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Disease specific enrichment of circulating let-7 family microRNA in MuSK + myasthenia gravis

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ABSTRACT

Myasthenia gravis (MG) patients with antibodies against the muscle specific tyrosine kinase (MuSK+) have predominantly involvement of cranio-bulbar muscles and do not display thymus pathology, as do acetylcholine receptor antibody seropositive (AChR+) MG patients. In search of novel biomarkers for MuSK+ MG, we evaluated circulating serum microRNAs. Four analyzed microRNAs were specifically elevated in MuSK+ MG patient serum samples: let-7a-5p, let-7f-5p, miR-151a-3p and miR-423-5p. The circulating microRNA profile in MuSK+ MG differs from the profile previously observed in the serum of AChR+ MG, thus indicating the etiological difference between these two entities. We propose that the identified microRNAs could serve as potential serum biomarkers for MuSK+ MG.

1. Introduction

Myasthenia gravis (MG) is a chronic autoimmune disorder, caused by an antibody-mediated attack against proteins of the neuromuscular junction (Berrih-Aknin, 2014). In a subtype of MG, antibodies (abs) target the postsynaptic muscle specific tyrosine kinase (MuSK+). MuSK+ MG, that represents approximately 5–7% of the MG spectrum, appears to be more common in Mediterranean countries and is predominantly characterized by involvement of cranio-bulbar muscles and an increased rate of muscle wasting (Evoli et al., 2003). MuSK abs differentiate themselves in that they are mainly of the IgG4 subtype compared to the more common form of MG with predominantly IgG1 subtype abs against the acetylcholine receptor (AChR+). The MuSK IgG4 abs inhibit agrin-dependent MuSK activation by interfering with Lrp4-MuSK binding, and this is considered one of the key effector mechanisms of the MuSK abs (Huijbers et al., 2013; Koneczny et al., 2013). The MuSK IgG4 abs vary from other IgG antibodies in various functional aspects; for example, IgG4 abs do not activate the complement system. Still, prominent B-cell pathology, that has been described in AChR+ MG, was recently reported in MuSK+ MG (Guptill et al., 2015).

MuSK abs are very specific and their detection in patients with objective signs of skeletal muscle fatigue and disturbed neuromuscular transmission confirms MG diagnosis. Furthermore, serum concentration of MuSK abs decreases upon immunosuppression and correlates with disease severity (Bartoccioni et al., 2006). Nevertheless, the MuSK ab titer does not necessarily predict the course of the disease and thus more reliable biomarkers are needed for individual treatment and clinical trials (Kaminski et al., 2012; Meriggioli and Sanders, 2012).

Mammalian microRNAs (miRNAs) are important small non-coding regulatory RNAs that impede gene expression by blocking translation of their target messenger RNAs (mRNAs) (Krol et al., 2010). Quantitative detection of cellular miRNAs can be used to describe disease status, as abnormal presence of certain miRNAs correlates with the pathogenesis of diseases such as cancer and diabetes (Esteller, 2011; Kasinski and Slack, 2011). In addition to their intracellular accumulation, miRNAs can also be released from the cells into the extracellular space. The released miRNAs enter the circulation packed into the exosomes, where the miRNAs are protected from degradation (Zhang et al., 2015). The detection of circulating miRNAs in patient biofluids has been considered a novel approach to detect progression of cardiovascular diseases and malignant growth (Creemers et al., 2012; Etheridge et al., 2011) as well as monitoring the disease state in multiple sclerosis (MS)

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(Gandhi et al., 2013). Recent studies in AChR + MG have identified miR-151-5p and miR-21-5p as disease-specific miRNAs with different accumulation patterns in immunosuppressed and non-immunosuppressed patient sera (Punga et al., 2014, 2015). Since MuSK + MG clinical phenotype is different from AChR + MG, it would be important to analyze the possible involvement of circulating miRNAs in this MG entity.

Thus, the aim of the present study was to identify serum circulating miRNAs specific for MuSK + MG patients.

