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RESEARCH ARTICLE

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Expression profiling and bioinformatics analysis of exosomal long noncoding RNAs in patients with myasthenia gravis by RNA sequencing

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Abstract

Background: Myasthenia gravis (MG) is an autoimmune disease mediated by acetylcholine receptor antibodies. Exosomes were shown to be involved in the immune modulation and autoimmune diseases. However, the expression and function of exosomal long noncoding RNAs (lncRNAs) in MG are still unclear.

Methods: We conducted high-throughput sequencing to detect the lncRNA profiles of serum exosomes in 6 MG patients (2 grade I, 2 grade IIa, and 2 grade IIb) and 6 healthy controls (HC). Then, differentially expressed (DE) lncRNAs with the greatest difference between the MG and HC groups were selected for further quantitative real-time polymerase chain reaction (qRT-PCR) validation in additional 30 MG patients and 10 HC. The DE lncRNAs were used to construct the coding/noncoding network and perform enrichment analysis.

Results: We identified 378 significantly upregulated and 348 significantly downregulated lncRNAs in MG patients compared with HC. The top 5 lncRNAs (NR_104677.1, ENST00000583253.1, NR_046098.1, NR_022008.1, and ENST00000581362.1) were validated and shown to be significantly increased in the serum exosome of MG, and the expression level of NR_046098.1 significantly increased with the MG grading. Enrichment analysis showed that DE genes mainly participated in the basic biological regulation of MG and immune-related pathways, such as autoimmune thyroid disease pathway and T-cell receptor signaling pathway. A specific lncRNA-miRNA-mRNA regulatory network associated with the 5 lncRNAs, 14 MG-related miRNAs and 30 mRNAs was constructed.

Conclusions: We conducted a comprehensive analysis of exosomal lncRNAs to reveal potential biomarkers for the MG diagnosis and severity assessment.

KEYWORDS
biomarkers, exosome, high throughput, long noncoding RNA, myasthenia gravis

1 | INTRODUCTION

Myasthenia gravis (MG) is a neuromuscular disorder that is characterized by the presence of autoantibodies, which mainly target the specific acetylcholine receptor (AChR). Many factors contribute to the pathogenesis of MG, including the genes, environment, immunology, thymic disorder, and age.[1] However, the mechanisms that trigger and maintain this chronic disease are still unknown. In view of the evidence indicating a potential function of the exosomes in the modulated immune responses, studying the exosomes' content will be of great importance to understand the pathogenesis of this disease and search for new biomarkers to monitor the disease status and determine the prognosis. The exosomes are small, lipid bilayer membrane vesicles of an endocytic origin, which are released by diverse types of cells under different physiological or pathological conditions.[2] They contain some bioactive molecules including the proteins, DNA, microRNAs (miRNAs) and mRNAs as well as long noncoding RNAs (lncRNAs). LncRNAs represent RNA transcripts with a length of more than 200 nucleotides, which are not translated into proteins.[3] Interestingly, they can be packaged into exosomes and serve as messengers in the cell-to-cell communication.[4] Therefore, the detection of circulating exosomes has been referred to as a "liquid biopsy" to reflect the latent capacity for a thorough examination of the structure and function of the donor cells.[5]

LncRNAs have recently emerged as a critical factor in several biological processes, such as cell growth, apoptosis, and migration, as well as the immune and inflammation response. It has been reported that these molecules play important roles participating in autoimmune disorders, neurological diseases and various types of cancer.[6] LncRNAs are also involved in the pathological processes, and they help to control the gene regulatory network of the interactions between host and pathogen.[7] The exosomes can protect the RNA from degradation by endogenous ribonucleases (RNases), thereby enhancing the stability of the exosomal RNA in the circulating blood.[8] Many researchers have studied lncRNAs and found that they can regulate the gene expression at various levels, almost at all stages. On the other hand, many lines of evidence also indicate that the occurrence of autoimmune diseases is related to mutations or disorders affecting lncRNAs.[9] Luo et al identified a smaller group of abnormal lncRNAs and mRNAs as potential diagnostic biomarkers for MG.[10] LncRNA MALAT-1 has been associated with alternative splicing and gene regulation.[11] As a competing endogenous RNA for miR-338-3p, MALAT-1 directly induced the MSL2 expression in MG, suggesting that it may serve as a therapeutic target for the MG treatment.[12]

Given the important role of lncRNAs in autoimmune diseases, and since the alterations of the exosomal lncRNA content in serum from MG patients remain unknown, the present research aims to survey the lncRNAs expression profiles in the serum-derived exosomes of MG patients to identify the differentially expressed exosomal lncRNAs compared with healthy controls and investigate their functions and possible roles in the pathogenesis of MG.

