma associated MG (TAMG)]; and antibody subtype (AChR, MuSK or LRP4) (Gilhus and Verschuuren, 2015; Meriggioli and Sanders, 2009). Although diagnostic measures are well defined, an optimal biomarker that correlates with MG severity over time is so far lacking for the LOMG patients, although greatly needed (Senatar et al., 2012, Rostedt Punga et al., 2015).

Small non-coding microRNAs (miRNAs) are involved in negative intracellular posttranscriptional mRNA regulation. Interestingly, extracellular circulating miRNAs have been detected in blood serum and plasma as well as in other human body fluids and some are elevated in EOMG (Punga and Punga, 2018). In particular, the miRNAs miR-150-5p and miR-21-5p are elevated in female AChR antibody positive (AChR+) EOMG patients and miR-150-5p levels decrease following thymectomy in EOMG (Punga et al., 2014). This finding was further strengthened by a recent report from the prospective Randomized Trial of Thyrmectomy in MG (MGTX), where AChR+ EOMG patients treated with thymectomy had lower levels of miR-150-5p after 2 years in parallel with a reduction of disease severity (Molin et al., 2018). The circulating miRNA profile in MuSK antibody positive (MuSK+) MG differs from AChR+ MG (Punga et al., 2016). However, miRNA data from patients with LOMG or associated thymoma are sparse (Nogales-Gadea et al., 2014), with no data available from prospective cohorts with samples obtained at diagnosis and during serial follow-up. The aim of this prospective longitudinal cohort study was to analyze the circulating miRNA profile in the serum among patients with generalized and ocular LOMG in relation to clinical findings and disease course. Our primary hypothesis was that certain miRNAs would be associated with LOMG and the clinical MG score in this subgroup. The secondary hypothesis was that some miRNAs are able to discriminate between LOMG patients with generalized or purely ocular MG.

2. Material and methods

2.1. Subjects

Between August 2014 and February 2017 we prospectively recruited 150 consecutive patients with a new diagnosis of MG from Nottingham, Birmingham and Oxford, UK. All patients had typical
2.3. Serum circulating miRNA expression analysis

Blood samples were collected in tubes without any additives, stored at room temperature at least 20 min and centrifuged at room temperature at 1200 rcf for 5 min. The samples were stored at −80 °C until further processing. Total RNA was isolated from 200 μl serum. After thawing the samples, they were centrifuged at 150 rcf for 5 min. We used the miRCURY™ RNA Isolation Kit-Biofluids (Exiqon #300112) and followed the manufacturer’s instructions. For serum cDNA synthesis, we used Universal cDNA Synthesis Kit II (Exiqon #203301) and 2 μl of isolated RNA was used for cDNA synthesis in 10 μl reaction mix.

2.4. Statistical analysis

Our primary hypothesis was that certain miRNAs would be associated with LOMG and the clinical MG score in this subgroup. To obtain miRNAs more similar to a normal distribution, we converted data to the logarithmic scale. Results for the miRNA levels were stated as mean ± SD. Paired two-tailed t-test was used to compare the values of miRNA at recruitment and 1 or 2 years after that. The Wilcoxon matched-pair test was used to compare non-parametric data (clinical score) at different time points. To preserve the type I error at 5%, the statistical tests for the primary hypothesis were performed sequentially and only if significant could the subgroup analysis and follow-up data of LOMG patients be compared without risk of error inflation. Rank correlation or Spearman correlation was performed to find correlation between continuous factors (age, disease duration, disease severity) and different miRNAs. Statistical significance was defined as p < 0.05.

3. Results

3.1. Circulating miRNA profile in LOMG discovery cohort

The discovery cohort consisted of four LOMG patients (2 males, age 67.8 ± 6.2 years) without any prior immunosuppression and MGC score 18 ± 4.1 and four healthy age-and sex matched controls (2 males, age 68.8 ± 6.2 years). No hemolysis was detected. Normalization was performed to miR-191-5p, as other suggested normalizing genes were not abundantly detected in all samples according to NormFinder (Genex) and in accordance with previous studies (Molin et al., 2018). Five miRNAs were found to be strongly elevated in the LOMG discovery cohort (Table 1, Supplementary Fig. 1).