2. Material and methods

2.1. Subjects

In this study we included 25 patients with MuSK + MG, in different stages of their disease, who were regularly followed at the Neurology Clinic of the Catholic University Hospital, Rome, Italy. At time of diagnosis, all patients had muscle weakness and fatigability, together with evidence of impaired neuromuscular transmission during low-frequency repetitive nerve stimulation and/or single-fiber EMG, and positive serum MuSK ab titers (>0.05 nM/l) in a standard radioimmunoprecipitation assay. Three patients never received any immunosuppressive therapy, 9 patients were treated with prednisone and 13 patients were treated with prednisone and other immunosuppressants; two of these patients received rituximab. At time for blood sampling six patients did not have any ongoing immunosuppression (Table 1). Three patients had undergone thymectomy and in all, histologic examination revealed a thymus that was histopathologically normal for their age. Serum samples were obtained from MG patients and from age and gender matched healthy controls after informed consent. These studies were approved by the local Ethics Committees [Uppsala-Sweden (Dnr. 2010/446) and Rome, Italy (authorization number ID P/529/CE/2011))]. Disease severity was classified according to the Myasthenia Gravis Foundation of America (MGFA) system (Jaretzki et al., 2000); clinical status at the time of the study was graded according to a semi-continuous disease severity score (DSS) (Niks et al., 2008).

The discovery set (N = 10) included five MuSK + MG patients, out of whom three were on immunosuppressive therapy (see Table 1). The discovery set also included five age- and gender-matched healthy Italian controls (HCs).

The validation set (N = 40) consisted of 20 MuSK + MG patients and 20 age-and gender-matched healthy controls. Demographics of the discovery and validation sets are shown in Table 1. All patients and healthy controls were of Italian ethnicity.

2.2. Serum circulating miRNA isolation

Blood samples were collected in tubes without any additives, stored at room temperature for at least 20 min, and then centrifuged at 4000 rpm at 20 °C for 5 min. Total RNA was isolated from 200 μl serum by using a miRCURY™ RNA Isolation Kit-Biofluids (Exiqon #300112), according to the manufacturer's instructions. Two microliters of isolated RNA sample was used for cDNA synthesis in 10 μl reaction mix using the Universal cDNA Synthesis Kit II (Exiqon #203301).

2.3. Serum circulating miRNA expression analysis

2.3.1. miRNA analysis using microRNA PCR panel

Initial miRNA detection experiments were performed on Serum/Plasma Focus microRNA PCR Panel (V4.M) using ExiLENT SYBR® Green master mix (Exiqon #203420) as recommended by the manufacturer. This panel covers the detection primers for 179 human miRNAs and was used for profiling the discovery set of five patients and five age- and gender-matched HCs. The qRT-PCR data from Serum/Plasma Focus microRNA PCR Panels were examined with GenEx software (Exiqon) according to the recommendations from Exiqon. In this process the inter-plate calibration, approved quality controls (RNA-spike-

Table 1
Clinical data on the 25 MuSK antibody seropositive patients assayed. Disease dur (duration) is stated in years from MG diagnosis. M, male; F, female; MGFA; Myasthenia Gravis Foundation of America; IS, immunosuppressive; pred; prednisone; AZA, azathioprine; CyA, cyclosporine; MMF, mycophenolate; RTX; rituximab; *pharmacological remission; #complete stable remission. **Treatments with rituximab were given. N-m failure; examination of neuromuscular transmission failure with neurophysiological methods. RNS, repetitive nerve stimulation; SFEMG, single fiber electromyography. −, normal; +, abnormal neuromuscular transmission; n.p., not performed. HC, healthy control. Current MuSK antibody (ab) titer is stated.