2 | MATERIALS AND METHODS

2.1 | Study subjects and sample collection

The MG patients were recruited from the First Affiliated Hospital of Zhejiang University of Traditional Chinese Medicine in the period from January 2019 to November 2019. The subjects were selected according to the diagnostic criteria of MG: conspicuous symptoms, clinical muscle fatigue with a positive neostigmine test, the evidence of a disturbed neuromuscular transmission represented by an abnormal single-fiber electromyography and AChR antibody being positive or negative. We selected 2 ocular MG patients (grade I), 2 mild generalized MG patients (grade IIa) and 2 severe generalized MG patients (grade IIb) for a discovery cohort according to the modified Osserman classification.[13] A fourth group (the discovery set) included 6 age- and sex-matched healthy participants (Group 4). The validation set (N = 40) consisted of 30 MG patients (in 3 groups as described above, 10 in each group) and 10 healthy controls. The exclusion criteria for all the discovery patients were as follows: having complications with other immune system diseases, the presence of tumor and systemic hematologic diseases or the presence of short-term infections and immunosuppressive treatments. The exclusion criteria for all the validation patients were the same as those in the discovery group, except for a few patients who had received immunotherapy. A part of the clinical data of all the patients and healthy controls were shown in Table 1. The serum samples were obtained from the MG patients and healthy blood donors after obtaining the signed informed consent from all the participants. The study was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Zhejiang Chinese Medical University.

2.2 | Extraction and identification of the serum exosome

The blood samples were placed at room temperature for 20 minutes and centrifuged for 20 minutes at 10 000 × *g*, the upper serum was then transferred to a new Eppendorf tube and stored at −80°C and set aside. The serum exosome was extracted using the Ribo™ Exosome Isolation Reagent following the manufacturer's instructions (RiboBio, Guangzhou, China). The specific steps were as follows: 1. Prepare the serum or thaw frozen serum samples on ice or at 4°C; 2. Transfer 1.4 mL supernatant into a new 2 mL microcentrifuge tube; 3. Gently mix Precipitation Buffer A and add 560 μL to the supernatant. Close the tube and vortex for 5 seconds; 4. Incubate for 60 minutes at 2-8°C; 5. Centrifuge at 1500 × *g* for 30 minutes at 20°C; 6. Remove the supernatant completely and discard it or save it to perform a separate analysis; 7. Add 240 μL Resuspension Buffer to the tube containing the pellet and resuspend by vortexing. In order to perform the scanning electron microscope (SEM) analysis, the exosomes were then fixed with 4% paraformaldehyde, a drop of exosomes (20 μL) was pipetted onto a grid that was coated with formvar and carbon, standing for 5 minutes at room temperature.

TABLE 1 Demographic data of subjects in the discovery and validation set

		MG patients		
	Healthy controls	I	IIa	IIb
Discovery set				
N	6	2	2	2
Age (y)	36, 35, 37, 39, 37, 38, 38	36, 38	37, 41	39, 40
Sex (N)	3/3 (F/M)	1/1 (F/M)	1/1 (F/M)	1/1 (F/M)
Positive AchR-Ab (N)	/	2	2	2
Immunosuppressive treatment (N)	/	0	0	0
Validation set				
N	10	10	10	10
Age (mean ± SD)	39.5 ± 7.1	42.2 ± 6.6	40.7 ± 9.7	40.3 ± 10.2
Sex (N)	5/5 (F/M)	5/5 (F/M)	6/4 (F/M)	4/6 (F/M)
Positive AchR-Ab (N)	/	7	7	7
Immunosuppressive treatment (N)	/	1	1	1

Abbreviations: AchR-Ab, acetylcholine receptor antibody; F, female; M, male.

The excess fluid was then removed with a piece of filter, and the sample was negatively stained with 3% (wt/vol) phosphotungstic acid (PTA) (pH 6.8) for 5 minutes. Next, the sample was dried under an electric incandescent lamp and analyzed using the scanning electron microscope (Nova NANOSEM 450, FEI, USA).

2.3 | Exosomal RNA isolation, library construction and sequencing

A total of 5 mL of serum was mixed with the RiboTM Exosome Isolation Reagent, and the exosomes' isolation was performed according to the manufacturer's instructions (RiboBio, Guangzhou, China). The total RNA was extracted from the exosomes by applying TRIzol. The exosomal RNA sequencing (RNA-Seq) experiment was conducted by the RiboBio company (Guangzhou, China). In brief, about 50 ng of the total RNA was applied to construct a library using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (Illumina, San Diego, CA) following the manufacturer's instructions. The libraries were then amplified and sequenced using the HiSeq Rapid SBS Kit V2 (50 cycles) and HiSeq Rapid SR Cluster Kit V2 on the HiSeqTM 2500 system.

2.4 | RNA-seq data analysis and differential expression analysis

The high-throughput sequencing (HTSeq) software was used to count the number of the reads that were mapped to each lncRNA. The Reads Per Million (RPM) mapped reads, including TopHat and TopHatFusion mapping, were exploited to compute the expression level of individual lncRNAs. The differential expression (DE) between the lncRNAs was assessed by the DEGseq algorithm, and the statistically significant DE genes were obtained using the DEGseq software with an adjusted *P*-value threshold of <0.05 and |log 2(fold_change) | >1.

