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Circulating microRNA plasma profile in MuSK+ myasthenia gravis

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Abstract

Muscle-specific tyrosine-kinase-antibody positive myasthenia gravis (MuSK+ MG) is an immunological subtype with distinctive pathogenic mechanisms and clinical features. The aim of this study was to analyze the circulating plasma microRNA profile of patients with MuSK+ MG. From the discovery cohort miR-210-3p, miR-324-3p and miR-328-3p were further analyzed in the validation cohort. We found a distinct plasma profile of miR-210-3p and miR-324-3p that were significantly decreased in MuSK+ MG patients compared to healthy controls (4.1 ± 1.4 vs 5.1 ± 1.4 , $p=0.006$ and 4.7 ± 1.0 vs 5.4 ± 1.3 , $p=0.02$). These findings reveal a distinct plasma miRNA profile in MuSK+ MG.

Keywords

muscle-specific tyrosine kinase; MuSK; myasthenia gravis; microRNA; biomarker

1. Introduction

Myasthenia gravis (MG) with antibodies to muscle-specific tyrosine kinase (MuSK+ MG) is a rare immunological subtype, representing 5–8% of the MG patients (Evoli et al., 2018). Unlike MG with antibodies against AChR (AChR+MG), MuSK+MG is considered a more homogenous disease (Evoli, Alboini, 2018). First, it has a high prevalence of more than 70% in female patients (Oh, 2009). Secondly, the disease typically starts at early age and rarely occurs after 60 years of age (Sanders et al., 2003a). Thirdly, the clinical features in many

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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patients are relatively distinct with dominating cranial and bulbar muscle weakness (Evoli, Alboini, 2018). Lastly, association with HLA class II DQ5 alleles have been confirmed in different studies (Marino et al., 2014). On the other hand, diagnostic challenges include less diurnal symptom fluctuations, unresponsiveness to acetylcholine esterase inhibitors and very focal findings of neuromuscular transmission failure (Evoli, Alboini, 2018). Biomarkers of disease activity are helpful in differentiating clinical response and outcome. MuSK antibodies are very specific but the titer does not predict the course of the disease (Kaminski et al., 2012, Meriggioli and Sanders, 2012). As circulating microRNAs (miRNAs) play important roles in several biological processes and are easily accessible in biosamples, they are considered to fulfill the requirements for potential biomarkers also in MG (Punga and Punga, 2017). In search of novel biomarkers for MuSK+ MG, miRNAs have been proposed as potential serum biomarkers for MuSK+ MG (Punga et al., 2016). Previous studies revealed a distinct elevation of 4 miRNAs: let-7a-5p, let-7f-5p, hsa-miR-151a-3p, hsa-miR-423-5p in MuSK+ MG patient sera (Punga, Bartoccioni, 2016).

Circulating miRNAs in blood associate with various pathophysiological conditions. As the majority of blood samples are stored as serum, miRNA profiles of MG have so far been studied only in serum (Nogales-Gadea et al., 2014, Punga and Punga, 2017). However, different expression and different types of miRNAs were found in the serum and plasma among pregnant women (Ge et al., 2015). Due to the previously reported work that serum and plasma cannot be automatically assumed to be interchangeable with regard to miRNA concentrations (McDonald et al., 2011), it is important to also analyze the plasma profile of MG patients. The aim of the study was therefore to analyze the circulating miRNA profile in plasma from patients with MuSK+ MG.

2. Methods

2.1. Subjects

We included patients with MuSK+ MG, in different stages of the disease, who were followed at the Duke University (Durham, NC) and the University of North Carolina - Chapel Hill (Chapel Hill, NC) Myasthenia Gravis Clinics. Twenty healthy subjects were used as controls. All patients had muscle weakness and fatigability, confirmed impairment of neuromuscular transmission and positive serum MuSK antibody titers (radioimmunoprecipitation assay). Disease severity was classified by Myasthenia Gravis Foundation of America (MGFA) class (Jaretzki et al., 2000) and assessed with the MG-manual muscle test (MMT) (Sanders et al., 2003b).

For the discovery cohort, we selected three MuSK+ MG immunosuppressive naive patients with moderate disease severity and three age- and gender-matched healthy controls. Two MuSK+ MG patients were black or African American and one was white; controls were white (N=2) and from other ethnicity (N=1). None of them was on current immunosuppressive treatment during sampling (Table 1). Data from the discovery cohort were further analyzed in a validation cohort of 33 MuSK+ MG patients (Table 2).