Pat #	Age/sex	Disease dur	Max MGFA grade	IS therapy during disease course	MuSK ab titer (nM)	MG grade	IS now	N-m failure RNS/ SFEMG	HC Age/sex
Discovery set (N = 10)									
1	59F	46	3b	None	4.7	3	No	+/+	59F
2	61F	9	3b	Pred, AZA	3.5	3	Yes	n.p/+	62F
3	39M	8	2b	Pred	0.5	1#	No	n.p/+	39M
4	47F	15	3b	Pred, CyA	6	2	Yes	−/+	50F
5	35F	22	5	Pred, AZA	12.3	3	Yes	+/+	36F
Validation set (N = 40)									
11	81F	10	2b	None	5.3	2	No	−/+	82F
12	59F	1	5	Pred, AZA	3.98	2	Yes	+/+	59F
13	60M	2	2b	Pred	6.4	1*	Yes	−/+	60M
14	64F	16	5	Pred, AZA	5.48	3	Yes	−/+	64F
15	62F	2	2b	None	1.5	2	No	−/+	62F
16	31M	3	3b	Pred	7.19	2	Yes	n.p./+	31M
17	24F	8	3b	Pred, AZA	2.8	2	Yes	−/+	20F
18	48M	18	3b	Pred	3	2	No	−/+	48M
19	28M	2	3b	Pred	0.5	2	Yes	+/n.p	30M
20	43M	5	3b	Pred, AZA, MMF**	8.08	3	Yes	+/+	41M
21	61M	6	3b	Pred, AZA, MMF	0.91	2	Yes	+/n.p.	61M
22	36F	16	3b	Pred	5.9	2	Yes	+/+	36F
23	38F	4	4b	Pred, AZA	0.9	2	Yes	+/+	38F
24	45F	9	5	Pred	0	1*	Yes	+/n.p.	47F
25	42F	4	5	Pred, AZA	39	3	Yes	−/+	43F
26	35M	10	3b	Pred	0.8	1#	No	−/+	34M
27	41F	1	4b	Pred, AZA, CyA, MMF	7.33	3	Yes	−/+	38F
28	34F	5	5	Pred, AZA, CyA**	5.95	3	Yes	+/n.p.	33F
29	27F	15	5	Pred, AZA	0	1*	Yes	+/+	28F
30	21F	1	3b	Pred	0.80	2	Yes	−/+	26F

in) and hemolysis test (see below) were included. Analyzing the suggested candidate genes using the "NormFinder and geNorm" applications available in the GenEx software, we chose reference miRNAs miR-191-5p and miR-103a-3p.

2.3.2. Screening of the individual miRNAs

The qRT-PCR analysis was performed using ExiLENT SYBR® Green master mix (Exiqon #203420) on custom made 384-well Pick-&-Mix microRNA PCR panel plates (Exiqon #203815) pre-coated with validated primer sets to amplify target miRNAs. The cDNA reactions were diluted 100× in ExiLENT SYBR® Green master mix before being applied to the Pick-&-Mix microRNA PCR panel plates. All qRT-PCR reactions were analyzed with the Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies) as described previously (Punga et al., 2014). The following quality controls were included on Pick-&-Mix microRNA PCR panel plates: inter-plate calibration (UniSp3), RNA extraction control (UniSp2 and UniSp4), cDNA synthesis control (UniSp6) and hemolysis test (miR-23a-3p–miR-451a) (all from Exiqon). The ΔCT value of hemolysis markers ($\Delta CT_{(hemolysis)} = CT_{(miR\text{-}23a\text{-}3p)} - CT_{(miR\text{-}451a)}$), was used to detect hemolysis. A ΔCT > 7 in serum samples indicates a high risk of hemolysis and therefore these samples were not used for further analysis.

Normalization of individual miRNA levels was done according to GenEx manual guidelines (Exiqon). Relative quantities for the discovery cohort 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 $2^{-\Delta\Delta CT}$, where ΔCT = (CT gene of interest − CT reference gene) of each sample, using miR-191-5p and miR-103a-3p as the reference miRNA (Schmittgen and Livak, 2008).