2.5 | Exosomal lncRNA expression with Quantitative real-time PCR (qRT-PCR) validation

We screened 5 exosomal lncRNAs with the greatest difference in the MG group compared with HC and verified them using the qRT-PCR, including the samples of an additional 10 grade I, 10 grade IIa, and 10 grade IIb MG patients, along with 10 healthy volunteers, and λ polyA was used as an external reference to quantify the extracted target genes. The diluted λ polyA was added to the RT-PCR reaction, adjusting the λ polyA amount to 10^2-10^7 copies per PCR reaction. The primers information is listed in Table 2. The

TABLE 2 Primer sequence for qRT-PCR

Primer name	Sequence (5′-3′)
NR_104677.1	F: AGCTATTCTACCCAGCCCCA
	R: AGCTGTTAGACAAGGGAGCG
ENST00000583253.1	F: TCCCTCAATGTGAGCAGTTTATCT
	R: CCCTGGATTTTCCTGGTATTTCTG
NR_046098.1	F: TCATTCTCTACAGCAAGCAG
	R: GAGAGACACAAGAAAATCCT
NR_022008.1	F: AGTGGTAGGAGGAGGGTTGGCT
	R: GCAAGGCTGGACCTCACGCT
ENST00000581362.1	F: TGGGTCGTCTTCCAGCCACAA
	R: GCTCTGAATTGTAGTCCGCCTGG

RNA was reverse-transcribed into cDNA by random primers at 42°C for 60 minutes and at 72°C for 10 minutes. The PCR amplification system included 10 μL 2 × SYBR Green mix, 6.8 μL cDNA, 0.8 μL upstream primer (5 μM), 0.8 μL downstream primer (5 μM), and 1.6 μL RNase-free H2O. The relative quantitative results of the lncRNA were obtained after the λ polyA correction.

2.6 | Construction of the exosomal lncRNA-miRNA-mRNA network

In order to further explore the potential function of the dysregulated exosomal lncRNAs as competing with miRNA to inhibit its regulation of the target genes, we drew the map of the lncRNA-miRNA-mRNA interaction network. First of all, we selected the five verified lncRNAs based on the highest expression difference in the MG groups, most miRNAs will then be perfectly matched with the target sequence with 5′ nucleotide 2-8. This region is called the seed region. Our prediction algorithm mainly considers the following points: the perfect complementary degree of the miRNA seed region, sequence conservation of the miRNA recognition elements and binding free energy of lncRNA-miRNA. The bioinformatics software of miRanda, PITA, and RNAhybrid were applied to jointly predict the miRNAs that may bind to the five lncRNAs. After obtaining the candidate miRNAs, the databases of miRDB, targetScan, CLIP-seq, and miRanda were used to further identify the miRNA-mRNA pairs. The prediction results of all the software were further screened and sorted out to reduce the false-positive rates, and their common prediction results were taken as the candidate target genes of the miRNAs. Based on this information, a lncRNA-miRNA-mRNA network was established using the Cytoscape online platform (https://www.cytoscape.org).

2.7 | Statistical analysis

The statistical analysis was performed using the IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8 (GraphPad, La Jolla, CA, USA). Heatmaps were obtained using the R package. Brown-Forsythe and Welch ANOVA tests were used to analyze the differences in the lncRNA expression levels among the three patient groups. A $P < 0.05$ was considered to be statistically significant.

3 | RESULTS

3.1 | Characterization of the serum exosomes

The exosomes were isolated from the serum of the MG patients and healthy controls. The 20-200 nm vesicles isolated from MG patients were identified as exosomes by electron microscope (Figure 1).

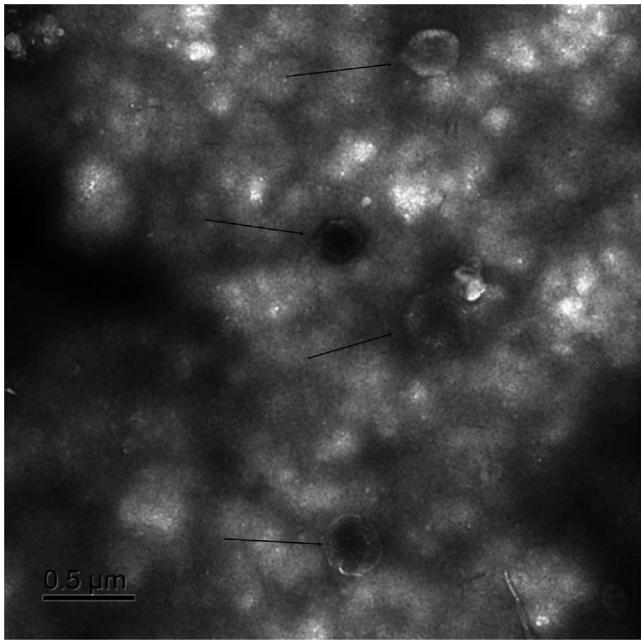

FIGURE 1 A representative electron microscopy image of serum-derived exosomes from an MG patient

