

RESEARCH ARTICLE

miR-30e-5p as predictor of generalization in ocular myasthenia gravis

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Abstract

Objective: To determine a predictive factor for the risk of conversion from ocular myasthenia gravis (OMG) to generalized MG (GMG) in a prospective study. **Methods:** RNA was isolated from serum samples and detection of micro-RNA (miRNA) expression analyzed with qPCR. In the discovery set, 179 human miRNAs were assayed for profiling of five OMG patients and four age- and gender-matched healthy controls. Based on the specific accumulation pattern of 19 miRNAs from the discovery set, in addition to miRNAs previously found elevated in generalized MG (GMG; miR-150-5p and miR-30e-5p), 21 miRNAs were subsequently analyzed in a validation cohort of 83 OMG patients (82 immunosuppression treatment naive; 49 male) within 3 months of diagnosis and at a follow-up visit (median duration 28 months from first visit). **Results:** Thirteen patients generalized 14.8 ± 12.0 months after the diagnosis and the majority (85%) belonged to the late onset MG group. Two miRNAs were significantly higher in secondary GMG (SGMG) patients compared to OMG patients with late onset MG: miR-30e-5p (9.1 ± 0.5 vs. 6.3 ± 0.9; $P < 0.0001$) and miR-150-5p (7.4 ± 1.1 vs. 6.4 ± 1.1; $P = 0.01$). The sensitivity for miR-30e-5p in differentiating OMG and SGMG was 96% in all OMG patients and 100% in late onset OMG patients. **Interpretation:** This is the first study to describe a potential predictive factor associated with the risk of generalization for patients with OMG. Raised levels (>8) of miR-30e-5p at initial presentation in patients with ocular MG symptoms, give a predictive cut-off for subsequent generalization of 96–100%.

Introduction

Myasthenia gravis (MG) is an autoimmune neuromuscular transmission disorder that causes fatigable skeletal muscle weakness. The prevalence ranges from 15 to 230 per million population^{1,2} and is increasing, in part likely owing to improved diagnosis. MG is commonly divided into clinical subgroups that are determined by for example age, that is, early onset MG (EOMG; ≤50 years of age) versus late onset MG (LOMG; >50 years of age) or antibody subtype.^{3,4} Furthermore, MG in all age groups (both EOMG and LOMG) can be divided according to clinical manifestations and muscle involved; mainly ocular MG (OMG) versus generalized MG (GMG).⁵ In

approximately half of MG patients, the presenting symptoms are purely ocular (ptosis and/or diplopia). Furthermore, involvement of the extraocular muscles occurs in the majority of patients at some point during their disease even when the onset involves other muscle groups.^{6,7} The diagnosis of OMG may pose difficulties, as approximately 50% are seronegative for antibodies directed against the acetylcholine receptor (AChR) or muscle specific tyrosine kinase (MuSK) and the clinical signs might initially be mild and difficult to detect.^{8,9} Previous retrospective studies reported that up to 80% of patients with purely ocular symptoms at onset develop secondary generalized MG (SGMG),^{5,7,8} most <2 years from disease onset, and hence those patients with pure ocular symptoms ≥2 years most

often remain as OMG, in particular older male patients.^{7,8} Previous studies have found subclinical abnormal neuromuscular transmission (increased jitter) also in limb muscles of patients with OMG¹⁰; however, predictive factors for the risk of conversion from OMG to SGMG have not been established, in part due to the retrospective nature of previous studies. Nevertheless, patients who are AChR antibody seropositive (AChR+) are likely to be at higher risk for conversion from OMG to SGMG¹¹ than AChR antibody seronegative (AChR-) patients.

