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RESEARCH

In silico investigation of potential COVID-19-associated microRNA signatures

Potansiyel COVID-19 ilişkili mikroRNA'ların bilgisayar ortamında araştırılması

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Abstract

Purpose: The global pandemic COVID-19, caused by the coronavirus SARS-CoV-2, is persistent despite the increasing vaccination rates, with new cases being reported per week. MicroRNAs, that is, non-coding RNA species that regulate gene expression at the post-transcriptional level, play a pivotal role in the SARS-CoV-2 life cycle, pathophysiology and host's anticoronaviral responses. The objective of this study was the in silico discovery of functionally associated miRNAs that likely co-regulate COVID-19-related genes

Materials and Methods: In the present study, an integrative bioinformatics approach was employed, including database searching, gene set enrichment analysis, network-based and microRNA target prediction methods, towards the discovery of epigenetic determinants of COVID-19.

Results: An intricate microRNA-target gene network was constructed, and a set of 8 highly interacting microRNAs, that potentially co-target and co-regulate key COVID-19-related genes, was detected. These miRNAs and their corresponding genes are likely involved in the host's response to SARS-CoV-2 infection.

Conclusion: The 8 functionally associated miRNAs could constitute a signature for COVID-19 diagnosis.

Keywords: bioinformatics; COVID-19 infection-associated genes; antiviral microRNAs; protein-protein interactions; gene set enrichment analysis; microRNA-gene network

Öz

Amaç: Koronavirüs SARS-CoV-2'nin neden olduğu küresel COVID-19 pandemisi, artan aşılama oranlarına rağmen varlığını sürdürmekte ve her hafta yeni vakalar rapor edilmektedir. Transkripsiyon sonrası seviyede gen ekspresyonunu düzenleyen kodlamayan RNA türleri olan mikroRNA'lar, SARS-CoV-2 yaşam döngüsünde, patofizyolojisinde ve konağın antikoronaviral yanıtlarında çok önemli bir rol oynarlar. Bu çalışmada, COVID-19 ile ilişkili genleri düzenlemesi muhtemel, işlevsel olarak ilişkili miRNA'ları in silico araştırıp bulmak amaçlanmıştır.

Gereç ve Yöntem: Bu çalışmada, COVID-19'un epigenetik belirleyicilerini bulmaya yönelik veri tabanı araştırması, gen seti zenginleştirme analizi ve internet tabanlı mikroRNA'ya yönelik hedef tahmin yöntemlerini içeren bütünleştirici bir biyoinformatik yaklaşım kullanılmıştır.

Bulgular: Karmaşık bir mikroRNA-hedef gen ağı oluşturularak, potansiyel olarak hedeflenen ve COVID-19 ile ilgili önemli genleri düzenleyen yüksek düzeyde etkileşime sahip 8 mikroRNA'dan oluşan bir dizi tespit edildi. Bu miRNA'lar ve bunlara karşılık gelen genler SARS-CoV-2 enfeksiyonuna verilen yanıtta rol oynayabilir.

Sonuç: İşlevsel olarak ilişkili 8 miRNA, COVID-19 tanısı için önemli bulgular olabilirler.

Anahtar kelimeler: biyoinformatik, COVID-19 enfeksiyonu ile ilişkili genler, antivirüs mikroRNA'lar, protein-protein etkileşimleri, gen seti zenginleştirme analiz,; mikroRNA-gen ağı

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INTRODUCTION

The ongoing global pandemic COVID-19, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), persists despite the vaccination efforts, with thousands of COVID-19 cases and a great number of deaths to be reported weekly[1]. Of note, the recently emerged SARS-CoV-2 variants BA.2.86 ('Pirola') and EG.5 (Eris) are highly infectious and transmissible strains[2-5]. Moreover, the growing incidence and prevalence, as well as the high transmissibility of the JN.1 strain, an offspring of BA.2.86, led WHO to declare it as a "variant of interest"[6,7]. SARS-CoV-2-mediated infection is characterized by activation of the innate immune response (the first line of immune defense) and hyperinflammation[8-10]. COVID-19 is associated with numerous complications and comorbidities, including common cold, pneumonia, blood coagulation, lymphopenia, acute respiratory distress syndrome (ARDS), tissue injury, multiple organ dysfunction, etc.[11-14].

