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Original Research Article

Identification and validation of lncRNA mutation hotspot SNPs associated with myasthenia gravis susceptibility

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ABSTRACT

Background: Myasthenia gravis (MG) is an autoimmune disorder caused by antibodies that target the postsynaptic muscle membrane. Recent evidence suggests that genetic variants and long noncoding RNAs (lncRNAs) play crucial roles in the pathogenesis of MG. The purpose of this study was to investigate the associations between lncRNA-related single-nucleotide polymorphisms (SNPs) and MG susceptibility in Chinese populations.

Methods: First, we identified lncRNA mutation hotspot regions based on the improved Kolmogorov−Smirnov test and the cumulative hypergeometric distribution principle. Next, we further identified lncRNA mutation hotspot SNPs by calculating conservative scores. Finally, experiments were conducted to verify the associations between lncRNA mutation hotspot SNPs and MG susceptibility. A total of 82 patients with MG and 82 healthy controls were recruited for genotyping of lncRNA mutation hotspot SNPs using the SNaPshot technique. Quantitative real-time PCR was used to investigate lncRNA expression in 34 patients with MG and 37 healthy controls.

Results: In the multistep calculation, 14 candidate SNPs of 3 lncRNAs (AL031686.1, NONHSAT028539.2 and AC245014.3) in MG were identified as mutation hotspot SNPs. The genotyping results of the 14 SNPs in our study revealed no statistically significant differences in the frequencies of genotypes and alleles between patients with MG and controls. However, in the lncRNA AL031686.1, rs1000383 and rs6094353 were in perfect linkage disequilibrium (LD) and were associated with an increased risk of ocular MG. Additionally, rs6094347 was associated with an increased risk of ocular MG. Nevertheless, no SNP was found to be associated with factors such as sex, age, the presence or absence of thymoma, or the genetic model of MG. Further experiments revealed that NONHSAT028539.2 expression was upregulated in peripheral blood mononuclear cells (PBMCs) from patients with MG compared with those from healthy controls.

Conclusion: In our study, we did not find an association between the 14 mutation hotspot SNPs of lncRNAs and susceptibility to MG. However, we observed that the rs6094347 and rs1000383/rs6094353 polymorphisms in the lncRNA AL031686.1 were associated with the risk of ocular MG.

1. Introduction

Myasthenia gravis (MG) is a T-cell dependent, B-cell-mediated autoimmune disease caused by autoantibodies that attack proteins of the postsynaptic membrane at the neuromuscular junction and impair neuromuscular transmission [1]. The main clinical manifestation of MG is fluctuating weakness of the skeletal muscles [2]. The most common autoantibody target is the acetylcholine receptor (AChR). Low-density lipoprotein receptor-related protein 4 (LRP4), muscle-specific kinase (MuSK) and agrin antibodies have also been implicated in MG [3–5]. At

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present, the etiology of MG is not completely clear. Previous studies have shown that its pathogenesis is related to genetic, environmental, and immune factors, among which genetic susceptibility plays an important role [6]. However, the precise mechanisms involved have not been fully elucidated.

Single-nucleotide polymorphisms (SNPs) are the most common genetic variants in the human genome. SNPs refer to nucleotide variations at specific positions in the genome and are usually defined by an incidence of variation greater than 1 % [7]. SNPs have become powerful tools with which to investigate the biological processes and genetic architecture of monogenic or complex diseases because of their high frequency and simple patterns of variation [8,9]. In addition, previous studies have reported that SNPs play important roles in the development of MG [10–12]. In general, SNPs occur much less frequently in coding regions of the genome than in noncoding regions, and the majority of the SNPs identified are located in noncoding regions, especially in lncRNAs [13,14]. These findings indicate that these molecules could be involved in disease susceptibility.

LncRNAs are generally defined as transcripts longer than 200 nucleotides without any protein-coding capabilities [15]. Although lncRNAs are not translated into proteins, increasing evidence has revealed several significant biological functions of lncRNAs, such as the regulation of allelic expression, organ or tissue development, metabolic processes, epigenetic regulation, transcription, posttranscriptional regulation, translational and posttranslational regulation [16–19]. It has been reported that lncRNAs can interact with DNA, RNA, and proteins to modulate the expression of protein-coding genes [20,21]. Current studies have shown that lncRNAs play important roles in regulating gene expression and are involved in the regulation of MG development. For example, in our previous study, we reported that the lncRNA SNHG16 regulates the expression of the key MG gene interleukin-10 by sponging let-7c-5p in a competitive endogenous RNA manner [22]. Furthermore, there is growing evidence that risk variants in lncRNAs may contribute to a range of diseases by influencing the expression of protein-coding genes or the expression levels of lncRNAs [23,24]. Therefore, it is conceivable that lncRNA-associated SNPs may affect lncRNA regulation. However, the exact mechanisms by which lncRNA variants (lncSNPs) play roles in MG disease remain unclear.

In the present study, we initially identified differentially expressed lncRNAs through lncRNA and mRNA microarray. We subsequently identified lncRNA mutation hotspot regions based on the improved Kolmogorov–Smirnov test and the cumulative hypergeometric distribution principle. We further verified lncRNA mutation hotspot SNPs by calculating conservative scores. Finally, experiments were conducted to validate the multistep calculation results, aiming to gain a better understanding of the relationship between lncSNPs and MG risk through a case–control study. In conclusion, we identified and verified the lncSNPs associated with the risk of MG, thus providing new insights into the specific roles of lncSNPs in MG.

2. Materials and methods

2.1. Identification of differentially expressed lncRNAs (DE lncRNAs) and acquisition of SNPs of DE lncRNAs

The microarray profile (GSE263220) was obtained from the sequencing data of our research group's previous study [25]. The DE lncRNAs and DE mRNAs were screened using fold change (FC) and Student's *t*-test, with thresholds set at FC > 1.5 and P < 0.05. A total of 2064 DE lncRNAs and 2374 DE mRNAs were identified. Then, SNP sites within the screened DE lncRNA regions were downloaded from the UCSC database (https://genome.ucsc.edu/), resulting in a total of 91, 276 SNP sites on 919 lncRNAs. Detailed annotation information for the lncRNAs was downloaded from the GENCODE database (https://www.gencodegenes.org/).

2.2. Distribution of SNPs on DE lncRNAs in MG

Like protein-coding genes, lncRNAs are composed of introns and exons. When a gene is transcribed, its pre-mRNA is processed so that only the exon sequence remains in the mature mRNA [26]. However, the number of exon blocks in each lncRNA is different. Therefore, the number of exons contained in these lncRNAs was calculated based on the annotation information and the start and end positions of their exons to further understand the structural characteristics of DE lncRNAs in MG. Studies have shown that SNPs are not evenly distributed throughout the genome and that their distribution varies greatly across different regions [7]. Hence, we calculated the number of SNPs in both the exon and intron regions based on their location information. Additionally, we utilized SNP density to analyse and compare the specific distribution characteristics of SNPs on lncRNAs. The SNP density was determined by dividing the number of SNPs by the total length of each lncRNA. We divided each exon and intron into 10 equal parts for each lncRNA and determined the average relative density of SNPs within each part. The above analyses were performed with R software.

