Construction of an miRNA-regulated drug-pathway network reveals drug repurposing candidates for myasthenia gravis

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Abstract. Myasthenia gravis (MG) is a rare debilitating autoimmune neuromuscular disorder. Many studies have focused on the mechanism and treatment strategies of MG. However, the exact pathogenesis of MG and effective treatment strategies remain unclear. Recent studies have indicated that microRNAs (miRNAs or miRs) can regulate the pathological pathways of MG, suggesting their potential role in novel treatments. In the present study, we created a comprehensive catalog of experimentally confirmed MG risk genes and miRNAs by manually mining published literature and public databases. Based on these genes and miRNAs, we identified 41 MG risk pathways and 105 approved drugs that can affect these pathways. Some important MG-related pathways, such as hsa04060 (cytokine-cytokine receptor interaction) and hsa05200 (pathway in cancer), were found to be regulated by MG risk miRNAs and drugs. Furthermore, we constructed an miRNA-regulated drug-pathway network and identified miRNAs and drugs that synergistically regulate key MG pathways and biological processes. We developed a drug repurposing strategy to identify 25 drug repurposing candidates for MG; several of these drugs, such as rituximab, adalimumab, sunitinib, and muromonab, have the potential to be novel MG treatment drugs. This study provides novel insight into the pathogenesis of MG and potential drug candidates for MG were identified.

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Introduction

Myasthenia gravis (MG) is a rare antibody-mediated autoimmune disease characterized by muscle fatigue manifested as ptosis, diplopia, dysarthria, dysphagia and limb weakness (1,2). Researchers have elucidated important mechanisms underlying MG (3). Current treatment strategies for MG mainly include cholinesterase inhibitors, immune-suppressants and thymectomy (4). However, various common side effects are major obstacles to the effective use of these drugs.

In recent years, many studies have revealed that microRNAs (miRNAs or miRs) are important regulators of MG pathogenesis. For example, miR-320a targets mitogen-activated protein kinase 1 (MAPK1) and regulates COX-2 expression through the ERK/nuclear factor-κB (NF-κB) pathway (5). Inhibition of miR-146a was found to reduce cell surface costimulatory molecules, such as CD40, CD80, CD86 and intracellular TLR4 and NF-κB levels in AChR-specific B cells (6). miR-181c was found to negatively regulate the production of pro-inflammatory cytokines interleukin-7 (IL-7) and IL-17 (7). Recently, as new potential therapeutic targets, miRNAs have attracted increased interest (8). For example, as miR-155 is involved in inflammation and immune diseases (9), therapies targeting miR-155 may be applicable to various autoimmune and inflammatory disorders. These findings indicate that miRNAs have potential influence on the pathways underlying MG pathogenesis and can thus affect the treatment of MG. However, most of these studies have focused on only one or a few miRNAs in cell lines or using limited samples.

The research and development of new drugs for MG is a time-consuming and labor-exhausting process. In recent years, drug repurposing has been a vital part of the drug discovery process that can uncover new indications for existing drugs (10). For example, Hu and Agarwal constructed a large scale disease-drug network for effective drug repositioning (11). One of our previous studies constructed a small molecule and miRNA association network for Alzheimer's disease (AD) (12). Ye *et al* proposed a disease-oriented strategy for evaluating the relationship between drugs and diseases based on their pathway profile (13). These studies are critical for drug discovery and development in future research. However, few studies have focused on drug repurposing for MG (14,15).

In the present study, we identified the risk pathways regulated by MG risk genes, miRNAs and drugs and constructed an miRNA-regulated drug-pathway network (MDPN). We analyzed the properties of the MDPN and determined how the miRNAs and drugs synergistically regulate MG risk pathways. Moreover, we identified drug repurposing candidates for MG and clarified their potential mechanisms as novel treatment drugs. Our results may provide a novel viewpoint with respect to the mechanism and treatment of MG.

Materials and methods

Human MG risk gene data. We manually read literature published before June 1, 2015 by searching the PubMed database using the terms [myasthenia gravis (MeSH Terms) and English (Language)]. The species of the risk genes was limited to '*Homo sapiens*'. We thoroughly read the 9,474 items returned by our searches and selected MG risk genes that met the following standards oulined in a previous study (16): i) the gene was presented in at least 5 MG samples (including blood samples and thymic tissue samples); ii) the gene was detected using reliable experimental methods, such as microarrays and RT-PCR; and iii) the gene was significantly differentially expressed (mRNA level or protein level). In addition, we also collected MG risk genes from current public databases, including the Genetic Association Database (GAD) (17), DisGeNET (18), Online Mendelian Inheritance in Man (OMIM) (19) and Functional Disease Ontology Annotation (FunDO) (20).

MG risk miRNAs and miRNA targets. Similar to the collection of MG risk genes, we also manually collected MG risk miRNAs from different sources, including the Human microRNA Disease Database (HMDD v2.0) (21), PhenomiR (22), and a literature search using the keywords 'miRNA' and 'myasthenia gravis' or 'microRNA' and 'myasthenia gravis' or 'miR' and 'myasthenia gravis' in PubMed. Furthermore, we downloaded the target genes of these MG risk miRNAs from five experimentally validated miRNA target databases, including miRWalk (23), miRTarBase (24), miR2Disease (25), miRecord (26) and miRSel (27).

Human drug target data. Drugs and their target genes were downloaded from DrugBank (version 4.0) (28), with the species of the drug targets restricted to '*Homo sapiens*'. Drugs with less than 5 target genes were excluded from this study.

Pathway analysis. The physiological pathways implicated in MG were determined through the functional enrichment of significant gene lists using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (29). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of the MG risk gene lists was calculated using a P-value cutoff of 0.05 (Fisher's exact test). In addition, the miRNA-related pathways and drug-related pathways were obtained using their target gene sets. Then, we constructed an MDPN consisting of drugs, miRNAs and their common MG risk pathways. Cytoscape 2.8.3 was utilized to visualize the network, and the network properties were analyzed using the Network Analysis plugin (30). Gene ontology (GO) biological process enrichment was performed using GO level 3 (FDR <0.05).