Table 1: Differentially expressed miRNAs in the late-onset MG discovery set.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>P-value</th>
<th>Change (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-106b-3p</td>
<td>0.007</td>
<td>4.8</td>
</tr>
<tr>
<td>hsa-miR-30e-5p</td>
<td>0.01</td>
<td>1.9</td>
</tr>
<tr>
<td>hsa-miR-223-3p</td>
<td>0.04</td>
<td>4.5</td>
</tr>
<tr>
<td>hsa-miR-140-5p</td>
<td>0.04</td>
<td>2.0</td>
</tr>
<tr>
<td>hsa-miR-199-3p</td>
<td>0.047</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Table 2: Demographic data of subjects in the longitudinal validation set. LOMG, late-onset myasthenia gravis; AChR +, acetylcholine receptor antibody positive; seronegative, no detectable serum antibodies against AChR, MuSK or LRP4; MGC, Myasthenia Gravis Composite score.

<table>
<thead>
<tr>
<th>Number</th>
<th>Generalized LOMG</th>
<th>Ocular LOMG</th>
<th>All LOMG</th>
</tr>
</thead>
<tbody>
<tr>
<td>At recruitment</td>
<td>50</td>
<td>23</td>
<td>73</td>
</tr>
<tr>
<td>After 1 year</td>
<td>49</td>
<td>19</td>
<td>68</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>69.6 ± 9.8</td>
<td>63.9 ± 9.5</td>
<td>67.1 ± 9.8</td>
</tr>
<tr>
<td>Gender (M:F)</td>
<td>32:18</td>
<td>16:7</td>
<td>48:25</td>
</tr>
<tr>
<td>Disease duration (months) (median, range)</td>
<td>1.8 (0–24)</td>
<td>4 (0–20.5)</td>
<td>2 (0–24)</td>
</tr>
<tr>
<td>Antibody subtype N (%)</td>
<td>AChR+ 48 (96%)</td>
<td>22 (96%)</td>
<td>70 (96%)</td>
</tr>
<tr>
<td>Seronegative</td>
<td>2 (4%)</td>
<td>1 (4%)</td>
<td>3 (4%)</td>
</tr>
<tr>
<td>Thymectomy N (%)</td>
<td>2 (4%)</td>
<td>2 (8%)</td>
<td>4 (5.5%)</td>
</tr>
<tr>
<td>Atrophy</td>
<td>1 (2%)</td>
<td>0</td>
<td>1 (1.3%)</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
<td>1 (4%)</td>
<td>1 (1.3%)</td>
</tr>
<tr>
<td>Thymoma</td>
<td>1 (2%)</td>
<td>1 (4%)</td>
<td>2 (2.7%)</td>
</tr>
<tr>
<td>MGC score</td>
<td>Before recruitment 10.4 ± 5.9</td>
<td>4.6 ± 1.9</td>
<td>8.7 ± 5.7</td>
</tr>
<tr>
<td>At recruitment</td>
<td>8.2 ± 5.8</td>
<td>3.0 ± 2.6</td>
<td>6.6 ± 5.6</td>
</tr>
<tr>
<td>After 1 year</td>
<td>2.7 ± 3.9</td>
<td>1.2 ± 1.8</td>
<td>2.3 ± 2.5</td>
</tr>
<tr>
<td>After 2 years</td>
<td>1.5 ± 3.2</td>
<td>1.0 ± 2.6</td>
<td>1.3 ± 3.0</td>
</tr>
<tr>
<td>Immunosuppressive treatment N (%)</td>
<td>At recruitment 11 (22%)</td>
<td>0 (0%)</td>
<td>11 (15.1%)</td>
</tr>
<tr>
<td>After 1 year</td>
<td>31 (63.3%)</td>
<td>3 (15.8%)</td>
<td>34 (50%)</td>
</tr>
<tr>
<td>After 2 years</td>
<td>10 (71.4%)</td>
<td>2 (28.6%)</td>
<td>12 (57.1%)</td>
</tr>
</tbody>
</table>

The discovery cohort consisted of four patients with generalized LOMG and four age- and gender-matched healthy controls (HCs). This selection was based on the severity of disease, as we included the patients with the highest MGC score and positive serum AChR antibody titers. All the subjects were Caucasians. The longitudinal validation set consisted of 73 patients with an LOMG diagnosis who had undergone at least one annual follow-up assessment, of whom 50 initially had generalized MG and 23 ocular MG.