2.3. Identification of mutation hotspot regions (MHRs) on lncRNAs

Single-nucleotide polymorphism sites with unusually high mutation frequencies are called mutation hotspots. These mutation hotspots often reflect the underlying mechanism of mutation, which is highly important to study [27]. Therefore, based on the sequence of the lncRNA, a region with a high density of mutations in the lncRNA can be identified as a mutation hotspot region (MHR). In this study, we calculated the MHRs of lncRNAs using an improved principle of the Kolmogorov–Smirnov test. The calculation principle is as follows: Each base site contained within each lncRNA sequence was evaluated. A positive score was assigned to positions with SNPs, whereas a negative score was given for positions without SNPs. This allowed for the determination of minimum and maximum cumulative scores for each lncRNA. The region between the highest and lowest scores was identified as the MHR of the lncRNA. The specific formula for calculating the MHR is as follows:

$$Score_k = \sum_{i}^{n} \left(\frac{m_{i,k}}{M_k} - \frac{1}{N_k} \right)$$

$$MHR = Score_{highest} - Score_{lowest}$$

In the formula, k represents the number of markers for all lncRNAs involved in calculating the mutation hotspot region score. Moreover, n denotes the total length of a lncRNA, and i indicates the position of each base passing through a lncRNA. Additionally, $m_{i,k}$ represents the number of SNPs occurring at position i, M_k represents the total number of SNPs on the current lncRNA, and Nk signifies the number of positions on the current lncRNA that have not undergone SNP mutations. $\frac{m_{i,k}}{M_k}$ is utilized as an impact score assigned to location i for a SNP mutation on this particular lncRNA, whereas $\frac{1}{N_k}$ serves as a score for a site without any mutations.

2.4. Screening of lncRNAs whose MHRs were significantly enriched

In accordance with the method of cumulative hypergeometric distribution, we evaluated the significance of SNP enrichment in MHRs. The improved hypergeometric formula is as follows:

$$p_{MHR} = 1 - \sum_{i=1}^{m-1} \frac{C\binom{m}{n} * C\binom{M-m}{N-n}}{C\binom{M}{N}}$$

In the formula, p_{MHR} represents the p value used to assess the enrichment significance of each lncRNA. The variable m denotes the number of SNPs

contained in the MHR of a given lncRNA, whereas *M* indicates the total number of sites with SNP variation within the entire lncRNA. Additionally, *n* signifies the number of bases contained in the MHR, and *N* represents the total number of bases within the lncRNA. Within this formula, *i* accumulates from 1 to m-1, representing the sum of probabilities for potential mutations within all MHRs smaller than that of the current lncRNA. By subtracting the accumulated probability from 1, a probability value can be obtained that takes into account the number of SNPs in the current MHR. A smaller p value indicates a more biologically significant enrichment of mutations in a lncRNA's MHR. In this study, we set the threshold for measuring the significance level of the p value at $P < 0.01$. The above analyses were conducted using Python software.

2.5. Identification of the conservation of lncRNAs and SNPs

The R package phastCons100way.UCSC.hg38 was used to calculate conservation scores. This annotation package stores the phascons conservation scores for the human genome (hg38) as annotated by UCSC. The aforementioned R package was used to calculate the conservation scores of lncRNAs in MHRs and non-MHRs and to identify lncRNAs with higher conservation scores in MHRs than in non-MHRs. This will allow for the identification of lncRNAs that are relatively more conserved in MHRs. Next, the top 3 lncRNAs with the highest conservation scores in MHRs were selected. Finally, the conservation scores of all SNP sites within these 3 lncRNAs' MHRs were calculated, and SNP sites with conservation scores greater than or equal to 0.7 were retained.

2.6. LncRNA-mRNA co-expression analysis

To identify lncRNA-mRNA interaction pairs, we performed co-expression correlations for lncRNA-mRNA by calculating Pearson correlation coefficients (PCC) based on the expression of three lncRNAs (AL031686.1, NONHSAT028539.2 and AC245014.3) and DE mRNAs. The threshold for lncRNA-mRNA interaction pairs was set to a PCC>0.5 and a P-value<0.05.

2.7. Functional enrichment analysis and pathway-gene interaction network construction

To explore the biological functions of lncRNAs, we applied gene ontology (GO) and the Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis using the R package "clusterProfiler". The GO and KEGG pathway databases originated from the "org.Hs.eg.db" package. The threshold for statistical significance was set at a p-value of 0.05 for selecting enriched GO terms and KEGG pathways. Based on the results of KEGG analysis, the top significant pathways and their enriched genes were used to construct the pathway-gene interaction network to identify key genes using the R packages "ggraph" and "igraph". The size of a node in the network is expressed by the degree of the node. The gene with a large degree of node is called key gene in the network. The results were visualized using the "ggplot2" R package.

2.8. Subjects and blood samples

In this study, blood samples were collected from a group of patients with MG and normal control volunteers at the Second Affiliated Hospital of Harbin Medical University. All the patients met the diagnostic criteria for MG [28]. This study was approved by the Ethics Committee of The Second Affiliated Hospital of Harbin Medical University, and all the subjects provided written informed consent. The study was conducted in accordance with the World Medical Association Declaration of Helsinki. Peripheral venous blood samples (5 ml) were obtained from each patient with MG and normal control volunteer in tubes containing ethylenediaminetetraacetic acid.

2.9. DNA extraction and genotyping

A total of 82 patients with MG (50 females; 32 males) and 82 healthy controls (49 females; 33 males) were collected for peripheral blood DNA extraction. The mean age of the MG group was 62.10 ± 12.12 years, whereas the mean age of the control group was 61.07 ± 10.29 years. Early-onset MG (EOMG <50 years) and late-onset MG (LOMG >50 years) were distinguished using an age cut-off of 50 years [29]. Among patients with MG, 15 had EOMG and 67 had LOMG; 23 were diagnosed with ocular MG (OMG) and 59 with general MG (GMG); 11 had thymoma, and 71 had no thymoma; and 54 were AChR antibody-positive, 3 were AChR antibody-negative, and 25 patients refused the MG-related antibody test. The clinical data of all patients and healthy controls are shown in Table S1. A Blood Genomic DNA Maxi Kit (GeneBetter Biotech, Beijing, China) was used to extract genomic DNA from each sample, which was then stored at −20 °C until analysis. The SNaPshot SNP assay was performed to detect dimorphism at the fourteen SNP loci. A segment of DNA surrounding the SNPs was amplified by PCR using Takara HotStarTaq (Takara). After purification using shrimp alkaline phosphatase and exonuclease I (Epicentre), the PCR products were subjected to a primer extension assay using a SNaPshot Multiplex Kit (ABI). The resulting primer extension products were analysed on an ABI 3130xl capillary electrophoresis DNA instrument using Gene Mapper 4.1 software (Applied Biosystems, Foster City, CA). To ensure quality control, researchers genotyped without knowledge of case or control status, and 5 % of the cases and controls were randomly selected and genotyped twice by different individuals, achieving a repeatability rate of 100 %. The PCR primer pairs and the SNaPshot multiplex single-base extension reaction primer sequences are shown in Table S2.