MicroRNAs (miRNAs) are small noncoding RNAs that have been postulated as potential diagnostic biomarkers of different autoimmune disorders including systemic lupus erythematosus, primary Sjögren's syndrome, systemic sclerosis, rheumatoid arthritis, and psoriasis.^{12,13} The first reports of miRNAs in MG emerged in 2012.¹⁴ Subsequent studies have presented a miRNA profiles in serum samples^{15–22} or peripheral blood mononuclear cells (PBMC)^{23–25} from MG patients. Punga et al. described distinct miRNA profiles in the subgroups of AChR+ MG and MuSK+ MG^{17–19} as well as recently also in LOMG.²⁰ Furthermore, miR-20b was found decreased in the serum of MG patients in a Chinese cohort and¹⁵ in another MG patient cohort with various clinical manifestations a set of seven miRNAs (miR-15b, miR-122, miR-140-3p, miR-185, miR-192, miR-20b, and miR-885-5p) were significantly lower in patients compared with healthy controls.¹⁶ Studies on the prognostic values of miRNAs have been performed predominantly within the oncology field thus far.²⁶ In MG, the miRNAs miRNA-150-5p and miRNA-21-5p are dysregulated among GMG patients with EOMG,¹⁸ although studies in OMG patients are lacking.

Serum antibodies and electrophysiology are important diagnostic biomarkers in MG diagnosis but do not correlate with the disease severity and are impractical in use as outcome measures.^{22,27} Therefore, the aim of the current study was to determine the circulating miRNA profile in serum among patients with OMG in order to identify candidate miRNAs that would serve as potential predictors of disease generalization.

Methods

Subjects

Between August 2014 and February 2017, 96 consecutive patients with a new diagnosis of OMG were prospectively recruited from UK centers (London, Nottingham, Birmingham and Oxford). For the discovery cohort, five OMG patients were selected and four age- and gender-matched healthy controls (HCs) at Uppsala University Hospital were used as controls in this set

(Table 1). All patients and controls were White European. Patient demographic details are shown in Table 2. For the validation cohort, the remaining patients were included.

Ocular myasthenia gravis was diagnosed based on subjective and objective clinical ocular manifestations of ptosis and/or diplopia, antibody detection, pharmacological, and/or electromyography test. The diagnosis of ocular myasthenia was made if the patient had classical ocular symptoms and signs of myasthenia (ptosis and/or diplopia) for at least 3 months, in the absence of an alternative diagnosis. Generalized MG symptoms were excluded at onset by clinical neurological examination. Most patients were AChR antibody positive, either in a radioimmunoprecipitation assay (RIA), or cell-based assay. The minority of patients who were antibody negative had either abnormal Single Fibre EMG studies, indicating disturbed neuromuscular transmission, or in a small number of cases, a positive response to treatment with acetylcholinesterase inhibitors.⁵ Patients with a confirmed diagnosis of MG were eligible if symptoms had been confined to the extraocular muscles for at least 3 months since symptom onset (OMG phenotype). As former studies have indicated, most OMG patients who develop GMG do so during the first 2 years, therefore all the patients were followed up for at least 2 years before classifying them as pure OMG or SGMG patients.

The study was approved by NRES Committee West Midlands – South Birmingham (12/WM/0414) and London (15/LO/0943) and the analysis of serum samples was approved by the Uppsala ethical standards committee on human experimentation (Dnr 2010/446/2). All patients provided signed, informed consent. Serum samples were taken and stored at -80°C , and myasthenia gravis composite (MGC) scores were recorded at recruitment and at each subsequent 12-month follow-up visit.

Circulating miRNA isolation

Blood samples were collected in tubes without any additives, stored at room temperature at least 20 min and centrifuged at room temperature at 1200 g 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 g for 5 min and RNA isolation was done with miRCURYTMRNA Isolation Kit-Biofluids (Exiqon #300112, Vedbaek, Denmark) according to the manufacturer's instructions. For serum cDNA synthesis, the Universal cDNA Synthesis Kit II (Exiqon #203301, Vedbaek, Denmark) and 2 μL of isolated RNA was used for cDNA synthesis in 10 μL reaction mix.