Association scores (AS) and significance levels between drugs and MG. Our drug repurposing strategy was developed based on two biological hypotheses: first, disease risk genes do not exert their functions independently but rather through regulating special pathways. Second, drugs exert therapeutic effects by regulating pathways involved in disease pathology rather than by directly targeting disease-associated genes. Drug pathways and MG risk pathways were obtained from the MDPN network. We calculated AS between the drugs and MG as described by the following formula (13):

$$S_{drugi,MG} = -\lg \sum \sqrt{p_{MG,k} \times p_{drugi,k}} \qquad (1)$$

Here, $p_{MG,k}$ is the enriched P-value of MG on pathway 'k'; $p_{drugi,k}$ is the enriched P-value of drug 'i' on pathway 'k'; k represents the pathway that is most significantly affected by both MG risk genes and drug 'i' targets.

Furthermore, to evaluate the specificity of the association between the drugs and MG, we performed random permutations of the pathways and calculated the Z scores of the drugs and MG. While retaining the pathways of the drugs, random pathway profiles of the drugs were generated by randomly sorting the pathway 10,000 times. For each random profile, $S_{random,drugi}$ was calculated according to formula (1). In this study, the Z-score was used to evaluate the specificity of the association between the drug and disease:

$$Z_{drugi,MG} = \frac{S_{drugi,MG} - average\ (S_{random,drugi})}{std\ (S_{random,drugi})} \qquad (2)$$

where the average ($S_{random,drugi}$) is the average association score between random cases and drug i, and std ($S_{random,drugi}$) is the standard variation of association between random cases and drug i.

Results

A comprehensive catalog of MG risk genes, miRNAs and pathways. We created a comprehensive catalog of 162 risk genes that have been experimentally confirmed to be associated with MG. Among these genes, 123 risk genes were manually collected by literature mining, and 39 risk genes were compiled from public databases. Based on these risk genes, we identified 45 MG risk pathways (P<0.05) using KEGG enrichment analysis. The top 25 pathways and their P-values are shown in Fig. 1A. According to the pathway classification of the KEGG database, all pathways were divided into seven categories (Fig. 1B). We found that most of the MG risk pathways are related to 'Human Diseases: Immune diseases', 'Organismal Systems: Immune system' and 'Human Diseases: Cancers', which implies that we can use immune pathways as well as cancerous pathways to functionally characterize MG. As a paraneoplastic phenomenon, MG is mainly caused by thymoma but can also be associated with other cancers, such as renal cell carcinoma (31). More importantly, hsa04060 (cytokine-cytokine receptor interaction) was the first significantly enriched pathway; 39 MG risk genes participated in this pathway, which plays key roles in MG (Fig. 1C). We also performed a functional enrichment analysis of these risk genes,

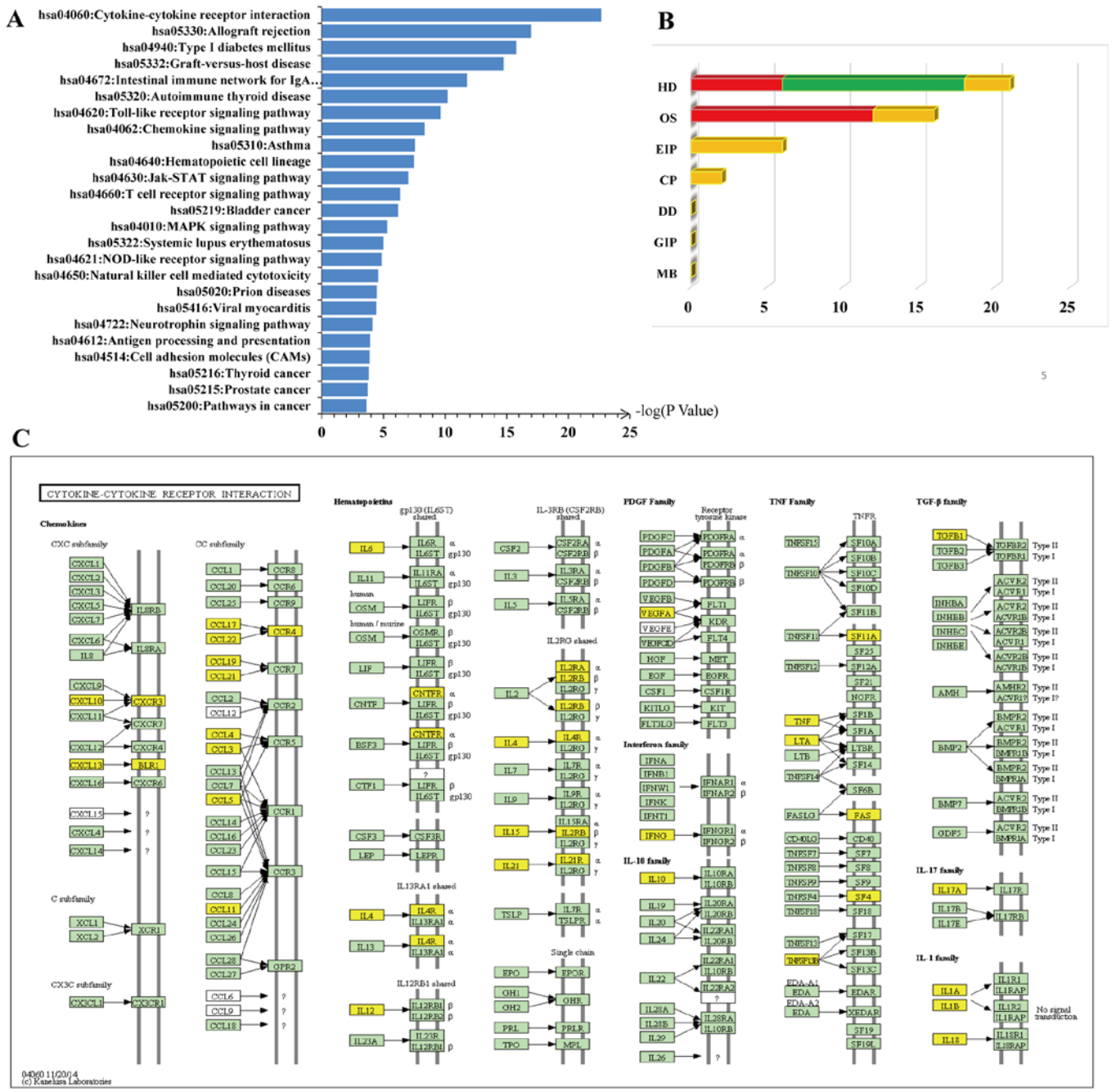

Figure 1. Myasthenia gravis (MG) risk pathways. (A) Top 25 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched by MG risk genes (P<0.05). (B) Classification of MG risk pathways. Red represents the pathways related to immune diseases and the immune system; green represents the pathways related to cancer diseases; yellow represents other diseases. (C) Dissection of one of the MG risk pathways (hsa04060: cytokine-cytokine receptor interaction). Proteins coded by MG risk genes are indicated with a yellow background.