2.10. RNA extraction, reverse transcription, and qRT−PCR

A total of 34 patients with MG (22 females; 12 males) and 37 healthy controls (26 females; 11 males) were collected for peripheral blood RNA extraction. The mean age of the MG group was 65.35 ± 12.39 years, whereas the mean age of the control group was 61.78 ± 8.58 years. Among patients with MG, 4 were EOMG and 30 were LOMG; 11 were diagnosed with OMG and 23 with GMG; 4 had thymoma; 30 had no thymoma; 24 were AChR antibody-positive; 1 was AChR antibody-negative; and 9 patients refused MG-related antibody tests. The clinical data of all patients and healthy controls are shown in Table S3. Peripheral blood mononuclear cells (PBMCs) were isolated using lymphocyte separation medium and stored at −80 °C until experiments. Total RNA was extracted from PBMCs using TRIzol reagent (SEVEN, Beijing, China) following the manufacturer's instructions. A Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) was used to reverse transcribe total RNA with the corresponding primers following the manufacturer's instructions. A FastStar Universal SYBR Green Master Kit (Roche, Basel, Switzerland) was then used to detect the expression levels of NONHSAT028539.2, AC245014.3 and AL031686.1 by quantitative real-time PCR (qRT−PCR). GAPDH was used as an internal control for lncRNAs. The qPCR results were quantified as the fold change between the expression of GAPDH and that of the target gene using formula $2^{-\triangle\triangle Ct}$. The primer sequences are provided in Table S4.

2.11. Statistical analysis

All the statistical analyses were performed with SPSS 27.0 (SPSS, Chicago, Illinois, USA). Hardy−Weinberg equilibrium (HWE) was tested using the chi-square test to compare the expected genotype frequencies with the observed genotype frequencies in healthy controls. The frequencies of genotypes and alleles were obtained through direct counting. The genotype and allele frequencies between the MG and control groups and the MG subgroups (sex, age, with or without thymoma, etc.) were compared using the chi-square test or Fisher's exact probability test, as appropriate. The relative risk associated with alleles and

genotypes was estimated as an odds ratio (OR) with a 95 % confidence interval (CI). For qRT−PCR data analysis, each sample group was tested three times. The experimental results are presented as the means ± standard deviations. Comparisons between two groups were analysed using the Mann−Whitney test. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Identification of DE lncRNAs in MG

The DE lncRNAs between MG and healthy control samples in the GSE263220 dataset were identified using the "limma" package of R, with a p value of <0.05 and an FC of 1.5 as the threshold. As shown in Fig. 1A and B, we obtained 1480 downregulated lncRNAs and 584 upregulated lncRNAs in MG.

3.2. Distribution characteristics of SNPs on the DE lncRNAs in MG

The largest distribution of lncRNAs was mainly concentrated in intervals containing 1–4 exons (Fig. 1C). Among all the 47,770 SNPs in the lncRNA region, 43,783 SNPs were located in intron regions of the lncRNAs, accounting for 92 % of all the SNPs. Only 8 % of the SNPs, i.e., a total of 3987 SNPs, were located in the exon regions of the lncRNAs (Fig. 1D). We found that lncSNPs tended to have relatively high distribution density at the interface between lncRNA exons and introns, which was consistent with the generally accepted view that mutations usually occur in clipping site regions [30].

3.3. Characteristics of mutation hotspot regions on lncRNAs in MG

Based on the improved KS test principle, 487 lncRNAs containing MHRs were screened from 2064 DE lncRNAs. We subsequently utilized the cumulative hypergeometric distribution method to assess whether lncSNPs were significantly enriched with MHRs. With a significance threshold of 0.01, a total of 188 MHRs significantly enriched on lncRNAs were identified. Next, the R package phasstcon100way.ucsc.hg38 was used to calculate the MHR and SNP conservation scores of the lncRNAs. After the multistep calculation, 14 candidate SNPs of 3 lncRNAs in MG were identified as mutation hotspot SNPs. These lncSNPs are most likely associated with MG. The basic information of these 14 SNP loci is shown in Table 1.

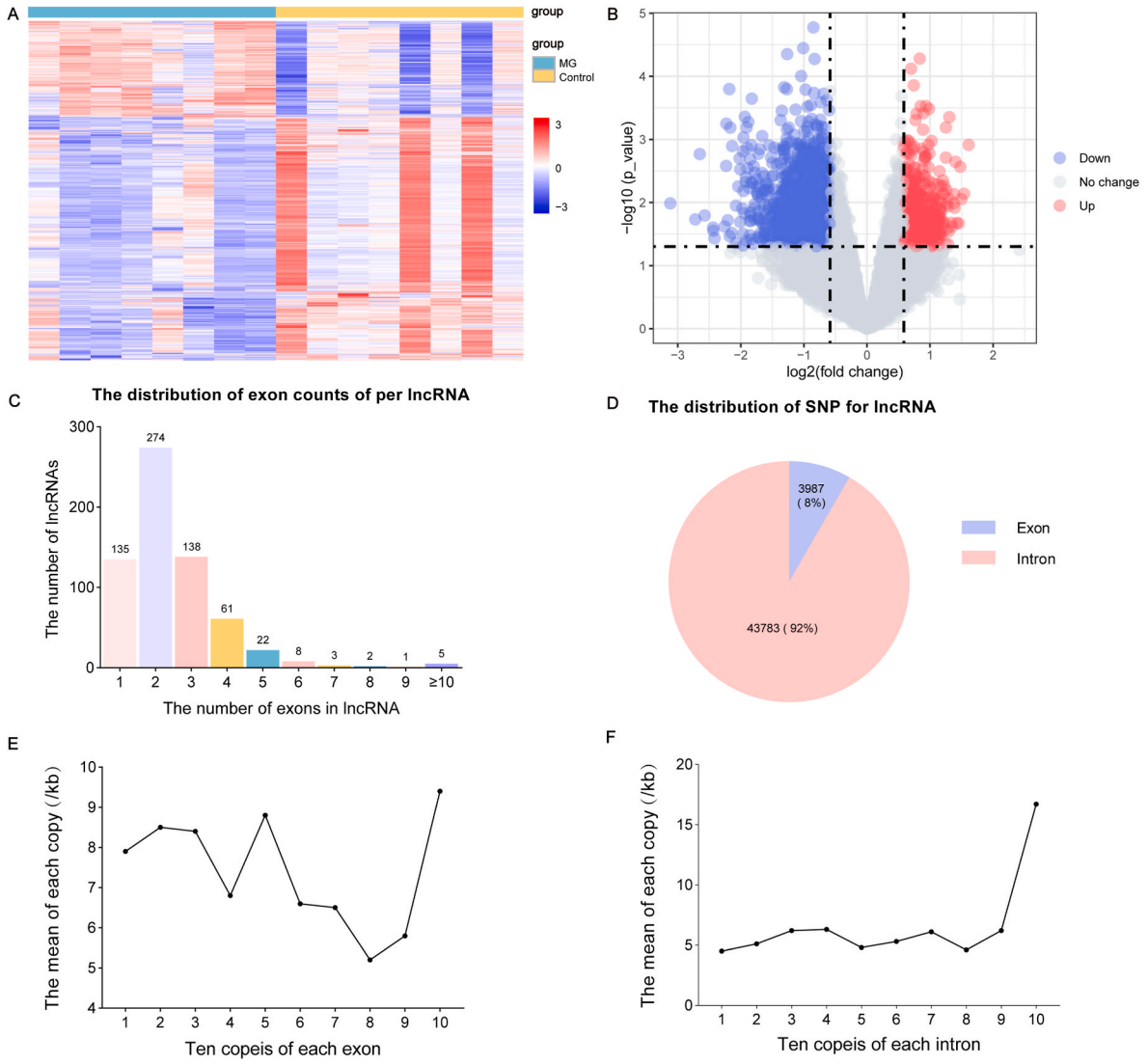

Fig. 1. Differentially expressed lncRNAs in MG and distribution characteristics of lncSNPs. (A) Heatmap of differentially expressed lncRNAs in MG; (B) volcano map of differentially expressed lncRNAs in MG; (C) number of exons on each lncRNA; (D) distribution of SNPs on exons and introns of lncRNAs; (E) average relative density of SNPs in 10 equally divided exons; (F) average relative density of SNPs in 10 equally divided introns.

