Journal	Brain Behavior and Immunity Integrative
Volume	Volume 5, January 2024, 100041
e-ISSN	2949-8341
DOI	https://doi.org/10.1016/j.bbii.2023.100041
Authors	Dwiki Fitri, Lalu Muhammad Irham, Nanik Sulistyani, Muhammad Ma'ruf, Anisa Nova Puspitaningrum, Wirawan Adikusuma, Maulida Mazaya, Rockie Chong
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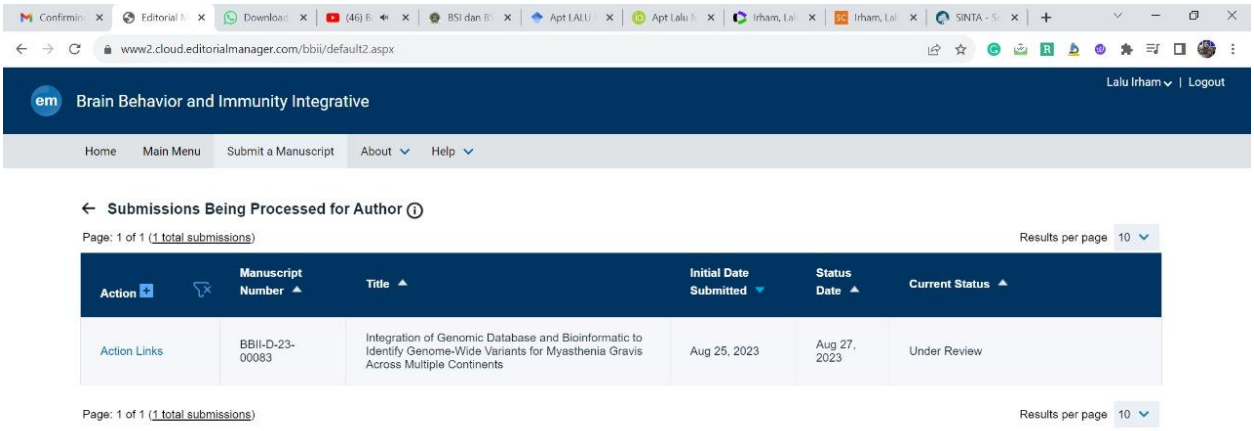
Dwiki Fitri, **Lalu Muhammad Irham*,** Nanik Sulistyani, Muhammad Ma'ruf, Anisa Nova Puspitaningrum, Wirawan Adikusuma, Maulida Mazaya, Rockie Chong , Integration of Genomics Database and Bioinformatics to Identify Genome-Wide Variants for Myasthenia Gravis Across Multiple Continents. Brain Behavior and Immunity Integrative, 24 Desember 2023: p. 100041. https://doi.org/10.1016/j.bbii.2023.100041

Manuscript Submission (25 August 2023)



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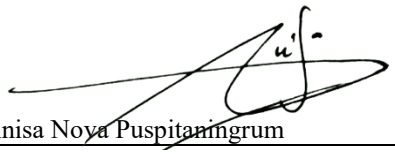
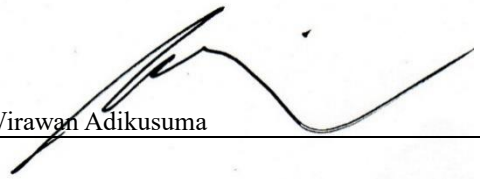
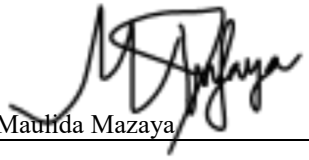
	 Anisa Noya Puspitaningrum
	 Wirawan Adikusuma
	 Maulida Mazaya
	 Rockie Chong
Author's signature	 Lalu Muhammad Irham
Date	August 24, 2023

Draft Manuscript saat submit ke Jurnal Brain Behavior and Immunity Integrative

Integration of Genomic Database and Bioinformatic to Identify Genome Variants for Myasthenia Gravis Across Multiple Continents

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ABSTRACT

Autoimmune disease is an immune response that damages the body's tissues, thereby disrupting the body's physiological functions. Myasthenia gravis is an autoimmune disease characterized by muscle weakness due to impaired neuromuscular transmission. Anyone can develop myasthenia gravis, but this condition is more common in women aged 20-30 and men over 50. Myasthenia gravis is a genetic disorder. This disease develops in old age when antibodies in the body attack the typical receptors in the muscles. To identify the genes that can affect myasthenia gravis in this study, we used several databases, including the GWAS Catalog, HaploReg Version 4.2, GTEX portal, and ensemble in particular, to identify the genomic variants and the expression of the *LTA* and *CTLA4* genes. This study shows that two variants (rs2071591 and rs231770) affect *LTA* expression in muscle and brain tissue and *CTLA4* expression in cell tissue in the testes. The allele frequency of each variant was then assessed in regional populations, including African, American, East Asian, European, and Southeast Asian. This study shows that the *LTA* and *CTLA4* genes have a higher frequency in African, East Asian, and European populations than in American and Southeast Asian populations. o It was concluded that the latter two populations may be relatively more susceptible to the autoimmune disease myasthenia gravis.

Keywords: Myasthenia Gravis, Autoimmune, Bioinformatics, Gene Variation.

1. Introduction

Myasthenia gravis (MG) is a neuromuscular disease characterized by voluntary muscle weakness (1) (2). This disease has different symptoms that vary in other patients depending on the degree of involvement of the striated muscles. The most common symptoms in patients with myasthenia gravis are ocular symptoms, which present as ptosis and diplopia. These symptoms usually occur

later in the day, and following activities such as watching TV or driving are more common. Excessive fatigue has been reported due to frequent exertion in patients with this disease. Myasthenia gravis is an autoimmune disease that connects nerves to muscles (3), produced by different antibodies against synaptic membrane proteins (4). It usually accounts for more than 85% of cases and is caused by a type of antibody to the skeletal muscle acetylcholine receptor (AChR-Ab) (5). However, components other than AChR, such as muscle-specific receptor tyrosine kinase or lipoprotein-associated protein 4 (LRP4), can also be targeted for autoimmune attacks (6).

Based on the mechanism of autoimmune disease and antibodies, molecular skeletal muscle invasiveness, thymus status, genetic characteristics, disease phenotype, and response to treatment, myasthenia gravis is divided into early and late ocular subtypes, seronegative, thymoma, LRP4. Diagnosis of the MG subtype influences treatment decisions and disease prognosis (7). Approximately 50% of patients with ocular MG develop generalized myasthenia gravis (GMG) over a 2-year, which affects other muscles and is manifested by visual weakness and symptoms (8).

According to a systematic population-based study, Car et al. (9) estimated the incidence and prevalence of MG to be 54 per million and 77.7 per million, respectively. However, significant changes have been reported in various studies. The incidence of this disease has been shown to range between 1.77 and 21.3 per million people and a prevalence of 15 to 179 million people (9). Many epidemiological studies, especially in Western Europe and Asia, report significant differences in the incidence and prevalence of MG. The incidence of myasthenia gravis ranges from 1.7 to 30 per million per year (10). This disease has two age peaks: 40-40 years, mainly affecting women, and the other 80-60 years, which occurs equally in men and women (4).

Myasthenia gravis (MG) is an autoimmune disease due to antibodies against muscle membrane components retained at the neuromuscular junction (5). MG is part of the paradigm of autoantibody-mediated disease. Antibodies to the acetylcholine receptor (AChR) are found in 85% of patients with generalized muscle weakness and in 50% of patients with pure ocular involvement (11). MG is an autoimmune disease that connects nerves to muscles (3), produced by different antibodies against synaptic membrane proteins (4). It occurs in more than 85% of cases and is caused by a type of antibody to the skeletal muscle acetylcholine receptor (AChR-Ab) (5). Gene variation often links disease development as well as pathogenesis, including MG. One website that discusses gene variances is the Genome-Wide Association Studies (GWAS) catalog (12). GWAS is a database with search results for Single Nucleotide Polymorphism (SNP) in several human genomes as a genetic marker for predicting a disease disorder (13). Utilizing the GWAS Catalog can help to pinpoint the specific SNPs linked to MG..

This study aims to investigate the variants associated with MG through an approach based on bioinformatics. In addition, gene expression profile patterns and allele frequencies of genetic variant populations were assessed using various databases. The results will enable future studies to determine whether these variants may be associated with multiple risks of MG infection, as well as MG progression and disease susceptibility.

In summary, MG is a neuromuscular disorder characterized by muscle weakness, with its manifestations varying among patients. Ocular symptoms, notably ptosis and diplopia, are common, often exacerbated by activities like watching TV or driving. MG results from autoimmune processes triggered by antibodies against synaptic membrane proteins, predominantly the acetylcholine receptor (AChR-Ab). The disease is categorized into subtypes, impacting treatment choices and prognosis. Epidemiologically, MG's incidence and prevalence vary across regions and age groups. Genetic variations play a significant role, with GWAS cataloging identifying relevant SNPs. This study employs bioinformatics to uncover MG-related genetic variants, offering insights into disease susceptibility and progression.

2. Method

Gene information can not only be used to identify disease-associated variants but can also be interpreted as actionable knowledge for that disease. MG is an autoimmune disease that affects the connection between nerves to muscles. This study used an approach based on bioinformatics to prioritize pathogenic variants that have the potential to cause MG. Detailed information regarding the study design has been visualized in **Figure 1**. To obtain data on variants associated with MG. We leveraged the GWAS Catalog National Human Genome Research Institute (NHGRI) Catalog Database http:// www.ebi.ac.uk/gwas (accessed 22 May 2023). Through this database, we used the keyword “Myasthenia Gravis” (MG) to derive the associated MG. MGs with 36 SNPs were obtained, and further analysis was performed using HaploReg (version 4.2). The GWAS catalog inclusion criteria used in this study were SNPs with a p-value $<10^{-8}$, resulting in 26 SNPs (17). This value is used to account for some of the tests in the GWAS catalog. These values are widely used to identify associations between variants and the similarity of genetic traits with adjacent gene expression (18). Next, an evaluation was made between the associations of various genetic variants and gene expression profiles using expression quantitative trait loci (QTL) using the GTEx Portal database http://www.gtexportal.org/home/ (19) (accessed May 22, 2023), which was found via gene expression from various networks. *LTA* and *CTLA4* gene variants in muscle tissue, cerebellum, and testicular cell tissue were obtained from the GTEx portal database. Then confirm the variant using the Ensembl Genome Browser https://www.ensembl.org/index.html (20) (accessed May 22, 2023). In addition, the allele frequencies of the MG-associated variants were evaluated in different populations, including African, American, East Asian, European, and Southeast Asian people. Samples from each region consisted of 1195 individuals (Africa), 567 individuals (America), 1112 individuals (East Asia), 693 individuals (Europe), and 554 individuals (Southeast Asia).

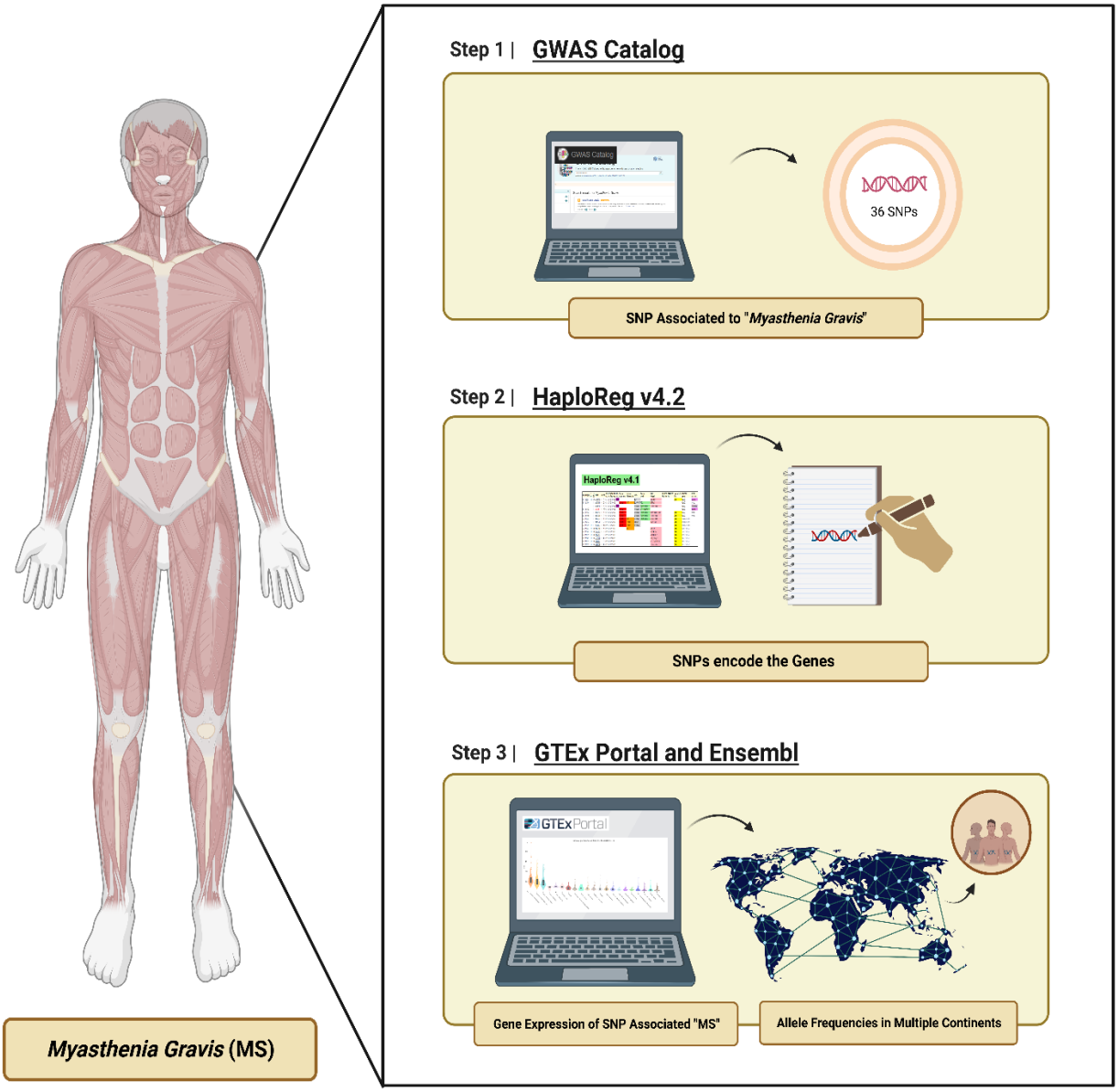

Figure 1. Bioinformatics work scheme for identification of gene variations associated with Myasthenia Gravis (MG)

3. Results

This study used the GWAS database for the first time to identify SNPs associated with MG. From the GWAS database, 36 SNPs associated with MG were found. Then 26 unique SNPs associated with MG were identified after removing all SNP duplications (Table 1.) Based on the number of SNPs obtained, the SNPs were forwarded using HaploReg version 4.2, with a p-value $<10^{-8}$.

Table 1. GWAS catalog result the duplicates removed are obtained from 26 SNPs with signification $<10^{-8}$

SNP	P-value
rs3093958	$4x10^{-42}$
rs9271375	$2x10^{-19}$
rs4369774	$6x10^{-19}$
rs111945767	$3x10^{-17}$
rs76815088	$6x10^{-16}$
rs4409785	$2x10^{-07}$
rs4574025	$7x10^{-14}$
rs35274388	$1x10^{-12}$
rs2476601	$2x10^{-12}$
rs2071591	$4x10^{-12}$
rs150881176	$1x10^{-11}$
rs231770	$9x10^{-11}$
rs4574025	$4x10^{-07}$
rs9963862	$4x10^{-07}$
rs12653117	$5x10^{-07}$
rs6914704	$2x10^{-06}$
rs4128527	$4x10^{-06}$
rs4518467	$4x10^{-06}$
rs2476601	$7x10^{-06}$
rs9266277	$7x10^{-10}$
rs6998967	$9x10^{-10}$
rs35274388	$1x10^{-09}$
rs4263037	$1x10^{-08}$
rs73007767	$4x10^{-08}$
rs2245569	$6x10^{-08}$
rs9270986	$6x10^{-08}$

Based on the data presented in Table 2, this study focused on two genomic variants of the same gene that qualified as biological risk SNPs in this MG study.

Table 2. Varian risk allele which codes two gen.

SNP	*Gencode*	*P-value*	*Allele Location*
rs2071591	*LTA*	$4x10^{-12}$	Missense
rs231770	*CTLA4*	$9x10^{-11}$	Missense

Using an integrative bioinformatics approach, two variants with missense mutations rs2071591 and rs231770 encoding the *LTA* and *CTLA4* genes were prioritized as MG biological risk SNPs. MG disease is characterized by muscle and tissue weakness that occurs when the immune system is impaired and produces antibodies that attack the tissues in the body (10). It was also reported that Lymphotoxin α (*LTA*) is a cytokine secreted by lymphocytes and is a member of the Tumor Necrosis Factor (*TNF*) family. *LTA* gene variations can contribute to threshold brain excitability, the spread of neural hyperexcitability (21).

In Renton's (2015), the gene for cytotoxic T lymphocyte-associated protein (*CTLA4*) was previously suggested as a cause of myasthenia gravis susceptibility. The *CTLA4* gene also multiplies when symptoms are present regardless of age, indicating that it is responsible for aberrant autoimmune responses that lead to neuromuscular junction dysfunction. *CTLA4* 45-kD

immunoglobulin is expressed by activated T cells and has a significant sequence identity with CD28 (22).

LTA gene expression in muscle and brain tissue.

In atherosclerotic plaques, intimal cells, some spindle-shaped or have globular, vacuolated cytoplasm, show immunoreactivity for *LTA* and galectin-2. Binds to adjacent portions of anti-tender muscle (SMC) cells. Galectin-2 and LTA are expressed in human smooth muscle cells and macrophages affected by atherosclerotic lesions (23).

In Feroni's (2022), states that *LTA* genes encode cytokines that can modulate many inflammatory, immunological, and antiviral responses. It has been postulated that the inflammatory process modulated by *LTA* may contribute to the propagation of neural hyperexcitability by acting as an initiation and maintenance factor during migraine attacks (24). These results agree with a study on 439 Korean migraine patients genotyped for several *LTA* gene polymorphisms (17). Migraines occur because the blood vessels in the brain experience dilation or expansion, the main form of headache, characterized by debilitating headache attacks and symptoms of autonomic nervous system dysfunction (18).

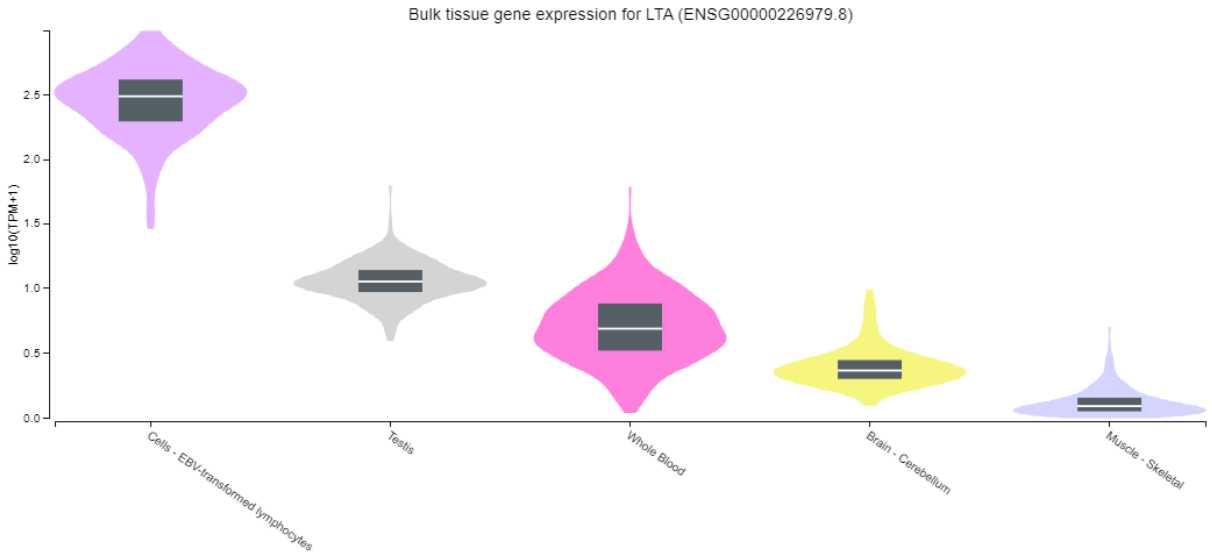

Figure 2. *LTA* gene expression associated with myasthenia gravis in several human tissues from the GTEx Portal.

Expression of the _CTLA4_ gene in cell tissue in the testis.

Our new analysis does not show the *CTLA4* location in the previous GWAS (rs231770). After tracing using HaploReg4.2, it was found that neighboring missenses may show variations in alleles that impact the risk of myasthenia gravis in various populations. Although these loci still make biological sense, more extensive studies are needed to prove that they are related to each other (19).

Another study in Vergoosen 2021 said that almost all MG-related genes were found in the testes and ectocervix. This study did not say a specific *CTLA4* gene existed in testicular tissue.

Still, the genes in question were *RAPSN* and *CHRNA1* expression mostly limited to skeletal muscle, with some additional words in the tibial nerve and the testicular and pituitary glands, respectively. Overall, the expression of MG-related genes is prominent in skeletal muscle and brain, but individual genes are also expressed in other tissues of the human body (23).

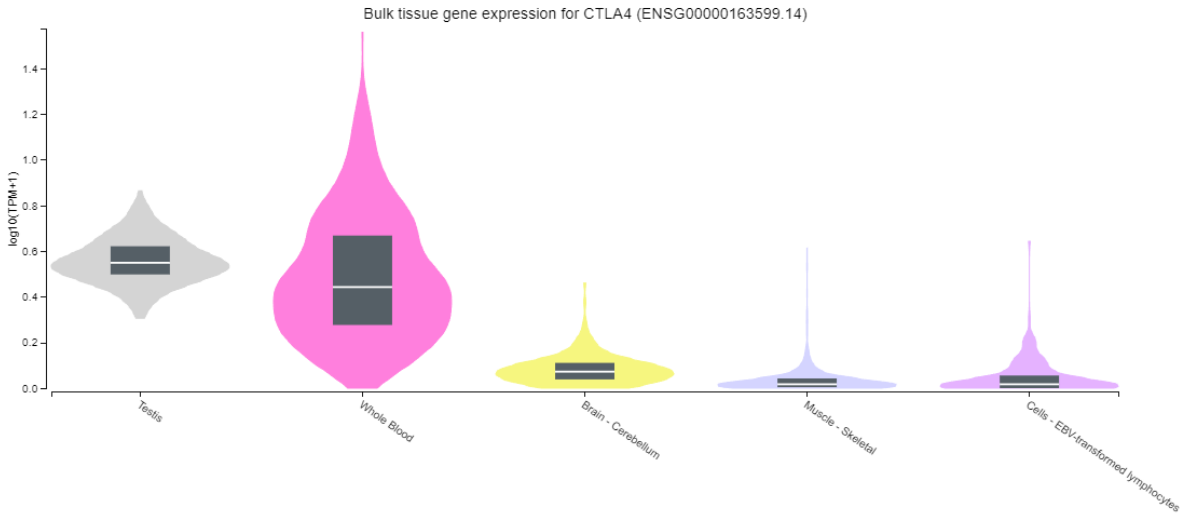

Figure 3. *CTLA4* gene expression associated with myasthenia gravis in several human tissues from the GTEx Portal.

Relationship between *LTA* and *CTLA4* genes with eQTL from GTEx portal database.

The GTEx Portal database aims to evaluate MG gene expression in various human tissues, including muscle tissue. Using the GWAS catalog database, we located 37 SNPs and identified genomic variation in *LTA* and *CTLA4* gene expression. We found 26 SNPs with the highest p values from this analysis. After this investigation, two statistically significant SNPs were found and prioritized. Based on an extended SNP count analysis using HaploReg version 4.2, we prioritized the two SNPs at risk for MG because the functional annotations of the SNPs were 10^{-8}. The following table shows the results of genetic variation.