2.4. 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. An unpaired two-tailed t-test of independent samples 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 significantly differentially expressed in the MG versus HC group. Exclusively miRNAs that were found to be reduced or elevated in each individual patient compared to his/her matched control were selected for the validation set analysis. Spearman Rank correlation was performed in order to determine the correlation coefficient between disease duration, MuSK ab titer, age and differentially expressed miRNAs. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Circulating miRNA profile in MuSK + MG patient sera in the discovery set (N = 10)

The discovery set included five MuSK + MG patients (mean age: 49 ± 10 years; 4 women) who had mean disease duration of 20 ± 15 years. In this cohort, age of MG onset ranged from 13 to 52 years of age. Three patients were currently on immunosuppressive treatment and two patients (#1 and #5) had undergone thymectomy (Table 1). These patients were age- and gender-matched with five healthy blood donors (mean age: 48 ± 10 years, 4 women) without any diagnosed disease. Patients #1,2,3 and 4 had no concomitant disease, whereas patient #5 had thyroiditis.

The first step was to evaluate the circulating miRNA profile in sera of patients with MuSK + MG. For this purpose, the discovery set samples of five MuSK + MG patients and five age- and gender-matched HCs were analyzed. Importantly, none of the discovery set serum samples showed significant signs of hemolysis (ΔCT < 6.5). Normalization of the miRNA expression was done using the recommended reference miRNAs (miR-191-5p and miR-103a-3p). Out of 179 tested miRNAs, 107 miRNAs showed amplification in more than 60% of the samples and were thus further analyzed. Twelve miRNAs were found to be strongly elevated or reduced in the MuSK + MG patients (Table 2). Out of these candidates, 10 miRNAs had p-values <0.05 after statistical analysis with t-test, visualized in the volcano plot (Fig. 1). These data were supported by principal component analysis (Supplemental Fig. 1), indicating different expression of the 10 miRNAs between MG patients and HC. Clustering of different miRNAs in a heat map analysis was in accordance with these data (Supplemental Fig. 2).

Table 2
Differentially expressed miRNAs in the discovery set. Selection of miRNAs of interest among the 107 miRNA validated from the discovery set of 179 miRNAs. Comparing five MG patients with MuSK antibodies (MuSK+) versus five healthy controls, miRNAs were selected when a p-value <0.05 was found on unpaired two-sided t-test and the miRNAs were elevated in each individual MuSK + MG patient compared to its matched healthy control (bold).

microRNA	Fold change (log) elevated	Fold change (log) reduced	p-Value
hsa-miR-151a-3p	2.63		0.000887
hsa-let-7f-5p	3.76		0.01040
hsa-miR-423-5p	4.30		0.0118
hsa-let-7d-3p	3.68		0.0178
hsa-miR-10b-5p	3.48		0.0247
hsa-miR-376c-3p	3.75		0.0324
hsa-let-7a-5p	2.03		0.0327
hsa-miR-484	3.17		0.0342
hsa-miR-409-3p	4.46		0.0351
hsa-miR-421	2.99		0.0447
hsa-miR-25-3p		1.95	n.s.
hsa-miR-140-3p		1.05	n.s.

3.2. Individual validation of the miRNA expression pattern (N = 40)

The six miRNAs that were found to be significantly altered between all individual MuSK + MG patients and HCs in the discovery set (Table 1) were further analyzed by qRT-PCR in sera from a larger Italian cohort. The validation cohort included 20 MuSK + MG patients (mean age: 44 ± 16 years; 13 women) who had mean disease duration of 7 ± 6 years. Age at disease onset in this MuSK + MG cohort ranged from 13 to 71 years of age. Sixteen MuSK + MG patients were immunosuppressed at the time of sampling and one had undergone