Table 1
Basic information of the included SNPs.

LncRNA name	Rs number	Chrom	Position	Alleles
AL031686.1	rs6094347	chr20	46462361	G > A
	rs279730	chr20	46463878	T > C
	rs24236	chr20	46464220	C > T
	rs1000383	chr20	46464282	G > A
	rs279729	chr20	46464486	A > G
	rs6094353	chr20	46471127	A > G
NONHSAT028539.2	rs12426399	chr12	54026126	G > T
	rs4759318	chr12	54026314	C > T
	rs7305320	chr12	54026411	C > T
	rs73313197	chr12	54031687	A > G
	rs10747689	chr12	54031773	C > T
	rs35606176	chr12	54033155	G > C
AC245014.3	rs587663752	chr1	145281204	C > T
	rs368561300	chr1	145281235	G > C

3.4. Distribution of genotypes and alleles of lncSNPs and the risk of MG

In this study, the polymorphisms rs7305320, rs73313197, and rs35606176 in the lncRNA NONHSAT028539.2, as well as rs587663752 and rs368561300 in the lncRNA AC245014.3, were not detected in any of the participants, including both patients with MG and controls. Therefore, these SNPs were excluded from further analysis. The genotypic and allelic frequencies of the other 9 SNPs in patients with MG and healthy controls are shown in Table 2.

In our study, we observed perfect linkage disequilibrium (LD) in the lncRNA AL031686.1 (Fig. 2A and B), and the SNPs rs279730 and rs279729 were in perfect LD (D' = 1, r2 = 1). In addition, perfect LD was also found between SNPs rs1000383 and rs6094353 (D' = 1, r^2 = 1). Additionally, we found that in NONHSAT028539.2 (Fig. 2C and D), SNPs rs12426399 and rs4759318 were also in perfect LD (D' = 1, r^2 = 1). Previous studies have shown that perfect LD indicates that these SNP markers have not been separated by recombination and share the same allele frequencies across populations [31]. Therefore, all of these pairs in perfect LD had the same allele frequencies in our study (Table 2).

In addition, in lncRNA AL031686.1, for SNP rs6094347, only two genotypes were detected (GG and GA); similarly, for rs1000383/rs6094353, two genotypes (GG and GA/AA and AG) were detected in this study. The allele frequencies of these SNPs were similar to those reported in the 1000Genomes_30x study for the East Asian population.

The nine SNPs genotyped in the control group were in Hardy–Weinberg equilibrium (HWE). However, neither the genotype nor allele frequencies of the nine SNPs were significantly different between patients with MG and controls.

3.5. Associations between the lncSNPs and MG clinical characteristics

To investigate the associations of the 9 SNPs with the clinical

Table 2
Genotype and allele frequencies of lncSNPs in patients with MG and controls.

LncRNA	SNP	Genotype Allele	MG N (%)	Control N (%)	P_{HWE}	P	OR (95 % CI)
AL031686.1	rs6094347	GG	58 (70.7)	54 (65.9)	0.062		1.000
		GA	24 (29.3)	28 (34.1)		0.502	0.798 (0.413–1.543)
		G	140 (85.4)	136 (82.9)	–		1.000
		A	24 (14.6)	28 (17.1)		0.545	0.833 (0.460–1.508)
	rs279730	TT	61 (74.4)	61 (74.4)	0.651		1.000
		TC	19 (23.2)	20 (24.4)		0.889	0.950 (0.462–1.954)
		CC	2 (2.4)	1 (1.2)		0.568	2.000 (0.177–22.639)
		T	141 (86.0)	142 (86.6)	–		1.000
		C	23 (14.0)	22 (13.4)		0.872	1.053 (0.561–1.975)
	rs24236	CC	55 (67.1)	56 (68.3)	0.326		1.000
		CT	25 (30.5)	25 (30.5)		0.958	1.018 (0.522–1.985)
		TT	2 (2.4)	1 (1.2)		0.558	2.036 (0.179–23.110)
		C	135 (82.3)	137 (83.5)	–		1.000
		T	29 (17.7)	27 (16.5)		0.769	1.090 (0.613–1.938)
	rs1000383	GG	63 (76.8)	57 (69.5)	0.103		1.000
		GA	19 (23.2)	25 (30.5)		0.290	0.688 (0.343–1.379)
		G	145 (88.4)	139 (84.8)	–		1.000
		A	19 (11.6)	25 (15.2)		0.331	0.729 (0.384–1.382)
	rs279729	AA	61 (74.4)	61 (74.4)	0.651		1.000
		AG	19 (23.2)	20 (24.4)		0.889	0.950 (0.462–1.954)
		GG	2 (2.4)	1 (1.2)		0.568	2.000 (0.177–22.639)
		A	141 (86.0)	142 (86.6)	–		1.000
		G	23 (14.0)	22 (13.4)		0.872	1.053 (0.561–1.975)
	rs6094353	AA	63 (76.8)	57 (69.5)	0.103		1.000
		AG	19 (23.2)	25 (30.5)		0.290	0.688 (0.343–1.379)
		A	145 (88.4)	139 (84.8)	–		1.000
		G	19 (11.6)	25 (15.2)		0.331	0.729 (0.384–1.382)
NONHSAT028539.2	rs12426399	GG	22 (26.8)	29 (35.4)	0.240		1.000
		GT	43 (52.4)	35 (42.7)		0.183	1.619 (0.795–3.299)
		TT	17 (20.7)	18 (22.0)		0.619	1.245 (0.525–2.954)
		G	87 (53.0)	93 (56.7)	–		1.000
		T	77 (47.0)	71 (43.3)		0.506	1.159 (0.750–1.792)
	rs4759318	CC	22 (26.8)	29 (35.4)	0.240		1.000
		CT	43 (52.4)	35 (42.7)		0.183	1.619 (0.795–3.299)
		TT	17 (20.7)	18 (22.0)		0.619	1.245 (0.525–2.954)
		C	87 (53.0)	93 (56.7)	–		1.000
		T	77 (47.0)	71 (43.3)		0.506	1.159 (0.750–1.792)
	rs10747689	CC	29 (35.4)	30 (36.6)	0.260		1.000
		CT	40 (48.8)	35 (42.7)		0.631	1.182 (0.597–2.340)
		TT	13 (15.9)	17 (20.7)		0.603	0.791 (0.327–1.915)
		C	98 (59.8)	95 (57.9)	–		1.000
		T	66 (40.2)	69 (42.1)		0.736	0.927 (0.597–1.440)

MG, myasthenia gravis; P_{HWE} = P value for Hardy-Weinberg Equilibrium; OR, odds ratio; 95 % CI, 95 % confidence interval.