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

	Age at diagnosis	Sex	Race	Disease duration at recruitment (months)	Serotype	MCG score	Treatment	Thymoma
Patient 1	43	M	Caucasian	0.4	Clustered AChR+	7	Pyridostigmine 60 mg × 3	No
Patient 2	64	F	Caucasian	1	AChR+	0	Pyridostigmine 60 mg × 3	No
Patient 3	61	M	Caucasian	1.5	AChR+	6	Pyridostigmine 30-30-60 mg	No
Patient 4	42	M	Caucasian	12	AChR+	1	Pyridostigmine 60 mg × 4	Yes
Patient 5	52	M	Caucasian	1.3	AChR+	0	Pyridostigmine 90-90-90-60 mg	Yes
Control 1	43	M	Caucasian	N/A	N/A	N/A	N/A	N/A
Control 2	66	F	Caucasian	N/A	N/A	N/A	N/A	N/A
Control 3	60	M	Caucasian	N/A	N/A	N/A	N/A	N/A
Control 4	52	M	Caucasian	N/A	N/A	N/A	N/A	N/A

miRNA expression analysis

We performed reverse transcription (RT) followed by real-time quantitative PCR using ExiLent SYBR[®] Green master mix (Exiqon #203421, Vedbaek, Denmark). MicroRNA expression was analyzed using the Serum/Plasma Focus microRNA PCR Panel (V4.M, Exiqon, Vedbaek, Denmark), containing primer sets of 179 human miRNAs, in the discovery cohort.

Thirteen selected primers detected from the discovery cohort were added to the PCR plates. The cDNA templates were diluted 50× in nuclease-free water before applying them to the 384-well PCR panel plates. We used control assays that were available in the miRNA qPCR panels: interplate calibration with UniSp3, quality of RNA isolation with UniSp2 and UniSp4, and cDNA synthesis control with UniSp6.

Cellular miRNA contamination was controlled in order to exclude hemolysis during the serum sample preparation. ΔCq (miR-23a–miR-451a) <7 represented non-hemolyzed samples. The reference candidates in the study were miR-103a-3p, miR-191-3p, miR-93-3p, and miR-423-4p. Comparative CT method was used to quantify miRNA using the formula $2^{-\Delta\Delta CT}$ where $\Delta CT = (CT \text{ gene of interest} - CT \text{ reference gene})$. Normalization was performed to miR-191-5p as other normalizing genes were not abundantly detected in all samples²⁸ and since it was recommended as normalizing gene by GenEx. Initial miRNA detection experiments that were performed on the Serum/Plasma Focus microRNA PCR Panel profiled the genes of interest from 179 different miRNAs. From these 30 genes that were significantly different among OMG patients compared to the HCs (Fig. 1), 12 were selected for further analysis based on earlier research in connection with miRNAs and autoimmunity. Specifically referring to previous data obtained with miR150-5p^{17,18} and miR-30e-5p in AChR+ EOMG and LOMG that also included OMG patients,²⁰ these two miRNAs were added to the list of the genes of interest in the validation cohort.

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 was performed, comparing OMG and control groups with the null hypothesis that the mean values of the different miRNAs were the same across OMG and control categories. Mann–Whitney *U* test was performed in case of nonparametric data. In the discovery set, candidate miRNAs were selected if they were found to be significantly different expressed in the OMG versus control group. In addition to those differentially expressed, only miRNAs that were found to be up- or downregulated in each individual patient were selected for the validation set analysis. The null hypothesis for the predictive sensitivity of the dysregulated miRNAs, using ROC curve, indicates a true area of 0.5. Spearman Rank correlation was performed to analyze correlation between continuous factors (age, disease duration, disease severity) and the miRNAs tested. Statistical significance was defined as $P < 0.05$.

Results

Specific circulating miRNA profile in the OMG discovery cohort

In the discovery cohort of five OMG patients and four sex- and age-matched healthy controls (Table 1), no hemolysis was detected. All the discovery cohort patients remained ocular for more than 2 years after diagnosis (from 2.5 to 4.8 years) and median followed-up time was median 52.5 months (IQR 30.2–59.0 months). Thirty miRNAs were strongly elevated in the OMG cohort (Table S1) and of these, 19 miRNAs, in addition to miR-150-5p and miR-30e-5p, were further analyzed by qRT-PCR in sera from a larger UK validation cohort of OMG patients.

Table 2. Demographic data of myasthenia gravis (MG) patients in the final validation cohort (including only samples that did not have hemolysis).