3.2 | Differentially expressed profiles of the exosomal lncRNA by RNA sequencing

We identified the differentially expressed exosomal lncRNAs using the fold-change filtering (|log 2(fold_change) | >1, $P < 0.05$). Compared with the healthy control group, a total of 378 significantly upregulated and 348 significantly downregulated exosomal lncRNAs were found in the MG patients (Figure 2A). There were 10 upregulated lncRNAs (NR_104677.1, ENST00000583253.1, NR_046098.1, NR_022008.1, ENST00000581362.1, NR_110616.1, ENST00000583253.1, ENST00000559292.2, ENST00000502636.1, ENST00000421020.1) and 10 downregulated ones (NR_003351.1, NR_046668.1, ENST00000526777.1, ENST00000547834.1, NR_037714.1, ENST00000567401.1, ENST00000457998.2, NR_033837.1, ENST00000601040.1, NR_027072.2). The most differentially expressed lncRNAs were used for the cluster analysis, and the heatmap showed the difference in the expression levels between the lncRNAs in the MG groups and those in the control group (Figure 2B).

3.3 | QRT-PCR validation and comparison of exosomal lncRNA levels among the three groups of patients

In order to validate the reliability of the sequencing data, we used the qRT-PCR method to evaluate the levels of 5 significant exosomal lncRNAs (NR_104677.1, ENST00000583253.1, NR_046098.1, NR_022008.1, and ENST00000581362.1) in the remaining cohort of 30 subjects from our initial collection. The qRT-PCR results demonstrated that the expression levels of these lncRNAs were in consistence with the results of the RNA sequencing (Figure 3A-C). In addition,