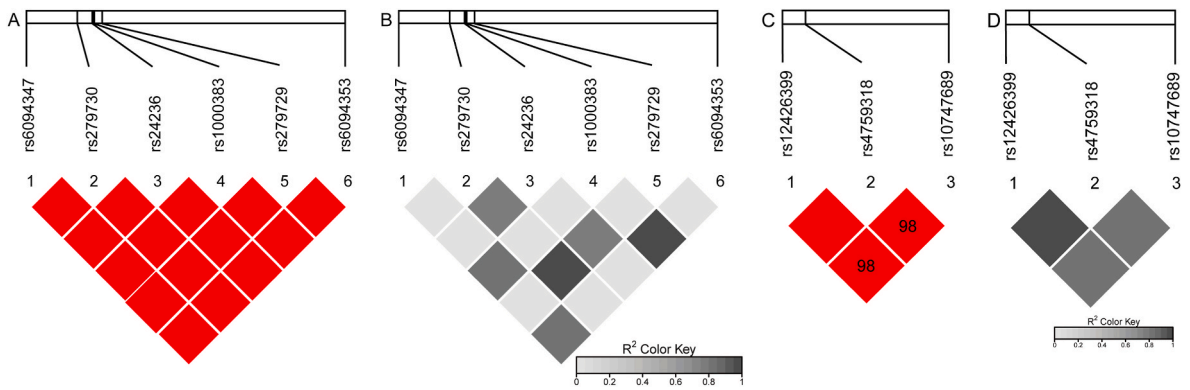

Fig. 2. Linkage disequilibrium (LD) patterns of single-nucleotide polymorphisms (SNPs) of (A)(B) AL031686.1 and (C)(D) NONHSAT028539.2 in a Chinese population. The rs number corresponds to the polymorphism name. The pairwise correlation between SNPs was measured as D′ and is shown (x100) in each diamond. Diamonds without values indicate D' = 1. The colour scheme is white, $r^2 = 0$; shades of grey, $0 < r^2 < 1$; black, $r^2 = 1$.

features of MG, further analysis was conducted to assess the relationships between the SNPs and sex, age, subgroup, and the presence or absence of thymoma in patients with MG.

As shown in Table 3, for lncRNA AL031686.1, in all 82 patients with MG, the frequencies of the rs2275913 GA genotype (P = 0.004, OR = 4.273, 95 % CI95 % CI = 1.518–12.028) and A allele (P = 0.010, OR = 3.118, 95 % CI95 % CI = 1.282–7.581) in the OMG group were significantly greater than those in the GMG group, indicating that these alleles will increase the risk of ocular MG. In addition, the frequencies of the GA/AG genotype (P < 0.001, OR = 5.844, 95 % CI95 % CI = 1.932–17.674) and A/G allele (P = 0.002, OR = 4.321, 95 % CI95 % CI = 1.611–11.595) in rs1000383/rs6094353 were significantly greater in the OMG group than in the GMG group, suggesting that the minor allele A/G may increase the susceptibility to ocular MG. Furthermore, for the lncRNA NONHSAT028539.2, a greater frequency of the GT/CT genotype of rs12426399/rs4759318 was observed in the GMG group, suggesting that the GT/CT genotype is more closely associated with general MG.

As shown in Table S5, there was no significant difference in lncSNP polymorphisms between female and male patients with MG, and there was also no significant difference in lncSNP polymorphisms between the EOMG and LOMG groups. Moreover, no significant differences in genotype or alleles of lncSNPs were observed in patients with MG with or without thymoma. These findings indicate that there is no association between the 9 SNP polymorphisms and sex, age, or the presence or absence of thymoma in patients with MG.

3.6. Expression of lncRNAs (NONHSAT028539.2, AC245014.3, AL031686.1) in MG

The expression levels of NONHSAT028539.2, AC245014.3 and AL031686.1 were evaluated by qRT-PCR in PBMCs from patients with MG and control subjects. NONHSAT028539.2 was upregulated in patients with MG compared with controls (P = 0.023, Fig. 3A). However, the expression of the lncRNA AC245014.3 was lower in MG samples than in control samples, but the difference was not statistically significant (P = 0.424, Fig. 3B). Although AL031686.1 was downregulated in the MG samples compared with the control samples, the difference was not statistically significant (P = 0.577, Fig. 3C).

3.7. Dissection of the potential mechanisms underlying the regulation of MG by lncRNAs with mutation hotspot SNPs

At first, as shown in Fig. 4A and B, we obtained 1425 up-regulated mRNAs and 949 down-regulated mRNAs from the GSE263220 dataset. Then, lncRNA-mRNA co-expression analysis was performed to explore the relationships among above identified three lncRNAs (AL031686.1, NONHSAT028539.2 and AC245014.3) and DE mRNAs in MG. Next, GO functional enrichment analysis revealed that the DE mRNAs co-expressed with these three lncRNAs were enriched in macroautophagy, interferon-mediated signaling pathway, neutrophil differentiation, and CD40 signaling pathway (Fig. 4C). These processes were involved in the immunological mechanism of MG pathogenesis. And these findings revealed the fundamental characteristics of co-expressed lncRNAs that may be involved in the pathogenesis of MG.

Furthermore, we performed KEGG pathway enrichment analysis and then constructed KEGG pathway-gene interaction network to identify key genes for MG. For the co-expression lncRNA AL031686.1, the KEGG pathways were mainly enriched in Endocytosis, Pathways of neurodegeneration - multiple diseases and Neurotrophin signaling pathway (Fig. 5A), and we identified three key genes (NFKB1, AKT2 and MAP3K5) in pathway-gene interaction network (Fig. 5B).Similarly, as shown in Fig. 5C, the KEGG pathways of the co-expression lncRNA NONHSAT028539.2 were mainly enriched in Pathways of neurodegeneration -multiple diseases, mTOR signaling pathway and Endocytosis, and four risk genes (AKT2, BID, NFKB1 and RAB5A) were identified in pathway-gene interaction network (Fig. 5D). In addition, for the co-expression lncRNA AC245014.3, the KEGG pathways were mainly enriched in Natural killer cell mediated cytotoxicity, Notch signaling pathway and cGMP-PKG signaling pathway (Fig. 5E), and we identified four risk genes (HLA-B, HLA-C, MAP2K2 and AKT2) in pathway-gene interaction network (Fig. 5F). These findings indicate that lncRNAs containing mutation hotspot SNPs may be associated with pathways that play crucial roles in the immunological pathogenesis of MG. In conclusion, we constructed the pathway-gene interaction network and identified several critical pathways and genes of co-expression lncRNAs associated, but the actual relationships between them need to be verified experimentally in future studies.

4. Discussion

MG is a neurological autoantibody-mediated disease, and the pathogenetic mechanisms and autoimmune processes involved are still unclear; however, the roles of genetic and environmental factors in its pathogenesis are widely recognized [32]. In addition, an extensive body of evidence now suggests that lncRNAs play regulatory roles in the immune system and can influence numerous autoimmune diseases and MG [33–35]. The identification of mutation hotspot SNPs in DE lncRNAs in MG could help us to elucidate the roles of genetic susceptibility in the progression of MG. Therefore, it is highly important to search for MG-susceptible lncRNAs and explore their functions from the perspective of genetics to understand the pathogenesis of MG.