and 256 GO terms were obtained (FDR value <0.05), including 236 biological processes (BP), 7 molecular functions (MF) and 13 cellular components (CC) (data not shown). MG risk genes are involved in significant BP, such as immune responses, positive regulation of immune system process, regulation of cell activation and proliferation, and inflammatory responses, which conforms with the immune pathogenesis of MG.

In addition, a catalog of 85 MG risk miRNAs was manually constructed. Among these miRNAs, 78 miRNAs and their 4,514 experimentally validated targets, as well as 12,575 miRNA-target interaction pairs, were collected. We identified miRNA-regulated risk pathways and constructed an miRNA-pathway regulating network for MG. In this network, miRNA-146a regulated the most MG risk pathways (data not shown), which suggested that miRNA-146a plays a significant role in the pathologic pathways of MG. Williams *et al* concluded that miRNA-146a is related to immune responses and inflammatory diseases through negatively regulating immune genes, such as IL-8 and RANTES (32). Zhang *et al* demonstrated that silencing miRNA-146a can improve clinical myasthenic symptoms in EAMG (33). Our results were consistent with previous studies.

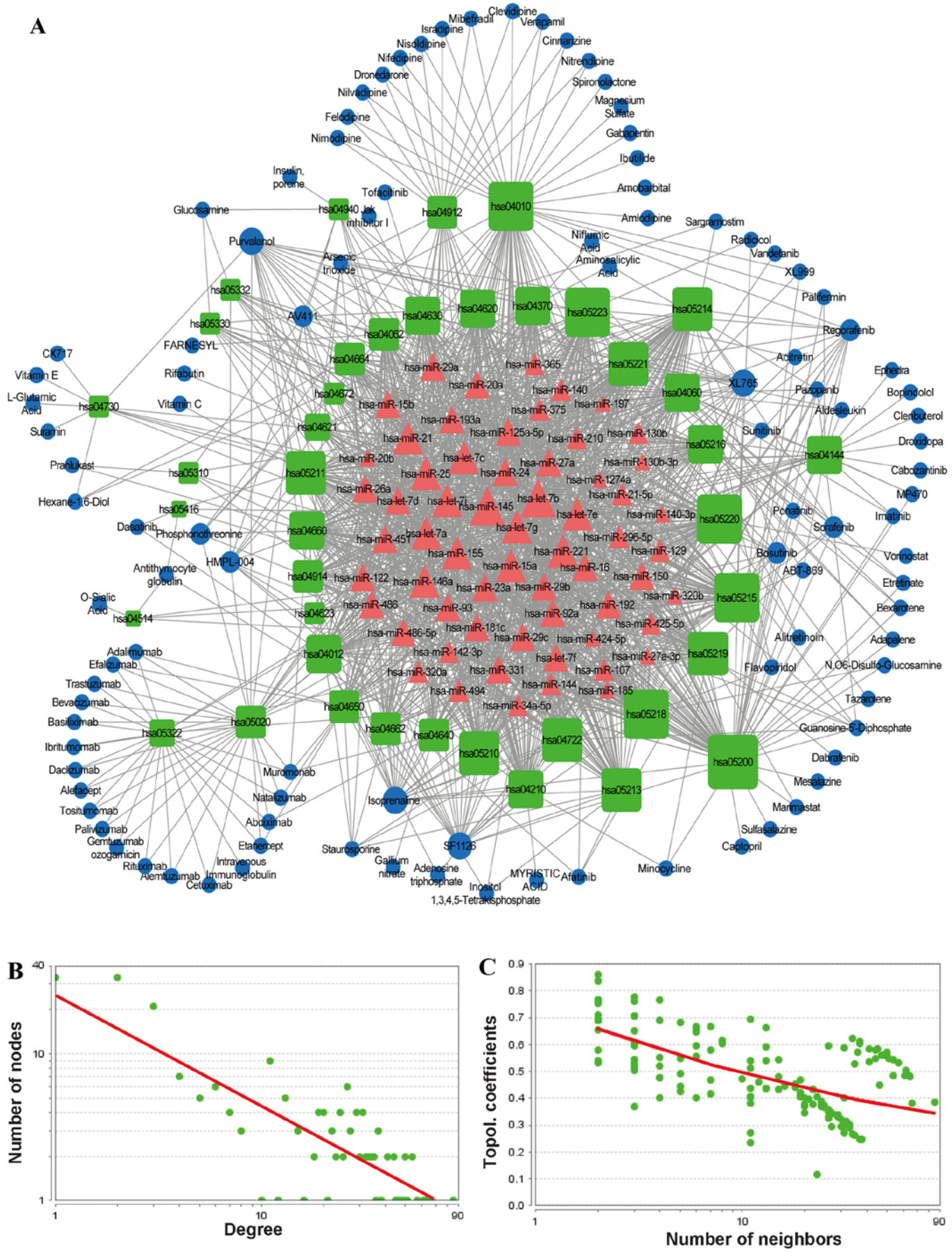

Figure 2. (A) miRNA-regulated drug-pathway network (MDPN) in myasthenia gravis (MG). Network organization of drug and pathway associations, as well as pathway and miRNA associations. Red triangles represent miRNAs, and green squares and blue ellipses indicate pathways and drugs, respectively. (B) Degree of distribution for all nodes in the MDPN. (C) Topological coefficients for all nodes in the MDPN.