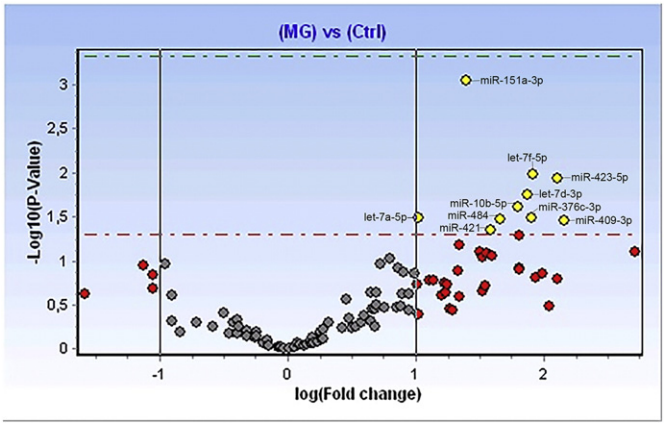

Fig. 1. Volcano plot of the 107 expressed miRNAs in the discovery set (N = 10). The differentially expressed miRNAs with p ≤ 0.05 on t-test (yellow dots; shown in Table 2) were further analyzed in the validation set. All dots under the red line (gray or red) did not differentiate between MuSK + MG patients (MG; N = 5) and healthy controls (Ctrl; N = 5). The Y-axis represents 10 log of the p-value and the X-axis represents log fold change of miRNA expression in the MG versus healthy controls.

thymectomy. Concomitant diseases were observed in five patients. Patient #11 had polycythemia, #21 had cerebral stroke, #26 and #30 had thyroiditis and #27 had cardiomyopathy. The patients were age- and gender matched with 20 HC (mean age: 44 ± 16 years; 13 women).

The haemolysis quote ($\Delta CT_{(haemolysis)} = CT_{(miR\text{-}23a\text{-}3p)} - CT_{(miR\text{-}451a)}$) was <6 in all MG and HC sera; hence all samples were further processed. All selected miRNAs of interest had a stable amplification in all of the individual samples with good expression (CT ≤ 34), except for one MuSK+ MG sample (M17; Table 1) and one healthy control (C41; Table 1), with reference CT > 35, and were thus excluded. Based on these results, we were able to validate four miRNAs that were differentially expressed in MuSK+ MG patients and HCs: miR-151a-3p ($p = 0.0212$; Fig. 2A), let-7a-5p ($p = 0.0464$; Fig. 2B), let-7f-5p ($p = 0.0050$; Fig. 2C) and miR-423-5p ($p = 0.0473$; Fig. 2D), whereas let-7d-3p ($p = 0.115$; Fig. 2E) and miR-409-3p ($p = 0.8234$; Fig. 2F) were not significantly altered in the validation cohort. The ROC curve (area under the curve; AUC) for these validated, deregulated miRNAs displayed the strongest association with MG for miR-151a-3p and miR-423-5p (both AUC of 0.740), followed by let-7f-5p (AUC of 0.726) and let-7a-5p (AUC of 0.659) (Fig. 3). Disease duration among the validation cohort of MuSK+ MG patients ranged from 1 to 18 years (mean 6.9 ± 6.0 years). We found no correlation between disease duration and expression level of any miRNA ($p > 0.05$). There was a correlation between MuSK ab titer and clinical score ($R = 0.67$; $p = 0.002$), however there was no significant correlation between individual miRNAs and MuSK ab titres or between miRNA levels and disease severity (data not shown).