At present, lncSNPs have been studied in cancer and immune diseases [36–38]. However, the majority of research on polymorphisms in

Table 3
Genotype and allele frequencies of lncSNPs in subgroups and thymic disease-associated MG.

LncRNA	SNP	Genotype Allele	Subgroups		P	OR (95 % CI)	Thymoma		P	OR (95 % CI)
			OMG	GMG			Yes	No		
AL031686.1	rs6094347	GG	11 (47.8)	47 (79.7)		1.000	8 (72.7)	50 (70.4)		1.000
		GA	12 (52.2)	12 (20.3)	**0.004**	**4.273 (1.518–12.028)**	3 (27.3)	21 (29.6)	0.876	0.893 (0.216–3.699)
		G	34 (73.9)	106 (89.8)		1.000	19 (86.4)	121 (85.2)		1.000
		A	12 (26.1)	12 (10.1)	**0.010**	**3.118 (1.282–7.581)**	3 (13.6)	21 (14.8)	0.887	0.910 (0.247–3.348)
	rs279730	TT	20 (87.0)	41 (69.5)		1.000	9 (81.8)	52 (73.2)		1.000
		TC	3 (13.0)	16 (27.1)	0.153	0.384 (0.100–1.474)	2 (18.2)	17 (23.9)	0.640	0.680 (0.134–3.459)
		CC	0	2 (3.4)	0.327	–	0	2 (2.8)	0.557	–
		T	43 (93.5)	98 (83.1)		1.000	20 (90.9)	121 (85.2)		1.000
		C	3 (6.5)	20 (16.9)	0.084	0.342 (0.096–1.212)	2 (9.1)	21 (14.8)	0.474	0.576 (0.125–2.649)
	rs24236	CC	19 (82.6)	36 (61.0)		1.000	8 (72.7)	47 (66.2)		1.000
		CT	4 (17.4)	21 (35.6)	0.089	0.361 (0.108–1.204)	3 (27.3)	22 (31.0)	0.759	0.801 (0.194–3.315)
		TT	0	2 (3.4)	0.309	–	0	2 (2.8)	0.561	–
		C	42 (91.3)	93 (78.8)		1.000	19 (86.4)	116 (81.7)		1.000
		T	4 (8.7)	25 (21.2)	0.060	0.354 (0.116–1.082)	3 (13.6)	26 (18.3)	0.593	0.704 (0.194–2.559)
	rs1000383	GG	12 (52.2)	51 (86.4)		1.000	10 (90.9)	53 (74.6)		1.000
		GA	11 (47.8)	8 (13.6)	**<0.001**	**5.844 (1.932–17.674)**	1 (9.1)	18 (25.4)	0.234	0.294 (0.035–2.463)
		G	35 (76.1)	110 (93.2)		1.000	21 (95.5)	124 (87.3)		1.000
		A	11 (23.9)	8 (6.8)	**0.002**	**4.321 (1.611–11.595)**	1 (4.5)	18 (12.7)	0.268	0.328 (0.042–2.590)
	rs279729	AA	20 (87.0)	41 (69.5)		1.000	9 (81.8)	52 (73.2)		1.000
		AG	3 (13.0)	16 (27.1)	0.153	0.384 (0.100–1.474)	2 (18.2)	17 (23.9)	0.640	0.680 (0.134–3.459)
		GG	0	2 (3.4)	0.327	–	0	2 (2.8)	0.557	–
		A	43 (93.5)	98 (83.1)		1.000	20 (90.9)	121 (85.2)		1.000
		G	3 (6.5)	20 (16.9)	0.084	0.342 (0.096–1.212)	2 (9.1)	21 (14.8)	0.474	0.576 (0.125–2.649)
	rs6094353	AA	12 (52.2)	51 (86.4)		1.000	10 (90.9)	53 (74.6)		1.000
		AG	11 (47.8)	8 (13.6)	**<0.001**	**5.844 (1.932–17.674)**	1 (9.1)	18 (25.4)	0.234	0.294 (0.035–2.463)
		A	35 (76.1)	110 (93.2)		1.000	21 (95.5)	124 (87.3)		1.000
		G	11 (23.9)	8 (6.8)	**0.002**	**4.321 (1.611–11.595)**	1 (4.5)	18 (12.7)	0.268	0.328 (0.042–2.590)
NONHSAT028539.2	rs12426399	GG	10 (43.5)	12 (20.3)		1.000	3 (27.3)	19 (26.8)		1.000
		GT	8 (34.8)	35 (59.3)	**0.022**	**0.274 (0.088–0.856)**	5 (45.5)	38 (53.5)	0.816	0.833 (0.180–3.863)
		TT	5 (21.7)	12 (20.3)	0.307	0.500 (0.131–1.907)	3 (27.3)	14 (19.7)	0.731	1.357 (0.238–7.754)
		G	28 (60.9)	59 (50.0)		1.000	11 (50.0)	76 (53.5)		1.000
		T	18 (39.1)	59 (50.0)	0.210	0.643 (0.321–1.286)	11 (50.0)	66 (46.5)	0.758	1.152 (0.469–2.828)
	rs4759318	CC	10 (43.5)	12 (20.3)		1.000	3 (27.3)	19 (26.8)		1.000
		CT	8 (34.8)	35 (59.3)	**0.022**	**0.274 (0.088–0.856)**	5 (45.5)	38 (53.5)	0.816	0.833 (0.180–3.863)
		TT	5 (21.7)	12 (20.3)	0.307	0.500 (0.131–1.907)	3 (27.3)	14 (19.7)	0.731	1.357 (0.238–7.754)
		C	28 (60.9)	59 (50.0)		1.000	11 (50.0)	76 (53.5)		1.000
		T	18 (39.1)	59 (50.0)	0.210	0.643 (0.321–1.286)	11 (50.0)	66 (46.5)	0.758	1.152 (0.469–2.828)
	rs10747689	CC	11 (47.8)	18 (30.5)		1.000	4 (36.4)	25 (35.2)		1.000

(*continued on next page*)

Table 3 (*continued*)

LncRNA	SNP	Genotype Allele	Subgroups		P	OR (95 % CI)	Thymoma		P	OR (95 % CI)
			OMG	GMG			Yes	No		
		CT	7 (30.4)	33 (55.9)	0.056	0.347 (0.115–1.051)	4 (36.4)	36 (50.7)	0.627	0.694 (0.159–3.041)
		TT	5 (21.8)	8 (13.6)	0.974	1.023 (0.266–3.928)	3 (27.3)	10 (14.1)	0.455	1.875 (0.354–9.930)
		C	29 (63.0)	69 (58.5)		1.000	12 (54.5)	86 (60.6)		1.000
		T	17 (37.0)	49 (41.5)	0.592	0.825 (0.409–1.665)	10 (45.5)	56 (39.4)	0.592	1.280 (0.518–3.161)