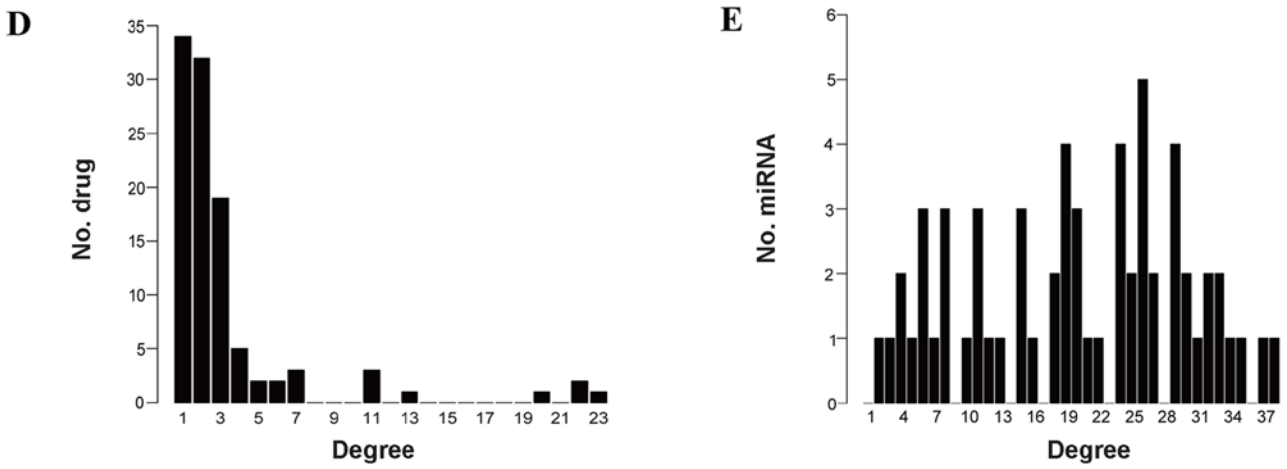

Figure 2. Continued. (D) Degree distribution of the drug nodes. (E) Degree distribution of the miRNA nodes.

Topological features of the MDPN. First, we obtained 891 drugs, 1,871 drug targets and 10,037 drug-target pairs from DrugBank. We then identified the statistically significant pathways of each drug based on its target genes. Second, we extracted drugs if their pathways overlapped with the pathways enriched by the MG risk genes and miRNA targets. Finally, we obtained 105 drugs that shared pathways with MG risk genes and miRNAs. Based on these drug-pathway and miRNA-pathway associations, we constructed an MDPN (Fig. 2A). The network contained 207 nodes and 1,575 interactions, which included 41 pathways, 61 MG risk miRNAs and 105 drugs.

We determined the topological characteristics of the network, including the degree distribution and topological coefficient. The degree distribution followed a power law distribution [$f_{(x)}=25.051x^{-0.757}$] using all of the nodes in the MDPN (Fig. 2B). The topological coefficient was computed to measure the extent to which a node shared links with the others in a network. As shown in Fig. 2C, with an increase in the node degree, the topological coefficient decreased. We also analyzed the degree distribution of drugs and miRNAs. The degree distribution of the drugs is displayed in Fig. 2D, which shows that the majority (92/105, 87.62%) of the drugs were connected by only a few pathways (no more than five). However, the degree distribution of the miRNAs was different, ranging from 2 to 37, which is displayed in Fig. 2E. There were few miRNAs associated with a small number of drugs, suggesting that these miRNAs may perturb multiple drug-related pathways and supporting the idea that miRNAs may be associated with drug sensitivity or resistance.

miRNAs and drugs synergistically regulate key MG pathways. Hsa05200 (pathway in cancer) was found to be regulated by the largest number of miRNAs and drugs after calculating the number of miRNAs and drugs for each pathway. There were 20 MG risk genes participating in the pathway (including HRAS, BRAF, IGF1, IL-6, BCL2, MMP2, MMP9, MAPK1, FAS, VEGF, HSP90B1, PTEN, AR, TGFB1, NRAS, MAX, IGF1R, KRAS, BAX and MYC) which indicated that this pathway is extremely important in MG pathogenesis and treatment. Therefore, we performed an in-depth dissection of this pathway and identified key genes co-regulated by MG risk miRNAs and drugs (Fig. 3). We found that many MG risk genes were targeted by drugs and miRNAs, indicating that miRNAs and drugs can synergistically regulate MG risk pathways by regulating these genes. There were five MG risk genes (RAS, RAF, MMPs, VEGF and IL-6) regulated by multiple drugs and miRNAs. For example, RAS was targeted by two miRNAs (let-7b and miR-494) and a drug [guanosine-5'-diphosphate (GDP)]. RAF was targeted by 11 miRNAs (let-7 family and miR-20a, miR-145, miR-221 and miR-192) and two drugs (regorafenib and sorafenib). Arimori and Song investigated the expression of RAS and RAF and found increased levels in the peripheral blood mononuclear cells of MG patients that were related to clinical exacerbations of MG (34). Moreover, RAS has been previously investigated as a popular drug target related to drug sensitivity or resistance (35).

Identification of novel drug repurposing candidates for MG. We performed a drug repurposing strategy and identified 25 drug repurposing candidates for MG with Z scores >1.96 (P<0.05) (Materials and methods). Clinical trials (www.ClinicalTrials.gov) and relevant scientific literature were investigated to assess the validity of the MG repurposing candidates. Among these 25 drugs, two predicted drugs (rituximab, intravenous immunoglobulin) were studied in MG clinical studies. In addition, there were three clinic trials that studied the treatment effects of rituximab on MG, especially refractory MG (ClinicalTrials.gov identifier: nos. NCT00619671, NCT02110706 and NCT00774462). There were five clinic trials connecting intravenous immunoglobulin (IVIG) and MG (ClinicalTrials.gov identifier: nos. NCT00306033, NCT00515450, NCT01179893, NCT00004682 and NCT00774462). We also identified the relationship between the candidate drugs and MG using PubMed. Among the 25 drugs, 19 drugs (76.0%) were directly related to the treatment of MG and/or other immune diseases. For example, adalimumab and etanercept have been investigated for treating MG. Rituximab is an anti-CD20 monoclonal antibody that can decrease plasma cells ($CD19^+$) and B cells ($CD20^+$) and thus suppress the production of antibodies (36). Díaz-Manera *et al* demonstrated that rituximab can produce long-lasting clinical benefits in patients with severe, drug-resistant MG (37). Studies have found that rituximab is beneficial for patients with MuSK antibody-positive MG (38) and can be a successful treatment