2.2. Serum circulating miRNA isolation

The reverse transcription (RT), followed by real-time PCR amplification, was performed using ExiLENT SYBR® Green master mix (Exiqon #203421). MicroRNA expression was analyzed using the Serum/Plasma Focus miRCURY LNA Universal RT microRNA PCR Panel (V4; Exiqon, Denmark) in the discovery cohort. In the validation cohort we added selected primers detected from the discovery cohort to the qPCR plates. The following steps have been described in detail previously (Punga et al., 2015, 2016, 2014). Briefly, cDNA was diluted 50× in nuclease free water. Internal technical controls included interplate calibration with UniSp3, quality of RNA isolation with UniSp2 and UniSp4, and cDNA synthesis control with UniSp6.

Hemolysis, cellular miRNA contamination was controlled through: ΔCq (miR-23a - miR-451a); a value lower than 7 represented samples without hemolysis. The reference candidates we used were miR-103a-3p, miR-191-3p, miR-93-3p and miR-423-5p. The ultimate selection to choose normalization to miR-191-3p was based on the results from NormFinder in the GenEx software (data not shown). Comparative CT method was used to quantify miRNA expression using the formula 2−ΔΔCT where ΔΔCT = (CT gene of interest – CT reference gene) (Schmittgen and Livak, 2008). The genes of interest were those with significant difference from the discovery cohort in addition to miR150-5p and miR-21-5p, based on previous data on these miRNAs.
3.2. Description of the validation cohort of LOMG

All the dysregulated miRNAs in LOMG patients were further analyzed by qRT-PCR longitudinally in an independent validation cohort. Additionally, we added miR-150-5p and miR-21-5p into the analysis as they have previously been found to be important MG-associated candidate biomarkers (Punga et al., 2015; Punga and Punga, 2018; Punga et al., 2014). Thus, miR-150-5p, miR-21-5p, miR30e-5p, miR-140-5p, miR-19b-3p and miR-223-5p were the miRNAs studied in all longitudinal subgroups. MiR-106b-3p was left out from further analysis as > 40% of samples had undetectable levels.

The validation cohort of LOMG patients had either generalized symptoms (111 samples) or ocular symptoms (60 samples). These samples were obtained from 50 LOMG patients with generalized

![Fig. 1. Longitudinal changes in circulating miRNAs among patients with generalized late-onset MG (LOMG) A) total B) men and C) women. Paired two-tailed t-test was used. ***p < 0.001; **p < 0.01; *p < 0.05. Results for the miRNA levels were stated as mean ± SD.](image-url)
symptoms drawn at recruitment (50 samples), at 1-year follow-up (49 samples) and at 2-year follow-up (14 samples) after recruitment, and from 23 LOMG patients with ocular symptoms taken at recruitment (23 samples), at 1-year follow-up (19 samples) and at 2-year follow-up (7 samples). One sample from the generalized LOMG group and 9 samples from the ocular LOMG group had hemolysis and were thus excluded from further analysis. Demographics of the 73 LOMG patients in the validation cohort, including antibody status, are shown in Table 2. The median duration of MG was 2.3 (range 0–24) months at recruitment and only 11 patients were on immunosuppressive treatment in the beginning of the study. All 11 patients who had started immunosuppression prior to recruitment blood sampling had received < 4 weeks of treatment (intravenous immunoglobulin, prednisolone, azathioprine).