Table 3. e-QTL's result for the Myasthenia gravis from GTEX portal database

SNP	***Gencode ID (ENSG00000-)***	***Gene symbol***	***P-value***	***NES***	***Tissue***	***Actions***
rs2071591	**ENSG00000226979.8**	***LTA***	**0.000065**	**0.29**	**Brain - Cerebellum**	**GG>GA>AA**
	ENSG00000226979.8	***LTA***	**0.00024**	**0.14**	**Muscle - Skeletal**	**GG>GA>AA**
rs231770	**ENSG00000163599.14**	***CTLA4***	**0.0000016**	**-0.28**	**Testis**	**CC>CT>TT**

Myasthenia gravis (MG) candidate variant allele frequencies across continents

After identifying candidate variants related to *LTA* and *CTLA4* gene expression, allele frequencies have been determined across populations of all continents as shown in (Table 4). Allele frequencies

for the four variants were evaluated in different people, including populations of 1195 individuals (Africa), 567 individuals (America), 1112 individuals (East Asia), 693 individuals (Europe), and 554 individuals (South East Asia). Using the Ensemble Genome Browser, we obtained allele frequencies in Africa, America, East Asia, Europe, and Southeast Asia (http://www.ensembl.org). Allele frequencies across populations differ for each *LTA* and *CTLA4* gene variant. Table 4 and **Figure 4.** shows the gene expression level at a higher frequency of the related allele (T) rs231770 than the corresponding allele (A) rs2071591. In the associated alleles (T) rs231770 and (A) rs2071591, the African and East Asian populations are much higher than the American, Southeast Asian, and European Asian people.

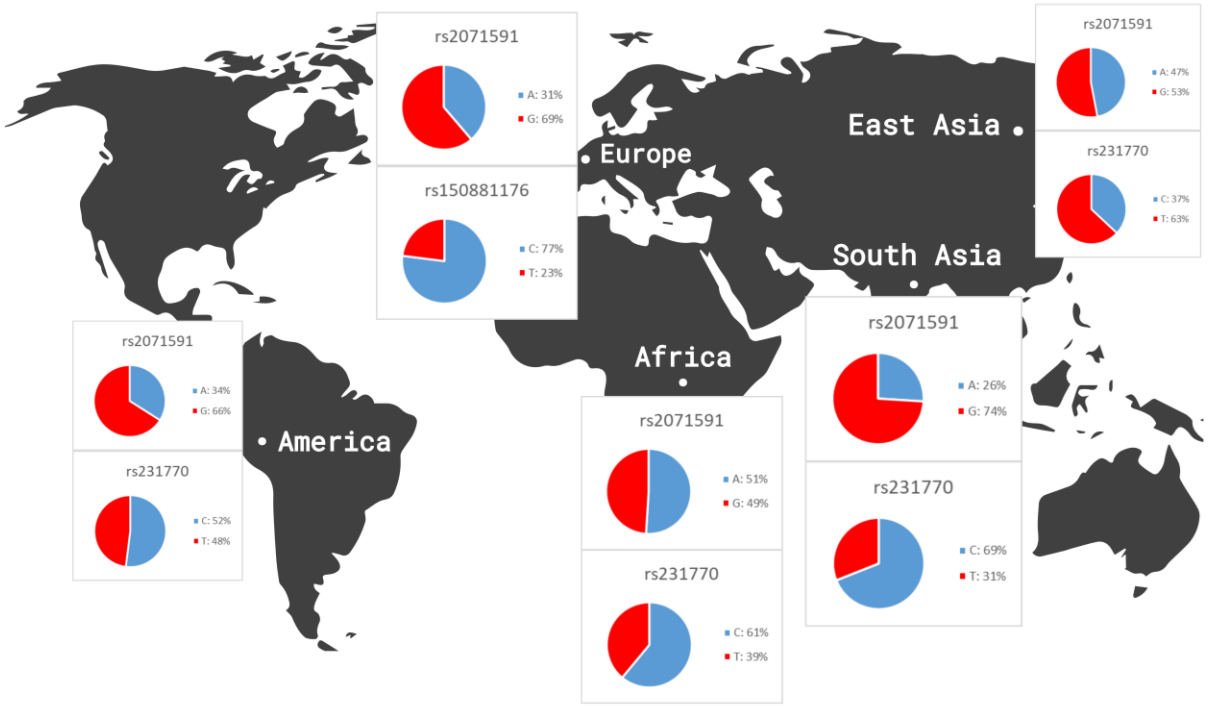

Figure 4. Summary of allele frequency analysis of *LTA* and *CTLA4* gene expression in Africa, America, East Asia, Europe, and Southeast Asia.

Table 4. Allele frequencies for SNPs examined in this study

SNP	Position (hg38)	Gene	Location	Alel		Frekuensi Alel (N)				
				Ref	Eff*	AFR	AMR	EAS	EUR	SAS
rs2071591	**Chr6 31548022**	*LTA*	**Missense**	**G**	**A**	**0.512 (677)**	**0.336 (233)**	**0.469 (473)**	**0.310 (312)**	**0.261 (255)**
rs231770	Chr2 203864430	*CTLA 4*	**Missense**	**C**	**T**	***0.392 (518)***	***0.481 (334)***	***0.634 (639)***	***0.379 (381)***	***0.306 (299)***

The higher frequency of the related allele is (T) rs231770 compared to the other related allele, namely (A) rs2071591. In the associated alleles (T) rs231770 and (A) rs2071591, the

African and East Asian populations are much higher than the American, Southeast Asian, and European Asian people. The rs2071591 allele indicated a differential variant prevalence contribution for *LTA* gene expression, while the rs231770 allele indicated a differential variant prevalence contribution for *CTLA4* gene expression.

Allele frequencies in all populations differ for each SNP, as shown in **Figure 4.** It is generally known that the A and T allele frequencies for rs2071591 and rs231770 also appear to have a higher frequency in Europe with alleles rs2071591 (47%), rs231770 (63%), East Asia with alleles rs2071591 (47%), rs231770 (63%), compared to America with alleles rs2071591 (34%), rs231770 (48%), Africa with alleles rs2071591 (51%), rs231770 (39%), Asia southeast with alleles rs2071591 (26%), rs231770 (31%).

In conclusion, a bioinformatic-based approach revealed pathogenic variants potentially associated with MG. We propose that this variant may be used for further studies to identify MG and prognosis diagnostic biomarkers. However, we acknowledge that there are limitations to the bioinformatics-based approaches used to investigate genetic variants associated with MG. One of the significant limitations is that not all variants necessarily have the gene to match them (i.e., non-coding variants); if these genes or genetic variants are present, they may not be suitable drug targets. Nonetheless, clinical validation is recommended as a next step to confirm our findings and better understand the underlying etiology and functional effects of MG disease.

4. Discussion.

This study used the GWAS database for the first time to identify SNPs associated with MG; from the GWAS database, 36 SNPs associated with MG were found. After removing all SNP duplications, we further identified 26 SNPs uniquely associated with MG. Based on the number of SNPs obtained, the SNPs are forwarded using HaploReg version 4.2, with a p-value $<10^{-8}$.

Using an integrative bioinformatics approach, two variants with missense mutations rs2071591 and rs231770 encoding the *LTA* and *CTLA4* genes were prioritized as MG biological risk SNPs. MG disease is characterized by muscle and tissue weakness that occurs when the body's immune system is impaired and produces antibodies that attack tissues (5). It was also reported that Lymphotoxin α (*LTA*) is a cytokine secreted by lymphocytes and is a member of the Tumor Necrosis Factor (TNF) family. *LTA* gene variation may contribute to threshold brain excitability and disseminated neural hyperexcitability (21).

A study by Feroni's in 2022posited that *LTA* genes encode cytokines that can modulate many inflammatory, immunological, and antiviral responses. It has been postulated that the inflammatory process modulated by *LTA* may contribute to the propagation of neural hyperexcitability by acting as an initiation and maintenance factor during migraine attacks (21). These results agree with a study on 439 Korean migraine patients genotyped for several *LTA* gene polymorphisms (17). Migraine occurs due to dilatation or expansion of the blood vessels in the brain, a significant form of headache characterized by debilitating headache attacks and symptoms of autonomic nervous system dysfunction (18).

The 2021 Vergoosen study said that almost all MG-related genes were found in the testes and ectocervix. This study did not say a specific *CTLA4* gene existed in testicular tissue. Still, the genes

in question were RAPSN and CHRNA1 expression mostly limited to skeletal muscle, with some additional words in the tibial nerve and the testicular and pituitary glands, respectively. Overall, the expression of MG-related genes is prominent in skeletal muscle and brain, but individual genes are also expressed in other tissues of the human body (23).

In Renton's 2015 study, the gene for cytotoxic T lymphocyte-associated protein (*CTLA4*) was previously suggested as a cause of myasthenia gravis susceptibility. The *CTLA4* gene also multiplies when symptoms are present regardless of age, indicating that it is responsible for aberrant autoimmune responses that lead to neuromuscular junction dysfunction. The 45-kD immunoglobulin *CTLA4* is expressed by activated T cells and has a significant sequence identity with CD28 (22).

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Consequently, this bioinformatics-driven approach has the potential to uncover pathogenic variants associated with MG. These findings propose avenues for further research in identifying diagnostic biomarkers for MG and its prognosis. However, we acknowledge limitations in bioinformatics-based methodologies for investigating genetic variants linked to MG. Notably, some variants might not align with functional genes (non-coding variants), and even when they do, they may not be viable drug targets. Thus, clinical validation remains a crucial next step to affirm our discoveries and gain deeper insights into the underlying causes and effects of MG..

5. Conclusion

In this study, we used state-of-the-art MG bioinformatics to analyze genomic databases showing distinct gene expression of the *LTA* and *CTLA4* genes in muscle, brain, and testicular cell tissues. Relevant gene variants include rs2071591, expressed in muscle and brain tissue, and rs231770, described in testicular cell tissue. Overall higher frequency in Europe with alleles rs2071591 (47%), rs231770 (63%), East Asia with alleles rs2071591 (47%), rs231770 (63%), compared to America with alleles rs2071591 (34%), rs231770 (48%), Africa with alleles rs2071591 (51%), rs231770 (39%), Southeast Asia with alleles rs2071591 (26%), rs231770 (31%).

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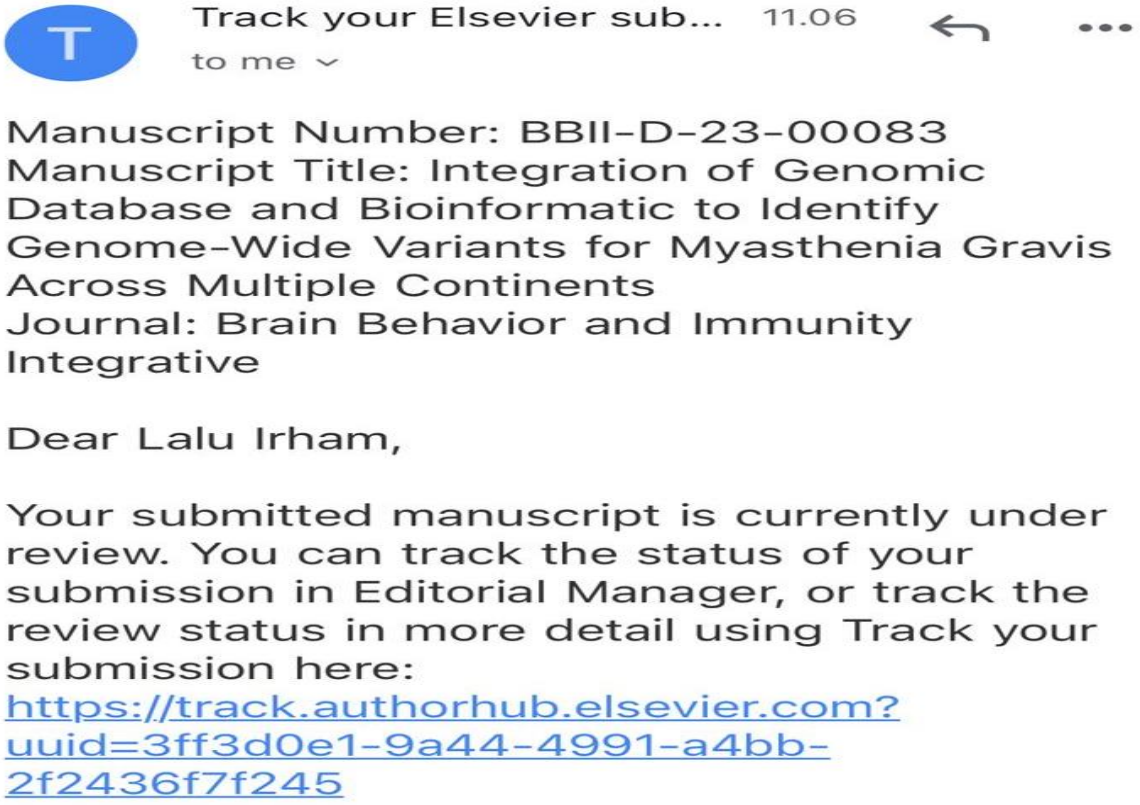

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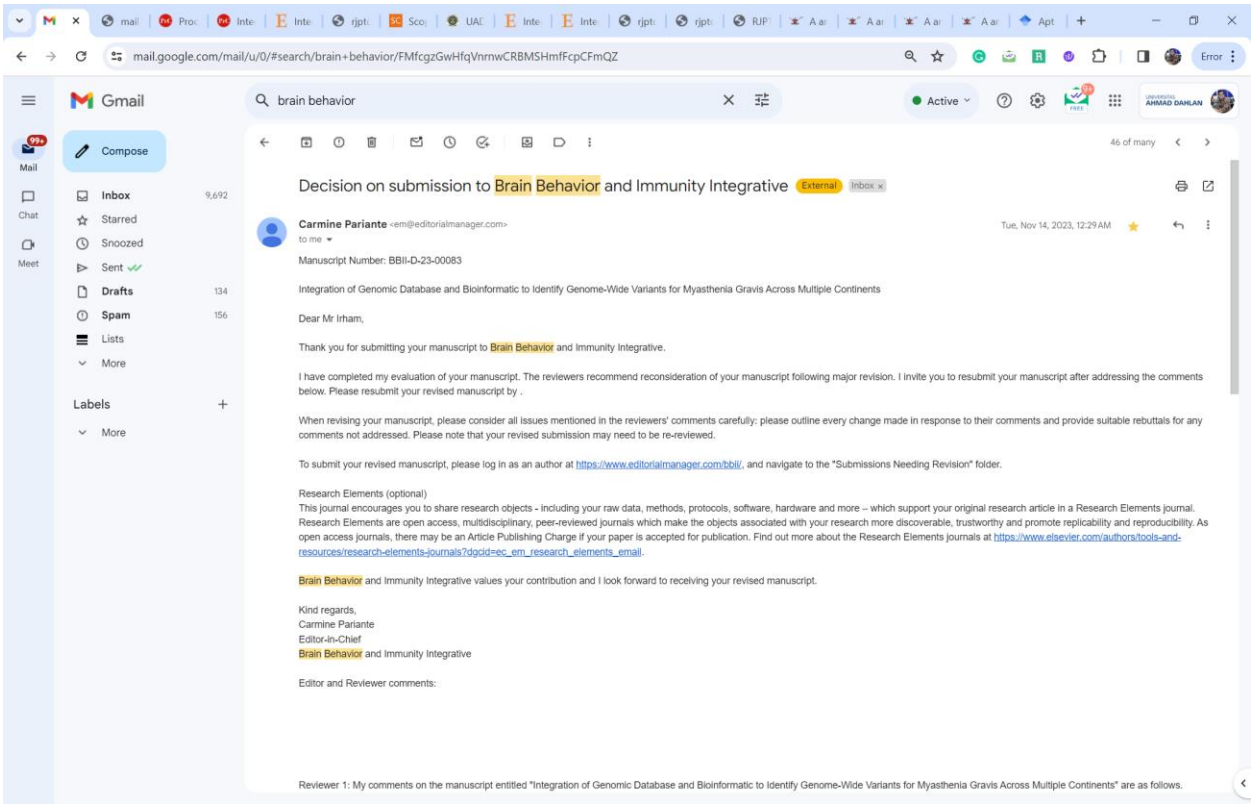

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Integration of Genomic Database and Bioinformatic to Identify Genome-Wide Variants for Myasthenia Gravis Across Multiple Continents

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Reviewer 2: This study examined the variants related to Myasthenia Gravis by using different databases. The results provided evidence to the risks of Myasthenia Gravis infection and disease susceptibility. However, there are some significant revisions that would need to be made before I recommend that the manuscript be considered for publication:

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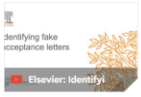

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Respon kepada reviewer jurnal:

November 15, 2023

Dear Editors,

Please find our attached revised manuscript, entitled “Integration of Genomic Database and Bioinformatic to Identify Genome-Wide Variants for Myasthenia Gravis Across Multiple Continents” which we are submitting for consideration for publication as an original research article in Brain Behavior and Immunity Integrative with manuscript number: BBII-D-23-00083. We are thankful for your kind encouragement regarding our manuscript. Herewith, we are sending this revised manuscript in accordance with the comments given by the reviewers. The revised parts of the manuscript are highlighted in yellow. Finally, we would like to thank you once again for giving us the opportunity to improve our manuscript. We very much hope that these revisions are adequate. We appreciate your review and assistance, and look forward to hearing from you.

Sincerely yours,

Apt Lalu Muhammad Irham M.Farm Ph.D.

Faculty of Pharmacy,

Universitas Ahmad Dahlan, Yogyakarta, Indonesia

Jl. Prof. DR. Soepomo SH, Warungboto,

Kec. Umbulharjo, Kota Yogyakarta, Daerah Istimewa Yogyakarta

Recommendation Reviewer 1:

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Q1: The idea of the manuscript is very good; the abstract section should also include limitations of the manuscript (questions left unanswered) along with future prospects.

A1: We appreciate the reviewer's comments. We now revised the abstract about this topic and problem in [Page 1, lines 31-33] The sentence is modified as follows :

"Therefore, variations in these genes not only offer insights into disease susceptibility, diagnostic and prognostic biomarkers but also open avenues for identifying candidate drug targets through genomic-driven drug repurposing"

Q2: Introduction section is unnecessarily very long; focus on your major objectives and approaches utilized in the study of your manuscript. Novelty should be included in this section with appropriate justification.

A2: We have removed the overly long introduction section and have summarized it. we have added updates to this research in [Page 1, lines 37-77] The introduction that we have summarized is as follows :

"Myasthenia gravis (MG) is a neuromuscular disease characterized by voluntary muscle weakness (Sanders, Wolfe, & Narayanaswami, 2017) (Gilhus & Verschuuren, 2015). This disease has different symptoms that vary in other patients depending on the degree of involvement of the striated muscles. The most common symptoms in patients with myasthenia gravis are ocular symptoms, which present as ptosis and diplopia. These symptoms usually occur later in the day, and following activities such as watching TV or driving are more common. Excessive fatigue has been reported due to frequent exertion in patients with this disease. Myasthenia gravis is an autoimmune disease that connects nerves to muscles (Murai, 2014), produced by different antibodies against synaptic membrane proteins (Benatar et al., 2016). It usually accounts for more than 85% of cases and is caused by a type of antibody to the skeletal muscle acetylcholine receptor (AChR-Ab) (Berrih-Aknin, Frenkian-Cuvelier, & Eymard, 2014). However, components other than AChR, such as muscle-specific receptor tyrosine kinase or lipoprotein-associated protein 4 (LRP4), can also be targeted for autoimmune attacks (Mehling et al., 2011).

Based on the mechanism of autoimmune disease and antibodies, molecular skeletal muscle invasiveness, thymus status, genetic characteristics, disease phenotype, and response to treatment, myasthenia gravis is divided into early and late ocular subtypes, seronegative, thymoma, LRP4. Diagnosis of the MG subtype influences treatment decisions and disease

prognosis (Kerty, Elsais, Argov, Evoli, & Gilhus, 2014). Approximately 50% of patients with ocular MG develop generalized myasthenia gravis (GMG) over a 2-year, which affects other muscles and is manifested by visual weakness and symptoms (Wang et al., 2017).

According to a systematic population-based study, Car et al. (Carr, Cardwell, McCarron, & McConville, 2010) estimated the incidence and prevalence of MG to be 54 per million and 77.7 per million, respectively. However, significant changes have been reported in various studies. The incidence of this disease has been shown to range between 1.77 and 21.3 per million people and a prevalence of 15 to 179 million people (Carr et al., 2010). Many epidemiological studies, especially in Western Europe and Asia, report significant differences in the incidence and prevalence of MG. The incidence of myasthenia gravis ranges from 1.7 to 30 per million per year (Breiner et al., 2016). This disease has two age peaks: 40-40 years, mainly affecting women, and the other 80-60 years, which occurs equally in men and women (Benatar et al., 2016).

In summary, MG is a neuromuscular disorder characterized by muscle weakness, with its manifestations varying among patients. Ocular symptoms, notably ptosis and diplopia, are common, often exacerbated by activities like watching TV or driving. MG results from autoimmune processes triggered by antibodies against synaptic membrane proteins, predominantly the acetylcholine receptor (AChR-Ab). The disease is categorized into subtypes, impacting treatment choices and prognosis. Epidemiologically, MG's incidence and prevalence vary across regions and age groups. Genetic variations play a significant role, with GWAS cataloging identifying relevant SNPs. This study aims to investigate the variants associated with MG through an approach based on bioinformatics, offering insights into disease susceptibility and progression. In addition, gene expression profile patterns and allele frequencies of genetic variant populations were assessed using various databases. The results will enable future studies to determine whether these variants may be associated with multiple risks of MG infection, as well as MG progression and disease susceptibility."

Q3: Similar problems found for method and material section, concise it.

A3: We appreciate the reviewer's comments. We have revised the section listed below in [Page 3, lines 79-97]. The method that we have summarized is as follows :

"This study employed a bioinformatics-based approach to prioritize pathogenic variants potentially linked to MG. A detailed outline of the study design is visually represented in Figure 1. To collect data on MG-associated variants, we leveraged the GWAS Catalog from the National Human Genome Research Institute (NHGRI) Database (http:// www.ebi.ac.uk/gwas), accessed on May 22, 2023. Employing the keyword "Myasthenia Gravis" (MG), we extracted information on variants associated with MG. We found a total 36 SNPs number of MG-associated variants. Next, subsequent analysis of MG-related SNPs was conducted using HaploReg (version 4.2)

HaploReg is a tool designed to analyze non-coding genome annotations from published GWAS or new variants. It aids in understanding the functional outcomes of GWAS results, predicting potential causal variants, identifying involved cell types, and predicting candidate target genes (Ward & Kellis, 2016) (Ward & Kellis, 2012). In this study, the GWAS catalog inclusion criteria for SNPs were those with a p-value $<10^{-8}$ (Lee et al., 2007). The investigation further entailed assessing associations between various genetic variants and gene expression profiles utilizing expression quantitative trait loci (QTL) available on the GTEx Portal database (http://www.gtexportal.org/home/) (Blauwendraat, 2022) accessed on May 22, 2023. Then, we confirmed the variant using the Ensembl Genome Browser (https://www.ensembl.org/index.html) (Ozaki et al., 2004) accessed on May 22, 2023. In addition, allele frequencies of the MG-associated variants were evaluated across diverse populations, encompassing African, American, East Asian, European, and Southeast Asian people."

Q4: Result and discussion should be sequentially discussed, check and remove the repetitions from this section.

A4: We appreciate the reviewer's comments. We have removed the discussion section and have combined the results and discussion sequentially in [Page 4, lines 103-230]. The results and discussion sections sequentially are as follows :

"This study used the GWAS database for the first time to identify SNPs associated with MG. From the GWAS database, 36 SNPs associated with MG were found. Then 26 unique SNPs associated with MG were identified after removing all SNP duplications (Table 1.) Based on the number of SNPs obtained, the SNPs were forwarded using HaploReg version 4.2, with a p-value $<10^{-8}$.