MicroRNAs (miRNAs) are endogenous, short (22-nucleotide) single-stranded non-protein-coding RNA molecules that can regulate gene expression at the post-transcriptional level through base-pairing with the 3' untranslated region (3'UTRs) of their target mRNAs; they subsequently suppress mRNA expression by mRNA degradation or translational inhibition. A single miRNA can potentially target multiple genes, and, conversely, a gene can be targeted by many miRNAs[15-16].

It has been demonstrated that miRNAs play a critical role in the coronaviral life cycle, pathogenesis and host antiviral responses[17,18]. SARS-CoV-2 infection-induced changes in the expression patterns of the host miRNAs can lead to the down-regulation of key genes involved in the immune response and, consequently, to immunosuppression or attenuated host immune surveillance, thereby enabling the coronavirus to evade the host's immune system[19-21]. SARS-CoV-2 can also co-opt host miRNAs implicated in immune response suppression (e.g., hsa-miR-939 and hsa-miR-146b), in order to facilitate coronaviral replication and subvert host immune responses[18,22]. Moreover, miRNAs encoded by the coronaviral genome can target genes involved in host immune response/inflammation, like IFN1-mediated signaling[23,24]. SARS-CoV-2 non-coding RNAs can also deplete the host's miRNA pool, by acting as miRNA sponges[25,26].

In a recent study, Ahmad *et al.* (2023) suggest that the host miRNAs that are differentially regulated during COVID-19 infection can serve as biomarkers for prognosis in COVID-19, as well as the design of novel miRNA-based antiviral agents[27]. According to Fayyad-Kazan and colleagues (2021), differentially expressed circulating miRNAs (e.g., miR-19a-3p, miR-19b-3p, and miR-92a-3p) are considered powerful biomarkers for the timely and accurate diagnosis of COVID-19[28]. In addition, deregulated plasma miRNAs were shown to have predictive and discriminatory potential for COVID-19 severity and mortality[29]. In a study by Farr *et al.* (2021), it was shown that three differentially expressed miRNAs (miR-423-5p, miR-23a-3p and miR-195-5p) could accurately discriminate COVID-19 patients from healthy controls[19]. Furthermore, low expression of hsa-let7b-5p was detected in naso-oropharyngeal specimens from COVID-19 patients and it is implicated in the regulation of the angiotensin-converting enzyme 2 (ACE2) and dipeptidyl peptidase-4 (DPP4) receptors[30].

Although the co-regulatory effects of host miRNAs during SARS-CoV-2 infection have been studied fragmentarily, a comprehensive network analysis of the host COVID-19-relevant genes and miRNAs has not been conducted. Therefore, our study aims to contribute to the investigation of potential miRNA determinants of the epigenetic regulation of genes that are prominently associated with COVID-19, in order to decipher the co-regulation activities of miRNAs exerted upon those genes during coronavirus infection. This study hypothesizes that by employing a bioinformatics approach to construct a miRNA-gene network, we could provide a fundamental framework for detecting host genes and epigenetic regulators that likely respond during SARS-CoV-2 infection in a coordinated way.

MATERIALS AND METHODS

Acquisition of COVID-19-related genes

To obtain a comprehensive list of genes significantly linked to COVID-19, the GeneCards database (https://www.genecards.org/)[31,32] (accessed March 2023) was searched by using the keywords "COVID-19" and "SARS-CoV-2"; in this way, the genes with relevance score ≥ 9 were extracted.