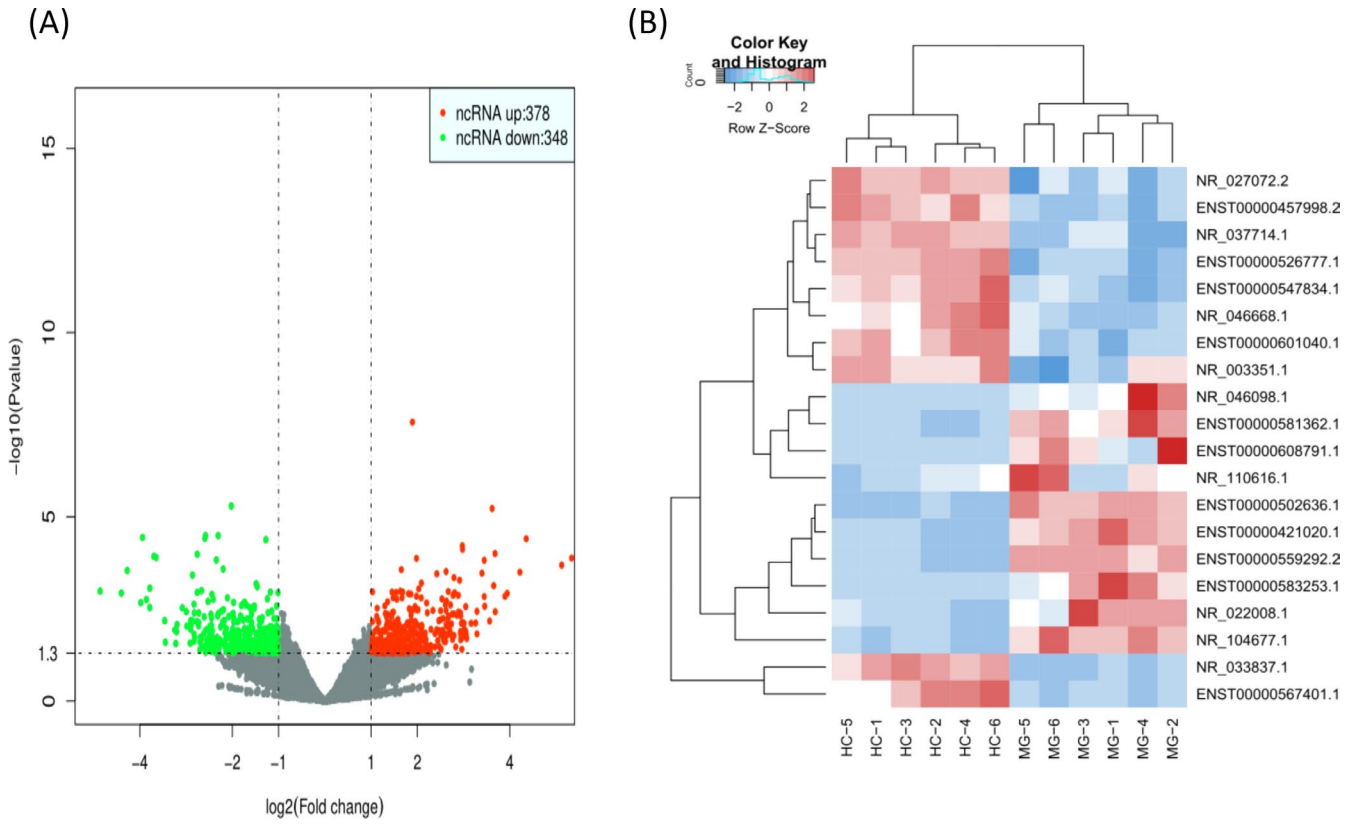

FIGURE 2 (A) The differentially expressed exosomal lncRNAs volcano plots between the MG patients and HC group. (B) Hierarchical cluster analysis of 20 lncRNAs with the largest difference. Each row represents a lncRNA, and each column represents an RNA sample. The red and blue colors indicate that the expression of the genes is relatively upregulated or downregulated, respectively

we compared the 5 exosomal lncRNAs in MG patients with different grades, and the expression of the exosomal lncRNA NR_046098.1 was significantly higher in the IIb group than in the IIa patients, and significantly higher in the IIa patients than in the I patients. The 4 other lncRNAs showed no significant difference among these groups (Figure 3D).

3.4 | Prediction of the lncRNA-miRNA interactions and the functions of their target genes

As shown in Figure 4, the core network comprises the relationships between 5 validated dysregulated lncRNAs, 14 MG-related miRNAs and 30 mRNAs. The green nodes represent the lncRNAs, the red nodes represent the miRNAs, and the purple ones denote the mRNAs. The results demonstrated that several critical lncRNAs played important roles in the pathological processes of MG through regulating a sequence of miRNAs and mRNAs.

3.5 | GO and KEGG pathway analysis of the differentially expressed exosomal lncRNAs in MG patients

To further clarify the function of the exosomal lncRNAs from serum-derived MG and find out which functional terms are enriched, we performed the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis on the differentially expressed mRNAs of the dysregulated lncRNAs between the MG and the control groups. The GO analysis was performed using the KOBAS 3.0 software and consists of three categories: cellular component (CC), molecular function (MF), and biological process (BP). The differentially expressed profiles of the mRNAs and different enrichment pathways were mapped using the KEGG and the KOBAS 3.0 software. The top 9 results of the GO enrichment analysis are shown in (Figure 5). Based on the GO analytical data, the most enriched CC terms were the MIS12/MIND type complex, lipid droplet, and the integral component of membrane; the main enriched BP terms were the detection of the stimulus involved in sensory perception, nervous system process, and G protein-coupled receptor signaling pathway; the most notably enriched MF terms were the G protein-coupled receptor activity, olfactory receptor activity, and transmembrane signaling receptor activity. These terms suggest that some lncRNAs are directly or indirectly involved in the basic biological regulation of MG. On the other hand, the KEGG pathway analysis was performed to show how several key pathways related to mRNAs showed significant differences in the functional changes between MG and control groups. The metabolic disorder, autoimmune thyroid disease, T-cell receptor signaling pathway, Toll-like receptor signaling pathway, and cytokine-cytokine receptor interaction were enriched in the immune-related pathways (Figure 6).