3.3. Prospective circulating miRNA analysis in generalized LOMG in correlation with clinical MG score

The MGC score declined significantly from recruitment to the 1-year follow-up (7.8 ± 1.4, p = 0.047). Further, miR-21-5p levels decreased significantly at 1-year follow-up (12.5 ± 1.3 to 11.9 ± 1.1, p = 0.0002) but there was then no subsequent increase at the 2-year follow-up (12.5 ± 1.4, p = 0.05). A similar pattern was found for miR-30e-5p (9.5 ± 1.1 to 8.7 ± 1.2, p < 0.0001; to 9.1 ± 1.8, p = 0.29) (Fig. 1; Supplementary Fig. 2), although not with the other analyzed miRNAs. Among patients who started taking immunosuppressants after recruitment (N = 20) or who were already on immunosuppressive treatment at baseline but increased immunosuppression (N = 11), miR-150-5p, miR-21-5p and miR-30e-5p decreased significantly at 1-year follow-up (Fig. 2A), whereas no change was seen at 2 years follow up (N = 10 available for follow-up). For patients who were not on immunosuppressive treatment, either at recruitment or during follow-up, (N = 20), miR-30e-5p, miR-21-5p and miR-19b-3p were reduced at 1-year follow-up (Fig. 2B), whereas no change was seen for miR-150-5p or other miRNAs (data not shown).

Further subgroup analysis revealed that the reduction of miR-150-5p, miR-21-5p and miR-30e-5p levels correlated positively with MGC only among male LOMG patients (p = 0.03, R = 0.25; p = 0.01, R = 0.30 and p = 0.01, R = 0.30 respectively), which was not seen for male LOMG patients.
female LOMG patients. As the treatment was initiated or optimized, the correlation between these variables disappeared as immunosuppressive treatment caused reduction of the miRNA levels. Further, we did not find any correlation between current age (mean 70.3 ± 9.4) or AChR antibody titer and differentially expressed miRNAs.

3.4. Longitudinal analysis of circulating miRNAs in ocular LOMG

The MGC score decreased significantly in ocular LOMG patients at the 1-year follow-up (Table 2, p = 0.02), without further change at the 2-year follow-up (p = 0.51). Further, among ocular LOMG patients, no correlation with all miRNA levels and MGC score and age was detected. Only miR-140-5p had the longitudinal pattern of reduction from baseline to the 1-year follow-up (6.5 ± 1.6 to 5.6 ± 1.2, p = 0.03) and thereafter increasing, although not significant (p = 0.12).

3.5. Comparison of patients with generalized and ocular LOMG

In analyzing patients with either generalized or ocular LOMG, we found that only miR-150-5p and miR-21-5p correlated positively with age (R = 0.25; p = 0.04 and R = 0.34; p = 0.003, respectively). In addition, miR-150-5p, miR-21-5p and miR-30e-5p also correlated positively with the MGC score in the entire LOMG cohort (Fig. 3). Still, further analysis elucidated that the correlation was mainly derived from the male ocular LOMG subgroup, probably since the number of male LOMG patients was much higher.

Finally, patients with generalized LOMG had higher levels of miR150-5p (p < 0.0001) and miR-21-5p (p = 0.0007) (Fig. 4) than those with ocular LOMG.

4. Discussion

Research on circulating miRNAs in the LOMG subgroup, especially longitudinal studies, is lacking. The current study is the first to longitudinally investigate circulating miRNAs in the sera of LOMG patients, recruited at the time of MG diagnosis. We analyzed 6 miRNAs in generalized and ocular LOMG patients that have been described in conjunction with different autoimmune diseases. MiR-150-5p and miR-21-5p, that we added into the analysis as important MG-associated biomarkers (Punga et al., 2015, 2014), are considered crucial regulators in T cell processes (Kroesen et al., 2015) since they are upregulated with T cell maturation and differentiation. MiR-21-5p is expressed at higher levels in T regulatory cells, which control the autoimmune response and inhibit autoimmunity (Kroesen et al., 2015). This miRNA has previously been reported as associated with other autoimmune disorders (SLE, MG, DM I) (Chen et al., 2016; Hu and O’Connell, 2013). The third miRNA that we analyzed in this subgroup was miR-30e-5p, which was previously found to be reduced among EOMG patients in comparisons to healthy controls (Punga et al., 2014). Another previous report found miR-30e-5p, miR-92a-3p and miR-223-3p in plasma to be altered in SLE patients (Kim et al., 2016). Further, the importance of miR-140-5p, miR-19b-3p and miR-223-5p has been mentioned in connection with other autoimmune diseases (Chen et al., 2016).