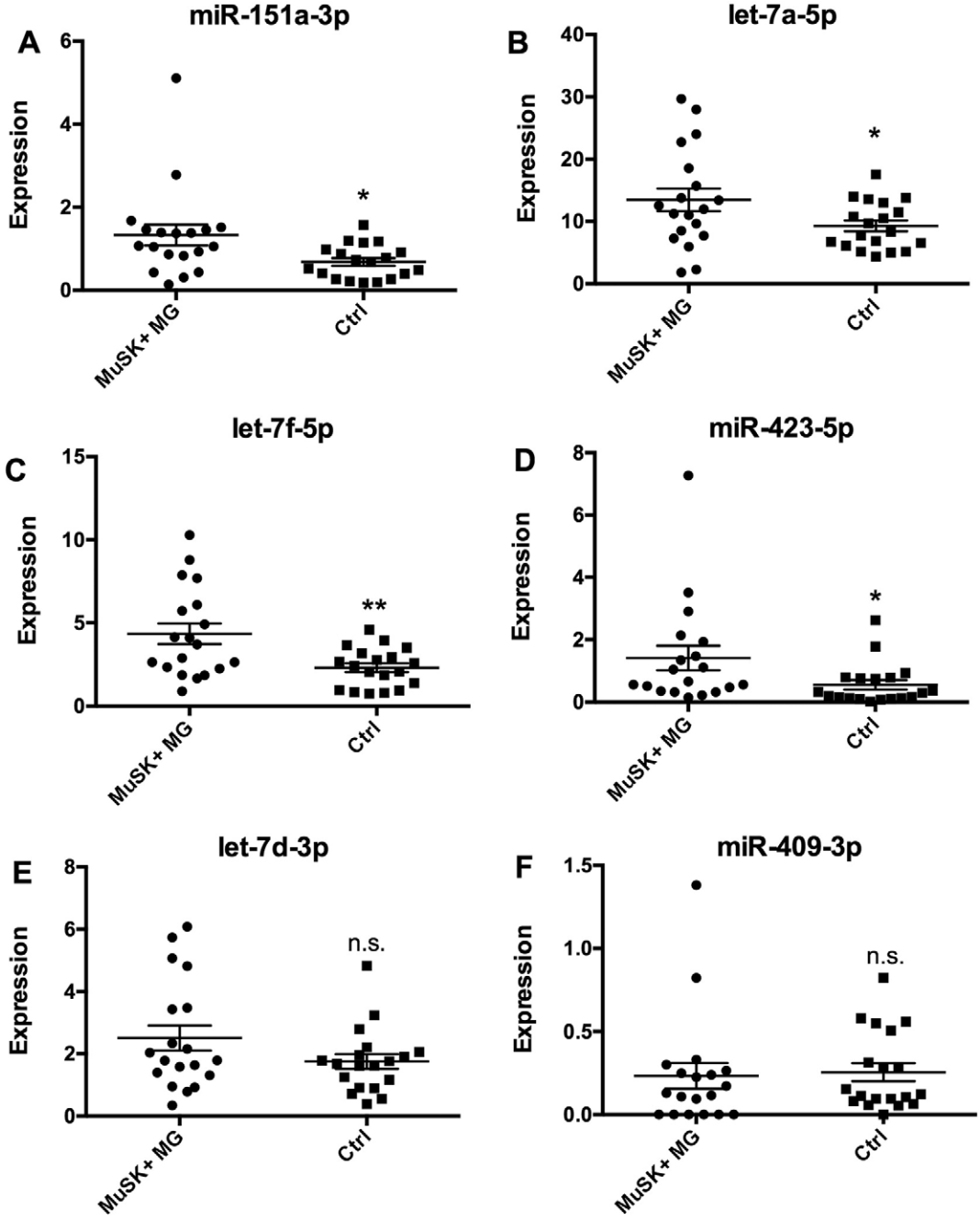

Fig. 2. Significantly altered miRNAs in MG patients (N = 19) compared to age- and gender-matched healthy blood donors (N = 19). Relative expression with individual values as well as median ± interquartile range (bars) are shown for the assayed miRNAs in the validation cohort: hsa-miR-151a-3p (A), let-7a-5p (B), let-7f-5p (C), miR-423-5p (D), let-7d-3p (E) and miR409-3p (F). *p ≤ 0.05; **p ≤ 0.01.