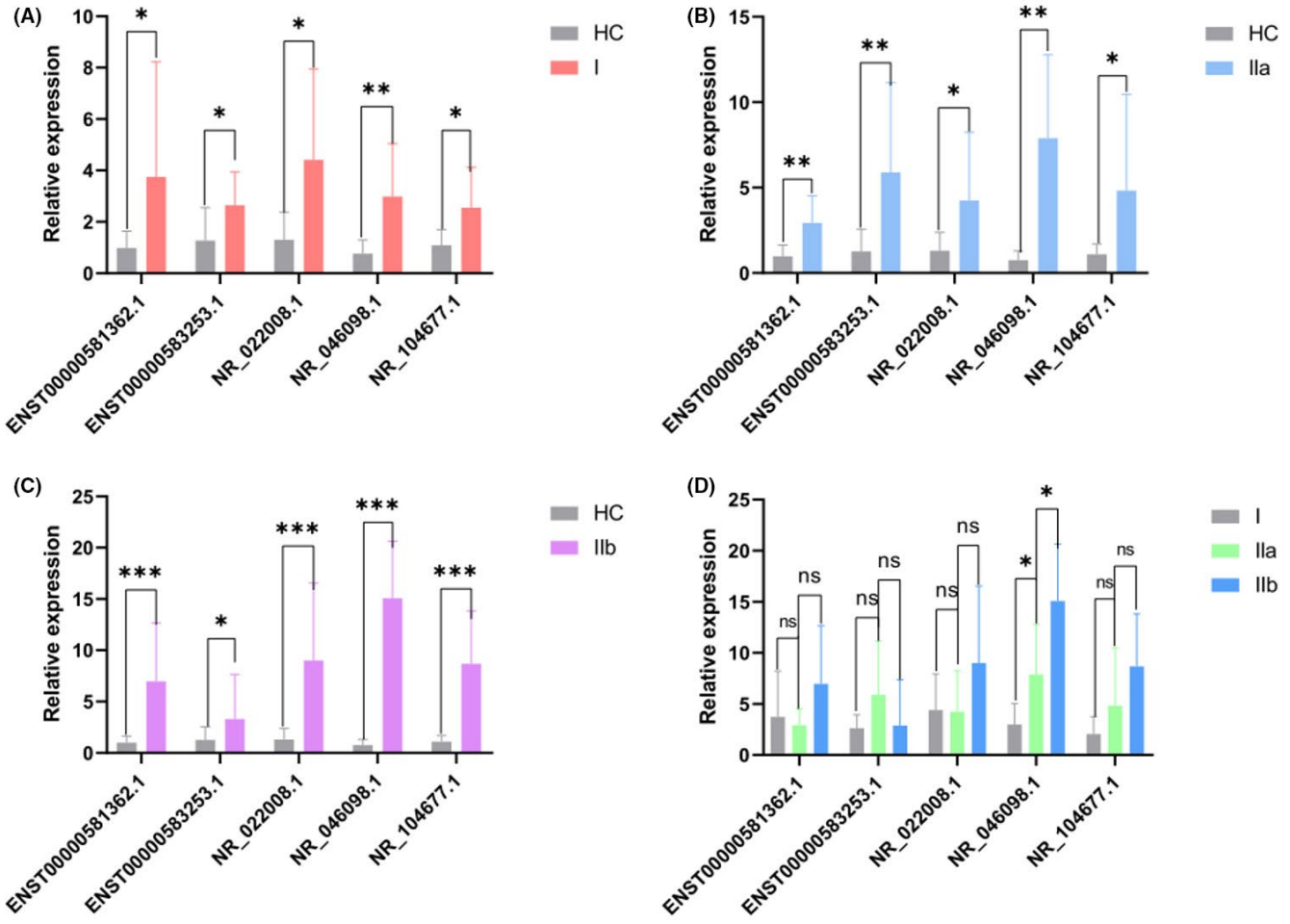

FIGURE 3 The qRT-PCR validation of 5 upregulated lncRNAs between MG patients (A-C) and HC. *P < 0.05, **P < 0.01, ***P < 0.001. (D) Comparison of expression levels of 5 lncRNAs in exosomes of MG patients with different degrees (IIb vs IIa, IIa vs I, both adjusted *P < 0.05)

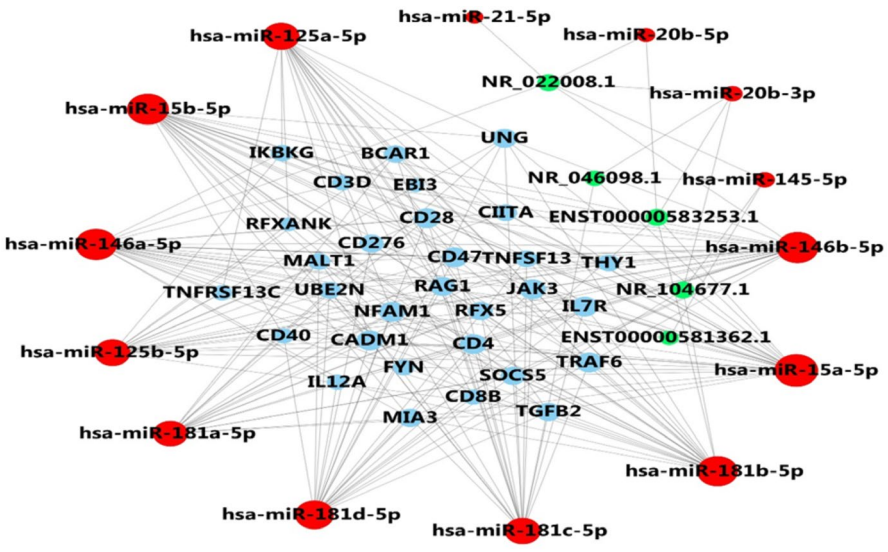

FIGURE 4 Construction of the dysregulated lncRNA-miRNA-mRNA network in MG patients. The green circles represent the lncRNAs, the red circles represent the miRNAs and the blue circles denote the mRNAs. Their regulatory relationships are displayed as lines between them

