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A novel infection- and inflammation-associated molecular signature in peripheral blood of myasthenia gravis patients

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ABSTRACT

Myasthenia gravis (MG) is a T-cell dependent autoimmune disorder of the neuromuscular junction, characterised by muscle weakness and fatigability. Autoimmunity is thought to initiate in the thymus of acetylcholine receptor (AChR)-positive MG patients; however, the molecular mechanisms linking intrathymic MG pathogenesis with autoreactivity *via* the circulation to the muscle target organ are poorly understood. Using whole-transcriptome sequencing, we compared the transcriptional profile of peripheral blood mononuclear cells from AChR-early onset MG (AChR-EOMG) patients with healthy controls: 178 coding transcripts and 229 long non-coding RNAs, including 11 pre-miRNAs, were differentially expressed. Among the 178 coding transcripts, 128 were annotated of which 17% were associated with the 'infectious disease' functional category and 46% with 'inflammatory disease' and 'inflammatory response-associated' categories. Validation of selected transcripts by qPCR indicated that of the infectious disease-related transcripts, *ETF1*, *NFKB2*, *PLK3*, and *PPP1R15A* were upregulated, whereas *CLC* and *IL4* were downregulated in AChR-EOMG patients; in the 'inflammatory' categories, *ABCA1*, *FUS*, and *RELB* were upregulated, suggesting a contribution of these molecules to immunological dysfunctions in MG. Data selection and validation were also based on predicted microRNA-mRNA interactions. We found that miR-612, miR-3654, and miR-3651 were increased, whereas miR-612-putative *AKAp12* and *HRH4* targets and the miR-3651-putative *CRISP3* target were downregulated in AChR-EOMG, also suggesting altered immunoregulation. Our findings reveal a novel peripheral molecular signature in AChR-EOMG, reflecting a critical involvement of inflammatory- and infectious disease-related immune responses in disease pathogenesis.

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Abbreviations: AChR, acetylcholine receptor; AChR-EOMG, acetylcholine receptor-early onset AChR-MG; AZA, azathioprine; IPA, Ingenuity Pathway analysis; lincRNAs, long non-coding intergenic RNAs; lncRNAs, long non-coding RNAs; MG, myasthenia gravis; miRNAs, microRNAs; pre-miRNAs, miRNA precursors; NMJ, neuromuscular junction; PBMCs, peripheral blood mononuclear cells; RNA-seq, RNA-sequencing.

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1. Introduction

Myasthenia gravis (MG), a T cell-dependent, B cell-mediated autoimmune disorder affecting the neuromuscular junction (NMJ), is characterised by the presence of autoantibodies against post-synaptic membrane proteins of striated skeletal muscle. These autoantibodies promote autoimmunity by disrupting neuromuscular transmission, resulting in clinical symptoms such as muscle weakness and abnormal fatigability (Berrih-Aknin and Le Panse, 2014). Antibodies directed against the acetylcholine receptor (AChR) of the post-synaptic NMJ are detected in approximately 80% of patients (Cavalcante et al., 2012), but other autoimmune targets, including the muscle kinase receptor and lipoprotein receptor-related protein 4, have also been observed in a variable proportion of seronegative patients (Hoch et al., 2001; Higuchi et al., 2011; Zhang et al., 2012). Several lines of evidence support the contribution of the thymus in the pathogenesis of MG. This lymphoid organ is commonly recognised as the main site of autosensitisation in most AChR-positive MG (AChR-MG) patients. These patients show histological and functional thymic abnormalities, including hyperplasia and thymoma, and in a high proportion of cases, thymectomy results in symptomatic improvement (Marx et al., 1996; Mantegazza et al., 2003; Berrih-Aknin et al., 2013).

MG is a chronic although treatable disease; therapeutic strategies take three different lines: (i) symptomatic therapies consisting of the use of acetylcholinesterase inhibitors, such as pyridostigmine bromide; (ii) immunosuppressive treatments, such as azathioprine (AZA) and steroids; and (iii) alteration of the natural history of the disease (*e.g.* thymectomy) (Mantegazza et al., 2011). However, in a substantial proportion of cases, these treatments do not lead to complete stable remission (Baggi et al., 2013) and may give rise to severe side effects, highlighting the need for more specific and effective therapies. More detailed characterisation of the immunological and molecular alterations leading to autoimmunity development and perpetuation would be helpful to guide this process.