Table 1. GWAS catalog result the duplicates removed are obtained from 26 SNPs with significance (p-value $<10^{-8}$)

SNP	P-value
rs3093958	$4x10^{-42}$
rs9271375	$2x10^{-19}$
rs4369774	$6x10^{-19}$
rs111945767	$3x10^{-17}$
rs76815088	$6x10^{-16}$
rs4409785	$2x10^{-07}$
rs4574025	$7x10^{-14}$

rs35274388	$1x10^{-12}$
rs2476601	$2x10^{-12}$
rs2071591	$4x10^{-12}$
rs150881176	$1x10^{-11}$
rs231770	$9x10^{-11}$
rs4574025	$4x10^{-07}$
rs9963862	$4x10^{-07}$
rs12653117	$5x10^{-07}$
rs6914704	$2x10^{-06}$
rs4128527	$4x10^{-06}$
rs4518467	$4x10^{-06}$
rs2476601	$7x10^{-06}$
rs9266277	$7x10^{-10}$
rs6998967	$9x10^{-10}$
rs35274388	$1x10^{-09}$
rs4263037	$1x10^{-08}$
rs73007767	$4x10^{-08}$
rs2245569	$6x10^{-08}$
rs9270986	$6x10^{-08}$

Based on the data presented in Table 2, this study focused on two genomic variants of the same gene that qualified as biological risk SNPs in this MG study.

Table 2. Varian risk allele which codes two genes

SNP	GENCODE	P-value	Allele Location
rs2071591	*LTA*	$4x10^{-12}$	Missense
rs231770	*CTLA4*	$9x10^{-11}$	Missense

Using an integrative bioinformatics approach, two variants with missense mutations rs2071591 and rs231770 encoding the *LTA* and *CTLA4* genes were prioritized as MG biological

risk SNPs. MG disease is characterized by muscle and tissue weakness that occurs when the immune system is impaired and produces antibodies that attack the tissues in the body (Benatar et al., 2016). It was also reported that Lymphotoxin α (*LTA*) is a cytokine secreted by lymphocytes and is a member of the Tumor Necrosis Factor (*TNF*) family. *LTA* gene variations can contribute to threshold brain excitability, the spread of neural hyperexcitability (Aurora & Welch, 2000).

In Renton's (2015), the gene for cytotoxic T lymphocyte-associated protein (*CTLA4*) was previously suggested as a cause of myasthenia gravis susceptibility. The *CTLA4* gene also multiplies when symptoms are present regardless of age, indicating that it is responsible for aberrant autoimmune responses that lead to neuromuscular junction dysfunction. *CTLA4* 45-kD immunoglobulin is expressed by activated T cells and has a significant sequence identity with CD28 (Renton et al., 2015).

LTA gene expression in muscle and brain tissue.

In atherosclerotic plaques, intimal cells, some spindle-shaped or have globular, vacuolated cytoplasm, show immunoreactivity for *LTA* and galectin-2. Binds to adjacent portions of anti-tender muscle (SMC) cells. Galectin-2 and LTA are expressed in human smooth muscle cells and macrophages affected by atherosclerotic lesions (Vergoossen, Keo, Mahfouz, & Huijbers, 2021).

In Feroni's (2022), states that *LTA* genes encode cytokines that can modulate many inflammatory, immunological, and antiviral responses. It has been postulated that the inflammatory process modulated by *LTA* may contribute to the propagation of neural hyperexcitability by acting as an initiation and maintenance factor during migraine attacks (Ma'ruf et al., 2023). These results agree with a study on 439 Korean migraine patients genotyped for several *LTA* gene polymorphisms (Lee et al., 2007). Migraines occur because the blood vessels in the brain experience dilation or expansion, the main form of headache, characterized by debilitating headache attacks and symptoms of autonomic nervous system dysfunction (Olesen, 2018).

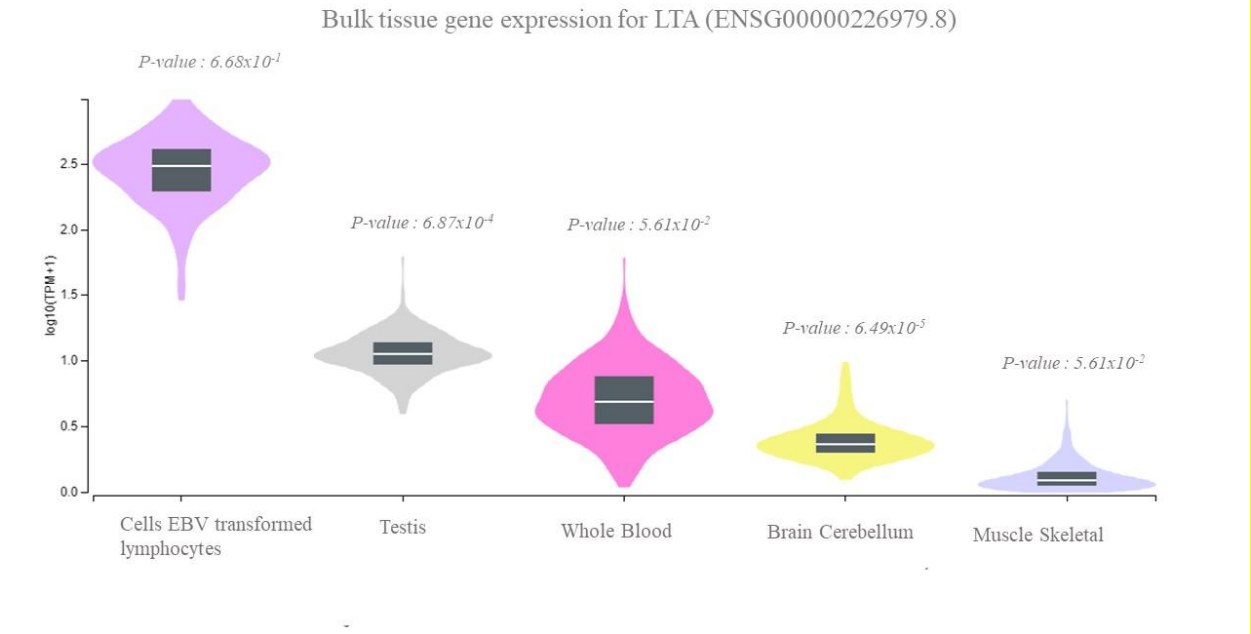


Figure 2. *LTA* gene expression associated with myasthenia gravis across several human tissues from the GTEx Portal.

Expression of the *CTLA4* gene in cell tissue in the testis.

Our new analysis does not show the *CTLA4* location in the previous GWAS (rs231770). After tracing using HaploReg4.2, it was found that neighboring missenses may show variations in alleles that impact the risk of myasthenia gravis in various populations. Although these loci still make biological sense, more extensive studies are needed to prove that they are related to each other (Blauwendraat, 2022).

Another study in Vergoosen 2021 said that almost all MG-related genes were found in the testes and ectocervix. This study did not say a specific *CTLA4* gene existed in testicular tissue. Still, the genes in question were *RAPSN* and *CHRNA1* expression mostly limited to skeletal muscle, with some additional words in the tibial nerve and the testicular and pituitary glands, respectively (Yasumizu et al., 2022). Overall, the expression of MG-related genes is prominent in skeletal muscle and brain, but individual genes are also expressed in other tissues of the human body (Vergoossen et al., 2021).

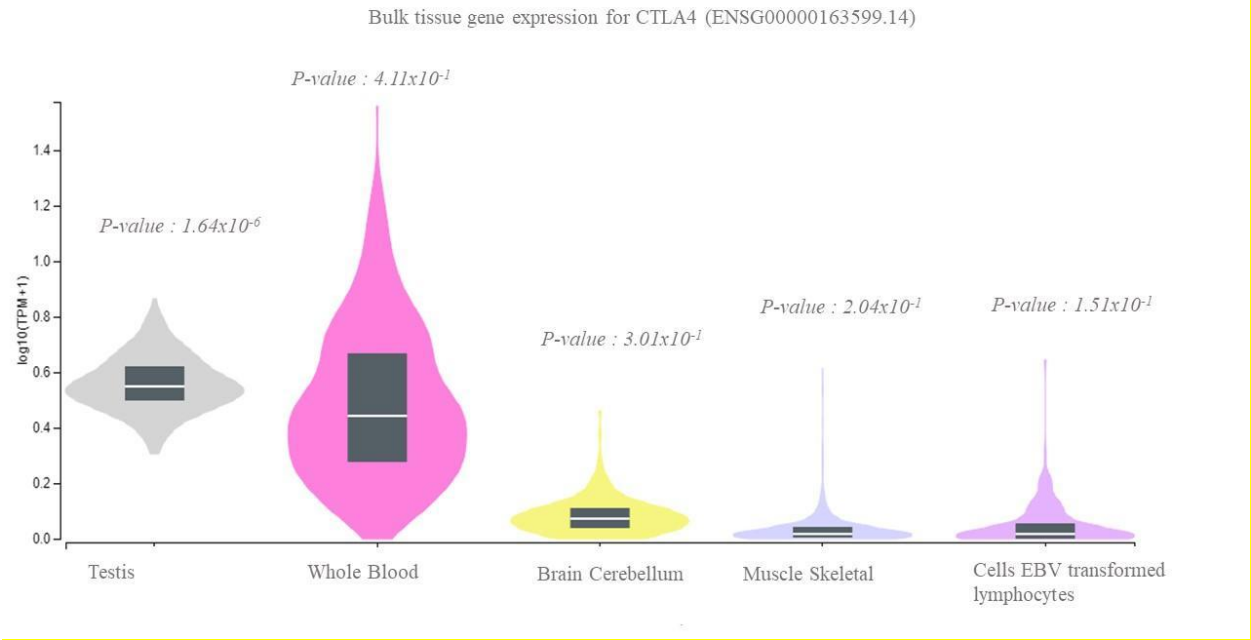


Figure 3. *CTLA4* gene expression associated with myasthenia gravis across several human tissues from the GTEx Portal.

Relationship between *LTA* and *CTLA4* genes with eQTL from GTEx portal database.

The GTEx Portal database aims to evaluate MG gene expression in various human tissues, including muscle tissue. Using the GWAS catalog database, we located 37 SNPs and identified genomic variation in *LTA* and *CTLA4* gene expression. We found 26 SNPs with the highest p values from this analysis. After this investigation, two statistically significant SNPs were found and prioritized. Based on an extended SNP count analysis using HaploReg version 4.2, we prioritized the two SNPs at risk for MG because the functional annotations of the SNPs were 10^{-8}.

According to (Yasumizu et al., 2022), the increase in *CHRNA1* expression tend to be lower when compared with other neuromuscular antigens such as *GABRA5* and *RYR3*. Although a moderate rise in *CHRNA1* expression seems sufficient to cause severe symptoms in MG, it is related to the availability of acetylcholine receptor antibodies and their crucial biological role. These findings provide valuable clues for understanding the pathogenesis of various autoimmune neurological diseases. The following Table 3 shows the results of genetic variation from MG.

Table 3. e-QTL's result for the Myasthenia gravis from GTEX portal database

SNP	GENCODE ID (ENSG00000-)	Gene symbol	P-value	NES	Tissue	Actions
rs2071591	226979.8	*LTA*	0.000065	0.29	Brain - Cerebellum	GG>GA>AA
	226979.8	*LTA*	0.00024	0.14	Muscle - Skeletal	GG>GA>AA
rs231770	163599.14	*CTLA4*	0.0000016	-0.28	Testis	CC>CT>TT

Myasthenia gravis (MG) candidate variant allele frequencies across continents

After identifying candidate variants related to *LTA* and *CTLA4* gene expression, allele frequencies have been determined across populations of all continents as shown in (Table 4). Allele frequencies for the four variants were evaluated in different people, including populations of 1195 individuals (Africa), 567 individuals (America), 1112 individuals (East Asia), 693 individuals (Europe), and 554 individuals (South East Asia). Using the Ensemble Genome Browser, we obtained allele frequencies in Africa, America, East Asia, Europe, and Southeast Asia (http://www.ensembl.org). Allele frequencies across populations differ for each *LTA* and *CTLA4* gene variant. Table 4 and Figure 4. shows the gene expression level at a higher frequency of the related allele (T) rs231770 than the corresponding allele (A) rs2071591. In the associated alleles (T) rs231770 and (A) rs2071591, the African and East Asian populations are much higher than the American, Southeast Asian, and European Asian people.

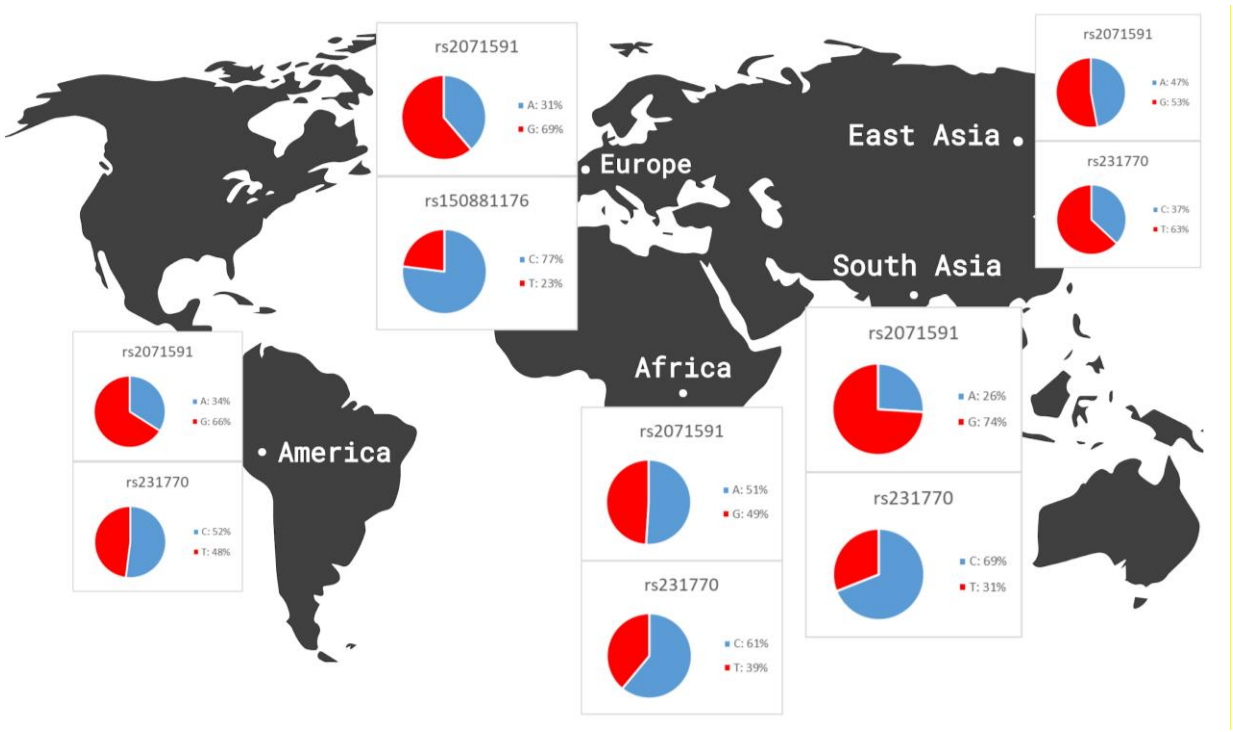


Figure 4. Summary of allele frequency analysis of *LTA* and *CTLA4* gene expression in Africa, America, East Asia, Europe, and Southeast Asia.

Table 4. Allele frequencies for SNPs examined in this study

SNP	Position (hg38)	Gene	P-value	Location	Allele		Allele Frequency (N)				
					Ref	Eff*	AFR	AMR	EAS	EUR	SAS
rs2071591	Chr6 31548022	*LTA*	$4x10^{-12}$	Missense	G	A	0.512 (677)	0.336 (233)	0.469 (473)	0.310 (312)	0.261 (255)
rs231770	Chr2 203864430	CTLA 4	$9x10^{-11}$	Missense	C	T	0.392 (518)	0.481 (334)	0.634 (639)	0.379 (381)	0.306 (299)

The higher frequency of the related allele is (T) rs231770 compared to the other related allele, namely (A) rs2071591. In the associated alleles (T) rs231770 and (A) rs2071591, the African and East Asian populations are much higher than the American, Southeast Asian, and European Asian people. The rs2071591 allele indicated a differential variant prevalence contribution for *LTA* gene expression, while the rs231770 allele indicated a differential variant prevalence contribution for *CTLA4* gene expression.

Allele frequencies in all populations differ for each SNP, as shown in Figure 4. It is generally known that the A and T allele frequencies for rs2071591 and rs231770 also appear to have a

higher frequency in Europe with alleles rs2071591 (47%), rs231770 (63%), East Asia with alleles rs2071591 (47%), rs231770 (63%), compared to America with alleles rs2071591 (34%), rs231770 (48%), Africa with alleles rs2071591 (51%), rs231770 (39%), Asia southeast with alleles rs2071591 (26%), rs231770 (31%).

Identification of gene variations that are unique and pathogenic for a disease is very interesting for clinical research and validation. Identification of these variants not only provide clues to disease susceptibility or as a diagnostic and prognostic biomarker (Irham, Adikusuma, & Perwitasari, 2022) but also can be used to discover candidate drug targets or what is known as drug repurposing (genomic driven drug repurposing) (Irham et al., 2020) (Afief et al., 2022). The authors hope that the discovery of the candidate gene variations found can be validated in clinical settings and can become a diagnostic and prognostic biomarker for myasthenia gravis disease.

In conclusion, a bioinformatics-based approach revealed pathogenic variants potentially associated with MG. We propose that this variant may be used for further studies to identify MG and prognosis diagnostic biomarkers. However, we acknowledge that there are limitations to the bioinformatics-based approaches used to investigate genetic variants associated with MG. Notably, not all variants necessarily correspond to identifiable gene (i.e., non-coding variants) and even when genes or genetic variants are present, they might not be suitable drug targets. Therefore, we recommend clinical validation to further corroborate our findings and gain deeper insight into the etiology and functional implications of MG disease."

Q5: Lack of coherence found in the discussion part of your manuscript; relate your findings with the already published recent articles.

A5: We appreciate the reviewer's comments. We have written according to input from reviewers in [page 8 lines 172-178] We have searched for recent articles (Yasumizu et al., 2022) related to myasthenia gravis and the discovered genes :

"According to (Yasumizu et al., 2022), the increase in *CHRNA1* expression tend to be lower when compared with other neuromuscular antigens such as *GABRA5* and *RYR3*. Although a moderate rise in *CHRNA1* expression seems sufficient to cause severe symptoms in MG, it is related to the availability of acetylcholine receptor antibodies and their crucial biological role. These findings provide valuable clues for understanding the pathogenesis of various autoimmune neurological diseases. The following Table 3 shows the results of genetic variation from MG."

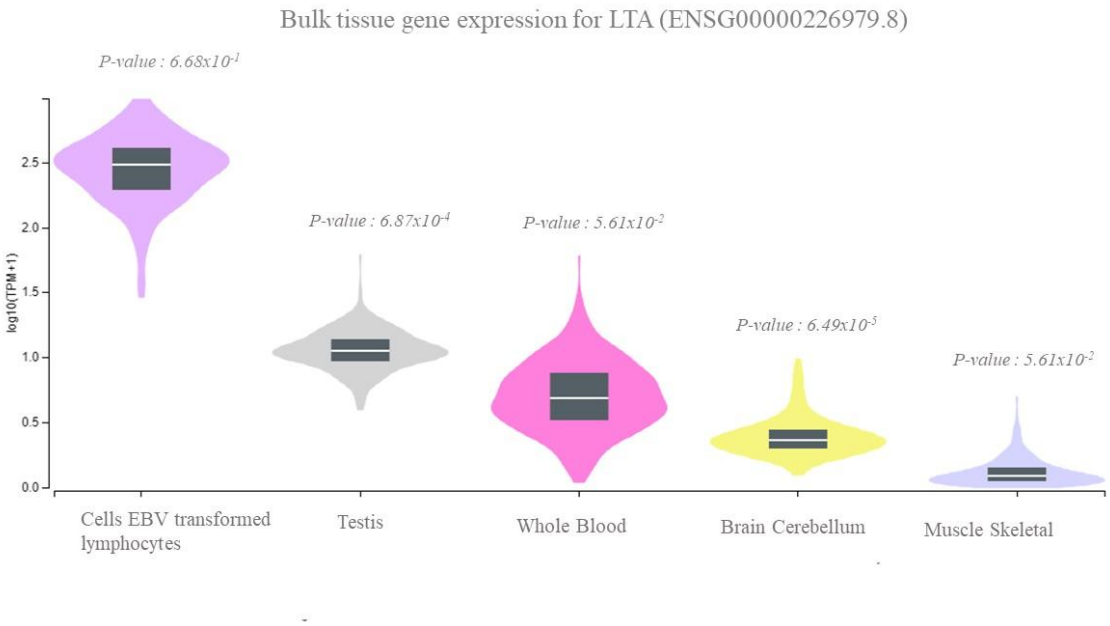
Q6: Include limitations and future prospects in the conclusion section of your manuscript.

A6: We appreciate the reviewer's comments. We have added according to the input in the conclusion section [Page 11, lines 240-242] The additional future prospects have been incorporated into the conclusion section :

"We recommend that the discovered candidate gene variations be validated clinically, which couldserve as diagnostic or prognostic biomarkers for MG."

Q7: Provide exact p value in each histograms

A7: We appreciate the reviewer's comments. We have added all the p values present in the histogram [Page 7, lines 144] and [Page 8, lines 161]



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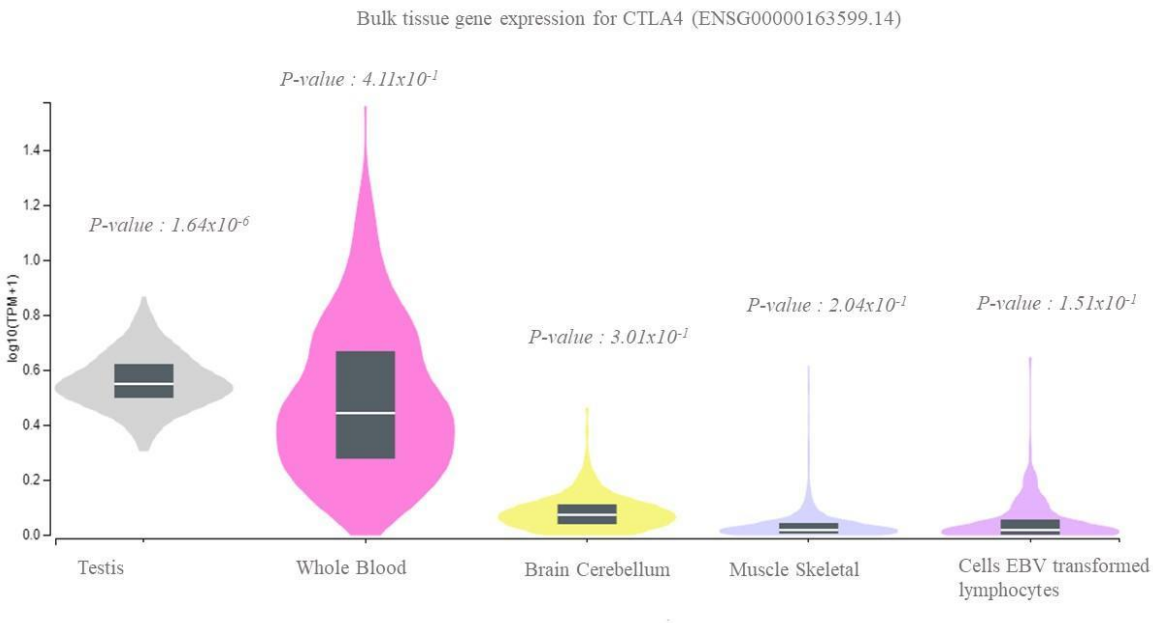

Figure 3. *CTLA4* gene expression associated with myasthenia gravis in several human tissues from the GTEx Portal.