	N = 83
Age at MG onset (years; mean \pm SD)	54.5 \pm 15.2
Duration of MG symptoms (months; median; IQR)	42.6 (32.1–52.8)
Gender N (%)	
Male	49 (59%)
Female	34 (41%)
Subgroups at study onset N (%)	
EOMG	32 (38.6%)
LOMG	51 (61.4%)
Subgroups at last follow-up N (%)	
OMG	70 (84.3%)
SGMG	13 (15.7%)
Antibody subgroup N (%)	
AChR+	57 (68.7%)
RIA	52 (91.2%)
CBA	5 (8.8%)
MuSK+	8 (9.6%)
RIA	0 (0%)
CBA	8 (100%)
LRP4+	2 (2.4%)
CBA	2 (100%)
Seronegative	16 (19.3%)
Initial MGC score (mean \pm SD)	3.7 \pm 2.7
Prednisone treatment N (%)	1 (1.2%)

IQR, interquartile range; EOMG, early onset MG; LOMG, late onset MG; OMG, ocular MG; SGMG, secondary generalized MG, AChR+, acetylcholine receptor antibody positive; RIA, radioimmunoassay; CBA, cell-based assay; MuSK, muscle specific tyrosine kinase; LRP4, lipoprotein receptor-related protein 4; seronegative, no detectable serum antibodies against AChR, MuSK or LRP4; MGC, MG Composite score. Duration of MG symptoms, time from first symptom to most recent follow-up.

Characteristics of the prospective validation cohort of OMG

In the validation cohort, the hemolysis quote was >7 in 12 samples and these were thus excluded from further analysis. The final validation cohort therefore consisted of 83 OMG patients (49 men and 34 women; Table 2). All patients had purely ocular symptoms within 3 months from the diagnosis. Thirteen patients generalized 14.8 ± 12.0 months (range 3–38.3) after the diagnosis and the majority (85%) belonged to the LOMG group. Initially, 68.7% of OMG patients were AChR+, 9.6% were MuSK+ and the remaining 19.3% were AChR–/MuSK–. Almost all (92%) patients whose disease generalized during the follow-up were AChR+ and one patient was MuSK+. Ninety-nine percent of patients were treatment-naïve at the time of sampling; one patient was given prednisolone before the blood sample was drawn,

however it was stopped 1 week before blood sampling. None of the patients that generalized had taken prednisone before the disease generalized. Five patients had thymectomy during follow-up (thymic hyperplasia in two, thymoma in two, and atrophic thymus in one). The mean MGC score at recruitment was 3.7 ± 2.7 , being significantly higher among patients whose disease generalized (5.5 ± 3.4 vs. 3.3 ± 2.5 , $P = 0.009$). Median duration of follow-up from first symptoms was 47.4 months with interquartile range (IQR) 34.0–62.4.

Median duration of symptoms had been shorter in SGMG patients before they were recruited (5.3 (IQR 4.2; 8.6) vs. 12.0 (5.5; 24) months, $P = 0.02$). Still, all the SGMG patients had only ocular symptoms at the recruitment.

Elevated circulating miRNA miR-30e-5p identifies OMG patients at risk for generalization

In comparing circulating miRNA levels among the entire patient cohort with OMG and SGMG, miR-30e-5p levels were significantly higher among SGMG patients (9.0 ± 0.5) than among OMG patients (6.5 ± 1.4 ; $P < 0.0001$; Fig. 2A). None of the remaining miRNAs showed significant difference between OMG and SGMG patients. Subgroup analysis in LOMG patients ($N = 50$) revealed that two miRNAs significantly differed between OMG and SGMG patients (Table 3), miR-30e-5p (6.3 ± 0.9 vs. 9.1 ± 0.5 ; $P < 0.0001$; Fig. 2B) and miR-150-5p (6.4 ± 1.1 vs. 7.4 ± 1.1 ; $P = 0.01$; Fig. 2C). The area under the ROC curve for miR-30e-5p was 0.96 (95% CI: 0.92–1.0; $P < 0.0001$; Fig. 2D) for all OMG patients and 1.0 for the LOMG cohort (95% CI: 1.0–1.0; $P < 0.0001$; Fig. 2E). Therefore, based on the different IQR values and the ROC curve, the predictive value for all OMG patients to generalize was 96% and for LOMG patients 100% for a cutoff >8 for miR-30e-5p, whereas no particular cut-off was identified for miR-150-5p due to overlap between the groups. For patients who had positive AChR ab on RIA, there was no difference in AChR ab titers between OMG ($N = 23$) and SGMG ($N = 10$) patients (124.6 ± 18.1 vs. 62.6 ± 27.2 ; $P = 0.07$); thus, we found no predictive value for generalization regarding ab titer (Figure S1).