The study was approved by the Duke University Institutional Review Board (#Pro00023267, Pro00062952). All patients provided informed consent.

2.2. Circulating miRNA isolation

The blood samples were collected in citrate-containing tubes and density gradient centrifugation was performed at 800g for 30 minutes. Separated plasma was aspirated and centrifuged again at 800g for 20 minutes. The samples were stored at -80°C until further processing. Total RNA was extracted from 200 μl plasma using miRCURYTM RNA Isolation Kit-Biofluids according to the manufacturer's instructions. For the reverse transcription reactions 2 μl of isolated RNA was used with universal cDNA Synthesis Kit II.

2.3. miRNA expression analysis

Following RNA isolation, real-time quantitative PCR (qRT-PCR) was performed using ExiLent SYBR Green Master Mix. MiRNA expression in the discovery cohort was analyzed using the Serum/Plasma Focus microRNA PCR Panel. Subsequent analysis in the validation cohort was performed for three selected individual primers detected from the discovery cohort. Interplate calibration was done with UniSp3, quality of RNA isolation with UniSp2 and UniSp4, and cDNA synthesis control with UniSp6.

Cellular miRNA contamination was controlled with hemolysis test Cq (miR23a - miR-451a) and values <7 represented non-hemolyzed samples and were thus included for further analysis. The relative expression level of each miRNA was normalized using miR-103a-3p, miR-191-5p, miR-93-3p and miR-423-3p as reference candidates. The final normalization was performed to miR-191-5p as this was the only miRNA that was abundantly detected in all samples, in accordance with previous studies (Marabita et al., 2016), and as it was recommended by the "NormFinder" function in the GenEx software.

Initial miRNA detection experiments in the discovery cohort profiled the genes of interest from 179 different miRNAs. Three of these genes were significantly different among MuSK + MG patients compared to healthy controls (Figure 1) and they were further analyzed in the validation cohort.

2.4. Statistical analysis

The numbers of miRNA expression were log transformed to obtain data more similar to a normal distribution. Unpaired two-tailed T-test of independent samples were performed, comparing the MuSK+ MG and control groups. Spearman rank correlation was used to determine the correlation coefficient between different continuous factors (age, disease duration) and miRNAs level. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. MiRNA profile in the discovery cohort

In the discovery cohort 3 female MuSK+ MG patients and 3 sex and age-matched healthy controls were analyzed (Table 1). No hemolysis was detected in any sample. Two miRNAs: miR-210-3p and miR-324-3p were strongly decreased and miR-328-3p was elevated in the MuSK+ MG cohort (Table 3) and they were further analyzed by qRT-PCR in plasma from the larger cohort of MuSK+ MG patients.

3.2. MuSK+ MG validation cohort

None of the samples were excluded from the analysis due to hemolysis. The final validation cohort included 33 MuSK+ MG patients with a marked female predominance of 88%. (Table 2). The age of MuSK+ MG patients at recruitment did not differ from the healthy controls ($p=0.92$). The age at onset was 20 to 70 years, similar among women and men (31.8 ± 15.1 vs 43.3 ± 7.6 , $p=0.15$). Most patients had mild MG disease (MGFA class II; table 2). The disease severity was not different between Caucasians and African American patients ($p=0.57$). Most of the patients in the validation cohort were on immunosuppressive treatment at recruitment (table 2) and 27% had another autoimmune disorder in addition to MG. Further, six patients had a concomitant cancer (two cases of breast cancer, one case of renal cell cancer, one case of thyroid cancer, one case of adrenal cortical cancer and one uterine cancer)

3.3. MiRNA profile in the validation cohort

MiR-210-3p was significantly lower among MuSK+ MG patients (4.1 ± 1.4) compared to the healthy controls (5.1 ± 1.4 , $p=0.006$). The same difference was found with miR-324-3p between MuSK+ MG (4.7 ± 1.0) and healthy controls (5.4 ± 1.3 , $p=0.02$). The third miRNA miR-328-3p did not differ significantly between MuSK+ MG and controls (Figure 2).

There was a trend for Caucasian MuSK+ MG patients to have higher miR-210-3p levels than African American patients (4.4 ± 1.2 vs 3.8 ± 1.5 , $p=0.22$) and a similar difference was seen for miR-324-3p (5.1 ± 1.0 vs 4.4 ± 1.0 , $p=0.05$). There was no correlation between miRNAs and any other clinical features, such as age, MuSK titer, MGFA severity class, MMT, medication type or disease duration ($p>0.05$ for all).