Protein-protein interaction network

The physical and functional associations among the protein products of the retrieved COVID-19-related genes were investigated and visualized through STRING (Search Tool for Retrieval of Interacting Genes/Proteins) v11.5[33], a knowledgebase of both experimentally supported or predicted, functional and/or physical, gene/protein association data derived from diverse resources. In order to enhance the reliability of the given interactions, a relatively high confidence interaction score (≥0.7) was set as cutoff; only those associations based on automated text mining, knowledge databases and experimental evidence were taken into consideration. The associations were further visualized and analyzed through the open-source platform Cytoscape (v.3.10.0)[34]. Moreover, the Cytoscape plugin cytoHubba[35], which allows network topological analysis by twelve local and global ranking algorithms (betweenness, bottleneck, clustering coefficient, closeness, degree, density of maximum neighborhood component, eccentricity, edge percolated component, maximum clique centrality, maximum neighborhood component, radiality, and stress), was utilized to select the top 26 nodes, by using the node-degree filter.

Functional enrichment analysis

Gene set enrichment analysis was conducted with the online tool WebGestalt (WEB-based GEne SeT AnaLysis Toolkit)[36,37] for the identification of statistically significant over-represented biological pathways in the COVID-19 genes under study. The WebGestalt parameters selected were "Organism of Interest": Homo sapiens, "Method of Interest": Over-Representation Analysis (ORA), "Functional database": pathway/Reactome for biological paths, "Select gene ID type": gene symbol, "Select Reference set": genome; the default advanced parameters were chosen, and only those pathways with a Benjamini-Hochberg-adjusted *p*-value[38] ≤ 0.05 were included in the analysis. Affinity propagation was applied to reduce the terms and cluster them into representative categories.

miRNA regulators of COVID-19 genes

The miRNAs potentially regulating the COVID-19 genes were investigated. To this end, both, the experimentally verified and predicted miRNAs targeting the COVID-19 genes were obtained by applying four state-of-the-art software tools: i) microT_CDS (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index) is based on the DIANA-microT-CDS algorithm to predict miRNA target genes[39]; ii) TargetScan (https://www.targetscan.org/vert_80/) searches for the presence of conserved sites matching the miRNA seed region[40]; iv) PITA (https://genie.weizmann.ac.il/pubs/mir07/) recognizes thermodynamically favorable miRNA-mRNA interactions[41]; iii) miRDB (http://mirdb.org/) predicts functionally annotated miRNA gene targets by machine learning approaches[42,43]. The miRNA-mRNA interactions retrieved from each tool were combined and the duplicates were removed. To enhance the accuracy of the prediction, only those miRNA-gene targets predicted by more than three methods were considered in this study.

Pairwise miRNA associations

The miRNA-miRNA relationships were collected from two different sources, MiRGOFS[44] and GOSemSim[45], which infer functional similarities between miRNA pairs based on the degree of functional relatedness of their corresponding target genes. The pairwise miRNA interactions from both sources were merged, and the duplicates were removed. Only those miRNA-miRNA interactions with a weight score above 0.85 (where "1.0" is the highest score) were chosen so as to enhance robustness.

Independent validation

The findings of this study were further compared against an independent transcriptomic dataset, available in the comprehensive online resource COVID19db[46]; the "differential expression" module was used, which provides gene expression profiling in whole blood derived from COVID-19 patients and healthy controls.

RESULTS

A total of 149 COVID-19-relevant genes were obtained from GeneCards (Table S1). A functional network of the products of those genes was generated, and 128 nodes appear to be highly interconnected (Figure 1). By examining the topological properties of the network, we identified those key nodes that are more relevant to the overall

function of the network and, therefore, biologically meaningful[47-48]. The top 26 nodes were detected in the protein-protein interaction network (Figure 1 and Table S1) based on the combined output of the twelve algorithms in cytoHubba; scores generated by all algorithms were assigned to each node in the network and, then, the top-ranked nodes were scored based on the degree method (i.e. the number of direct connections of a given node to its neighbor nodes).