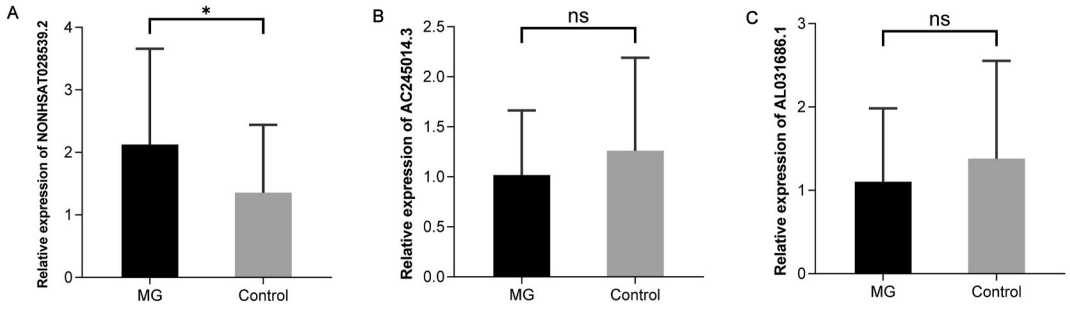

Fig. 3. LncRNA NONHSAT028539.2, AC245014.3 and AL031686.1 expression in MG. (A) NONHSAT028539.2 was upregulated, whereas (B) AC245014.3 and (C) AL031686.1 were downregulated in PBMCs from patients with MG compared with those from healthy controls. The experiment was repeated at least three times, and the data are presented as the means ± SDs. *p < 0.05. ns indicates no significant difference.

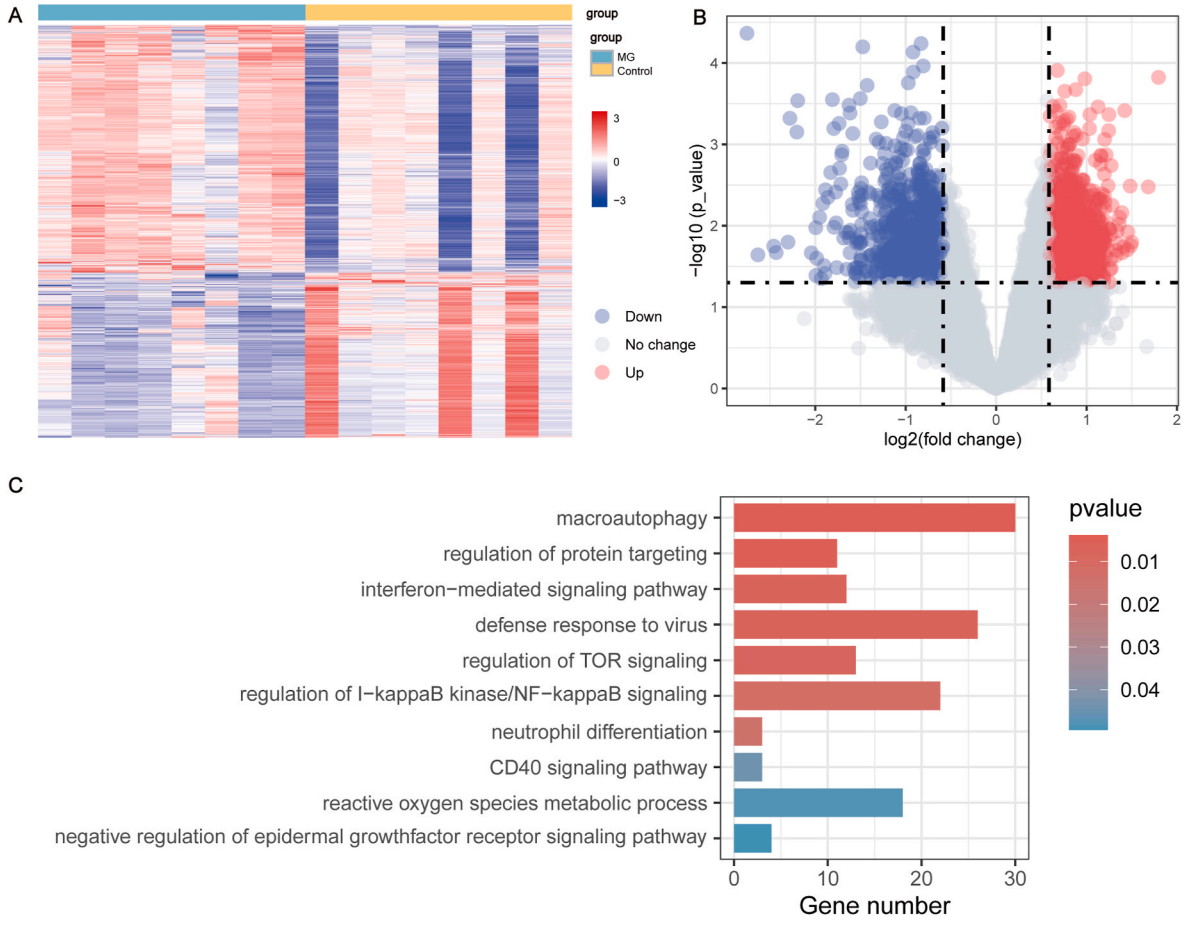

Fig. 4. Differential expression mRNAs in MG and GO functional enrichment analysis. (A) Heatmap of differentially expressed mRNAs in MG; (B) volcano map of differentially expressed mRNAs in MG; (C) The top 10 enriched GO_BP terms for co-expressed mRNAs of the AL031686.1, NONHSAT028539.2 and AC245014.3.