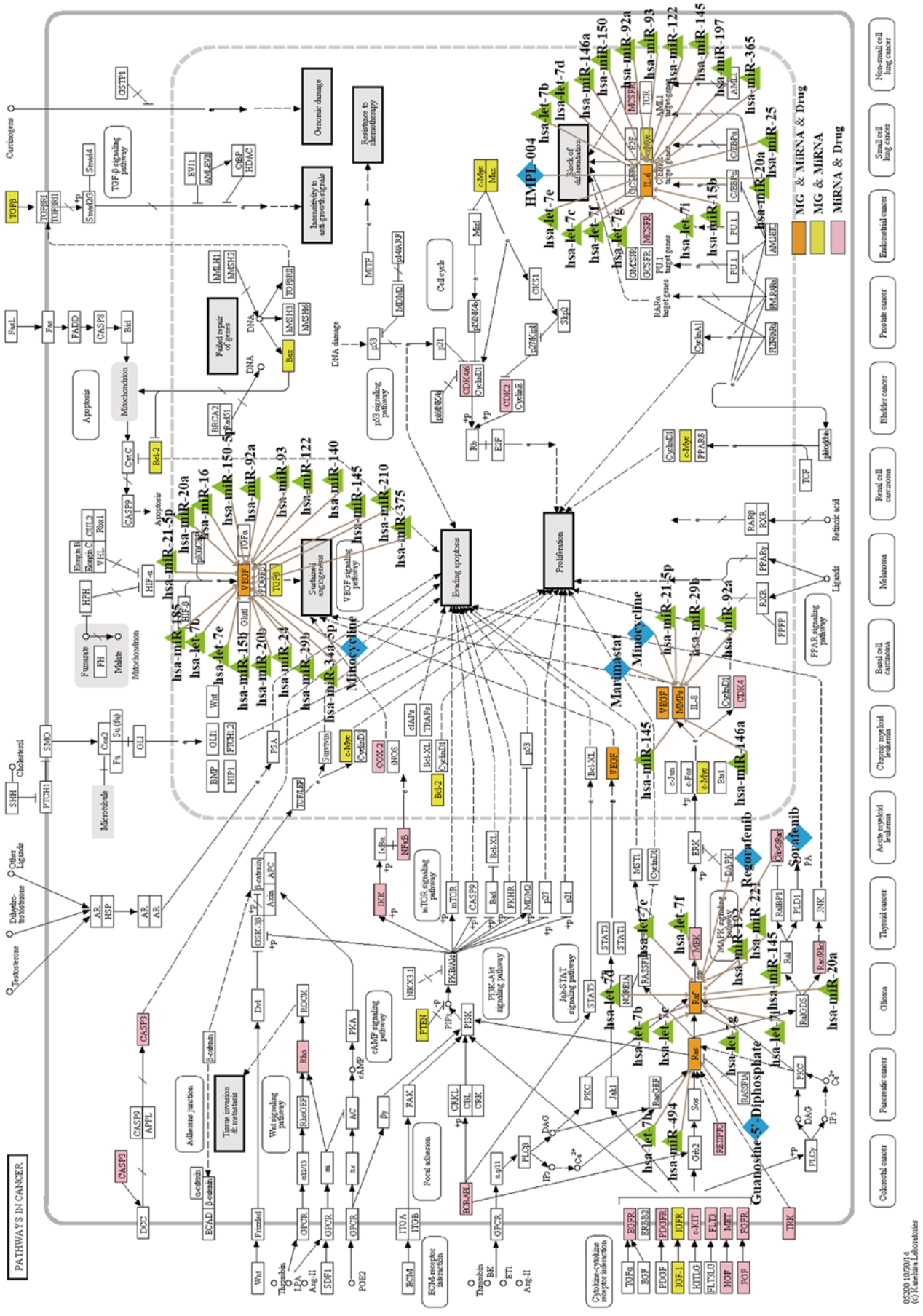

Figure 3. Identifying myasthenia gravis (MG) risk genes regulated by drugs and miRNAs in the hsa05200 pathway. Orange represents the genes co-regulated by MG, MG risk miRNAs and drugs. Yellow represents the genes regulated by MG and MG risk miRNAs. Pink represents the genes regulated by MG risk miRNAs and drugs. Blue diamonds represent the drugs, whereas green triangles represent the miRNAs.

Table I. Description of the 13 candidate drugs and their related references.

Drug candidates	Description	Refs.
Alemtuzumab	Used for the treatment of relapsing-remitting multiple sclerosis and rheumatoid arthritis.	(43-45)
Bevacizumab	Bevacizumab has been reported to induce remission of psoriasis and psoriatic arthritis. Bevacizumab has been reported to ameliorate vascular and T-cell responses during experimental autoimmune encephalomyelitis.	(46-48)
Cetuximab	Cetuximab has been reported to promote remission of rheumatoid arthritis.	(49)
Trastuzumab	Trastuzumab has been reported to be used in inflammatory breast cancer.	(50)
Efalizumab	Used for the treatment of plaque psoriasis and Crohn's Disease.	(51,52)
Insulin	Used for the treatment of type 1 diabetes, which is considered an immune disease.	(53)
Gemtuzumab ozogamicin	Used for the treatment of acute myeloid leukemia, which is a cancer of immune system.	(54)
Basiliximab	Basiliximab has been reported to promote prolonged remission of ulcerative colitis.	(55)
Palivizumab	Palivizumab has been reported to inhibit RSV-induced neurogenic-mediated inflammation.	(56)
Muromonab	Muromonab is used for the prevention of organ rejection.	(57)
Abciximab	Abciximab is used to alleviate vascular inflammation caused by both acute coronary syndromes and injury after percutaneous coronary intervention.	(58)
Tositumomab	Used for the treatment of non-Hodgkin's lymphoma, which is a cancer of immune system.	(59)
Ibritumomab	Used for the treatment of non-Hodgkin's lymphoma, which is a cancer of immune system.	(60)

for refractory generalized MG (39). Sunitinib has been reported to improve MG when it appears with thymomas (40). Another 13 candidate drugs were reported to treat other immune and inflammatory diseases, such as rheumatoid arthritis, psoriatic arthritis, psoriasis and multiple sclerosis, supporting their potential use in MG (Table I).