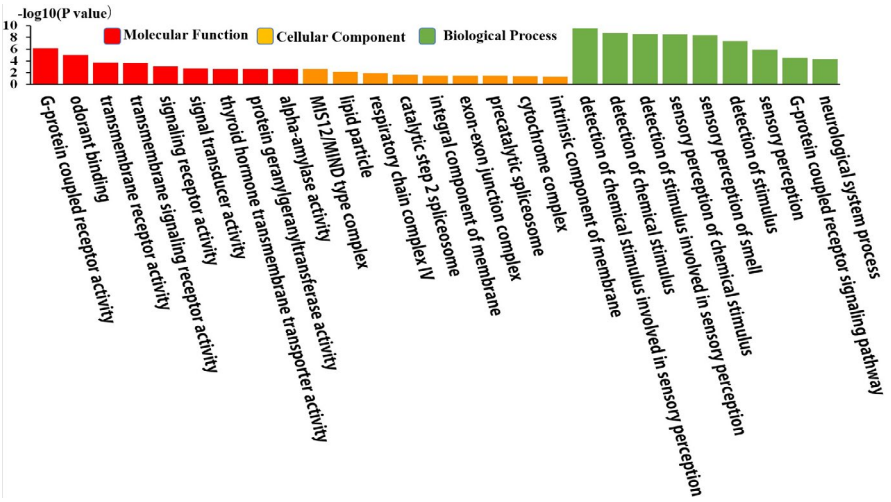

FIGURE 5 The GO terms with the highest enrichment (including the biological process, cellular component and molecular function) among the 27 major entities with changed mRNAs in MG were displayed. The abscissa represents various GO entries, and the ordinate represents the -log 10 (*P* value)

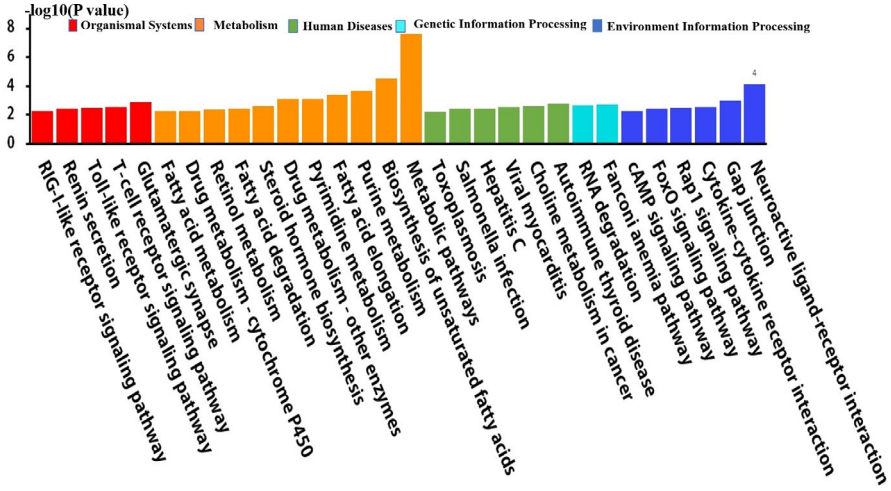

FIGURE 6 KEGG analysis of the differentially expressed mRNAs. 29 pathways with significantly changed functions including organismal systems, metabolism, human diseases, genetic information processing and environment information processing. The abscissa represents the name of the pathway, and the ordinate represents the -log 10 (*P* value)

4 | DISCUSSION

In this work, we investigated differentially expressed lncRNAs in the peripheral blood of MG patients. Our results might provide potential biomarkers for the diagnosis of MG and the function of lncRNAs in the pathogenesis of this disease. In order to provide an impartial profile evaluation, we isolated serum-derived exosomes from three groups of MG patients according the Osserman classification and used the RNA-seq technology. We identified and obtained the exosomal lncRNA profiles for the first time and validated the 5 exosomal lncRNAs, which had not been reported in the MG patients using the qRT-PCR. We observed that the level of the exosomal lncRNA NR_046098.1 significantly increased with the severity of MG.

To better understand how lncRNAs work, we sought the mRNAs related to the activation of the immune system, which are regulated by all the MG-related miRNAs and constructed a network representing the lncRNA-miRNA-mRNA relationships to investigate the biological mechanism of MG. It can be noticed that there are some immune-related target genes that are regulated by hsa-mir-146, hsa-mir-15, hsa-mir-181 and so on. In the network, these potential predicted target genes include the CD molecular series, TNFRSF13C, IL, and many more. The prediction showed that the lncRNAs of ENST00000583253.1, NR_104677.1, NR_022008.1, NR_037714.1, and ENST00000581362.1 can all target 4–5 miRNAs related to immune activation. For instance, miR-15a has been implicated in MG and the immunological disorders. It has been previously reported to be significantly downregulated and to promote the production of the cytokine IL-15 in experimental MG mice.[14] It has also been suggested that all members of the miR-15 cluster were decreased in various phenotypes of MG. Additionally, miR-15a regulates the abnormal immune response by targeting CXCL10 in MG.[15] Cytokines and chemokines are related to the imbalance of Th1/Th2, and they regulate the immune responses in the case of inflammation. Besides, miR-146a is considered to be an important autoimmunity regulation mechanism. The abnormal expression of miR-146a may play a crucial role in regulating the AChR specific B cells, such as the B-cell surface molecule CD40, CD86, and intracellular NF-κB and TLR4 proteins; the machine-processing of miR-146a in the autoimmune diseases mainly focused on the TLR4/NF-κB signaling pathway[16]; thus, these exosomal lncRNAs might act as miRNAs sponges, competitively affecting the miRNA binding to mRNA and being