Q8: Akt and Erk signalling (PMID: 30707354) Wnt-catenine signalling (PMID: 37489441) plays very important role in the progression of neurological diseases. Discuss these 2 pubmed is in your manuscript and cite and relate it.

A8: "We appreciate the reviewer's comments. After we read these two articles, it can be concluded that myasthenia gravis has nothing to do with these two articles because myasthenia gravis is not included in the neurodegenerative category. Myasthenia Gravis is an autoimmune disorder that affects neuromuscular function, so we are unable to add citations to this article in our article".

Q9: Some cited references are not related to their text content. Cross check each and every reference and their associated text in the manuscript.

A9: "We appreciate the reviewer's comments. We will delete the inappropriate part of the sentence".

Q10: Complete editorial checking will be needed for your manuscript.

A10: “We appreciate the reviewer's comments. We have corrected all the feedback that reviewers have given us”

Recommendation Reviewer 2:

This study examined the variants related to Myasthenia Gravis by using different databases. The results provided evidence to the risks of Myasthenia Gravis infection and disease susceptibility. However, there are some significant revisions that would need to be made before I recommend that the manuscript be considered for publication

Answer:

Q1: It would be helpful to provide some more introductions about the possible mechanism of the differences in the incidence and prevalence of MG in different countries for some readers who don't know much about this field.

A1: **We have appreciated the reviewer in correcting our article. We have written according from reviewers in [page 2 lines 56-64]**

"According to a systematic population-based study, Car et al. (Carr, Cardwell, McCarron, & McConville, 2010) estimated the incidence and prevalence of MG to be 54 per million and 77.7 per million, respectively. However, significant changes have been reported in various studies. The incidence of this disease has been shown to range between 1.77 and 21.3 per million people and a prevalence of 15 to 179 million people (Carr et al., 2010). Many epidemiological studies, especially in Western Europe and Asia, report significant differences in the incidence and prevalence of MG. The incidence of myasthenia gravis ranges from 1.7 to 30 per million per year (Breiner et al., 2016). This disease has two age peaks: 40-40 years, mainly affecting women, and the other 80-60 years, which occurs equally in men and women (Benatar et al., 2016)."

Q2: This study tried to synthesize the available literature to evaluate the possible variants related to MG. However, population variables, which significantly influence the prevalence of MG, may also have an interaction effect with the variants.

A2: **Thank you for the comments. We have written according to input from reviewers regarding the population we encountered in this article in [page 10 lines 210-215]**

"Allele frequencies in all populations differ for each SNP, as shown in Figure 4. It is generally known that the A and T allele frequencies for rs2071591 and rs231770 also appear to have a higher frequency in Europe with alleles rs2071591 (47%), rs231770 (63%), East Asia with alleles rs2071591 (47%), rs231770 (63%), compared to America

with alleles rs2071591 (34%), rs231770 (48%), Africa with alleles rs2071591 (51%), rs231770 (39%), Asia southeast with alleles rs2071591 (26%), rs231770 (31%).**"**

Q3: The authors discussed the positive results. However, these negative findings may be a key reference for future studies investigating.

A3: **We appreciate the reviewer's comments. We have written down the input provided by reviewers regarding this matter in [page 10 lines 224-225]**

"We propose that this variant may be used for further studies to identify MG and prognosis diagnostic biomarkers."

Q4: The authors provided detailed explanations of the results. However, practical implications of the findings are totally missing.

A4: **Thank you for the comments. We have written the implications of this research according to input from reviewers in [page 10 lines 216-222]**

"Identification of gene variations that are unique and pathogenic for a disease is very interesting for clinical research and validation. Identification of these variants not only provide clues to disease susceptibility or as a diagnostic and prognostic biomarker (Irham, Adikusuma, & Perwitasari, 2022) but also can be used to discover candidate drug targets or what is known as drug repurposing (genomic driven drug repurposing) (Irham et al., 2020) (Afief et al., 2022)."

Draft yang sudah direvisi sesuai dengan masukan dari para reviewer:

Integration of Genomics Database and Bioinformatics to Identify Genome-Wide Variants for Myasthenia Gravis Across Multiple Continents

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ABSTRACT

Autoimmune disease is an immune response that damages the body's tissues, thereby disrupting the body's physiological functions. Myasthenia gravis represents one such condition characterized by muscle weakness due to impaired neuromuscular transmission. While it can affect anyone, it tends to be more prevalent among women aged 20-30 and men over 50. This disease, deemed a genetic disorder, typically emerges in old age when antibodies target receptors in the muscles. In this study, we sought to identify the genes that can affect myasthenia gravis by leveraging several databases, including the GWAS Catalog, HaploReg Version 4.2, GTEX portal, and ensemble. Specifically, our focus was on exploring genomic variants and the expression of the *LTA* and *CTLA4* genes. Our findings reveal that two variants (rs2071591 and rs231770) impact *LTA* expression in both muscle and brain tissue, while affecting *CTLA4* expression in testicular cell tissue. Subsequently, we assessed the allele frequency of these variants across regional populations, namely African, American, East Asian, European, and Southeast Asian. This study demonstrates that the *LTA* and *CTLA4* genes have a higher frequency in African, East Asian, and European populations compared to American and Southeast Asian populations. Consequently, our finding suggests that the latter two populations might have relatively higher susceptibility to the autoimmune disease myasthenia gravis. Therefore, variations in these genes not only offer insights into disease susceptibility, diagnostic and prognostic biomarkers but also open avenues for identifying candidate drug targets through genomic-driven drug repurposing.

Keywords: Myasthenia Gravis, Autoimmune, Bioinformatics, Genetic Variation.

1. Introduction

Myasthenia gravis (MG) is a neuromuscular disease characterized by voluntary muscle weakness (Sanders, Wolfe, & Narayanaswami, 2017) (Gilhus & Verschuuren, 2015). This disease has different symptoms that vary in other patients depending on the degree of involvement of the striated muscles. The most common symptoms in patients with myasthenia gravis are ocular symptoms, which present as ptosis and diplopia. These symptoms usually occur later in the day, and following activities such as watching TV or driving are more common. Excessive fatigue has been reported due to frequent exertion in patients with this disease. Myasthenia gravis is an autoimmune disease that connects nerves to muscles (Murai, 2014), produced by different antibodies against synaptic membrane proteins (Benatar et al., 2016). It usually accounts for more than 85% of cases and is caused by a type of antibody to the skeletal muscle acetylcholine receptor (AChR-Ab) (Berrih-Aknin, Frenkian-Cuvelier, & Eymard, 2014). However, components other than AChR, such as muscle-specific receptor tyrosine kinase or lipoprotein-associated protein 4 (LRP4), can also be targeted for autoimmune attacks (Mehling et al., 2011).

Based on the mechanism of autoimmune disease and antibodies, molecular skeletal muscle invasiveness, thymus status, genetic characteristics, disease phenotype, and response to treatment, myasthenia gravis is divided into early and late ocular subtypes, seronegative, thymoma, LRP4. Diagnosis of the MG subtype influences treatment decisions and disease prognosis (Kerty, Elsais, Argov, Evoli, & Gilhus, 2014). Approximately 50% of patients with ocular MG develop generalized myasthenia gravis (GMG) over a 2-year, which affects other muscles and is manifested by visual weakness and symptoms (Khan & Wang, 2020).

According to a systematic population-based study, Car et al. (Carr, Cardwell, McCarron, & McConville, 2010) estimated the incidence and prevalence of MG to be 54 per million and 77.7 per million, respectively. However, significant changes have been reported in various studies. The incidence of this disease has been shown to range between 1.77 and 21.3 per million people and a prevalence of 15 to 179 million people (Carr et al., 2010). Many epidemiological studies, especially in Western Europe and Asia, report significant differences in the incidence and prevalence of MG. The incidence of myasthenia gravis ranges from 1.7 to 30 per million per year(Breiner et al., 2016). This disease has two age peaks: 40-40 years, mainly affecting women, and the other 80-60 years, which occurs equally in men and women (Benatar et al., 2016).

In summary, MG is a neuromuscular disorder characterized by muscle weakness, with its manifestations varying among patients. Ocular symptoms, notably ptosis and diplopia, are common, often exacerbated by activities like watching TV or driving. MG results from autoimmune processes triggered by antibodies against synaptic membrane proteins, predominantly the acetylcholine receptor (AChR-Ab). The disease is categorized into subtypes, impacting treatment choices and prognosis. Epidemiologically, MG's incidence and prevalence vary across regions and age groups. Genetic variations play a significant role, with GWAS cataloging identifying relevant SNPs. This study aims to investigate the variants associated with MG through an approach based on bioinformatics, offering insights into disease susceptibility and progression. In addition, gene expression profile patterns and allele frequencies of genetic variant populations were assessed using various databases. The results will enable future studies to determine whether

these variants may be associated with multiple risks of MG infection, as well as MG progression and disease susceptibility.

2. Method

This study employed a bioinformatics-based approach to prioritize pathogenic variants potentially linked to MG. A detailed outline of the study design is visually represented in Figure 1. To collect data on MG-associated variants, we leveraged the GWAS Catalog from the National Human Genome Research Institute (NHGRI) Database (http:// www.ebi.ac.uk/gwas), accessed on May 22, 2023. Employing the keyword "Myasthenia Gravis" (MG), we extracted information on variants associated with MG. We found a total 36 SNPs number of MG-associated variants. Next, subsequent analysis of MG-related SNPs was conducted using HaploReg (version 4.2) HaploReg is a tool designed to analyze non-coding genome annotations from published GWAS or new variants. It aids in understanding the functional outcomes of GWAS results, predicting potential causal variants, identifying involved cell types, and predicting candidate target genes (Ward & Kellis, 2016) (Ward & Kellis, 2012). In this study, the GWAS catalog inclusion criteria for SNPs were those with a p-value $<10^{-8}$ (Lee et al., 2007). The investigation further entailed assessing associations between various genetic variants and gene expression profiles utilizing expression quantitative trait loci (QTL) available on the GTEx Portal database (http://www.gtexportal.org/home/) (Blauwendraat, 2022) accessed on May 22, 2023. Then, we confirmed the variant using the Ensembl Genome Browser (https://www.ensembl.org/index.html) (Ozaki et al., 2004) accessed on May 22, 2023. In addition, allele frequencies of the MG-associated variants were evaluated across diverse populations, encompassing African, American, East Asian, European, and Southeast Asian people.

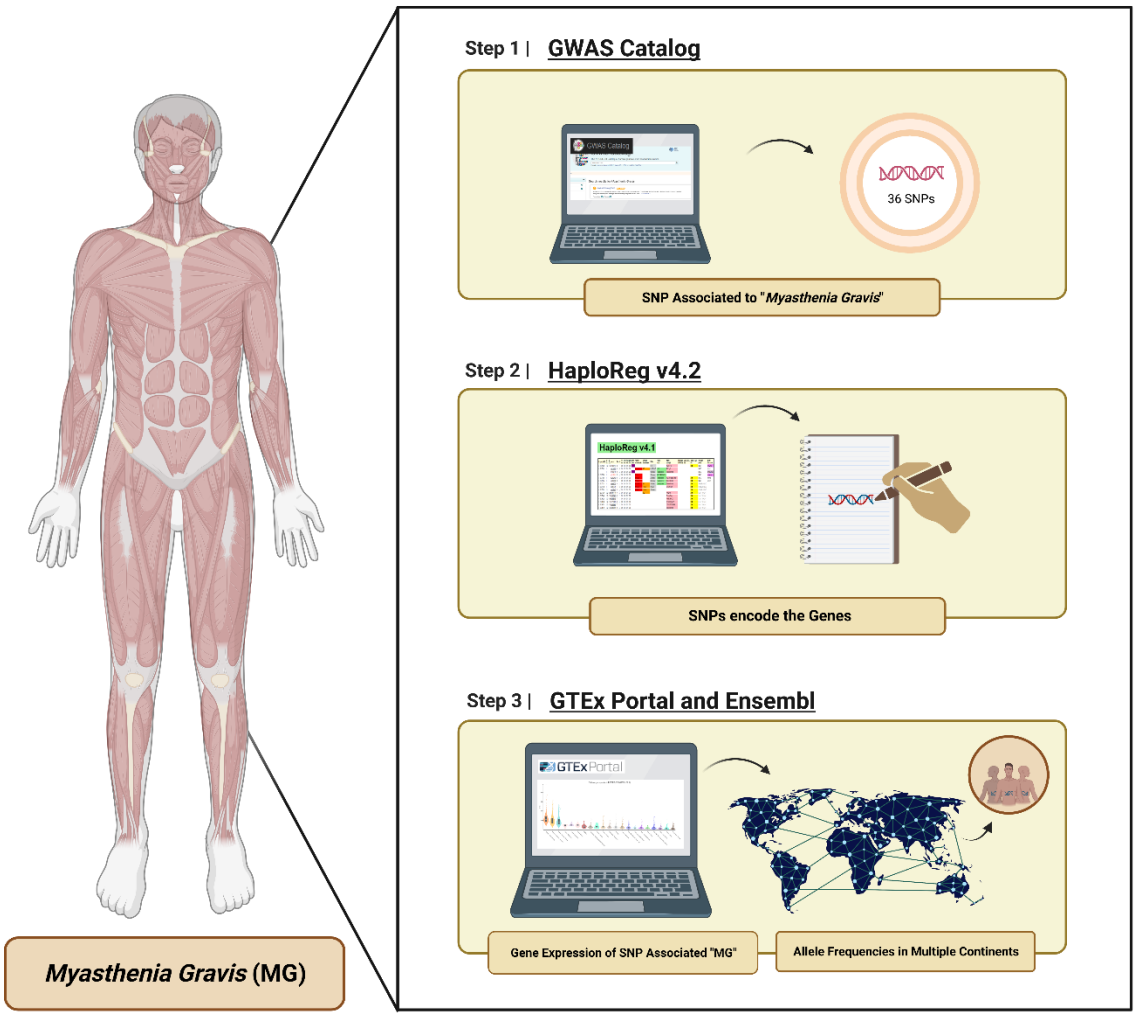

Figure 1. Bioinformatics workflow for the identification of genetic variations associated with Myasthenia Gravis (MG)

3. Results

This study utilized the GWAS database to identify SNPs associated with MG. From the GWAS database, 36 SNPs associated with MG were identifieden with 26 unique SNPs associated with MG further identified after removing all SNP duplications (Table 1.) Based on the number of SNPs obtained, the SNPs were forwarded using HaploReg version 4.2, with a p-value $<10^{-8}$.

Table 1. GWAS catalog result the duplicates removed are obtained from 26 SNPs with significance (p-value $<10^{-8}$)

SNP	P-value
rs3093958	$4x10^{-42}$
rs9271375	$2x10^{-19}$
rs4369774	$6x10^{-19}$

rs111945767	$3x10^{-17}$
rs76815088	$6x10^{-16}$
rs4409785	$2x10^{-07}$
rs4574025	$7x10^{-14}$
rs35274388	$1x10^{-12}$
rs2476601	$2x10^{-12}$
rs2071591	$4x10^{-12}$
rs150881176	$1x10^{-11}$
rs231770	$9x10^{-11}$
rs4574025	$4x10^{-07}$
rs9963862	$4x10^{-07}$
rs12653117	$5x10^{-07}$
rs6914704	$2x10^{-06}$
rs4128527	$4x10^{-06}$
rs4518467	$4x10^{-06}$
rs2476601	$7x10^{-06}$
rs9266277	$7x10^{-10}$
rs6998967	$9x10^{-10}$
rs35274388	$1x10^{-09}$
rs4263037	$1x10^{-08}$
rs73007767	$4x10^{-08}$
rs2245569	$6x10^{-08}$
rs9270986	$6x10^{-08}$

Based on the data presented in Table 2, this study focused on two genomic variants of the same gene that qualified as biological risk SNPs in this MG study.

Table 2. Varian risk allele which codes two genes

SNP	GENCODE	P-value	Allele Location
rs2071591	*LTA*	$4x10^{-12}$	Missense
rs231770	*CTLA4*	$9x10^{-11}$	Missense

Using an integrative bioinformatics approach, two variants with missense mutations rs2071591 and rs231770 encoding the *LTA* and *CTLA4* genes were prioritized as MG biological risk SNPs. MG disease is characterized by muscle and tissue weakness that occurs when the immune system is impaired and produces antibodies that attack the tissues in the body (Benatar et al., 2016). It was also reported that Lymphotoxin α (*LTA*) is a cytokine secreted by lymphocytes and is a member of the Tumor Necrosis Factor (*TNF*) family. *LTA* gene variations can contribute to threshold brain excitability, the spread of neural hyperexcitability (Aurora & Welch, 2000).

In Renton's (2015), the gene for cytotoxic T lymphocyte-associated protein (*CTLA4*) was previously suggested as a cause of myasthenia gravis susceptibility. The *CTLA4* gene also multiplies when symptoms are present regardless of age, indicating that it is responsible for aberrant autoimmune responses that lead to neuromuscular junction dysfunction. *CTLA4* 45-kD immunoglobulin is expressed by activated T cells and has a significant sequence identity with CD28 (Renton et al., 2015).

LTA gene expression in muscle and brain tissue.

In atherosclerotic plaques, intimal cells, some spindle-shaped or have globular, vacuolated cytoplasm, show immunoreactivity for *LTA* and galectin-2. Binds to adjacent portions of anti-tender muscle (SMC) cells. Galectin-2 and LTA are expressed in human smooth muscle cells and macrophages affected by atherosclerotic lesions (Vergoossen, Keo, Mahfouz, & Huijbers, 2021).

In Feroni's (2022), states that *LTA* genes encode cytokines that can modulate many inflammatory, immunological, and antiviral responses. It has been postulated that the inflammatory process modulated by *LTA* may contribute to the propagation of neural hyperexcitability by acting as an initiation and maintenance factor during migraine attacks (Ma'ruf et al., 2023). These results agree with a study on 439 Korean migraine patients genotyped for several *LTA* gene polymorphisms (Lee et al., 2007). Migraines occur because the blood vessels in the brain experience dilation or expansion, the main form of headache, characterized by debilitating headache attacks and symptoms of autonomic nervous system dysfunction (Olesen, 2018).

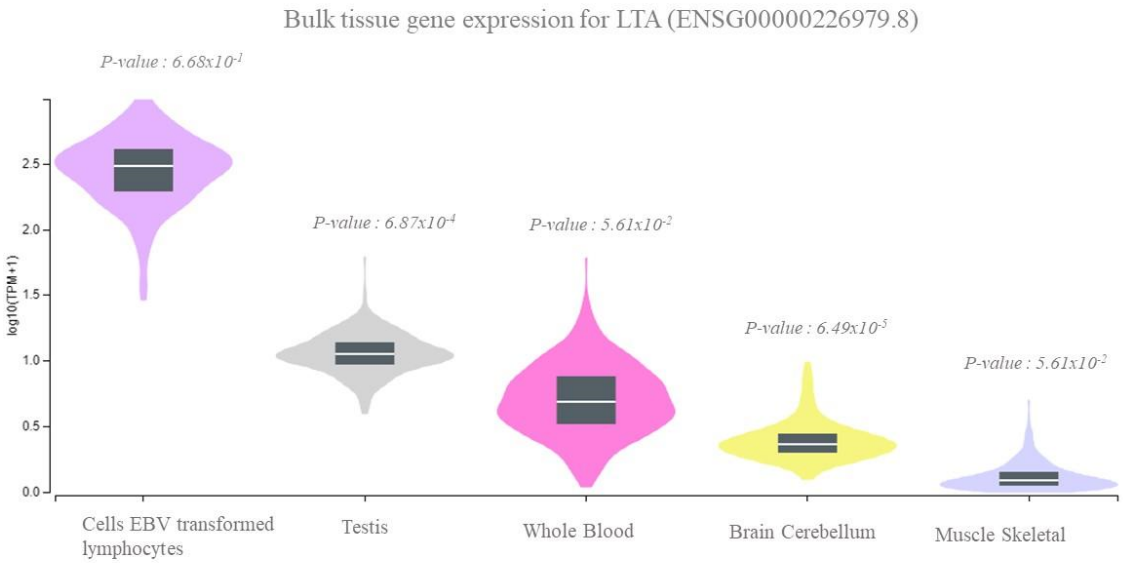

Figure 2. *LTA* gene expression associated with myasthenia gravis across several human tissues from the GTEx Portal.

Expression of the *CTLA4* gene in cell tissue in the testis.

Our new analysis does not show the *CTLA4* location in the previous GWAS (rs231770). After tracing using HaploReg4.2, it was found that neighboring missenses may show variations in alleles that impact the risk of myasthenia gravis in various populations. Although these loci still make biological sense, more extensive studies are needed to prove that they are related to each other (Blauwendraat, 2022).

Another study in Vergoosen 2021 said that almost all MG-related genes were found in the testes and ectocervix. This study did not say a specific *CTLA4* gene existed in testicular tissue. Still, the genes in question were *RAPSN* and *CHRNA1* expression mostly limited to skeletal muscle, with some additional words in the tibial nerve and the testicular and pituitary glands, respectively (Yasumizu et al., 2022). Overall, the expression of MG-related genes is prominent in skeletal muscle and brain, but individual genes are also expressed in other tissues of the human body (Vergoossen et al., 2021).

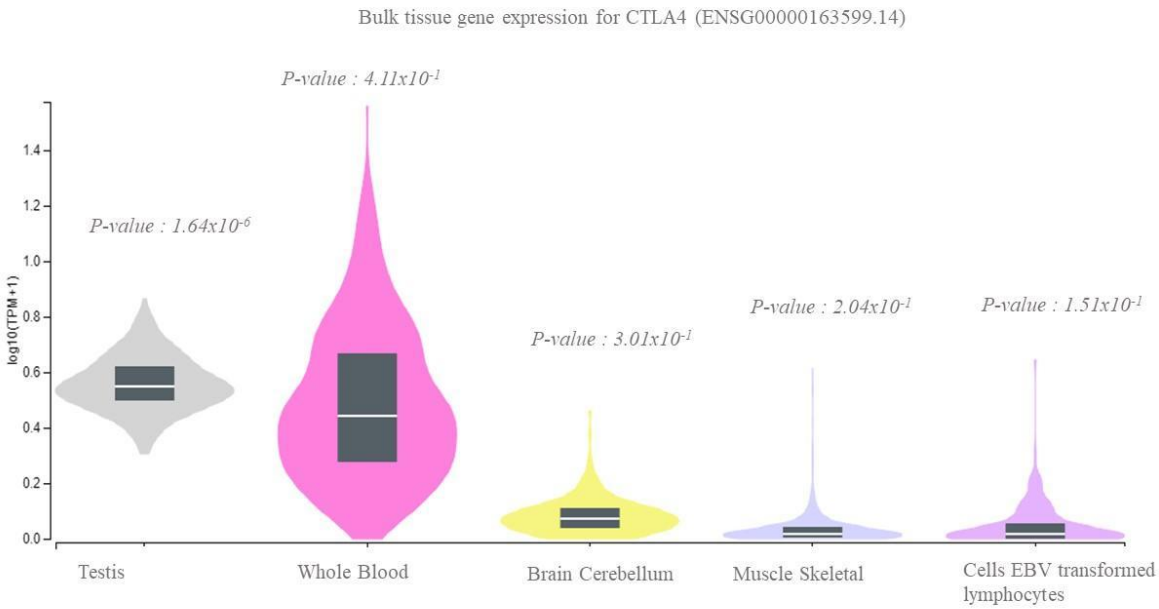

Figure 3. *CTLA4* gene expression associated with myasthenia gravis across several human tissues from the GTEx Portal.

Relationship between *LTA* and *CTLA4* genes with eQTL from GTEx portal database.

The GTEx Portal database aims to evaluate MG gene expression in various human tissues, including muscle tissue. Using the GWAS catalog database, we located 37 SNPs and identified genomic variation in *LTA* and *CTLA4* gene expression. We found 26 SNPs with the highest p values from this analysis. After this investigation, two statistically significant SNPs were found and prioritized. Based on an extended SNP count analysis using HaploReg version 4.2, we prioritized the two SNPs at risk for MG because the functional annotations of the SNPs were 10^{-8}.