Mechanism dissection of novel drug candidates and miRNAs in MG. To illustrate the complex relationship and underlying mechanisms among drugs, drug targets, MG risk genes, MG risk miRNAs and pathways, we constructed a layered network that can provide a convenient way for investigating detailed information regarding these associations. The links between every two layers were as follows: i) drug action on drug targets; ii) the influence of drug targets on MG risk pathways; iii) the effect of pathways on MG risk genes; iv) MG risk miRNA targets on MG risk genes; v) MG risk gene association with MG. We constructed the layered network between rituximab and MG to reveal the potential treatment effects of rituximab in MG (Fig. 4). Two pathways (hsa05322: systemic lupus erythematosus and hsa05020: prion diseases) were inferred, connecting rituximab and MG. These two pathways influenced MG risk genes, mainly IL-6, IL-1A, IL-1B, MAPK1, tumor necrosis factor (TNF), BAX and IFNG. The gene IL-6, which encodes a cytokine that functions in inflammation and the maturation of B cells, was targeted by MG-related miRNAs, such as let 7 family, miR-150, miR-145, miR-122, miR-421 and miR-25 (Fig. 4). The gene MAPK1 encodes a member of the MAP kinase family and is an integration point for multiple biochemical signals, which are involved in a variety of cellular processes, such as proliferation, differentiation and development. MAPK1 was targeted by miR-25, miR-129, miR-494, miR-15b, miR-24, miR-320a and miR-146a. The TNF gene encodes a multifunctional pro-inflammatory cytokine that belongs to the tumor necrosis factor superfamily and is targeted by the let 7 family, miR-16, miR-363, miR-25, miR-20a, miR-122, miR-140, miR-150 and miR-146a. This suggested that the function of genes can be influenced by abnormal expression of disease-related miRNAs. Similarly, we constructed a layered network among adalimumab (Fig. 5), sunitinib (Fig. 6), muromonab (Fig. 7) and MG. Sunitinib and ABT-869 targeted the same pathways, including hsa04060 (cytokine-cytokine receptor interaction), hsa04144 (endocytosis), hsa04640 (hematopoietic cell lineage) and hsa05200 (pathway in cancer), indicating that the two drugs can function through similar pathways, again providing evidence for drug combination.

Discussion

MG is a rare disease, and the current treatments for this disease do not meet the expectations of affected patients. Oral cholinesterase inhibitors, which can inhibit the activity of the enzyme acetylcholinesterase (AChE) and increase the level of acetylcholine available for binding at the NMJ, are the first-line treatment in patients with MG (14). However, AChE inhibitors rarely induce complete relief of MG symptoms and barely affect disease progression. Plasma exchange (PE) and intravenous immunoglobulin are used for the treatment of MG exacerbations to achieve a rapid clinical response by reducing the concentrations of circulating antibodies (41). However, both therapies are expensive and can induce side-effects, including hypotension and paresthesias from citrate-induced hypocalcaemia. Corticosteroids (CSs) were the first immune-suppressants (ISs)

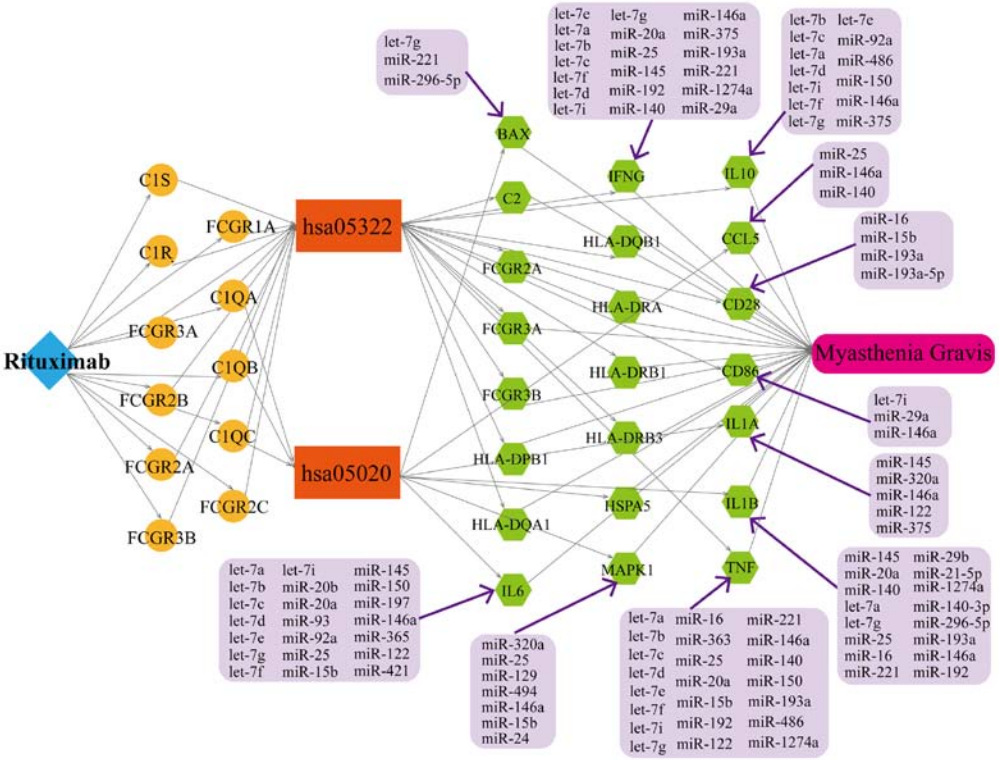

Figure 4. Layered network between rituximab and myasthenia gravis (MG). Diamond indicates drug, circles indicate drug target genes, rectangles indicate the MG risk pathways, and hexagons indicate MG risk genes that regulate the MG risk pathway. Many MG risk genes carry validated miRNA targets.