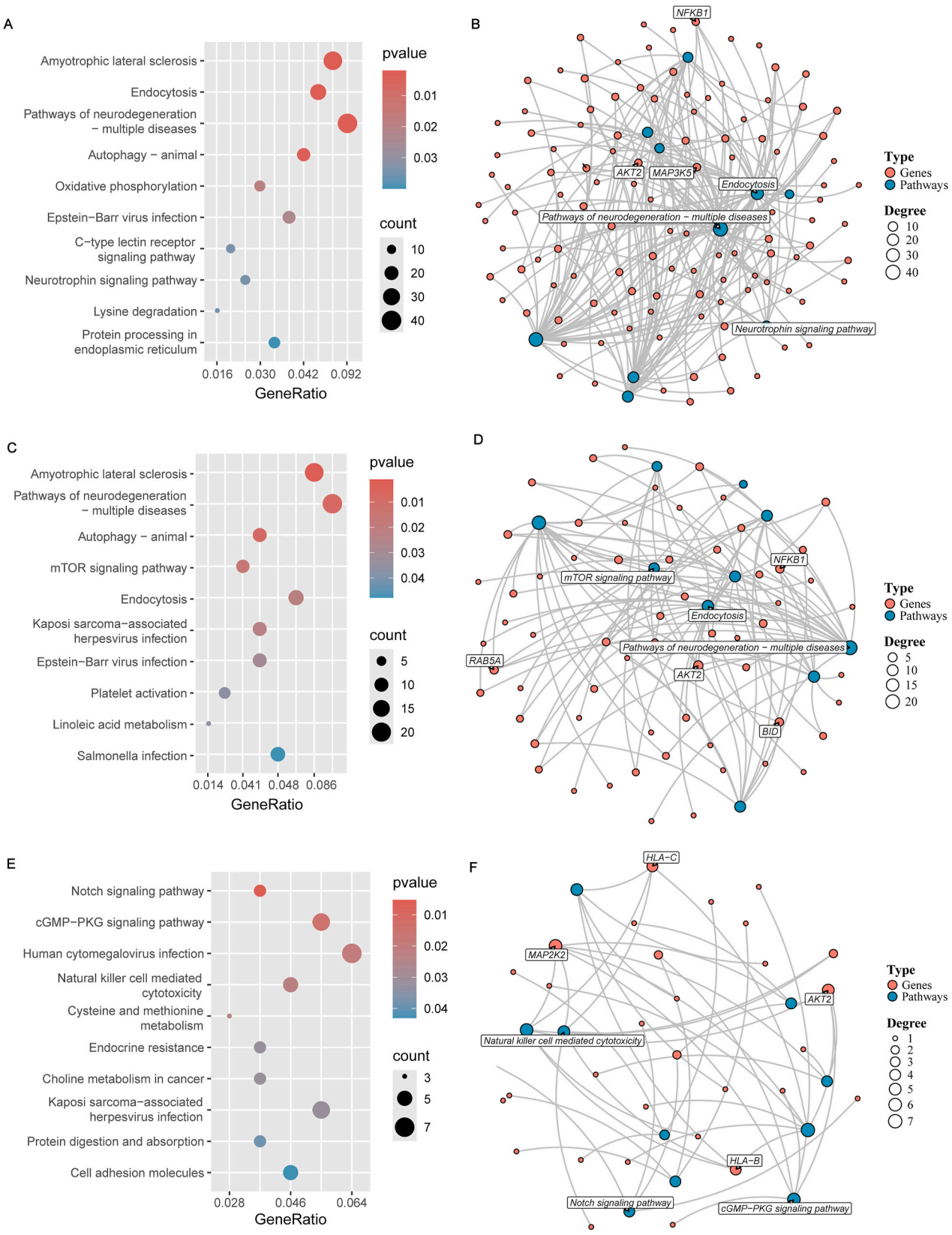

Fig. 5. KEGG enrichment analysis and pathway-gene interaction network construction. (A) The top 10 most enriched KEGG pathways for co-expressed mRNAs of AL031686.1; (B)The KEGG pathway-gene interaction network for co-expressed mRNAs of AL031686.1; (C) The top 10 most enriched KEGG pathways for co-expressed mRNAs of NONHSAT028539.2; (D)The KEGG pathway-gene interaction network for co-expressed mRNAs of NONHSAT028539.2; (E) The top 10 most enriched KEGG pathways for co-expressed mRNAs of AC245014.3; (F)The KEGG pathway-gene interaction network for co-expressed mRNAs of AC245014.3.

MG has focused on the gene level. For example, Agah E et al. studied the correlation between NLRP3 gene polymorphisms and MG [39]. A study in Turkey revealed that the PTPN22 R620W gene polymorphism was significantly associated with late-onset MG [40]. However, there are very few studies on lncRNA polymorphisms in MG. Wang et al. analysed lncRNA polymorphisms in MG by constructing competitive endogenous RNA networks and pathways but did not conduct biological experiments for further verification [41]. To date, only a few studies have investigated and verified the associations between lncRNA polymorphisms and MG. Therefore, we used bioinformatics analysis combined with multistep calculations to identify lncSNPs in MG and verified them through case–control experiments.

By improving the Kolmogorov–Smirnov test and the principle of cumulative hypergeometric distribution, lncRNA mutation hotspot regions can be identified, and further identification of lncRNA mutation hotspot SNPs can be achieved through the calculation of conservation scores. We subsequently conducted a case–control study to validate the relationships between 14 mutation hotspot SNPs in the lncRNAs AL031686.1, NONHSAT028539.2, and AC245014.3 and the risk of MG in the Chinese population for the first time. The genotyping of nine SNPs in our study revealed no statistically significant differences in genotypic distribution between patients with MG and controls.

Several clinical variables can influence the analysis of gene polymorphisms. Previous research has demonstrated that sex plays a significant role in the association between genetic polymorphisms and susceptibility to MG [42]. Therefore, to investigate the relationship between the clinical features of MG and SNPs, further analyses were conducted to evaluate the correlations between SNPs and MG based on sex, age, subgroup, and the presence or absence of thymoma. In our study, the polymorphisms of 9 SNPs were not associated with the clinical characteristics of age, sex, or the presence of thymoma in patients with MG. The genotype and allele frequencies of rs2275913 and rs1000383/rs6094353 in the lncRNA AL031686.1 were significantly different between ocular MG and general MG, suggesting that the genetic polymorphisms of these SNPs might be restricted to specific subgroups of MG.

MG can be classified into ocular MG and general MG based on the location of the affected muscles [43]. Our research revealed that in the lncRNA AL031686.1, the genotype and allele frequencies of rs2275913 and rs1000383/rs6094353 were greater in ocular MG, indicating that these SNPs are strongly associated with ocular MG susceptibility. Genetic and muscle gene expression studies have implicated dysregulated muscle atrophy signalling and mitochondrial metabolism pathways as pathogenetic mechanisms of ophthalmoplegia [44]. Therefore, we speculate that these SNPs may influence the development of ocular MG by affecting the signalling pathways involved in regulating ophthalmoplegia. Further validation is needed to determine their specific functions. Moreover, qRT-PCR experiments revealed that NONHSAT028539.2 expression was upregulated in patients with MG compared with controls. However, the expression levels of lncRNA AC245014.3 and AL031686.1 were not significant, which may be attributed to the limited sample size in our study. Future research should aim to expand the sample size for more comprehensive analysis.

5. Conclusion

In summary, in the present study, we first screened DE lncRNAs in MG by bioinformatics analysis. The risk-associated SNPs on the lncRNAs were subsequently identified using multistep calculations of mutation hotspot regions. Finally, biological experiments were conducted to analyse the correlations between lncSNPs and MG disease and to further verify the expression of lncRNAs. The SNPs rs2275913 and rs1000383/rs6094353 of the lncRNA AL031686.1 were associated with susceptibility to ocular MG in the Chinese Han population. Our research is the first to provide evidence of relationships between lncSNPs and MG susceptibility. In the future, lncRNA polymorphisms may provide valuable information for predicting the prognosis of and drug treatment effects for patients with MG in China.

CRediT authorship contribution statement

Ni He: Writing – original draft, Validation, Formal analysis, Data curation. **Liting Tian:** Data curation. **Jingnan Jin:** Data curation. **Yue Liu:** Methodology. **Lifang Li:** Formal analysis. **Xiaokun Wang:** Data curation. **Danyang Li:** Formal analysis. **Xia Wang:** Formal analysis. **Xiaoju Li:** Investigation. **Zihong Chen:** Validation. **Lanxin Zhang:** Validation. **Lukuan Qiao:** Validation. **Shangwei Ning:** Methodology, Conceptualization. **Lihua Wang:** Resources, Conceptualization. **Jianjian Wang:** Writing – review & editing, Supervision, Resources, Conceptualization.

Availability of data and materials

Datasets generated using microarray analysis are available at NCBI Gene Expression Omnibus Accession GSE263220.

Ethics approval and consent to participate

The studies involving human participants were approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University (YJSKY2022-242) and performed in accordance with the latest version of the Helsinki Declaration. The patients/participants provided their written informed consent to participate in this study.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests (e.g., any funding for the research project)/personal relationships (e.g., the author is an employee of a profitable company) which may be considered as potential competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ncrna.2024.12.012.

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