We did not find any correlation between miRNAs and any other clinical features, such as age, MGC score or disease duration ($P > 0.05$ for all).

Discussion

Depending on the distribution of fatigable skeletal muscle weakness in relation to extraocular muscles, MG can be

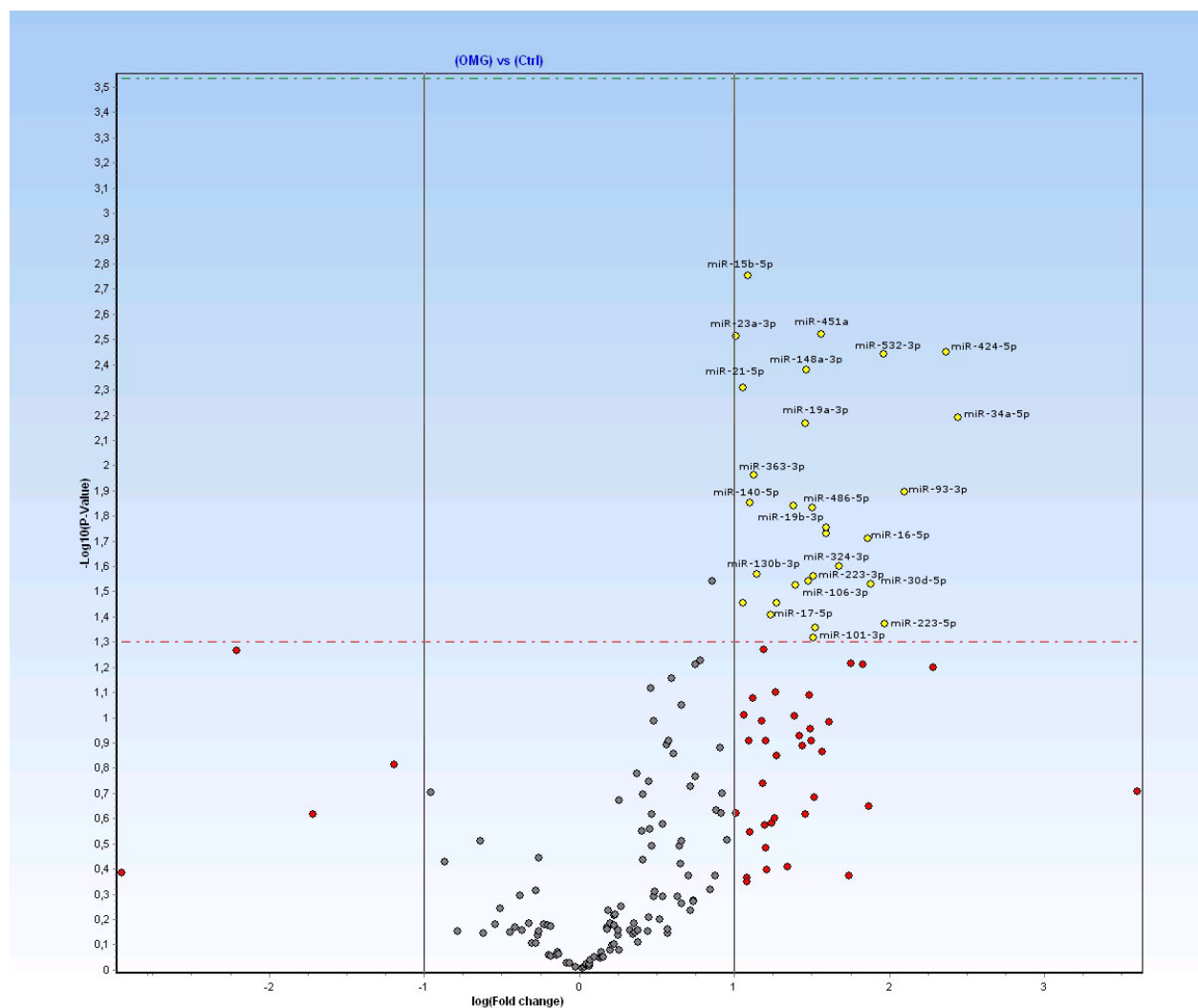


Figure 1. Volcano plot of the differentially expressed microRNAs (miRNAs) in the discovery cohort of ocular myasthenia gravis (OMG) patients compared to healthy controls (HC). The significantly altered miRNAs in OMG compared to healthy HC are indicated.

classified into the subgroups OMG or GMG. The number of patients converting from OMG to GMG varies widely, between 30% and 80%, in retrospective studies.^{6,29} This large range likely reflects the heterogeneity of patient groups and the retrospective nature of the studies. The patients in this cohort were recruited prospectively at around the time of diagnosis, and followed up for 2 years or more. Many OMG patients in this cohort were identified early due to the clinical set up in London, where patients with ocular symptoms self-present to the Eye Emergency Department and are then referred onwards to the Neuro-Ophthalmology department.

To the best of our knowledge, this is the first study that has identified a potential predictive biomarker that

can predict which OMG patients will generalize. Prognostic markers to stratify patients into high- or low-risk of conversion from OMG to GMG are important and may even guide future therapeutic decisions.³⁰ This is particularly relevant clinically as some retrospective studies have raised the possibility that starting treatment with immunosuppression early may alter the risk of conversion from OMG to GMG.^{9,30} None of the patients who generalized were on prednisone treatment. Our study provides evidence that miR-30e-5p could serve as a potential predictive biomarker for the future conversion from OMG to GMG, at least in LOMG. The risk of generalization was 100% in OMG cases with levels of miR-30e-5p >8 shortly after disease onset. This is in line with a very