We started the study by analyzing six circulating miRNAs in LOMG patients. Interestingly, we found that in addition to the previously detected miR-150-5p and miR-21-5p; miR-30e-5p levels also decreased significantly at the 1-year follow-up in parallel with improved MGC score. It is crucial to note that as immunosuppressive treatment was initiated after recruitment of most MG patients in this study, many patients were on immunosuppressive treatment at the 1-year follow-up and the MGC score was considerably reduced at that time point. The change in pattern that we found with some miRNAs, in particular miR-150-5p and miR-21-5p, is thus likely due commencement of immunosuppression (Punga et al., 2015). These results, including the reduction of miR-150-5p after immunosuppressive treatment, are in support of a very recent study (Molin et al., 2018), where reduced levels of miR-150-5p longitudinally accompanied the beneficial clinical effect of thymectomy in MG. Another aspect noted for these particular miRNAs was that sex seems to influence the miRNA profile differently during the course of LOMG. We initiated follow-up of most MG patients in this study, many patients were on immunosuppressive treatment at the 1-year follow-up and the MGC score was considerably reduced at that point. The change in pattern that we found with some miRNAs, in particular miR-150-5p and miR-21-5p, is thus likely due commencement of immunosuppression (Punga et al., 2015). These results, including the reduction of miR-150-5p after immunosuppressive treatment, are in support of a very recent study (Molin et al., 2018), where reduced levels of miR-150-5p longitudinally accompanied the beneficial clinical effect of thymectomy in MG. Another aspect noted for these particular miRNAs was that sex seems to influence the miRNA profile differently during the course of LOMG. In fact, miR-21-5p was significantly reduced in male LOMG patients after 1 year, whereas miR-150-5p was significantly reduced in female LOMG patients. Intriguingly, miR-30e-5p was reduced in patients who started immunosuppression, however a significant reduction was also noted in patients with no immunosuppression. This possibly suggests different mechanisms behind
the miRNA profile of circulating miRNAs. The older patients in our study most likely had atrophic thymuses for which thymectomy has not been considered beneficial (Weis et al., 2018). Only 5% of patients underwent thymectomy (thymoma removed in two patients) and therefore no conclusion could be drawn on the relation between thymus status and circulating miRNA levels in the LOMG cohort. This initial decline in miRNA levels at the 1-year follow-up was followed by a slight, although not significant, increase in miRNA levels at 2-year follow-up. A possible explanation for this became apparent when separating the analysis into patients who were immunosuppression naïve and those treated with immunosuppression before recruitment. LOMG patients on immunosuppressive treatment demonstrated the same decline in miRNAs level at both the 1-year and 2-year follow-up, without the subsequent increase that was discovered among patients who were immunosuppression naïve. Therefore, the cause of the latter increase that we noticed in the entire group likely depends on the group of patients without previous immunosuppression. Contrary to previous studies with disease duration up to 19 years (Punga et al., 2015), our patients without previous immunosuppression naïve. This enabled us to re-examine the situation of miRNAs in the beginning of the disease and then in the disease course in relation to clinical disease severity. However, the number of samples at the 2-year follow-up was relatively low, and thus, the increase in miRNA levels found at this time point needs to be interpreted with caution.

In conclusion, we found a specific pattern of miRNA expression in the subgroup of LOMG patients. MiR-150-5p and miR-21-5p were the two miRNAs that correlated with age and were higher among patients with generalized LOMG in comparison to ocular LOMG. Additionally, miR-30e-5p had a very distinct elevated pattern in LOMG compared to generalized LOMG. The positive correlation between miR-150-5p, miR-21-5p and miR-30e-5p with MGC score, in particular among male LOMG patients, strengthen the potential role of these miRNAs as biomarkers in follow-up of the clinical course in MG.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2018.05.003.

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