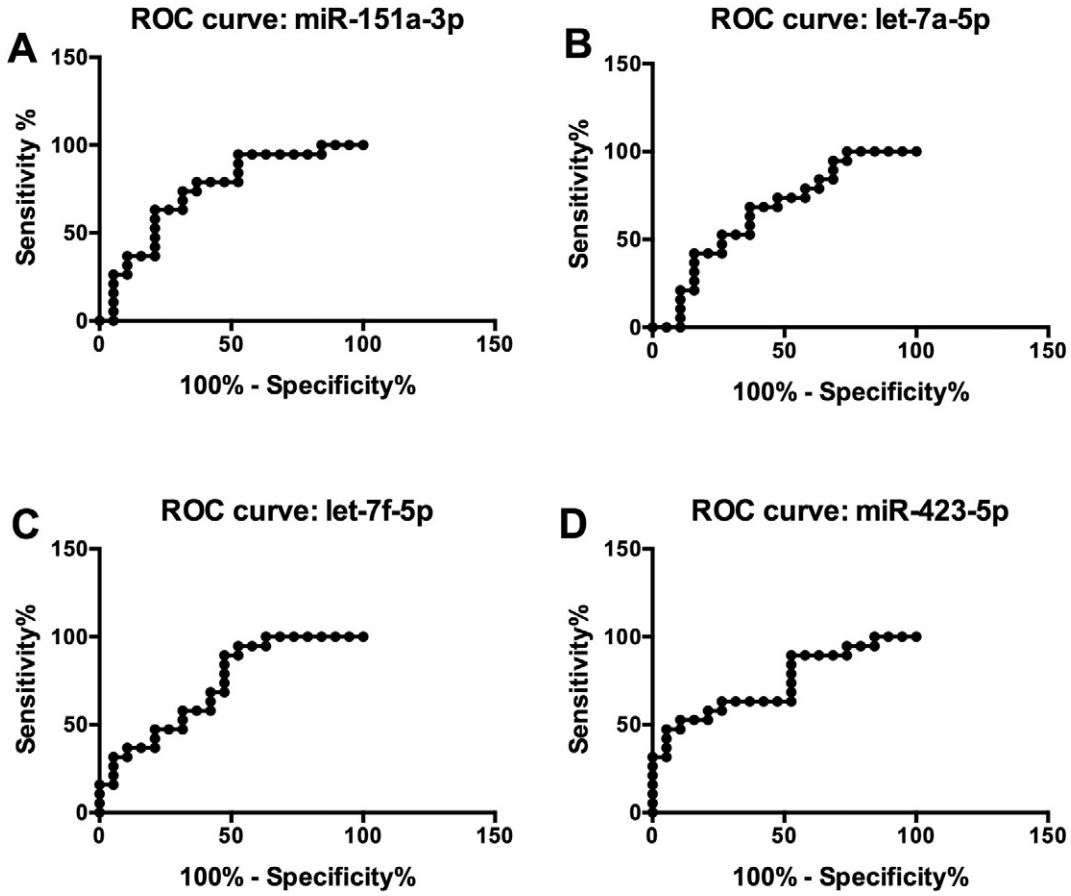

Fig. 3. ROC curve of all altered miRNAs, indicating sensitivity of each miRNA for MG: miR-151a-3p (A), let-7a-5p (B), let-7f-5p (C) and miR-423-5p (D).

4. Discussion

In the present report we have analyzed the presence and abundance of circulating miRNAs in MuSK+ MG patient sera. Out of 179 analyzed miRNAs, four miRNAs: let-7a-5p, let-7f-5p, miR-151a-3p and miR-423-5p, showed significant enrichment in MuSK+ MG patient sera. Previous reports observed elevated levels of circulating miR-150-5p and miR-21-5p in the sera of AChR+ MG patients, with lower levels in immunosuppressed patients (Punga et al., 2015). Additionally, miR-150-5p was reduced upon thymectomy in correlation with clinical improvement (Punga et al., 2014). In contrast to AChR+ MG, thymus pathology is very uncommon in MuSK+ MG, where the thymus does not seem to play a pathogenic role (Marx et al., 2013). We chose not to include AChR+ patients in this study as a third comparator, since the previous study on circulating miRNA profile in AChR+ MG did not indicate any changed profile of any of the miRNAs let-7a-5p, let-7f-5p, miR-151a-3p or miR-423-5p (Punga et al., 2014). Together with the difference between IgG1 AChR abs and IgG4 MuSK abs, it is not unexpected that AChR+ MG and MuSK+ MG are associated with different circulating serum miRNAs.