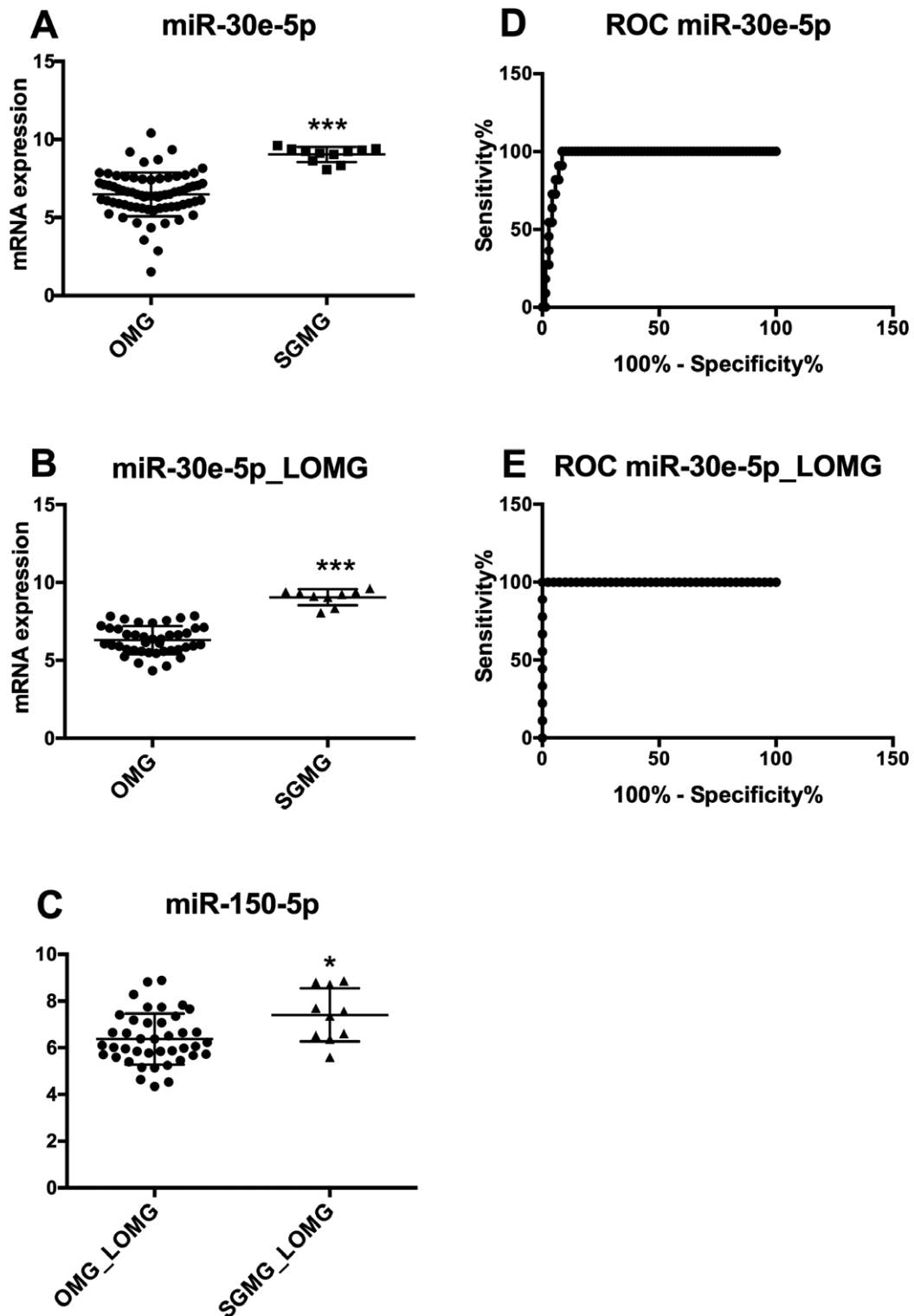


Figure 2. Relative mRNA expression of differently altered miRNAs miR-30e-5p in all OMG patients (A). Levels of miR-30e-5p (B) and miR-150-5p (C) in late-onset OMG patients were analyzed with unpaired *T* test between the OMG and SGMG groups. ROC curves of miR-30e-5p, indicating sensitivity for OMG versus SGMG in the entire cohort (D) and in the late onset MG (LOMG) cohort (E). OMG, ocular myasthenia gravis; SGMG, secondary generalized myasthenia gravis. * $P < 0.05$; *** $P < 0.001$.

Table 3. Mean circulating longitudinal miRNAs levels among ocular and secondary generalized LOMG patients.

	OMG (N = 40)	SGMG (11)	P value
hsa-miR-15b-5p	5.9 ± 1.9	5.8 ± 1.4	0.93
hsa-miR-21-5p	11.3 ± 1.3	11.6 ± 0.9	0.40
hsa-miR-424-5p	8.2 ± 1.8	7.3 ± 1.0	0.11
hsa-miR-532-3p	4.0 ± 1.6	3.8 ± 0.9	0.75
hsa-miR-148a-3p	7.6 ± 1.4	7.8 ± 1.1	0.71
hsa-miR-34a-5p	3.2 ± 1.9	3.5 ± 3.1	0.83