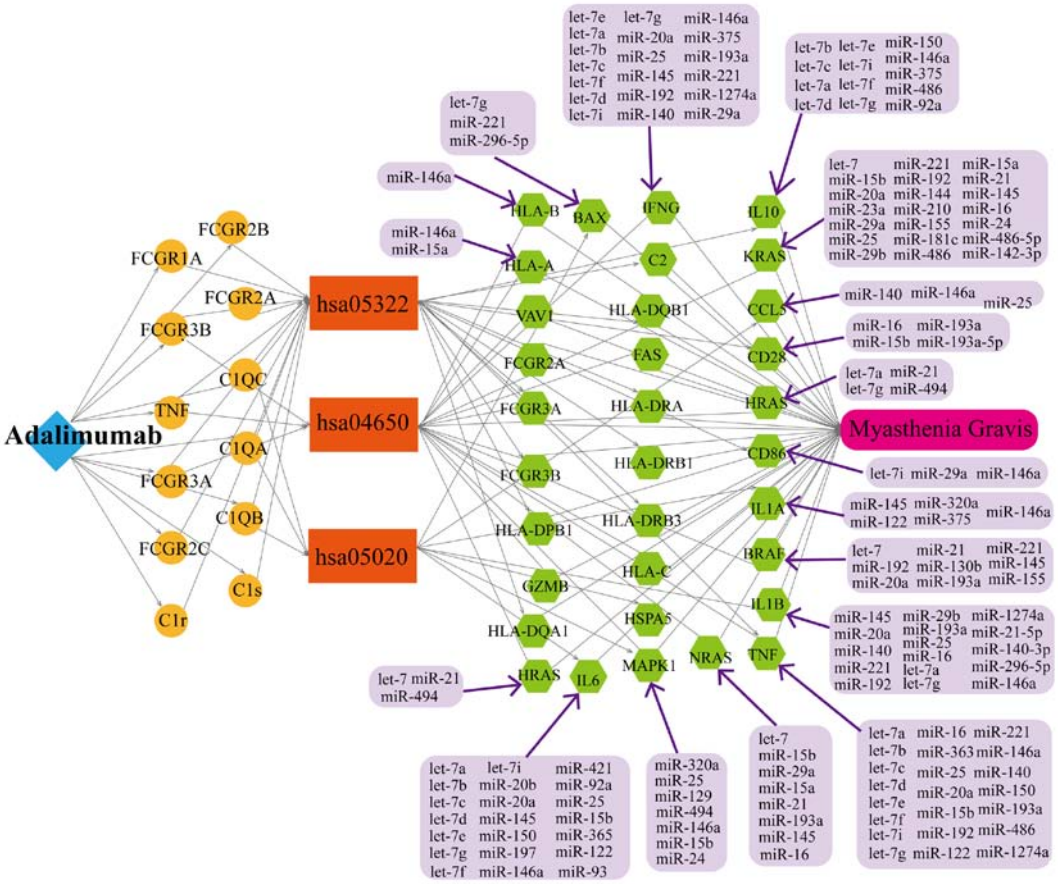

Figure 5. Layered network between adalimumab and MG. Diamond indicates drug, circles indicate drug target genes, rectangles indicate the MG risk pathways, and hexagons indicate MG risk genes that regulate the MG risk pathway. Many MG risk genes carry validated miRNA targets.

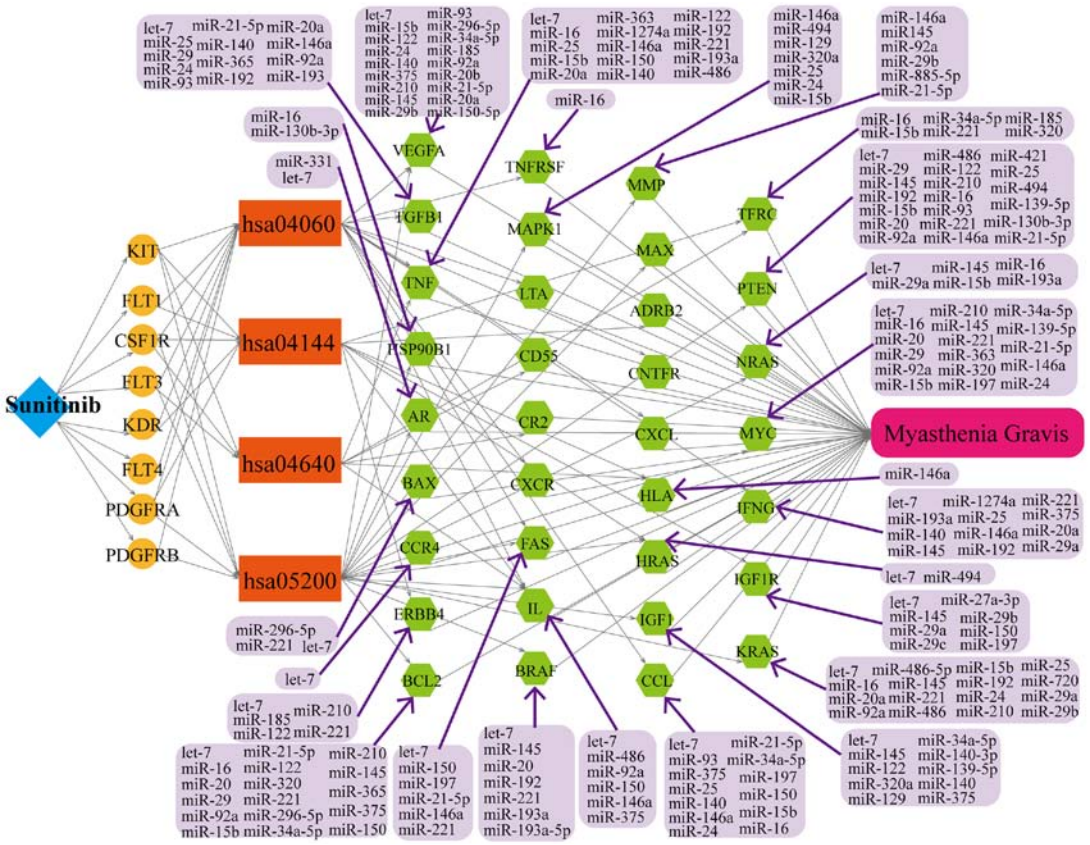

Figure 6. Layered network between sunitinib and myasthenia gravis (MG). Diamond indicates drug, circles indicate drug target genes, rectangles indicate the MG risk pathways, and hexagons indicate MG risk genes that regulate the MG risk pathway. Many MG risk genes carry validated miRNA targets.