The members of the let-7 miRNA family are among the most studied miRNAs since they possess a functional impact on various cellular processes including embryogenesis, neuronal development and glucose metabolism (Gurtan et al., 2013; Patterson et al., 2014; Zhu et al., 2011). Twelve different let-7 family members (let-7a-1, let-7a-2, let-7a-3, let-7b, let-7c, let-7d, let-7e, let-7f-1, let-7f-2, let-7 g, let-7i, and miR-98) are expressed in human cells (Roush and Slack, 2008). In addition to their intracellular roles, several members of the let-7 miRNA family have been shown to be incorporated into exosomes and actively secreted into the extracellular environment in the gastric cancer cell line AZ-P7a (Ohshima et al., 2010). Quantification of the circulating miRNAs in ovarian cancer and in non-small-cell lung carcinoma patient sera has shown that let-7b and let-7f are significantly underrepresented in these patient sera (Chung et al., 2013; Silva et al., 2011). In contrast, the present study identified selective enrichment of two let-7 miRNA family members, let-7a-5p and let-7f-5p, in MuSK+ MG sera. In accordance with our data, members of the let-7 miRNA family are highly elevated in the sera of patients with multiple sclerosis (MS) compared to healthy individuals (Gandhi et al., 2013). Especially, circulating serum let-7a shows significant enrichment in patients with secondary progressive MS (Gandhi et al., 2013). Thus, the enrichment of circulating serum let-7 miRNAs might be a selective feature that patients with MuSK+ MG and MS have in common.

One function of the let-7 miRNAs in the immune response is T cell activation through stimulation of the toll-like receptor 7 (TLR7) (Wang et al., 2011). Intriguingly, engagement of TLR7 in $CD4^+$ T cells actually induces unresponsiveness of T cells (Dominguez-Villar et al., 2015). One confounding factor in our cohort was the large number of immunosuppressed patients, since immunosuppression is known to attenuate the Th1 response in MuSK+ MG (Yilmaz et al., 2015). This problem is difficult to counteract, considering the relatively small international cohort of MuSK+ MG patients. On the contrary, lower levels of let-7 miRNAs were previously reported in peripheral blood mononuclear cells from MG patients compared to healthy controls (Jiang et al., 2012). Nevertheless, in this particular study the authors did not present antibody status of the MG patients, thus not allowing any comparison with our data. A previous Spanish study examined circulating miRNA of MG patients, however in this study only AChR+ patients were included and thus we cannot make direct comparisons to circulating miRNA profiles of our MuSK+ patients (Nogales-Gadea et al., 2014).

The other serum circulating miRNAs associated with MuSK + MG were miR-151a–3p and miR-423-5p. Unlike the established immune-miRNA, miR-150-5p (Kroesen et al., 2015), miR-151-3p is instead known as an oncomir (Chiyomaru et al., 2012). Increased levels of circulating miR-151–3p are considered a positive indicator, and thus useful biomarker in clinical trials for breast cancer (Krell et al., 2012). In addition, circulating miR-151-3p levels have been found to be reduced immediately following aerobic exercise (Nielsen et al., 2014).

The last elevated miRNA, miR-423-5p, has been confirmed to be elevated in plasma from patients with heart failure and to be predictive of its severity (Tijsen et al., 2010). Nevertheless, none of the patients in our study had any signs of heart failure. Regarding concomitant diseases, three patients had thyroiditis and three others had cardiomyopathy, cerebral stroke and polycythemia respectively. This low prevalence of concomitant diseases does not make us suspect that the observed miRNA profile was due to another disease process than MuSK + MG. The main limitation of the present study is that the group of MuSK + patients consists of a cross-sectional sample with relatively long disease duration as well as immunosuppressive treatment in the majority of patients. Although we did not find any particular correlation between disease duration and any of the expressed miRNAs, both long disease duration and concomitant medication could serve as potential confounders that may impact on outcome. Further, although all patients had a normal thymus as is commonly seen in MuSK + MG (Marx et al., 2013), we cannot exclude that thymectomy impacted the miRNA profile in a few patients.

In summary, the present study defined four circulating miRNAs that were elevated in MuSK + MG subtype. The identified miRNAs: let-7a-5p, let-7f-5p, miR-151a-3p and miR-423-5p, all could serve as potential biomarkers in addition to ab titres in MuSK + MG. Therefore, we suggest further validation of these biomarkers in a larger international cohort with a longitudinal follow-up study for diagnostic and therapeutic purposes.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jneuroim.2016.01.003.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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