hsa-miR-19a-3p	8.7 ± 1.7	8.6 ± 1.0	0.86
hsa-miR-19b-3p	10.7 ± 1.7	11.3 ± 1.3	0.30
hsa-miR-140-5p	5.6 ± 1.9	5.2 ± 1.5	0.64
hsa-miR-30e-5p	6.3 ± 0.9	9.1 ± 0.5	<0.0001
hsa-miR-223-3p	13.0 ± 1.5	12.6 ± 2.3	0.58
hsa-miR-150-5p	6.4 ± 1.1	7.4 ± 1.1	0.01
hsa-miR-223-5p	3.6 ± 1.8	3.1 ± 0.7	0.47

OMG, ocular myasthenia gravis; SGMG, secondary generalized myasthenia gravis; LOMG, late-onset myasthenia gravis. P-values in bold indicate significantly different miRNAs between the groups.

recent study that indicated miR-30e-5p as a potential biomarker in LOMG.²⁰ This particular miRNA has previously been found downregulated in patients with AChR+ EOMG patients compared to healthy controls.¹⁷ Consequently, one reason as to why miR-30e-5p was not significantly elevated in the OMG discovery cohort compared to the healthy controls could be that two of these OMG patients had EOMG and not LOMG, as was the case for the majority of the validation cohort.