Growing evidence points to the presence of a chronic inflammatory state in the MG thymus, likely due to persistent viral replications, which could alter innate immune responses and lead to the breakdown of self-tolerance, triggering autoimmune reactions (Cufi et al., 2013; Cavalcante et al., 2011a, 2013; Cordiglieri et al., 2014). Increased expression and production of cytokines in peripheral blood cells is also observed, further supporting a contribution of inflammation to MG pathogenesis (Yilmaz et al., 2015; Conti-Fine et al., 2006). However, a comprehensive understanding of the immunological alterations occurring in the periphery of MG patients, and the link between thymic inflammation and muscle autoreactivity is lacking.

Recent developments in sequencing technology are opening the door to comprehensive and high-throughput approaches for understanding the molecular basis of disease, including in autoimmunity. RNA-sequencing (RNA-seq) has improved the sensitivity for detection of novel (dysregulated) coding transcripts, non-coding transcripts, and splicing variants (Marioni et al., 2008). This technology has been used to detect novel long non-coding RNAs (lncRNAs) that are associated with immune-related functions in psoriatic skin, suggesting an involvement of these lncRNAs in the pathogenesis of psoriasis (Tsoi et al., 2015). Recently, next-generation sequencing was used to identify a number of novel disease-associated genes and isoforms from the transcriptomes of synovial fibroblasts of rheumatoid arthritis patients, contributing to better understand of the molecular mechanisms of disease (Heruth et al., 2012).

Here, we exploit whole-transcriptome sequencing to study the transcriptional profile of peripheral blood mononuclear cells of early-onset (≤50 years old) AChR-positive MG (AChR-EOMG) patients, who represent the most studied MG clinical subgroup (Berrih-Aknin and Eymard, 2014). We selected a clinically well-defined and homogeneous cohort of patients to reduce the impact of external confounding factors with the aim of identifying a molecular signature of disease that could be used to aid understanding of the development of peripheral autoimmunity in MG.

2. Materials and methods

2.1. Patients and specimen collection

The study included nineteen MG patients (14 females, 5 males, mean age at blood collection ± SD: 41.21 ± 12.81 years old) and 12 age- and sex-matched healthy controls (8 females, 4 males, mean age ± SD: 38.42 ± 9.99 years old) with no autoimmune diseases or signs of infection. Written informed consent was obtained from each patient and control. The study was approved by the Ethics Committee of the Neurological Institute 'Carlo Besta'. MG patients with early-onset (≤50 years old) of symptoms and positivity for anti-AChR antibodies were selected. Clinical severity was assessed based on the Myasthenia Gravis Foundation of America (MGFA) classification (Jaretzki et al., 2000).

At the time of blood collection, 13 AChR-EOMG patients were untreated or treated only with acetylcholinesterase inhibitors, and 6 patients were treated with AZA and/or acetylcholinesterase inhibitors; 7 patients were thymectomised (hereinafter referred as post-thymectomy), whereas the remaining 12 did not undergo thymic removal (hereinafter referred as pre-thymectomy) before sampling. Among the pre-thymectomy patients, two patients (MG1 and MG3) had thymoma diagnosed by computed tomography. Of the post-thymectomy patients, four (MG7, MG8, MG9, and MG10) had thymic hyperplasia, two had a normally involuted thymus (MG11 and MG19), and one (MG18) had thymoma. For the post-thymectomy patients, the time interval range between thymectomy and blood collection was <11 years, except for MG11 (25 years). Unfortunately, pre- and post-thymectomy samples from the same patients were not available. None of the patients presented signs of infections at time of blood collection with the exception of one patient (MG11) who had chronic hepatitis C. Recent infections were reported for MG12 who had H1N1 influenza A virus infection two months before sampling and MG1 who had mononucleosis approximately 6 months before sampling (two months before MG onset). Clinical characteristics of patients and healthy controls are summarised in Table 1 and Supplementary Table S1, respectively. Whole blood was collected in EDTA-supplemented tubes from patients and controls, and peripheral blood mononuclear cells (PBMCs) were then isolated using Lymphoprep (Axis-Shield, Dundee, Scotland) according to the manufacturer's recommendations. PBMCs were frozen in FBS containing 10% DMSO (Euroclone, Milan, Italy) and stored in liquid nitrogen until use.

2.2. RNA isolation

Total RNA was extracted from PBMCs using the mirVana mRNA extraction kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. RNA quality was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and RNA integrity number (RIN) was calculated for each sample (mean ± SD: 7.68 ± 1.49). RNA was then quantified using Quant-iT RiboGreen RNA Reagent (Thermo Fisher Scientific).

2.3. Whole-transcriptome sequencing

Total RNA from 11 AChR-EOMG patients (9 females, 2 males, mean age ± SD: 37.1 ± 8.8 years old) and 6 healthy controls (4

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