According to (Yasumizu et al., 2022), the increase in *CHRNA1* expression tend to be lower when compared with other neuromuscular antigens such as *GABRA5* and *RYR3*. Although a moderate rise in *CHRNA1* expression seems sufficient to cause severe symptoms in MG, it is related to the availability of acetylcholine receptor antibodies and their crucial biological role. These findings provide valuable clues for understanding the pathogenesis of various autoimmune neurological diseases. The following Table 3 shows the results of genetic variation from MG.

Table 3. e-QTL's result for the Myasthenia gravis from GTEX portal database

SNP	GENCODE ID (ENSG00000-)	Gene symbol	P-value	NES	Tissue	Actions
rs2071591	226979.8	*LTA*	0.000065	0.29	Brain - Cerebellum	GG>GA>AA

	226979.8	*LTA*	0.00024	0.14	Muscle - Skeletal	GG>GA>AA
rs231770	163599.14	*CTLA4*	0.0000016	-0.28	Testis	CC>CT>TT

Myasthenia gravis (MG) candidate variant allele frequencies across continents

After identifying candidate variants related to *LTA* and *CTLA4* gene expression, allele frequencies have been determined across populations of all continents as shown in (Table 4). Allele frequencies for the four variants were evaluated in different people, including populations of 1195 individuals (Africa), 567 individuals (America), 1112 individuals (East Asia), 693 individuals (Europe), and 554 individuals (South East Asia). Using the Ensemble Genome Browser, we obtained allele frequencies in Africa, America, East Asia, Europe, and Southeast Asia (http://www.ensembl.org). Allele frequencies across populations differ for each *LTA* and *CTLA4* gene variant. Table 4 and Figure 4. shows the gene expression level at a higher frequency of the related allele (T) rs231770 than the corresponding allele (A) rs2071591. In the associated alleles (T) rs231770 and (A) rs2071591, the African and East Asian populations are much higher than the American, Southeast Asian, and European Asian people.

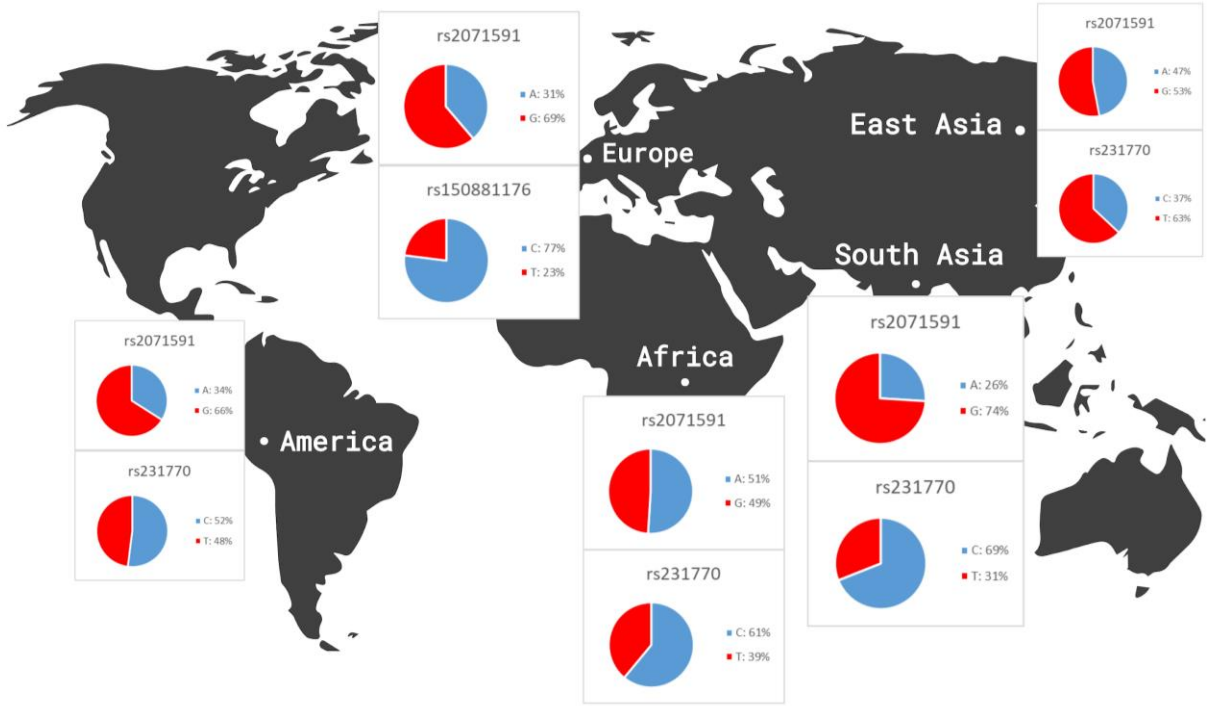

Figure 4. Summary of allele frequency analysis of *LTA* and *CTLA4* gene expression in Africa, America, East Asia, Europe, and Southeast Asia.

Table 4. Allele frequencies for SNPs examined in this study

SNP	Position (hg38)	Gene	P-value	Location	Allele		Allele Frequency (N)				
					Ref	Eff*	AFR	AMR	EAS	EUR	SAS
rs2071591	Chr6 31548022	*LTA*	$4x10^{-12}$	Missense	G	A	0.512 (677)	0.336 (233)	0.469 (473)	0.310 (312)	0.261 (255)
rs231770	Chr2 203864430	CTLA 4	$9x10^{-11}$	Missense	C	T	0.392 (518)	0.481 (334)	0.634 (639)	0.379 (381)	0.306 (299)

The higher frequency of the related allele is (T) rs231770 compared to the other related allele, namely (A) rs2071591. In the associated alleles (T) rs231770 and (A) rs2071591, the African and East Asian populations are much higher than the American, Southeast Asian, and European Asian people. The rs2071591 allele indicated a differential variant prevalence contribution for *LTA* gene expression, while the rs231770 allele indicated a differential variant prevalence contribution for *CTLA4* gene expression.

Allele frequencies in all populations differ for each SNP, as shown in Figure 4. It is generally known that the A and T allele frequencies for rs2071591 and rs231770 also appear to have a higher frequency in Europe with alleles rs2071591 (47%), rs231770 (63%), East Asia with alleles rs2071591 (47%), rs231770 (63%), compared to America with alleles rs2071591 (34%), rs231770 (48%), Africa with alleles rs2071591 (51%), rs231770 (39%), Asia southeast with alleles rs2071591 (26%), rs231770 (31%).

Identification of gene variations that are unique and pathogenic for a disease is very interesting for clinical research and validation. Identification of these variants not only provide clues to disease susceptibility or as a diagnostic and prognostic biomarker (Irham, Adikusuma, & Perwitasari, 2022) but also can be used to discover candidate drug targets or what is known as drug repurposing (genomic driven drug repurposing) (Irham et al., 2020) (Afief et al., 2022). The authors hope that the discovery of the candidate gene variations found can be validated in clinical settings and can become a diagnostic and prognostic biomarker for myasthenia gravis disease.

In conclusion, a bioinformatics-based approach revealed pathogenic variants potentially associated with MG. We propose that this variant may be used for further studies to identify MG and prognosis diagnostic biomarkers. However, we acknowledge that there are limitations to the bioinformatics-based approaches used to investigate genetic variants associated with MG. Notably, not all variants necessarily correspond to the identifiable gene (i.e., non-coding variants). ven when genes or genetic variants are present, they might not be suitable drug targets. Therefore, we recommend further clinical validation to corroborate our findings and gain deeper insight into the etiology and functional implications of MG disease.

4. Conclusion

In this study, we utilized a state-of-the-art bioinformatics approach to analyze genomic databases revealing distinct gene expressions of the *LTA* and *CTLA4* genes across muscle, brain, and testicular cell tissues for MG disease. Prominent gene variants include rs2071591, expressed in muscle and brain tissue, and rs231770, prevalent in testicular cell tissue. Notably, these variants

exhibit an overall higher frequency in Europe with alleles rs2071591 (47%), rs231770 (63%), East Asia with alleles rs2071591 (47%), rs231770 (63%), in comparison to America with alleles rs2071591 (34%), rs231770 (48%), Africa with alleles rs2071591 (51%), rs231770 (39%), and Southeast Asia with alleles rs2071591 (26%), rs231770 (31%). We recommend that the discovered candidate gene variations be validated clinically, which could serve as diagnostic or prognostic biomarkers for MG.

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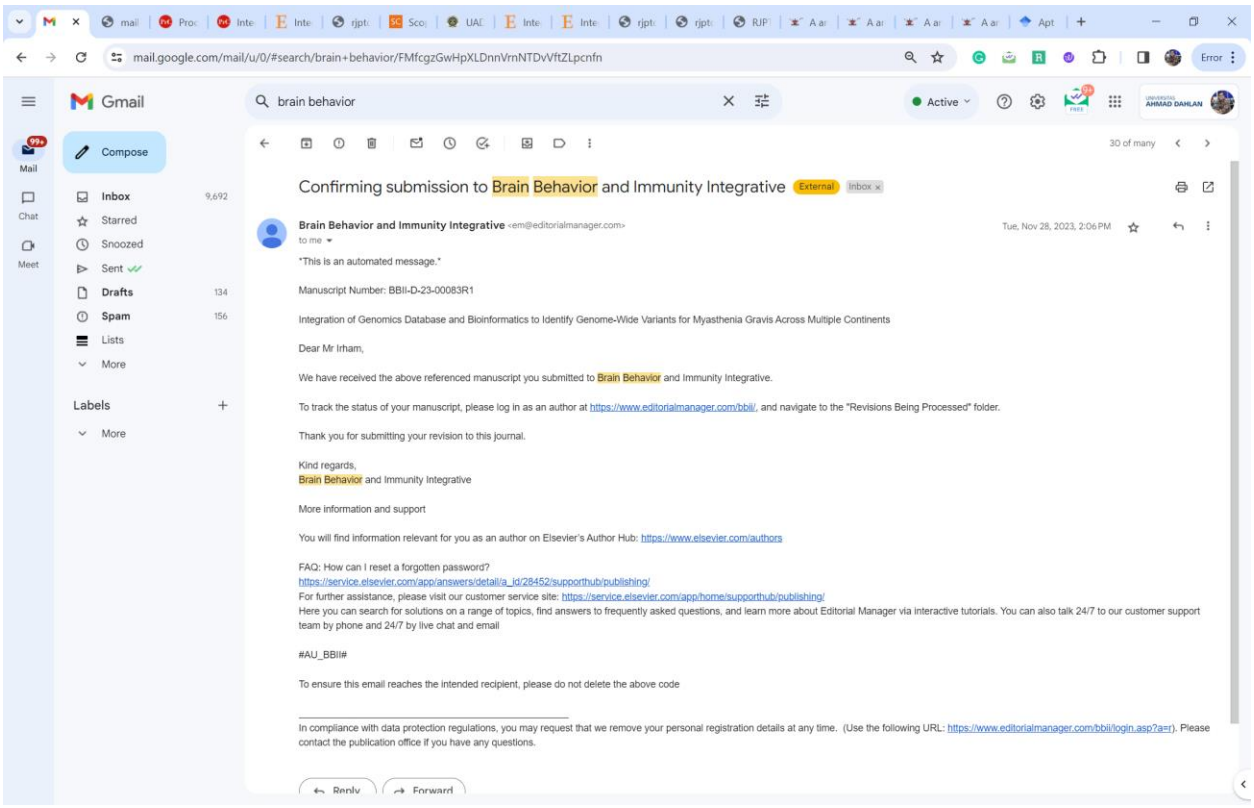

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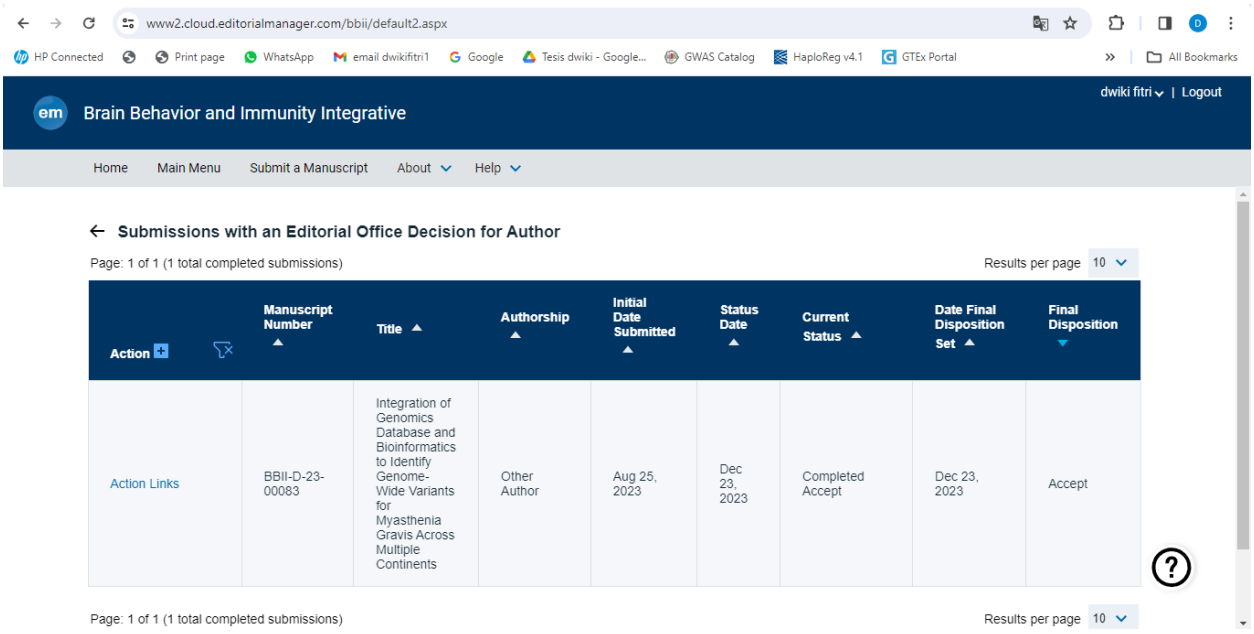

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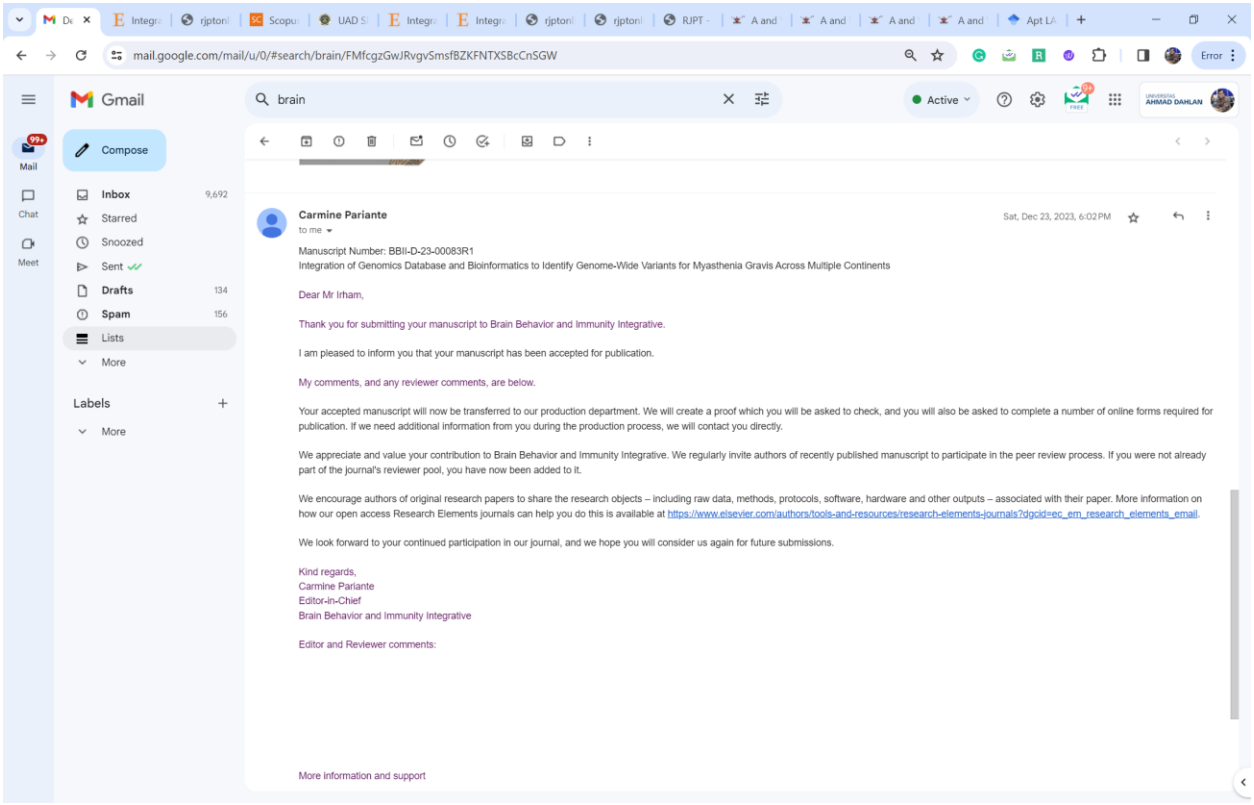

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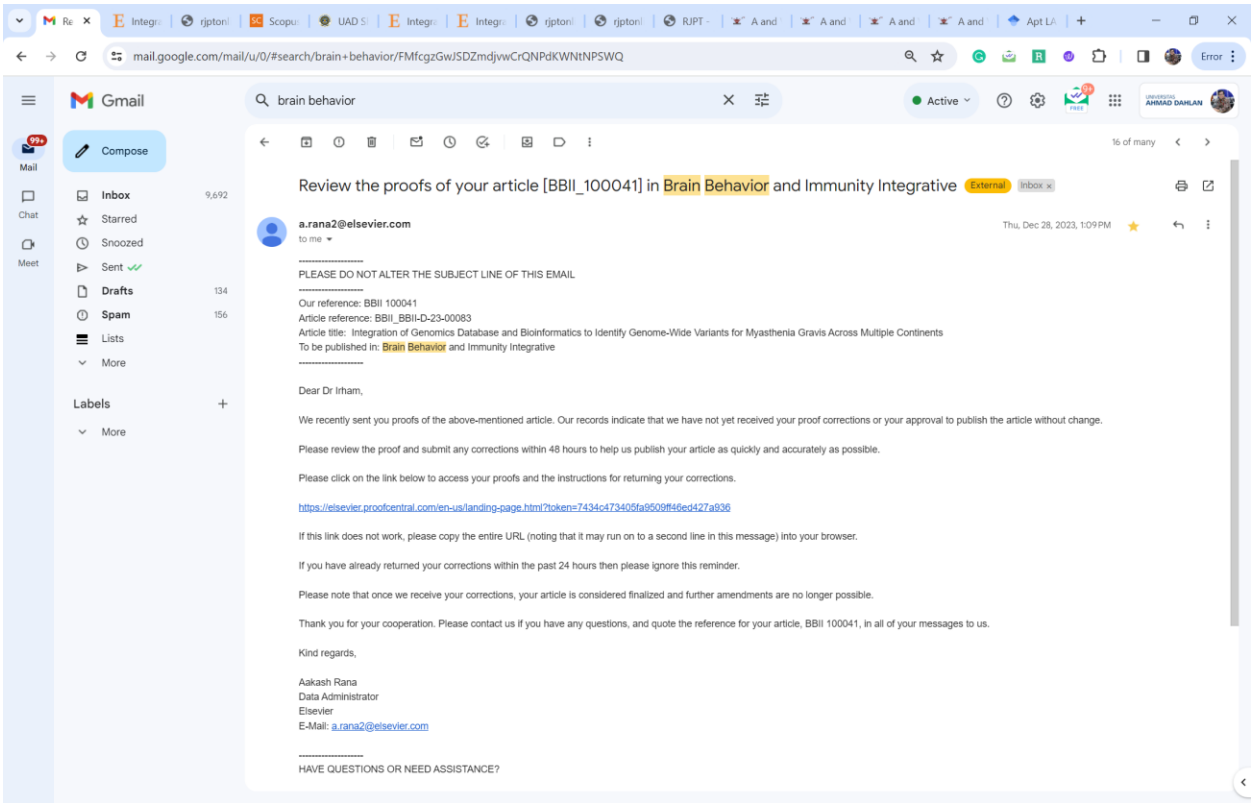

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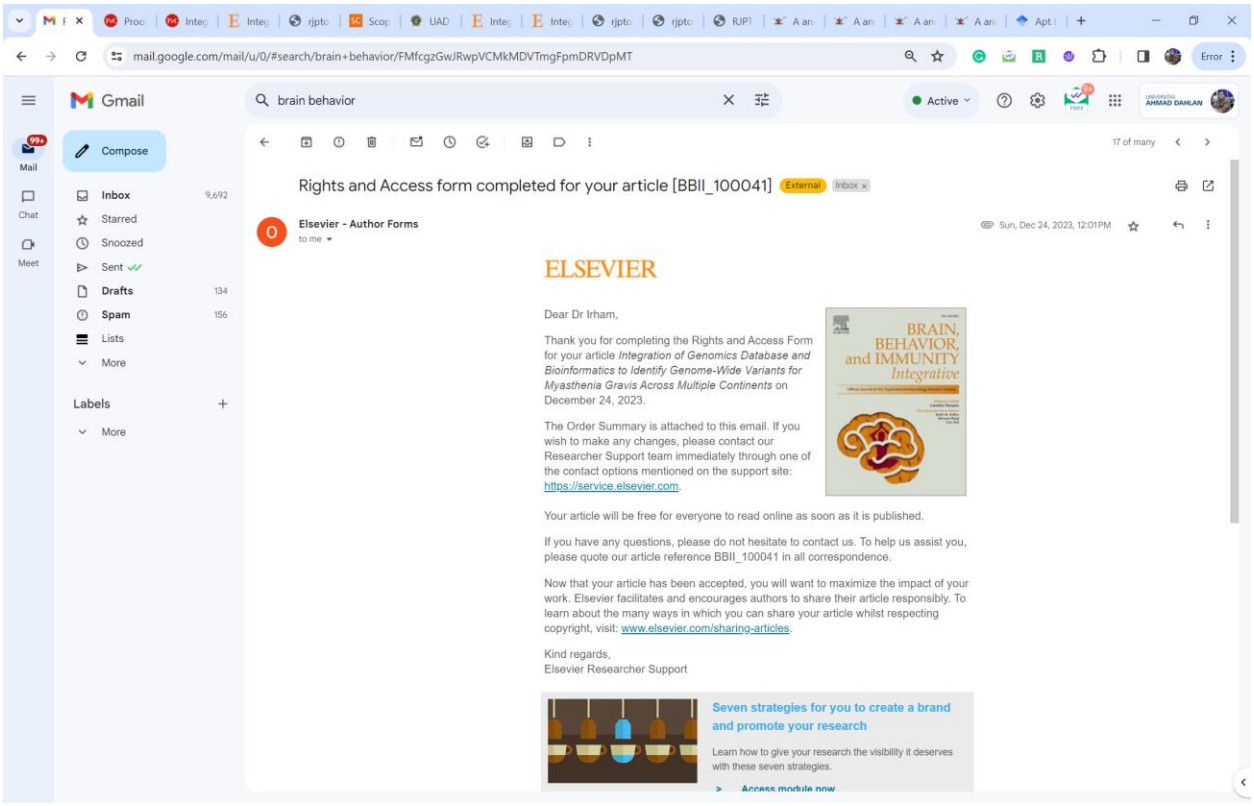