4. Discussion

We analyzed circulating miRNAs in MuSK+ MG patient plasma and found a few striking differences in comparison to healthy controls. Out of 179 different miRNAs three were different in MuSK+ MG patients' plasma. MiR-210-3p and miR-324-3p, which were lower in the discovery cohort, were also significantly lower in the validation cohort.

Circulating miRNAs are easily accessible and have a potential role as biomarkers in different diseases, including MG. Previous studies detected three dysregulated miRNAs, miR-150-5p, miR-21-5p and miR-30e-5p, in serum of AChR+ early-onset MG, late-onset MG and ocular MG subgroups (Punga et al., 2015, Punga et al., 2014, Sabre et al., 2018). The only previous study that analyzed MuSK+MG patient serum samples showed significant enrichment of let-7a-5p, let-7f-5p, miR-151a-3p and miR-423-5p (Punga, Bartoccioni, 2016).

Mir-210-3p, that was lower in our MuSK+ MG patients' plasma, is involved in various biological processes. It is upregulated by hypoxia and as this is the most common neoplastic microenvironment for solid tumors, it has been found to be elevated in several cancers (Bavelloni et al., 2017). Nevertheless, miR-210-3p can also act as tumor suppressor. Our study found that miR-210-3p was significantly lower among MuSK+MG patients. Eighteen percent of patients had had cancer diagnosis, however no distinct correlation with miRNA levels was found in these patients.

Limited information is available on the biological functions of miR-324-3p; however, it is overexpressed among patients with Parkinson disease and multiple system atrophy (Vallelunga et al., 2014). Its role as a tumor suppressor has also been proposed (Stappert et al., 2013). Interestingly, miR-324-3p was reported as a possible new biomarker in the diagnosis of osteoporosis (Feichtinger et al., 2018). They found that this miRNA level decreased in serum of patients with recent osteoporotic fractures. The third miR-328-3p that we studied has been shown to be significantly up-regulated in multiple sclerosis (Yang et al., 2017). MiR-328-3p was also down-regulated in the serum of women with postmenopausal osteoporotic fractures (Chen et al., 2016). This miRNA was the only one that was upregulated in the discovery cohort, but no difference remained in the validation cohort. Only one patient had an osteoporosis diagnosis in our cohort, although it was not systematically investigated in all patients and some could be undiagnosed. Still, one third had another autoimmune disease in addition to MG. Thyroid diseases and AChR+ MG are the most common coexisting disorders (Gilhus, 2016). The frequency of a second autoimmune disease had been reported 13–22% (Evoli, Alboini, 2018, Gilhus et al., 2015).

Serum and plasma are not interchangeable with regard to miRNA concentrations (McDonald, Milosevic, 2011) and intriguingly, contradictory results have been reported in this field. Some studies observed higher miRNA concentrations in serum samples compared to the corresponding plasma samples, whereas other studies reported higher miRNA concentrations in plasma than serum of healthy volunteers (Wang et al., 2012). Therefore, similar sample types, but also measurement platforms, are important to compare miRNA data (Wang, Yuan, 2012). Our results highlight the importance of these factors in miRNA studies; especially since previous studies on serum miRNA in MuSK+ MG have only found increased levels of miRNAs, which is the opposite to plasma (Punga, Bartoccioni, 2016).

There are a number of factors that might affect the measurement of circulating miRNAs. During the coagulation process, miRNA may be released from blood cells. In addition, platelet contamination, blood tube type, phlebotomy protocols or sample-processing protocols could cause the differences (McDonald, Milosevic, 2011, Wang, Yuan, 2012). Hemolysis produces artificially high miRNA concentration. In order to eliminate this confounder, we excluded possible hemolysis by comparing the level of miR-451a highly expressed in red blood cells with a miR-23a unaffected by hemolysis (Blondal et al., 2013).

Gender-associated molecular differences have been reported in serum and plasma samples (Guo et al., 2016, Sharma and Eghbali, 2014). As MG subtypes have gender-differences, this may be an important factor that has affected the miRNA results (Guo, Liang, 2016). Therefore, we cannot exclude that the results are involved in some gender-associated functions.

Race also varies among the MG subtypes. Striking regional and racial differences in the positive rate of MuSK antibodies have been reported (Oh, 2009, Oh et al., 2009). In the US, black women have been more likely to be affected by MuSK+ MG (Guptill and Sanders, 2010, Guptill et al., 2011). In terms of regional differences, MuSK+ MG rates are lower in the northern latitudes of Europe, though no such evidence has been observed in US or Asia (Oh, 2009). Our study included patients from the US and the majority of them were African

American women. Although the control group consisted predominantly of Caucasians, the different ethnicity groups did not show a statistically significant difference in miRNA pattern.