involved in the immune-related context of action and the regulation of the pathological process of MG. In the target genes of our ceRNA network, the gene of TNFRSF13C (TNF Receptor Superfamily Member 13C) is a protein-coding gene; it has been proposed that abnormally high levels of the B-cell-activating factor (BAFF) enhance the B-cell survival and may contribute to the pathogenesis of MG.[17] The protein encoded by CD28 (CD28 molecule) is essential for the T-cell proliferation and survival, cytokine production, and T-helper type-2 development.[18] Therefore, we infer that the tuples of NR_104677.1-miR-15b-5p-TNFRSF13C, NR_022008.1-miR-146a-5p-CD28, ENST00000581362.1-miR-146a-5p-TNFRSF13C (all up-down-up) and others are potential MG-related lncRNAs-miRNA-mRNA interactions.

In this work, the most enriched CC GO terms in the predicted target genes of the lncRNAs mainly include the following: GO:0000444 (MIS12/MIND type complex); GO:0005811 (lipid droplet), an intracellular non-membrane-bounded organelle comprising a matrix of coalesced lipids that are surrounded by a phospholipid monolayer. It may be explained that lipids and proteins are located in the cellular organelles, which have specific protein phenotypes to perform special functions. Regarding the BP GO terms, the enriched terms concentrate on GO:0050906 (detection of the stimulus involved in sensory perception); GO:0050877 (nervous system process); GO:0007186 (G protein-coupled receptor signaling pathway); GO:0050906 describes the series of events that are involved in the sensory perception in which a sensory stimulus is received and converted into a molecular signal. For example, when motoneurons receive signals from the nervous system, they release the signal molecule (acetylcholine) to the synapse of the cell, activate the M or N receptor of the postsynaptic membrane, transmit the signals to the cell through the receptor and open the downstream cascade reaction. As for the enriched MF GO terms, they included GO:0004930 (G protein-coupled receptor activity); GO:0004930 and GO:0004984 (olfactory receptor activity), which belong to GO:0004888 (transmembrane signaling receptor activity), which in turn refers to the combination with an extracellular or intracellular signal and transmitting the signal from one side of the membrane to the other side to initiate a change in the cell activity or state as part of the signal transduction. These GO terms are also closely related to the biological processes of MG.

We also performed the KEGG pathway analysis to annotate the differentially expressed mRNAs in the exosome that were mainly enriched in the metabolic pathways, autoimmune thyroid disease pathway, T-cell receptor signaling pathway, Toll-like receptor signaling pathway, and cytokine-cytokine receptor interaction, which are all directly or indirectly relevant to the pathogenesis of MG. For example, the glycolipid metabolism is closely related to the metabolic pathway, and the glucose and lipid molecules are involved in the immune reaction process. Its mechanism in MG may be that when muscle dysfunction occurs, the sensitivity of skeletal muscles to insulin decreases, and their ability to consume sugar and fat decreases, thus increasing the risk of abnormal glucose and lipid metabolism.[19] MG and the autoimmune thyroid disease are both autoimmune diseases of a specific organ with a concrete pathological role of the antibodies.[20] Besides, the disorder of the immune system and the implications of susceptibility genes such as the HLA and CTLA4 genes are the common pathogenic mechanisms.[21] CD40LG and HLA-DRB5 were predicted to be targeted in the autoimmune thyroid disease pathway (hsa05320). It is reported that the IFNG-AS1 lncRNA could decrease the expression of HLA-DRB and influence the levels of CD40L, and the CD4+ T-cell activation in MG patients relies on affecting the HLA-DRB1 expression to some extent.[22] An abnormal CD4+T-cell activation is known to play an important role in the pathogenesis of MG. T-cell receptor (TCR) signaling pathway is implicated in the functioning of T cells and formation of the immunological synapse. CD40LG is primarily expressed on the surface of the activated CD4+ T cells and it binds to CD40 on the B cells to offer an essential signal to initiate the immune response, including the B-cell activation and differentiation and pathogenic autoantibody production.

Our study still has several limitations. Firstly, MG is a rare disease, and relatively few cases participated in the validation; thus, the comparison results of the three groups are very preliminary and need further confirmation in a larger cohort. Secondly, we did not conduct follow-up experiments demonstrating the relevance of the identified RNAs function.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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