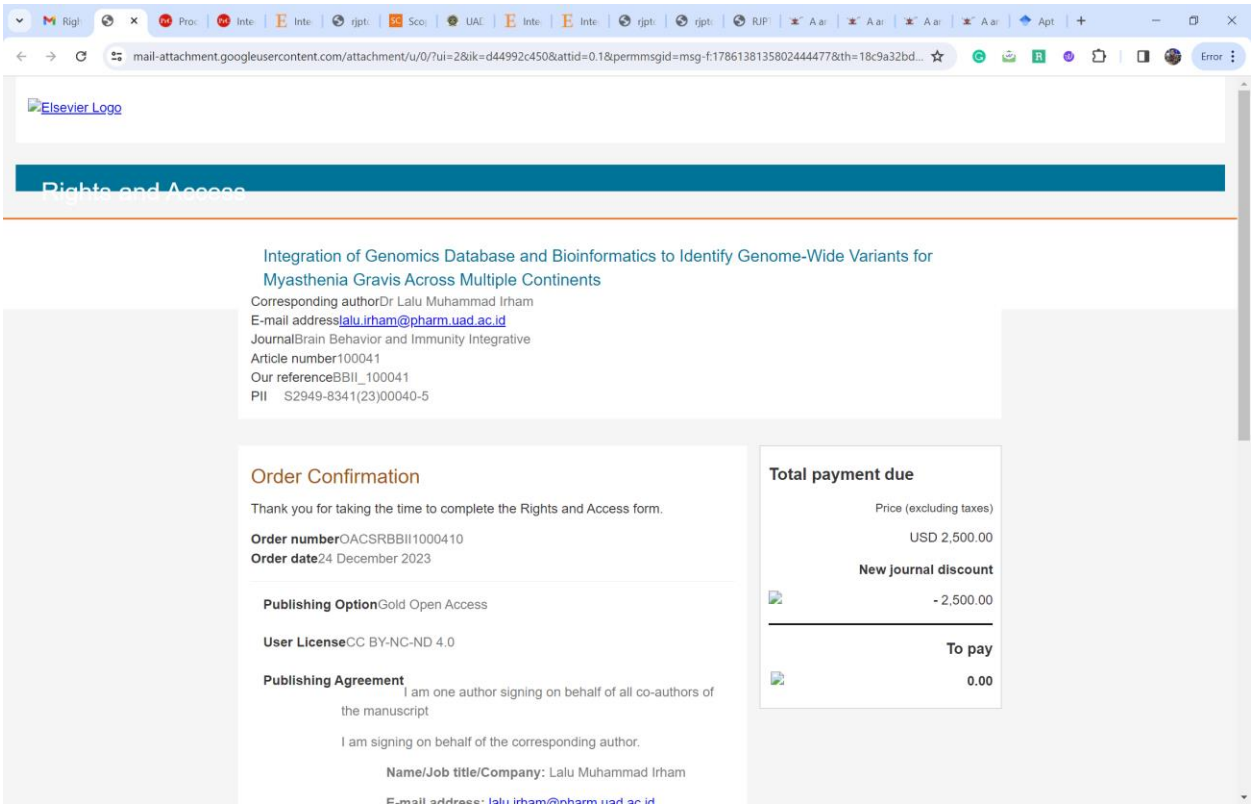

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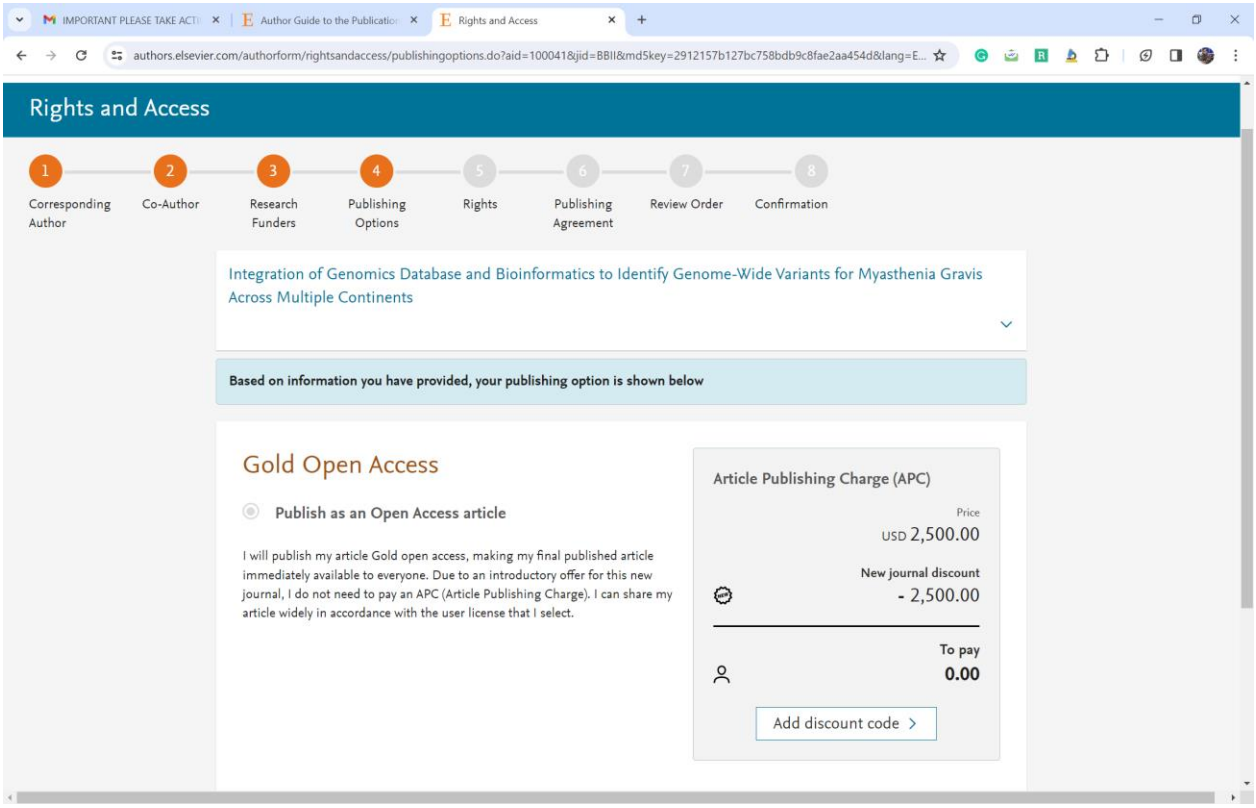

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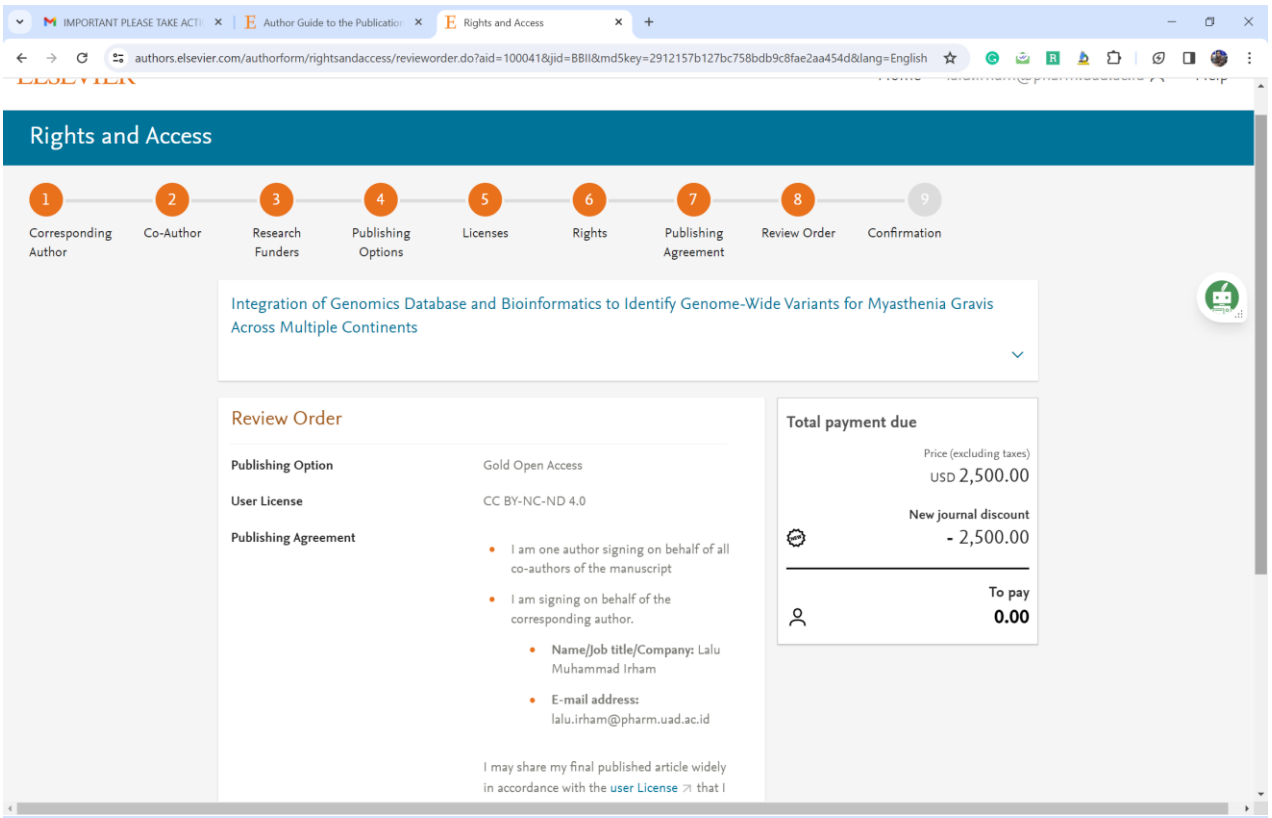
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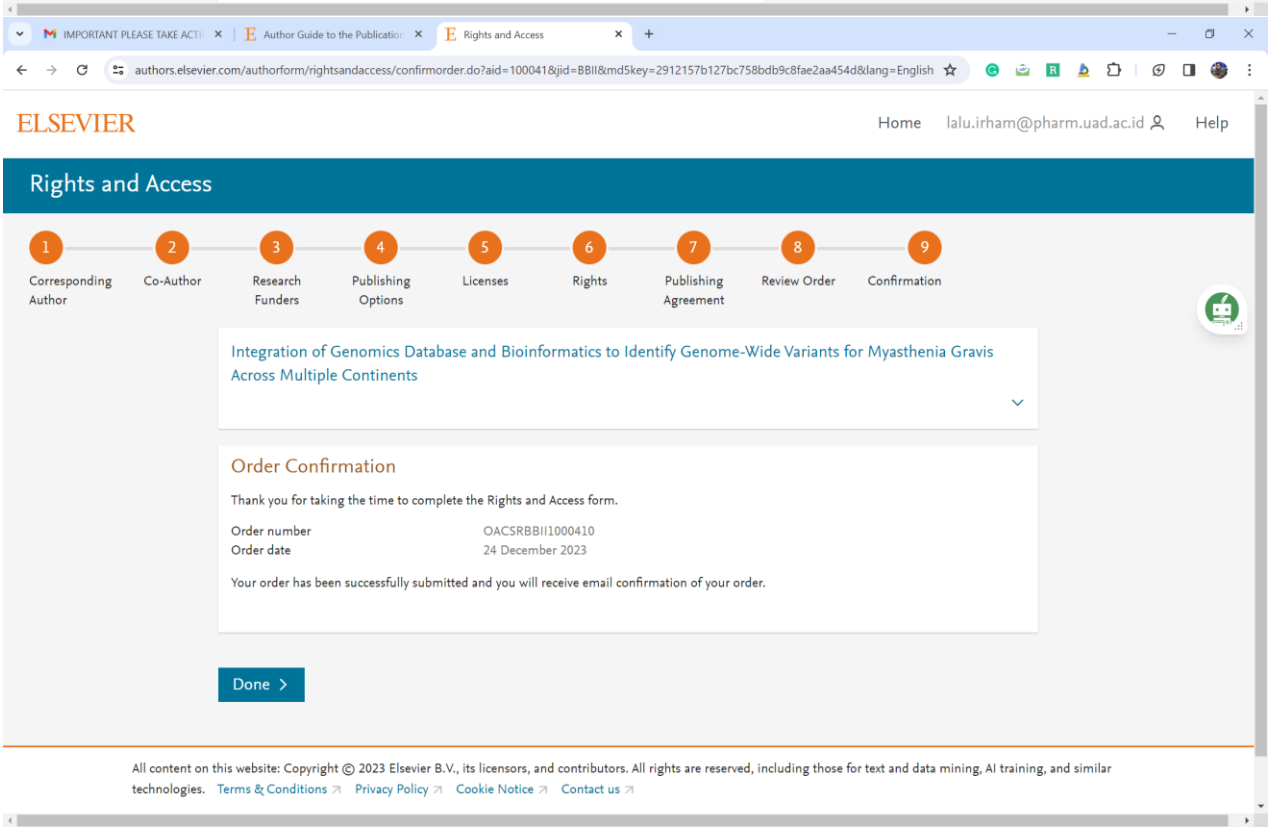
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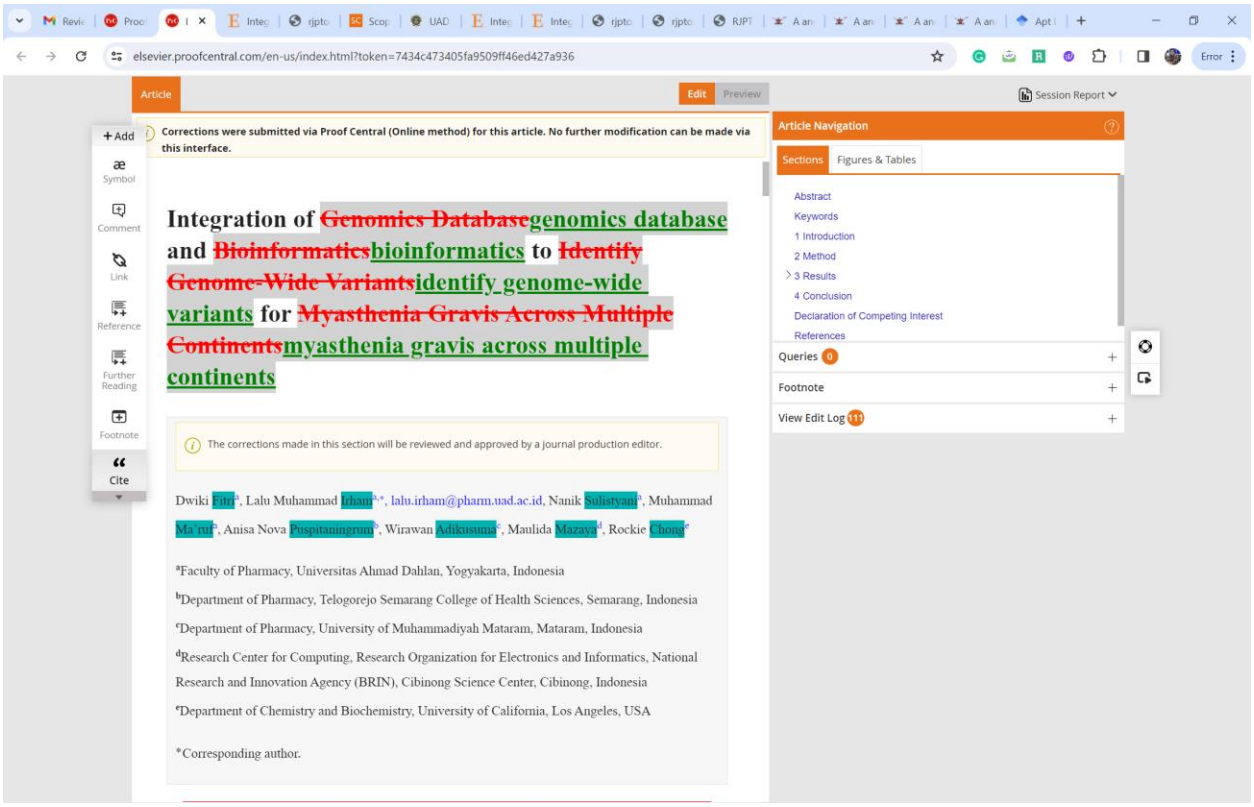
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Integration of genomics database and bioinformatics to identify genome-wide variants for myasthenia gravis across multiple continents

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ABSTRACT

Autoimmune disease is an immune response that damages the body's tissues, thereby disrupting the body's physiological functions. Myasthenia gravis represents one such condition characterized by muscle weakness due to impaired neuromuscular transmission. While it can affect anyone, it tends to be more prevalent among women aged 20–30 and men over 50. This disease, deemed a genetic disorder, typically emerges in old age when antibodies target receptors in the muscles. In this study, we sought to identify the genes that can affect myasthenia gravis by leveraging several databases, including the GWAS Catalog, HaploReg, GTEx portal, and Ensembl. Specifically, our focus was on exploring genomic variants and the expression of the *LTA* and *CTLA4* genes. Our findings reveal that two variants (rs2071591 and rs231770) impact *LTA* expression in both muscle and brain tissue, while affecting *CTLA4* expression in testicular cell tissue. Subsequently, we assessed the allele frequency of these variants across regional populations, namely African, American, East Asian, European, and Southeast Asian. This study demonstrates that the *LTA* and *CTLA4* genes have a higher frequency in African, East Asian, and European populations compared to American and Southeast Asian populations. Consequently, our finding suggests that the latter two populations might have relatively higher susceptibility to the autoimmune disease myasthenia gravis. Therefore, variations in these genes not only offer insights into disease susceptibility, diagnostic or prognostic biomarkers, but also open up avenues for identifying candidate drug targets through genomic-driven drug repurposing.

1. Introduction

Myasthenia gravis (MG) is a neuromuscular disease characterized by voluntary muscle weakness (Sanders et al., 2017; Gilhus and Verschuuren, 2015). This disease has different symptoms that vary in other patients depending on the degree of involvement of the striated muscles. The most common symptoms in patients with myasthenia gravis are ocular symptoms, which present as ptosis and diplopia. These symptoms usually occur later in the day, and following activities such as watching TV or driving are more common. Excessive fatigue has been reported due to frequent exertion in patients with this disease. MG is an autoimmune disease that connects nerves to muscles (Murai, 2014), produced by different antibodies against synaptic membrane proteins (Benatar et al., 2016). It usually accounts for more than 85% of cases and is caused by a type of antibody to the skeletal muscle acetylcholine receptor (AChR-Ab) (Berrih-Aknin et al., 2014). However, components other than AChR, such as muscle-specific receptor tyrosine kinase or lipoprotein-associated protein 4 (LRP4), can also be targeted for autoimmune attacks (Mehling et al., 2011).

Based on the mechanism of autoimmune disease and antibodies, molecular skeletal muscle invasiveness, thymus status, genetic characteristics, disease phenotype, and response to treatment, MG is divided into early and late ocular subtypes, seronegative, thymoma, LRP4. Diagnosis of the MG subtype influences treatment decisions and disease prognosis (Kerty et al., 2014). Approximately 50% of patients with ocular MG develop generalized myasthenia gravis (GMG) over a 2-year,

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https://doi.org/10.1016/j.bbii.2023.100041
Received 25 August 2023; Received in revised form 28 November 2023; Accepted 23 December 2023

which affects other muscles and is manifested by visual weakness and symptoms (Khan and Wang, 2020).

According to a systematic population-based study, (Carr et al., 2010) estimated the incidence and prevalence of MG to be 54 per million and 77.7 per million, respectively. However, significant changes have been reported in various studies. The incidence of this disease has been shown to range between 1.77 and 21.3 per million people and a prevalence of 15 to 179 million people (Carr et al., 2010). Many epidemiological studies, especially in Western Europe and Asia, report significant differences in the incidence and prevalence of MG. The incidence of MG ranges from 1.7 to 30 per million per year (Breiner et al., 2016). This disease has two age peaks: 40–40 years, mainly affecting women, and the other: 80–60 years, which occurs equally in men and women (Benatar et al., 2016).

In summary, MG is a neuromuscular disorder characterized by muscle weakness, with its manifestations varying among patients. Ocular symptoms, notably ptosis and diplopia, are common, often exacerbated by activities like watching TV or driving. MG results from autoimmune processes triggered by antibodies against synaptic membrane proteins, predominantly the acetylcholine receptor (AChR-Ab). The disease is categorized into subtypes, impacting treatment choices and prognosis. Epidemiologically, MG's incidence and prevalence vary across regions and age groups. Genetic variations play a significant role, with GWAS cataloging relevant variants. This study aims to investigate the variants associated with MG through an approach based on bioinformatics, offering insights into disease susceptibility and progression. In addition, gene expression profile patterns and allele frequencies of genetic variant populations were assessed using various databases. The results will enable future studies to determine whether these variants may be associated with multiple risks of MG infection, as well as MG progression and disease susceptibility.

2. Method

This study employed a bioinformatics-based approach to prioritize pathogenic variants potentially linked to MG. A detailed outline of the study design is visually represented in Fig. 1. To collect data on MG-associated variants, we leveraged the GWAS Catalog from the National Human Genome Research Institute (NHGRI) Database (http://www.ebi.ac.uk/gwas), accessed on May 22, 2023. Employing the keyword "Myasthenia Gravis" (MG), we extracted information on variants associated with MG. We found a total 36 single nucleotide polymorphisms (SNPs) number of MG-associated variants. Next, subsequent analysis of MG-related SNPs was conducted using HaploReg (version 4.2) HaploReg is a tool designed to analyze non-coding genome annotations from published GWAS or new variants. It aids in understanding the functional outcomes of GWAS results, predicting potential causal variants, identifying involved cell types, and predicting candidate target genes (Ward and Kellis, 2016, 2012). In this study, the GWAS catalog inclusion criteria for SNPs were those with a p-value $< 10^{-8}$ (Lee et al., 2007). The investigation further entailed assessing associations between various genetic variants and gene expression profiles utilizing expression quantitative trait loci (eQTL) available on the GTEx Portal database (http://www.gtexportal.org/home/) (Blauwendraat, 2022) accessed on May 22, 2023. Then, we confirmed the variant using the Ensembl Genome Browser (https://www.ensembl.org/index.html) (Ozaki et al., 2004) accessed on May 22, 2023. In addition, allele frequencies of the MG-associated variants were evaluated across diverse populations, encompassing African, American, East Asian, European, and Southeast Asian people.

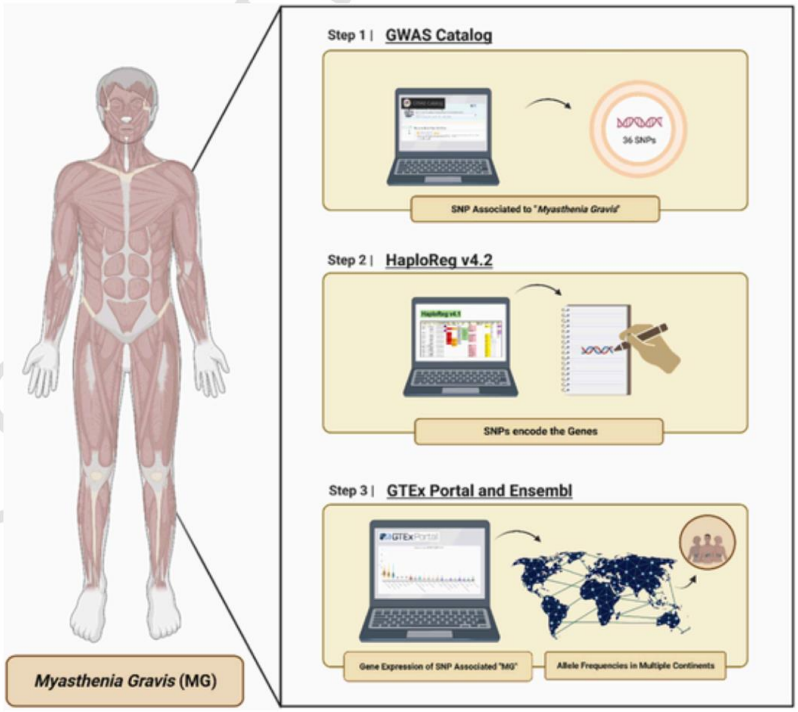

Fig. 1. Bioinformatics workflow for the identification of genetic variations associated with Myasthenia Gravis (MG).

3. Results

This study utilized the GWAS database to identify SNPs associated with MG. From the GWAS database, 36 SNPs associated with MG were identified with 26 unique SNPs associated with MG further identified after removing all SNP duplications (Table 1). Based on the number of SNPs obtained, the SNPs were forwarded using HaploReg version 4.2, with a *p*-value $< 10^{-8}$.