It is plausible that a number of limitations could have influenced the results obtained. The quite high prevalence of concomitant disease is a possible limitation as the miRNA profile could be affected by another autoimmune process. Still, none of the dysregulated miRNAs found in this study have been described with the other autoimmune diseases our patients had and no correlation was found with the diseases and analyzed miRNAs. The study was not longitudinal and therefore changes in miRNAs level, correlation with disease activity or changes in treatment could not be analyzed. Further, although the initial miRNA analysis in the discovery cohort was done in patients that were immunosuppressive naïve, most of the patients in the validation cohort were on immunosuppressive medication. This could serve as a potential confounder that may impact the outcome. These limitations related to disease heterogeneity are inherent to studying a disease as rare as MuSK+ MG. Coordinated multicenter biosample collection may be able to reduce these limitations in the future.

In summary, as far as we are aware, this is the first plasma-based miRNA study of MuSK+ MG patients that extends our knowledge of MG biomarkers. The identified markers, miR-210-3p and miR-324-3p, have a specific profile in the plasma of MuSK+MG patients. These miRNAs may have future use as a diagnostic marker or provide important clues about disease pathogenesis that can be pursued in future studies. As the study included only MuSK + MG patients' plasma from a specific US region, further validation in a larger intercontinental cohort comparing plasma and serum is warranted.

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Highlights

- We determined the plasma profile of circulating microRNAs in MuSK+ MG patients
- MiR-210-3p and miR-324-3p are downregulated in MuSK+ MG plasma
- The plasma microRNA profile in MuSK+ MG differs from the serum profile

Figure 1.

Volcano plot of the differentially expressed plasma microRNA (miRNAs) in the discovery cohort of MuSK+ myasthenia gravis (MuSK+MG) patients compared to healthy controls (HC). The significantly altered miRNAs in MuSK+MG compared to healthy HC are indicated. The y-axis displays Log10(p-value) and the x-axis presents log2(Fold change).

Figure 2.

Relative miRNA expression of candidate miRNAs from the validation cohort in MuSK+ myasthenia gravis compared to healthy controls. A) miR-210-3p, B) miR-324-3p and C) miR-328-3p.

Table 1.

Clinical data on the subjects in the discovery set.

	Age/sex	Disease duration (years)	MGFA grade	Immunosuppressive treatment	Healthy controls Age/sex
MuSK+ MG patients (N=3)					
1	48F	40	IIB	No	45F
2	31F	5	IIIB	No	31F
3	55F	20	IIA	No	57F

Table 2.

Descriptive summary of subjects in the validation cohort. MGFA, Myasthenia Gravis Foundation of America; MMT, manual muscle test.

	MuSK+ patients (N=33)	Controls (N=20)
Gender N (%)		
Female	29 (88%)	12 (60%)
Male	4 (12%)	8 (40%)
Time since diagnosis in years (median; range)	11.7 (0.9 – 39.8)	-
Age (mean $\pm$ SD)	44.9 $\pm$ 15.8	44.5 $\pm$ 13.5
Ethnicity N (%)		
Caucasian	12 (36.4%)	16 (80%)
Black/African American	19 (57.6%)	1 (5%)
Other	1 (3%)	3 (15%)
Missing	1 (3%)	-
MGFA score		
0	2 (6%)	-
I	7 (21.2%)	-
IIa/IIb	16 (48.5%)	-
IIIa/mb	6 (18.2%)	-
IVb	1 (3%)	-
V	1 (3%)	-
MMT (mean $\pm$ SD)	9.9 $\pm$ 9.4	-
Other autoimmune disorders		
Hypothyroidism	4 (12%)	-
Asthma	3 (9%)	-
Rheumatoid arthritis	1 (3%)	-
Crohn disease	1 (3%)	-
Cancer diagnosis	6 (18.2%)	-
Immunosuppressive treatment N (%)	25 (75.8%)	-
Corticosteroids	16 (48.5%)	-
Azathioprine	3 (9.1%)	-
Mycophenolate	11 (33.3%)	-
Cyclosporine	1 (3.0%)	-
Rituximab	3 (9.1%)	-

Table 3.

Differentially expressed miRNAs in the discovery set.

microRNA	Fold change (log)	p-value
hsa-miR-21G-3p	-3.0	0.02
hsa-miR-328-3p	2.5	0.02
hsa-miR-324-3p	-2.0	0.04

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