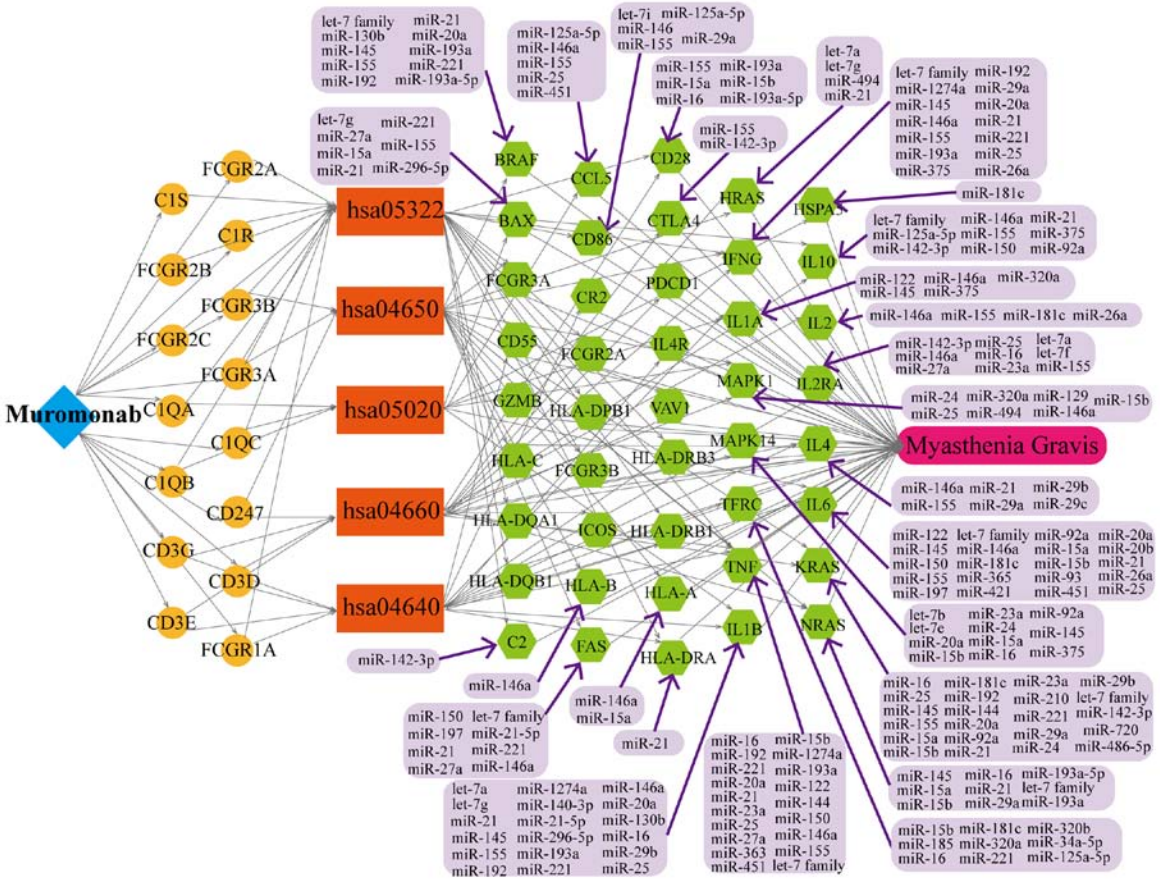

Figure 7. Layered network between muromonab and myasthenia gravis (MG). Diamond indicates drug, circles indicate drug target genes, rectangles indicate the MG risk pathways, and hexagons indicate MG risk genes that regulate the MG risk pathway. Many MG risk genes carry validated miRNA targets.

to be used in MG and remain the most commonly used immune-targeted therapy. However, common side-effects include Cushing's syndrome, osteoporosis, weight gain, hyperglycemia, diabetes, hypertension, gastritis or ulcers (4). Therefore, there is an urgent need for effective drugs that hit new MG targets.

In this study, we comprehensively selected MG risk genes and miRNAs and identified the MG risk pathways enriched by these genes and miRNAs. The MG risk pathways were mainly related to immunity and neoplasms, which may explain why MG sometimes occurs as a paraneoplastic syndrome associated with thymoma (42). Then, we identified drugs that targeted risk pathways with MG risk genes and miRNAs. We also analyzed how the miRNAs and drugs synergistically regulated MG risk pathways. We constructed an miRNA-regulated drug-pathway network of MG, which can help us understand the risk pathways regulated by MG risk miRNAs and drugs. Our method comprehensively considers the common pathways between drugs and MG rather than their common targets. Among the 25 drug repurposing candidates, 19 (76.0%) were related to MG or other immune diseases, supporting by clinical trials or related literature. Kim *et al* (14) identified treatments for MG based on its immune pathogenesis and suggested that treatment of MG should be according to the clinical features or subtypes of the disease, the severity of the disease and the activities of daily living of individual patients. Lewis (15) identified new potential immunotherapies for MG through the literature mining current treatments for immune diseases. Rituximab can target B cells by directly targeting CD20 molecules, which is consistent with our results. Whereas Lewis (15) and Kim *et al* (14) considered the pathology of MG based on its immune aspects, our study was based on the pathways that were enriched by MG risk genes and miRNAs.

The risk pathways of MG not only featured immune aspects but were also related to cancer and other diseases. It is unreasonable to characterize this disease from only an immune aspect; treatment based on immune pathology is not sufficient, which may explain why some patients do not respond to immune therapy. Therefore, identifying repurposing candidate drugs for MG based on the common pathways and drugs associated with this disease is more comprehensive and reliable. To determine whether these repurposing candidate drugs are applicable for the treatment of MG, well-designed experiments or clinical trials should be conducted.

In conclusion, we comprehensively collected the risk genes and miRNAs of MG and identified MG risk pathways to characterize the mechanism of and treatment strategy for MG. We also constructed an MDPN to better understand the relationship among genes, pathways, and drugs and to identify promising drug repurposing candidates for MG.

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