Based on the data presented in Table 2, this study focused on two genomic variants of the same gene that qualified as biological risk SNPs in this MG study. Using an integrative bioinformatics approach, two variants rs2071591 and rs231770 respectively encoding the *LTA* and *CTLA4* genes were prioritized as MG biological risk SNPs. Although these two variants does not show dbSNP functional annotation with missense mutation, however, by using HaploReg version 4.2 we identified the variant of rs2071591 near with rs1041981 variants which has missense mutation based on the criterion $r^2 > 0.8$. While for the variant with rs231770 near with the variant rs231775 which has missense mutation. MG disease is characterized by muscle and tissue weakness that occurs when the immune system is impaired and produces antibodies that attack the tissues in the body (Benatar et al., 2016). It was also reported that Lymphotoxin α (*LTA*) is a cytokine secreted by lymphocytes and is a member of the Tumor Necrosis Factor (*TNF*) family. *LTA* gene variations can contribute to threshold brain excitability, the spread of neural hyperexcitability (Aurora and Welch, 2000).

In Renton's et al. (2015), the gene for cytotoxic T lymphocyte-associated protein (*CTLA4*) was previously suggested as a cause of MG susceptibility. The *CTLA4* gene also multiplies when symptoms are present regardless of age, indicating that it is responsible for aberrant autoimmune responses that lead to neuromuscular junction dysfunction. *CTLA4* 45-kD immunoglobulin is expressed by activated T cells and has a significant sequence identity with CD28 (Renton et al., 2015).

Table 1
GWAS catalog results are obtained from 26 SNPs with significance (p-value $<10^{-8}$).

SNP	*p*-value
rs3093958	4×10^{-42}
rs9271375	2×10^{-19}
rs4369774	6×10^{-19}
rs111945767	3×10^{-17}
rs76815088	6×10^{-16}
rs4409785	2×10^{-07}
rs4574025	7×10^{-14}
rs35274388	1×10^{-12}
rs2476601	2×10^{-12}
rs2071591	4×10^{-12}
rs150881176	1×10^{-11}
rs231770	9×10^{-11}
rs4574025	4×10^{-07}
rs9963862	4×10^{-07}
rs12653117	5×10^{-07}
rs6914704	2×10^{-06}
rs4128527	4×10^{-06}
rs4518467	4×10^{-06}
rs2476601	7×10^{-06}
rs9266277	7×10^{-10}
rs6998967	9×10^{-10}
rs35274388	1×10^{-09}
rs4263037	1×10^{-08}
rs73007767	4×10^{-08}
rs2245569	6×10^{-08}
rs9270986	6×10^{-08}

Table 2
The variant risk allele which codes two genes

SNP	GENCODE	*p*-value
rs2071591	*LTA*	4×10^{-12}
rs231770	*CTLA4*	9×10^{-11}

3.1. LTA gene expression in muscle and brain tissues

In atherosclerotic plaques, intimal cells, some spindle-shaped or have globular, vacuolated cytoplasm, show immunoreactivity for *LTA* and galectin-2. Binds to adjacent portions of anti-tender muscle (SMC) cells. Galectin-2 and *LTA* are expressed in human smooth muscle cells and macrophages affected by atherosclerotic lesions (Vergoossen et al., 2021).

In Feroni's (2022), states that *LTA* genes encode cytokines that can modulate many inflammatory, immunological, and antiviral responses. It has been postulated that the inflammatory process modulated by *LTA* may contribute to the propagation of neural hyperexcitability by acting as an initiation and maintenance factor during migraine attacks (Ma'ruf et al., 2023). These results agree with a study on 439 Korean migraine patients genotyped for several *LTA* gene polymorphisms (Lee et al., 2007). Migraines occur because the blood vessels in the brain experience dilation or expansion, the main form of headache, characterized by debilitating headache attacks and symptoms of autonomic nervous system dysfunction (Olesen, 2018) (Fig. 2).

3.2. Expression of the CTLA4 gene in the testis

Our new analysis does not show the *CTLA4* location in the previous GWAS (rs231770). After tracing using HaploReg version 4.2, it was found that the SNP rs231770 near with the variant rs231775 which is missense mutation according to the criterion $r^2 >= 0.8$, the SNP may show variations in alleles that impact the risk of MG in various populations. Although these loci still make biological sense, more extensive studies are needed to prove that they are related to each other (Blauwendraat, 2022).

Another study in Vergoossen 2021 said that almost all MG-related genes were found in the testes and ectocervix. This study did not say a specific *CTLA4* gene existed in testicular tissue. Still, the genes in question were *RAPSN* and *CHRNA1* expression mostly limited to skeletal muscle, with some additional words in the tibial nerve and the testicular and pituitary glands, respectively (Yasumizu et al., 2022). Overall, the expression of MG-related genes is prominent in skeletal muscle and brain, but individual genes are also expressed in other tissues of the human body (Vergoossen et al., 2021) (Fig. 3).

3.3. Relationship between LTA and CTLA4 genes with eQTL from the GTEx portal database

The GTEx Portal database aims to evaluate MG gene expression in various human tissues, including muscle tissue. Using the GWAS catalog database we found 26 SNPs with the highest *p*-values from this analysis. Based on the *P*-value criterion, two statistically significant SNPs were found and prioritized. Based on an extended SNP count analysis using HaploReg version 4.2, we prioritized the two SNPs at risk for MG because the functional annotations of the SNPs were $<10^{-8}$.

According to (Yasumizu et al., 2022), the increase in *CHRNA1* expression tend to be lower when compared with other neuromuscular antigens such as *GABRA5* and *RYR3*. Although a moderate rise in *CHRNA1* expression seems sufficient to cause severe symptoms in MG, it is related to the availability of acetylcholine receptor antibodies and their crucial biological role. These findings provide valuable clues for understanding the pathogenesis of various autoimmune neurological diseases. The following Table 3 shows the results of genetic variation from MG.

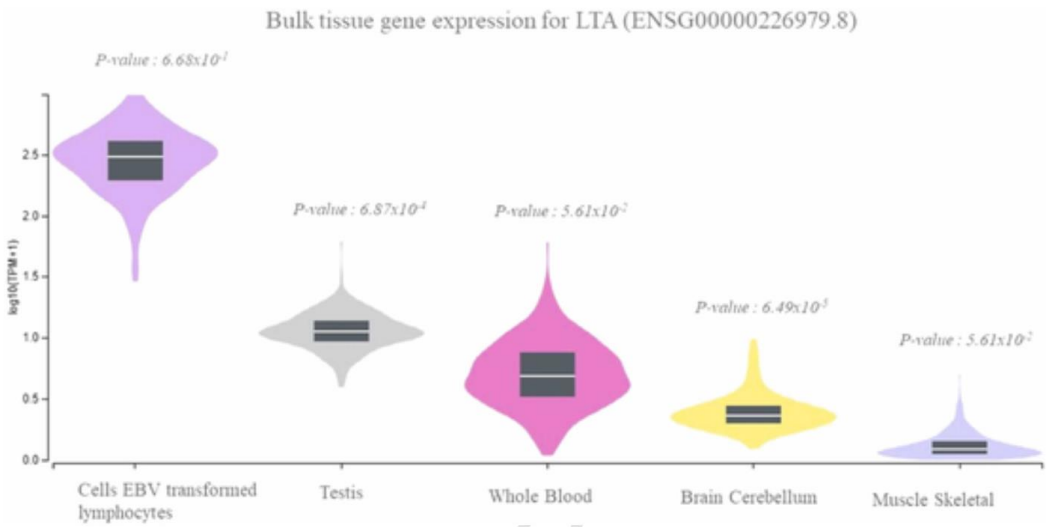

Fig. 2. *LTA* gene expression associated with myasthenia gravis across several human tissues from the GTEx Portal.

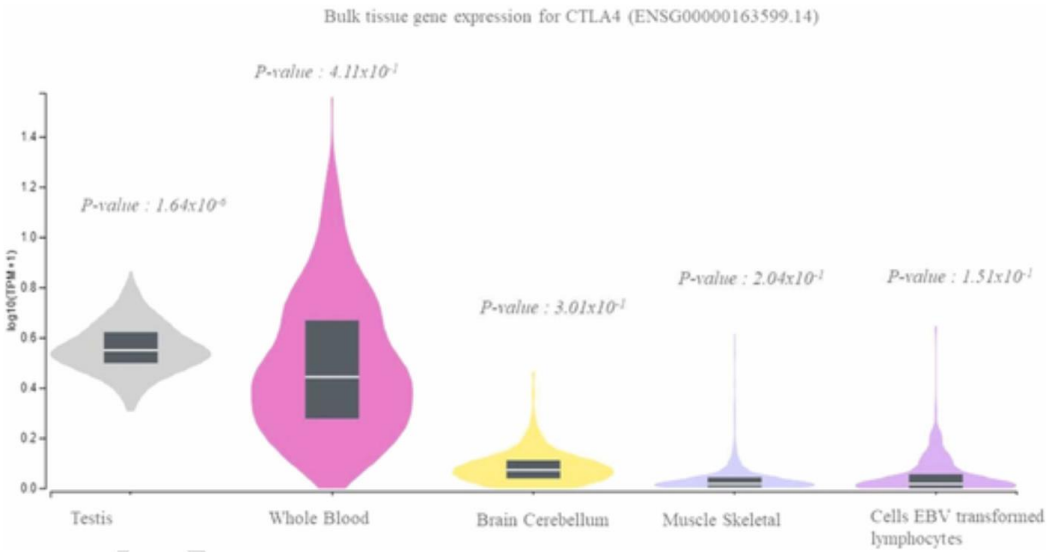

Fig. 3. CTLA4 gene expression associated with myasthenia gravis across several human tissues from the GTEx Portal.

3.4. Myasthenia gravis (MG) candidate variant allele frequencies across continents

After identifying candidate variants related to *LTA* and *CTLA4* gene expression, allele frequencies have been determined across populations of all continents, as shown in (Table 4). Allele frequencies for the two variants were evaluated in multiple continents, including populations of 1195 individuals (Africa), 567 individuals (America), 1112 individuals (East Asia), 693 individuals (Europe), and 554 individuals (South East Asia). Using the Ensemble Genome Browser, we obtained allele frequencies in Africa, America, East Asia, Europe, and Southeast Asia (http://www.ensembl.org). Allele frequencies across populations differ for each *LTA* and *CTLA4* gene variant. Table 4 and Fig. 4. shows the gene expression level at a higher frequency of the related allele (T) rs231770 than the corresponding allele (A) rs2071591. In the associated alleles (T) rs231770 and (A) rs2071591, the African and East Asian populations are much higher than the American, Southeast Asian, and European Asian people.

The higher frequency of the related allele is (T) rs231770 compared to the other related allele, namely (A) rs2071591. In the associated alleles (T) rs231770 and (A) rs2071591, the African and East Asian populations are much higher than the American, Southeast Asian, and European Asian people. The rs2071591 allele indicated a differential variant prevalence contribution for *LTA* gene expression, while the rs231770 allele indicated a differential variant prevalence contribution for *CTLA4* gene expression.

Table 3
e-QTL's result for the Myasthenia gravis from GTEx portal database.

SNP	GENCODE ID (ENSG00000-)	Gene symbol	*p*-value	NES	Tissue	Actions
rs2071591	226979.8	*LTA*	0.000065	0.29	Brain - Cerebellum	GG > GA > AA
	226979.8	*LTA*	0.00024	0.14	Muscle - Skeletal	GG > GA > AA
rs231770	163599.14	*CTLA4*	0.0000016	-0.28	Testis	CC > CT > TT

Allele frequencies in all populations differ for each SNP, as shown in Fig. 4. It is generally known that the A and T allele frequencies for rs2071591 and rs231770 also appear to have a higher frequency in Europe with alleles rs2071591 (47%), rs231770 (63%), East Asia with alleles rs2071591 (47%), rs231770 (63%), compared to America with alleles rs2071591 (34%), rs231770 (48%), Africa with alleles rs2071591 (51%), rs231770 (39%), Asia southeast with alleles rs2071591 (26%), rs231770 (31%).

Identification of gene variations that are unique and pathogenic for a disease is very interesting for clinical research and validation. Identification of these variants not only provide clues to disease susceptibility or as a diagnostic and prognostic biomarker (Irham et al., 2022) but also can be used to discover candidate drug targets or what is known as drug repurposing (genomic-driven drug repurposing) (Irham et al., 2020; Afief et al., 2022). The authors hope that the discovery of the candidate gene variations can be validated in clinical settings and can become a diagnostic and prognostic biomarker for MG disease.

In conclusion, a bioinformatics-based approach revealed pathogenic variants potentially associated with MG. We propose that this variant may be used for further studies to identify MG and prognosis diagnostic biomarkers. However, we acknowledge that there are limitations to the bioinformatics-based approaches used to investigate genetic variants associated with MG. Notably, not all variants necessarily correspond to the identifiable gene (i.e., non-coding variants) Even when genes or genetic variants are present, they might not be suitable drug targets. Therefore, we recommend further clinical validation to corroborate our findings and gain deeper insight into the etiology and functional implications of MG disease.

4. Conclusion

In this study, we utilized a state-of-the-art bioinformatics approach to analyze genomic databases revealing distinct gene expressions of the *LTA* and *CTLA4* genes across muscle, brain, and testicular cell tissues for MG disease. Prominent gene variants include rs2071591, expressed in muscle and brain tissue, and rs231770, prevalent in testicular cell tissue. Notably, these variants exhibit an overall higher frequency in Eu-

Table 4
Allele frequencies for SNPs examined in this study.

SNP	Position (hg38)	Gene	*p*-value	Allele		Allele Frequency (N)				
				Ref	Eff*	AFR	AMR	EAS	EUR	SAS
rs2071591	Chr6 31548022	*LTA*	4×10^{-12}	G	A	0.512 (677)	0.336 (233)	0.469 (473)	0.310 (312)	0.261 (255)
rs231770	Chr2 203864430	CTLA 4	9×10^{-11}	C	T	0.392 (518)	0.481 (334)	0.634 (639)	0.379 (381)	0.306 (299)

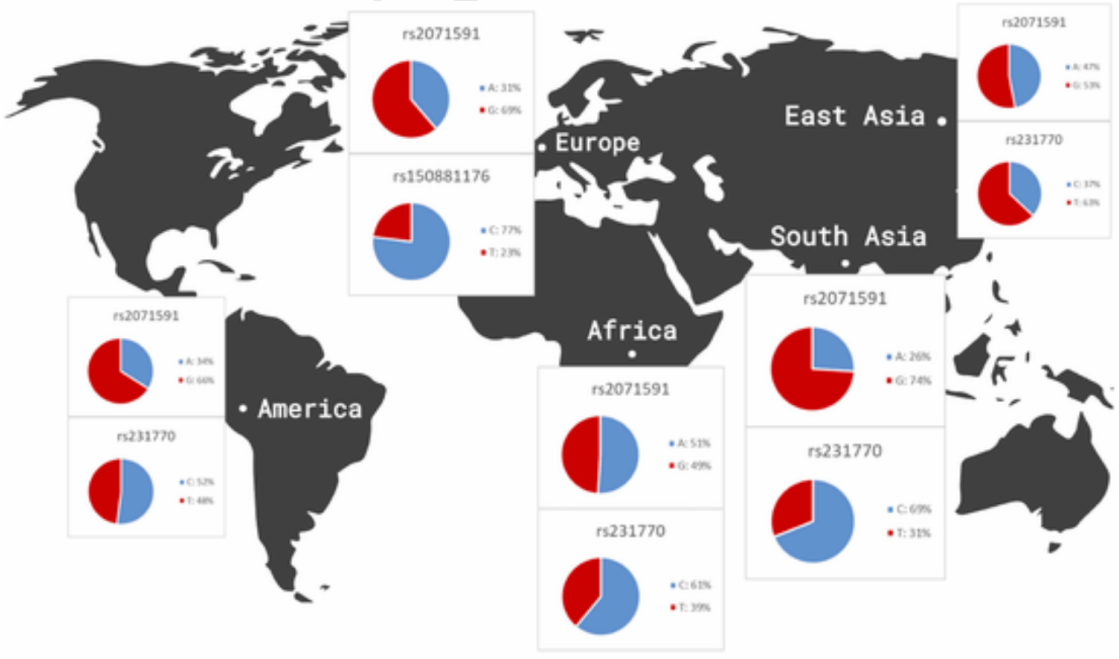

Fig. 4. Summary of allele frequency analysis of *LTA* and *CTLA4* gene expression in Africa, America, East Asia, Europe, and Southeast Asia.

rope with alleles rs2071591 (47%), rs231770 (63%), East Asia with alleles rs2071591 (47%), rs231770 (63%), in comparison to America with alleles rs2071591 (34%), rs231770 (48%), Africa with alleles rs2071591 (51%), rs231770 (39%), and Southeast Asia with alleles rs2071591 (26%), rs231770 (31%). We recommend that the discovered candidate gene variations be validated clinically, which could serve as diagnostic or prognostic biomarkers for MG.

Declaration of Competing Interest

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this study

Acknowledgement

We would like to thank all the lecturers in the Master Program at the Faculty of Pharmacy Universitas Ahmad Dahlan for their dedication to introducing Genomics and Bioinformatics for the author through Bioinformatics and Individualized medicine courses.

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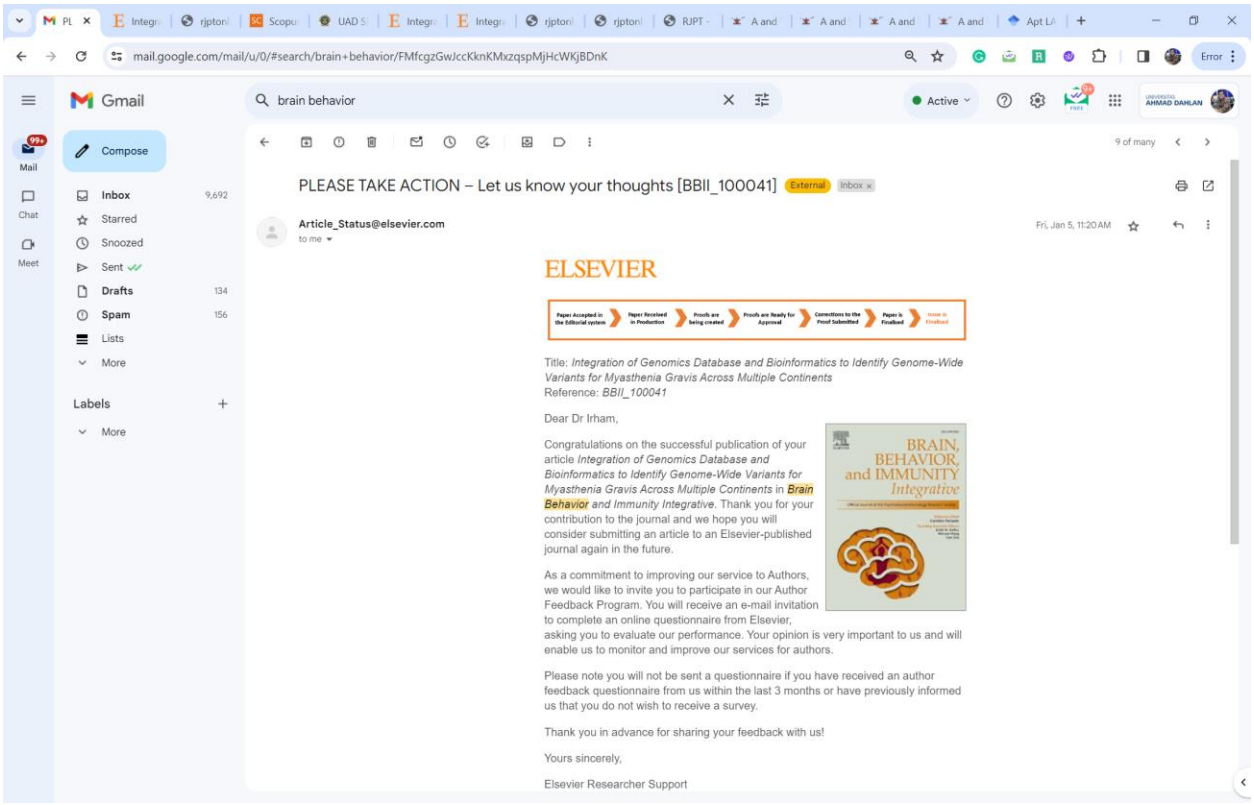
Acknowledgment setelah melakukan proses galley proof



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Integration of genomics database and bioinformatics to identify genome-wide variants for myasthenia gravis across multiple continents

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ARTICLE INFO

Keywords:
Myasthenia gravis
Autoimmune
Bioinformatics
Genetic variation

ABSTRACT

Autoimmune disease is an immune response that damages the body's tissues, thereby disrupting the body's physiological functions. Myasthenia gravis represents one such condition characterized by muscle weakness due to impaired neuromuscular transmission. While it can affect anyone, it tends to be more prevalent among women aged 20–30 and men over 50. This disease, deemed a genetic disorder, typically emerges in old age when antibodies target receptors in the muscles. In this study, we sought to identify the genes that can affect myasthenia gravis by leveraging several databases, including the GWAS Catalog, HaploReg, GTEx portal, and Ensembl. Specifically, our focus was on exploring genomic variants and the expression of the *LTA* and *CTLA4* genes. Our findings reveal that two variants (rs2071591 and rs231770) impact *LTA* expression in both muscle and brain tissue, while affecting *CTLA4* expression in testicular cell tissue. Subsequently, we assessed the allele frequency of these variants across regional populations, namely African, American, East Asian, European, and Southeast Asian. This study demonstrates that the *LTA* and *CTLA4* genes have a higher frequency in African, East Asian, and European populations compared to American and Southeast Asian populations. Consequently, our finding suggests that the latter two populations might have relatively higher susceptibility to the autoimmune disease myasthenia gravis. Therefore, variations in these genes not only offer insights into disease susceptibility, diagnostic or prognostic biomarkers, but also open up avenues for identifying candidate drug targets through genomic-driven drug repurposing.

1. Introduction

Myasthenia gravis (MG) is a neuromuscular disease characterized by voluntary muscle weakness (Sanders et al., 2017; Gilhus and Verschuuren, 2015). This disease has different symptoms that vary in other patients depending on the degree of involvement of the striated muscles. The most common symptoms in patients with myasthenia gravis are ocular symptoms, which present as ptosis and diplopia. These symptoms usually occur later in the day, and following activities such as watching TV or driving are more common. Excessive fatigue has been reported due to frequent exertion in patients with this disease. MG is an autoimmune disease that connects nerves to muscles (Murai, 2014), produced by different antibodies against synaptic membrane proteins (Benatar et al., 2016). It usually accounts for more than 85% of cases and is caused by a type of antibody to the skeletal muscle acetylcholine receptor (AChR-Ab) (Berrih-Aknin et al., 2014). However, components other than AChR, such as muscle-specific receptor tyrosine kinase or lipoprotein-associated protein 4 (LRP4), can also be targeted for autoimmune attacks (Mehling et al., 2011).

Based on the mechanism of autoimmune disease and antibodies, molecular skeletal muscle invasiveness, thymus status, genetic characteristics, disease phenotype, and response to treatment, MG is divided into early and late ocular subtypes, seronegative, thymoma, LRP4. Diagnosis of the MG subtype influences treatment decisions and disease

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https://doi.org/10.1016/j.bbii.2023.100041
Received 25 August 2023; Received in revised form 28 November 2023; Accepted 23 December 2023
Available online 24 December 2023

prognosis (Kerty et al., 2014). Approximately 50% of patients with ocular MG develop generalized myasthenia gravis (GMG) over a 2-year, which affects other muscles and is manifested by visual weakness and symptoms (Khan and Wang, 2020).

According to a systematic population-based study, (Carr et al., 2010) estimated the incidence and prevalence of MG to be 54 per million and 77.7 per million, respectively. However, significant changes have been reported in various studies. The incidence of this disease has been shown to range between 1.77 and 21.3 per million people and a prevalence of 15 to 179 million people (Carr et al., 2010). Many epidemiological studies, especially in Western Europe and Asia, report significant differences in the incidence and prevalence of MG. The incidence of MG ranges from 1.7 to 30 per million per year (Breiner et al., 2016). This disease has two age peaks: 40–40 years, mainly affecting women, and the other: 80–60 years, which occurs equally in men and women (Benatar et al., 2016).