In the previously analyzed cohort of LOMG patients, miR-30e-5p levels were not significantly different between SGMG and OMG patients. Here, we found that miR-30e-5p levels significantly differed between OMG and SGMG patients in the LOMG cohort. One reason for this could be that patients who rapidly develop GMG have a different pathogenesis of MG from those that remain with OMG.⁹ Furthermore, the SGMG patients in our previous study were generalized already at recruitment and were longitudinally compared to OMG patients.²⁰

Intriguingly, the low-density lipoprotein receptor-related protein 6 (LRP6), one of the critical co-receptors for Wnts (a family of genes that encode secretory glycoproteins), is a direct target of miR-30e.³¹ The Wnt signaling pathway is involved in development, proliferation, differentiation, adhesion and cellular polarity. Importantly, the canonical Wnt/ β -catenin signaling can regulate multiple steps of myogenesis, including cell proliferation as well as myoblast fusion and homeostasis.³² In a transcriptome analysis of pig skeletal muscles, miR-30e-5p was associated with the muscle protein PPARGC1A that plays an important role in mitochondrial biogenesis.³³

Although the exact role of miR-30e-5p in humans needs further elaboration, elevated levels of miR-30e-5p could hypothetically reduce the expression of important proteins involved in maintaining skeletal muscle homeostasis and metabolism.

In general, at least 3 months duration of isolated ocular symptoms is considered a reasonable time period to diagnose OMG.^{7,9} Nevertheless, the question to be asked is whether there is a window of inhibiting generalization. Considering that there is the potential to modify the progression to GMG with corticosteroids³⁴ and SGMG develops in up to 50% of OMG patients within 1 year,³⁵ how would one know the best intervention strategy? The suggestion for a minimum of 3 months to define patients as OMG was proposed in 1989 by Oosterhuis.³⁶ Therefore, we included patients whose ocular symptoms had extended at least 3 months or who did not generalize earlier than 3 months. Most of the patients who generalized (~90%) during the follow-up period were AChR+, and thus our study supported previous findings of older age at onset as well as AChR antibody seropositivity as factors associated with a greater likelihood of generalization.^{9,37} Nevertheless, we did not find any cut-off regarding AChR ab titer in the SGMG group compared to the OMG group. In addition, we also found 10% MuSK+ OMG patients, although in contrast to Evoli et al.³⁸ only one patient progressed to secondary GMG during the follow-up. We did not find a difference in sex regarding generalization frequency in our study.

Both miR-150-5p and miR-21-5p are considered to be important regulators of T-cells and are upregulated with T-cell maturation and differentiation.³⁹ MiR-150-5p was significantly lower among OMG patients in comparison to the patients who developed GMG, whereas no such difference was found for miR-21-5p.

One limiting factor of the study was the lack of correlation between miRNA levels and MGC scores. This may have been due to the delay between recruitment and blood sampling of >3 months in 9/45 (20%) patients in the London cohort. Also, the percentage of OMG patients generalizing was relatively low. Nevertheless, this was a true finding in that this was a prospective study of unselected OMG patients, almost all of whom had been followed up for 2 years or more to detect signs of generalization. The samples were taken when patients had only ocular symptoms, so the differences in miRNAs could be regarded as indeed prognostic.

In conclusion, this is the first study that found a potential predictor of which patients who develop GMG from OMG. We propose miR-30e-5p as this predictor, with 96% sensitivity in all OMG patients and 100% sensitivity in late onset OMG patients.

Acknowledgments

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Conflict of Interest

None declared.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Acetylcholine receptor antibody (AChR ab) titer as measured by radioimmunoprecipitation assay in nM. OMG, ocular myasthenia gravis; SGMG, secondary generalized myasthenia gravis.

Table S1. Differentially expressed miRNAs in ocular MG discovery set. Significantly different microRNAs (in bold) were selected for further analysis in validation set ($P < 0.05$).