In summary, MG is a neuromuscular disorder characterized by muscle weakness, with its manifestations varying among patients. Ocular symptoms, notably ptosis and diplopia, are common, often exacerbated by activities like watching TV or driving. MG results from autoimmune processes triggered by antibodies against synaptic membrane proteins, predominantly the acetylcholine receptor (AChR-Ab). The disease is categorized into subtypes, impacting treatment choices and prognosis. Epidemiologically, MG's incidence and prevalence vary across regions and age groups. Genetic variations play a significant role, with GWAS cataloging relevant variants. This study aims to investigate the variants associated with MG through an approach based on bioinformatics, offering insights into disease susceptibility and progression. In addition, gene expression profile patterns and allele frequencies of genetic variant populations were assessed using various databases. The results will enable future studies to determine whether these variants may be associated with multiple risks of MG infection, as well as MG progression and disease susceptibility.

2. Method

This study employed a bioinformatics-based approach to prioritize pathogenic variants potentially linked to MG. A detailed outline of the study design is visually represented in Fig. 1. To collect data on MG-associated variants, we leveraged the GWAS Catalog from the National Human Genome Research Institute (NHGRI) Database (http://www.ebi.ac.uk/gwas), accessed on May 22, 2023. Employing the keyword "Myasthenia Gravis" (MG), we extracted information on variants associated with MG. We found a total 36 single nucleotide polymorphisms (SNPs) number of MG-associated variants. Next, subsequent analysis of MG-related SNPs was conducted using HaploReg (version 4.2) HaploReg is a tool designed to analyze non-coding genome annotations from published GWAS or new variants. It aids in understanding the functional outcomes of GWAS results, predicting potential causal variants, identifying involved cell types, and predicting candidate target genes (Ward and Kellis, 2016, 2012). In this study, the GWAS catalog inclusion criteria for SNPs were those with a p-value $< 10^{-8}$ (Lee et al., 2007). The investigation further entailed assessing associations between various genetic variants and gene expression profiles utilizing expression quantitative trait loci (eQTL) available on the GTEx Portal database (http://www.gtexportal.org/home/) (Blauwendraat, 2022) accessed on May 22, 2023. Then, we confirmed the variant using the Ensembl Genome Browser (https://www.ensembl.org/index.html) (Ozaki et al., 2004) accessed on May 22, 2023. In addition, allele frequencies of the MG-associated variants were evaluated across diverse populations, encompassing African, American, East Asian, European, and Southeast Asian people.

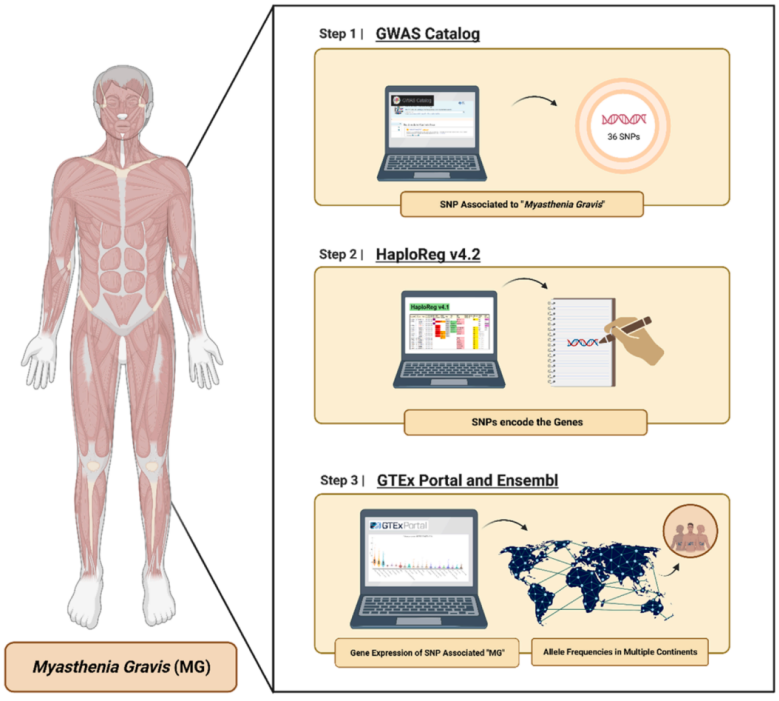

Fig. 1. Bioinformatics workflow for the identification of genetic variations associated with Myasthenia Gravis (MG).

3. Results

This study utilized the GWAS database to identify SNPs associated with MG. From the GWAS database, 36 SNPs associated with MG were identified with 26 unique SNPs associated with MG further identified after removing all SNP duplications (Table 1). Based on the number of SNPs obtained, the SNPs were forwarded using HaploReg version 4.2, with a *p*-value $< 10^{-8}$.

Based on the data presented in Table 2, this study focused on two genomic variants of the same gene that qualified as biological risk SNPs in this MG study. Using an integrative bioinformatics approach, two variants rs2071591 and rs231770 respectively encoding the *LTA* and *CTLA4* genes were prioritized as MG biological risk SNPs. Although these two variants does not show dbSNP functional annotation with missense mutation, however, by using HaploReg version 4.2 we identified the variant of rs2071591 near with rs1041981 variants which has missense mutation based on the criterion $r^2 > 0.8$. While for the variant with rs231770 near with the variant rs231775 which has missense mutation. MG disease is characterized by muscle and tissue weakness that occurs when the immune system is impaired and produces antibodies that attack the tissues in the body (Benatar et al., 2016). It was also reported that Lymphotoxin α (*LTA*) is a cytokine secreted by lymphocytes and is a member of the Tumor Necrosis Factor (*TNF*) family. *LTA* gene variations can contribute to threshold brain excitability, the spread of neural hyperexcitability (Aurora and Welch, 2000).

In Renton's et al. (2015), the gene for cytotoxic T lymphocyte-associated protein (*CTLA4*) was previously suggested as a cause of MG susceptibility. The *CTLA4* gene also multiplies when symptoms are present regardless of age, indicating that it is responsible for aberrant autoimmune responses that lead to neuromuscular junction dysfunction. *CTLA4* 45-kD immunoglobulin is expressed by activated T cells and has a significant sequence identity with CD28 (Renton et al., 2015).

3.1. LTA gene expression in muscle and brain tissues

In atherosclerotic plaques, intimal cells, some spindle-shaped or have globular, vacuolated cytoplasm, show immunoreactivity for *LTA* and galectin-2. Binds to adjacent portions of anti-tender muscle (SMC) cells. Galectin-2 and *LTA* are expressed in human smooth muscle cells and macrophages affected by atherosclerotic lesions (Vergoossen et al., 2021).

In Feroni's (2022), states that *LTA* genes encode cytokines that can modulate many inflammatory, immunological, and antiviral responses. It has been postulated that the inflammatory process modulated by *LTA* may contribute to the propagation of neural hyperexcitability by acting as an initiation and maintenance factor during migraine attacks (Ma'ruf et al., 2023). These results agree with a study on 439 Korean migraine patients genotyped for several *LTA* gene polymorphisms (Lee et al., 2007). Migraines occur because the blood vessels in the brain experience dilation or expansion, the main form of headache, characterized by debilitating headache attacks and symptoms of autonomic nervous system dysfunction (Olesen, 2018) (Fig. 2).

Table 1
GWAS catalog results are obtained from 26 SNPs with significance (p-value $<10^{-8}$).

SNP	*p*-value
rs3093958	4×10^{-42}
rs9271375	2×10^{-19}
rs4369774	6×10^{-19}
rs111945767	3×10^{-17}
rs76815088	6×10^{-16}
rs4409785	2×10^{-07}
rs4574025	7×10^{-14}
rs35274388	1×10^{-12}
rs2476601	2×10^{-12}
rs2071591	4×10^{-12}
rs150881176	1×10^{-11}
rs231770	9×10^{-11}
rs4574025	4×10^{-07}
rs9963862	4×10^{-07}
rs12653117	5×10^{-07}
rs6914704	2×10^{-06}
rs4128527	4×10^{-06}
rs4518467	4×10^{-06}
rs2476601	7×10^{-06}
rs9266277	7×10^{-10}
rs6998967	9×10^{-10}
rs35274388	1×10^{-09}
rs4263037	1×10^{-08}
rs73007767	4×10^{-08}
rs2245569	6×10^{-08}
rs9270986	6×10^{-08}

Table 2
The variant risk allele which codes two genes

SNP	GENCODE	*p*-value
rs2071591	*LTA*	4×10^{-12}
rs231770	*CTLA4*	9×10^{-11}

3.2. Expression of the CTLA4 gene in the testis

Our new analysis does not show the *CTLA4* location in the previous GWAS (rs231770). After tracing using HaploReg version 4.2, it was found that the SNP rs231770 near with the variant rs231775 which is missense mutation according to the criterion $r^2 >= 0.8$, the SNP may show variations in alleles that impact the risk of MG in various populations. Although these loci still make biological sense, more extensive studies are needed to prove that they are related to each other (Blauwendraat, 2022).

Another study in Vergoossen 2021 said that almost all MG-related genes were found in the testes and ectocervix. This study did not say a specific *CTLA4* gene existed in testicular tissue. Still, the genes in question were *RAPSN* and *CHRNA1* expression mostly limited to skeletal muscle, with some additional words in the tibial nerve and the testicular and pituitary glands, respectively (Yasumizu et al., 2022). Overall, the expression of MG-related genes is prominent in skeletal muscle and brain, but individual genes are also expressed in other tissues of the human body (Vergoossen et al., 2021) (Fig. 3).

3.3. Relationship between LTA and CTLA4 genes with eQTL from the GTEx portal database

The GTEx Portal database aims to evaluate MG gene expression in various human tissues, including muscle tissue. Using the GWAS catalog database we found 26 SNPs with the highest *p*-values from this analysis. Based on the *P*-value criterion, two statistically significant SNPs were found and prioritized. Based on an extended SNP count analysis using HaploReg version 4.2, we prioritized the two SNPs at risk for MG because the functional annotations of the SNPs were $<10^{-8}$.

According to (Yasumizu et al., 2022), the increase in *CHRNA1* expression tend to be lower when compared with other neuromuscular antigens such as *GABRA5* and *RYR3*. Although a moderate rise in *CHRNA1* expression seems sufficient to cause severe symptoms in MG, it is related to the availability of acetylcholine receptor antibodies and their crucial biological role. These findings provide valuable clues for understanding the pathogenesis of various autoimmune neurological diseases. The following Table 3 shows the results of genetic variation from MG.

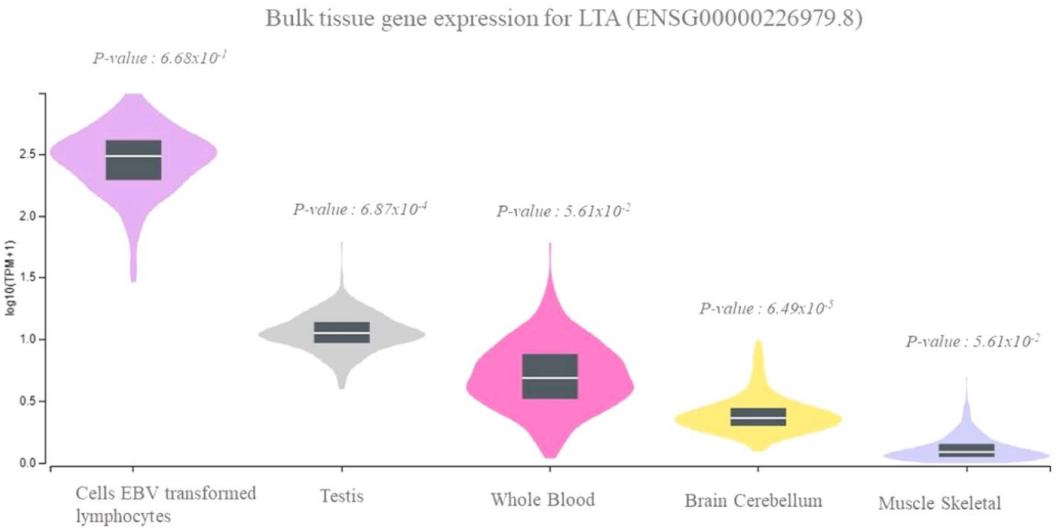

Fig. 2. *LTA* gene expression associated with myasthenia gravis across several human tissues from the GTEx Portal.

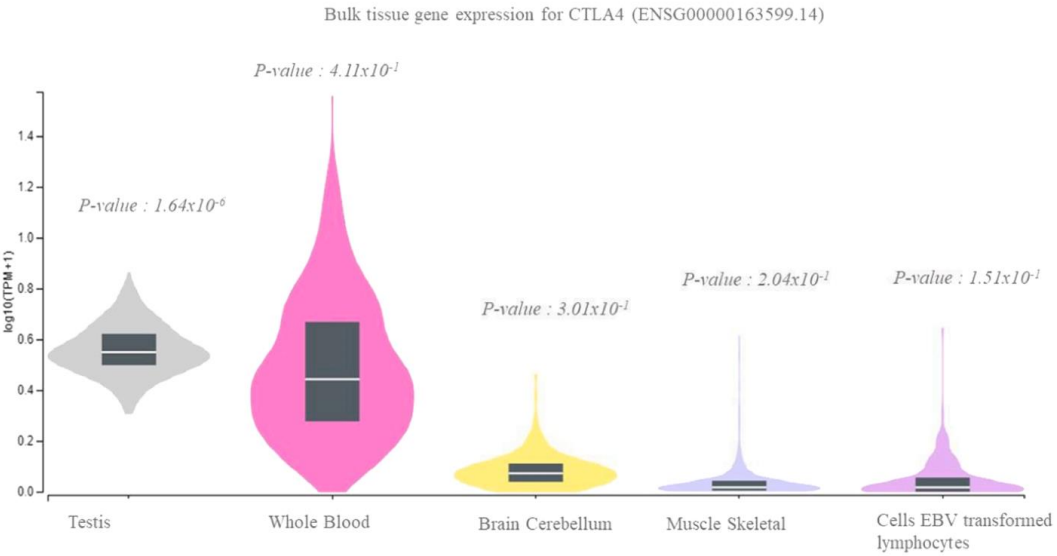

Fig. 3. CTLA4 gene expression associated with myasthenia gravis across several human tissues from the GTEx Portal.

Table 3
e-QTL's result for the Myasthenia gravis from GTEx portal database.

SNP	GENCODE ID (ENSG00000-)	Gene symbol	*p*-value	NES	Tissue	Actions
rs2071591	226979.8	*LTA*	0.000065	0.29	Brain - Cerebellum	GG>GA>AA
	226979.8	*LTA*	0.00024	0.14	Muscle - Skeletal	GG>GA>AA
rs231770	163599.14	*CTLA4*	0.0000016	-0.28	Testis	CC>CT>TT

3.4. Myasthenia gravis (MG) candidate variant allele frequencies across continents

After identifying candidate variants related to *LTA* and *CTLA4* gene expression, allele frequencies have been determined across populations of all continents, as shown in (Table 4). Allele frequencies for the two variants were evaluated in multiple continents, including populations of 1195 individuals (Africa), 567 individuals (America), 1112 individuals

Table 4
Allele frequencies for SNPs examined in this study.

SNP	Position (hg38)	Gene	*p*-value	Allele		Allele Frequency (N)				
				Ref	Eff*	AFR	AMR	EAS	EUR	SAS
rs2071591	Chr6 31548022	*LTA*	4×10^{-12}	G	A	0.512 (677)	0.336 (233)	0.469 (473)	0.310 (312)	0.261 (255)
rs231770	Chr2 203864430	*CTLA 4*	9×10^{-11}	C	T	0.392 (518)	0.481 (334)	0.634 (639)	0.379 (381)	0.306 (299)

(East Asia), 693 individuals (Europe), and 554 individuals (South East Asia). Using the Ensemble Genome Browser, we obtained allele frequencies in Africa, America, East Asia, Europe, and Southeast Asia (http://www.ensembl.org). Allele frequencies across populations differ for each *LTA* and *CTLA4* gene variant. Table 4 and Fig. 4. shows the gene expression level at a higher frequency of the related allele (T) rs231770 than the corresponding allele (A) rs2071591. In the associated alleles (T) rs231770 and (A) rs2071591, the African and East Asian populations are much higher than the American, Southeast Asian, and European Asian people.

The higher frequency of the related allele is (T) rs231770 compared to the other related allele, namely (A) rs2071591. In the associated alleles (T) rs231770 and (A) rs2071591, the African and East Asian populations are much higher than the American, Southeast Asian, and European Asian people. The rs2071591 allele indicated a differential variant prevalence contribution for *LTA* gene expression, while the rs231770 allele indicated a differential variant prevalence contribution for *CTLA4* gene expression.

Allele frequencies in all populations differ for each SNP, as shown in Fig. 4. It is generally known that the A and T allele frequencies for rs2071591 and rs231770 also appear to have a higher frequency in Europe with alleles rs2071591 (47%), rs231770 (63%), East Asia with alleles rs2071591 (47%), rs231770 (63%), compared to America with alleles rs2071591 (34%), rs231770 (48%), Africa with alleles rs2071591 (51%), rs231770 (39%), Asia southeast with alleles rs2071591 (26%), rs231770 (31%).

Identification of gene variations that are unique and pathogenic for a disease is very interesting for clinical research and validation. Identification of these variants not only provide clues to disease susceptibility or as a diagnostic and prognostic biomarker (Irham et al., 2022) but also can be used to discover candidate drug targets or what is known as drug repurposing (genomic-driven drug repurposing) (Irham et al., 2020; Afief et al., 2022). The authors hope that the discovery of the candidate gene variations can be validated in clinical settings and can become a diagnostic and prognostic biomarker for MG disease.

In conclusion, a bioinformatics-based approach revealed pathogenic variants potentially associated with MG. We propose that this variant may be used for further studies to identify MG and prognosis diagnostic biomarkers. However, we acknowledge that there are limitations to the bioinformatics-based approaches used to investigate genetic variants associated with MG. Notably, not all variants necessarily correspond to the identifiable gene (i.e., non-coding variants). Even when genes or genetic variants are present, they might not be suitable drug targets. Therefore, we recommend further clinical validation to corroborate our findings and gain deeper insight into the etiology and functional implications of MG disease.

4. Conclusion

In this study, we utilized a state-of-the-art bioinformatics approach

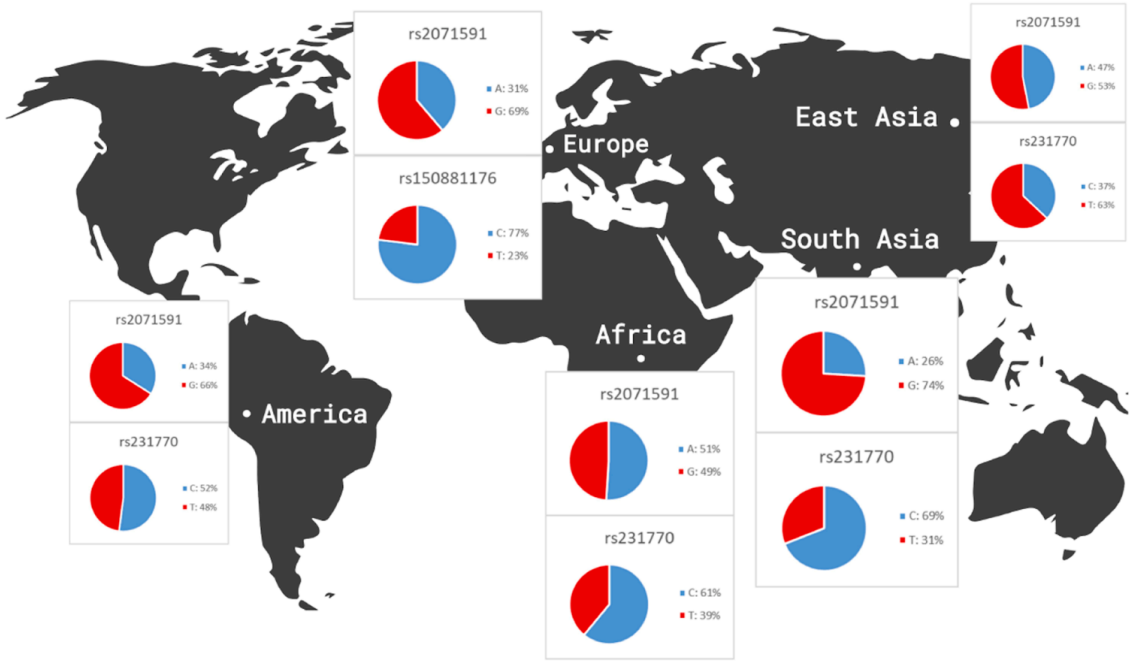

Fig. 4. Summary of allele frequency analysis of *LTA* and *CTLA4* gene expression in Africa, America, East Asia, Europe, and Southeast Asia.

to analyze genomic databases revealing distinct gene expressions of the *LTA* and *CTLA4* genes across muscle, brain, and testicular cell tissues for MG disease. Prominent gene variants include rs2071591, expressed in muscle and brain tissue, and rs231770, prevalent in testicular cell tissue. Notably, these variants exhibit an overall higher frequency in Europe with alleles rs2071591 (47%), rs231770 (63%), East Asia with alleles rs2071591 (47%), rs231770 (63%), in comparison to America with alleles rs2071591 (34%), rs231770 (48%), Africa with alleles rs2071591 (51%), rs231770 (39%), and Southeast Asia with alleles rs2071591 (26%), rs231770 (31%). We recommend that the discovered candidate gene variations be validated clinically, which could serve as diagnostic or prognostic biomarkers for MG.

Declaration of Competing Interest

The authors disclose no conflict of interest.

Acknowledgements

We would like to thank all the lecturers in the Master Program at the Faculty of Pharmacy Universitas Ahmad Dahlan for their dedication to introducing Genomics and Bioinformatics for the author through Bioinformatics and Individualized Medicine Courses.

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Brain Behavior and Immunity Integrative 5 (2024) 100041

Contents lists available at ScienceDirect

Brain Behavior and Immunity Integrative

journal homepage: www.editorialmanager.com/bbii

Integration of genomics database and bioinformatics to identify genome-wide variants for myasthenia gravis across multiple continents

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ARTICLE INFO

Keywords:
Myasthenia gravis
Autoimmune
Bioinformatics
Genetic variation

ABSTRACT

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1. Introduction

Myasthenia gravis (MG) is a neuromuscular disease characterized by voluntary muscle weakness (Sanders et al., 2017; Gilhus and Verschuuren, 2015). This disease has different symptoms that vary in other patients depending on the degree of involvement of the striated muscles. The most common symptoms in patients with myasthenia gravis are ocular symptoms, which present as ptosis and diplopia. These symptoms usually occur later in the day, and following activities such as watching TV or driving are more common. Excessive fatigue has been reported due to frequent exertion in patients with this disease. MG is an autoimmune disease that connects nerves to muscles (Murai, 2014), produced by different antibodies against synaptic membrane proteins (Benatar et al., 2016). It usually accounts for more than 85% of cases and is caused by a type of antibody to the skeletal muscle acetylcholine receptor (AChR-Ab) (Berrih-Aknin et al., 2014). However, components other than AChR, such as muscle-specific receptor tyrosine kinase or lipoprotein-associated protein 4 (LRP4), can also be targeted for autoimmune attacks (Mehling et al., 2011).

Based on the mechanism of autoimmune disease and antibodies, molecular skeletal muscle invasiveness, thymus status, genetic characteristics, disease phenotype, and response to treatment, MG is divided into early and late ocular subtypes, seronegative, thymoma, LRP4. Diagnosis of the MG subtype influences treatment decisions and disease

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https://doi.org/10.1016/j.bbii.2023.100041
Received 25 August 2023; Received in revised form 28 November 2023; Accepted 23 December 2023
Available online 24 December 2023