The terms over-represented in the genes, the products of which are involved in the protein association network (Figure 1), are mainly associated with immune signaling pathways, e.g., cytokine, interleukin, interferon, Toll-like receptor, MYD88, NF-kF, T-cell receptor (TCR)-mediated signaling cascades (Figure 2). Notably, 109 (out of 128) and all 26 top-ranking genes are found in the over-represented Reactome pathways, further highlighting their prominent role in COVID-19 pathophysiology.

The differential expression profiles of several COVID-19-related top-26 genes (Table S1) between SARS-CoV-2-infected patients and healthy controls were further investigated through the "differential expression" module of COVID19db[46]. Among those, the cytokines *IL10*, *IL18* and *CXC10* are up-regulated in the COVID-19 group (Figure 3), consistent with their pro-inflammatory effects[49-51].

As shown in Figure 4, 22 of the top-ranking genes are potentially targeted by more than 8 miRNAs, namely hsa-miR-1276, hsa-miR-3121-3p, hsa-miR-338-5p, hsa-miR-340-5p, hsa-miR-5692a, hsa-miR-570-3p, hsa-miR-664a-3p and hsa-miR-7-5p.

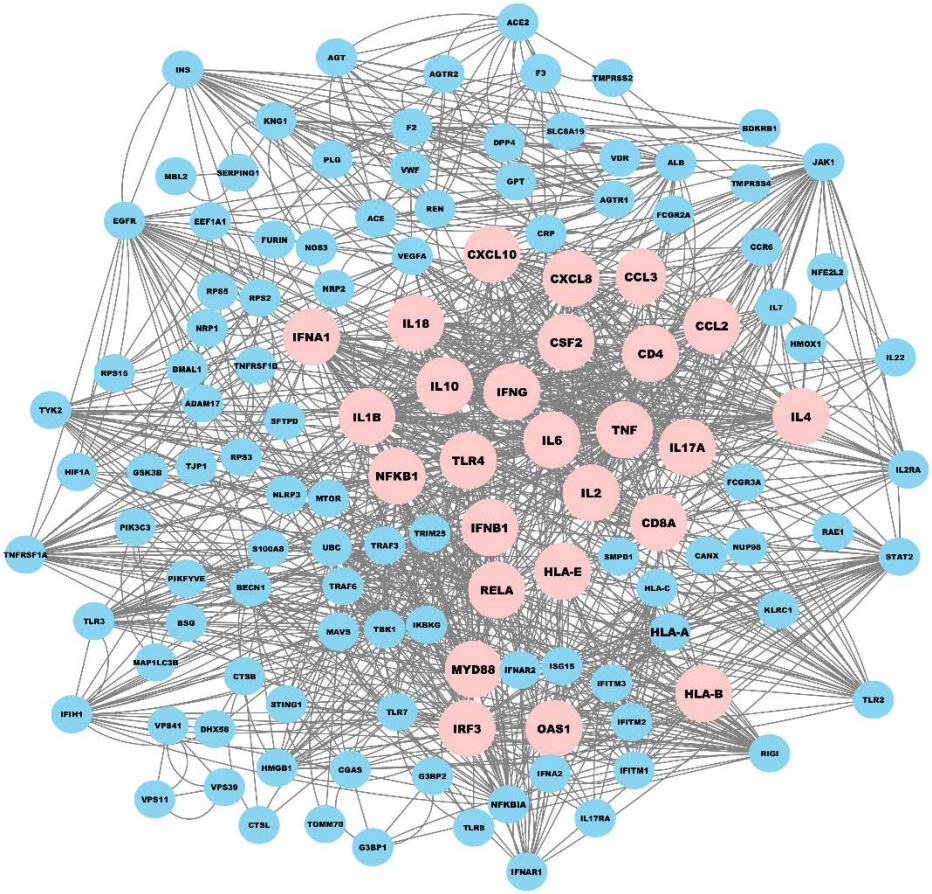

Figure 1. Interaction network of the COVID-19 proteins. The nodes denote genes/gene products, and the edges represent functional associations. The nodes corresponding to the top-ranking gene products are indicated by light red fill color.

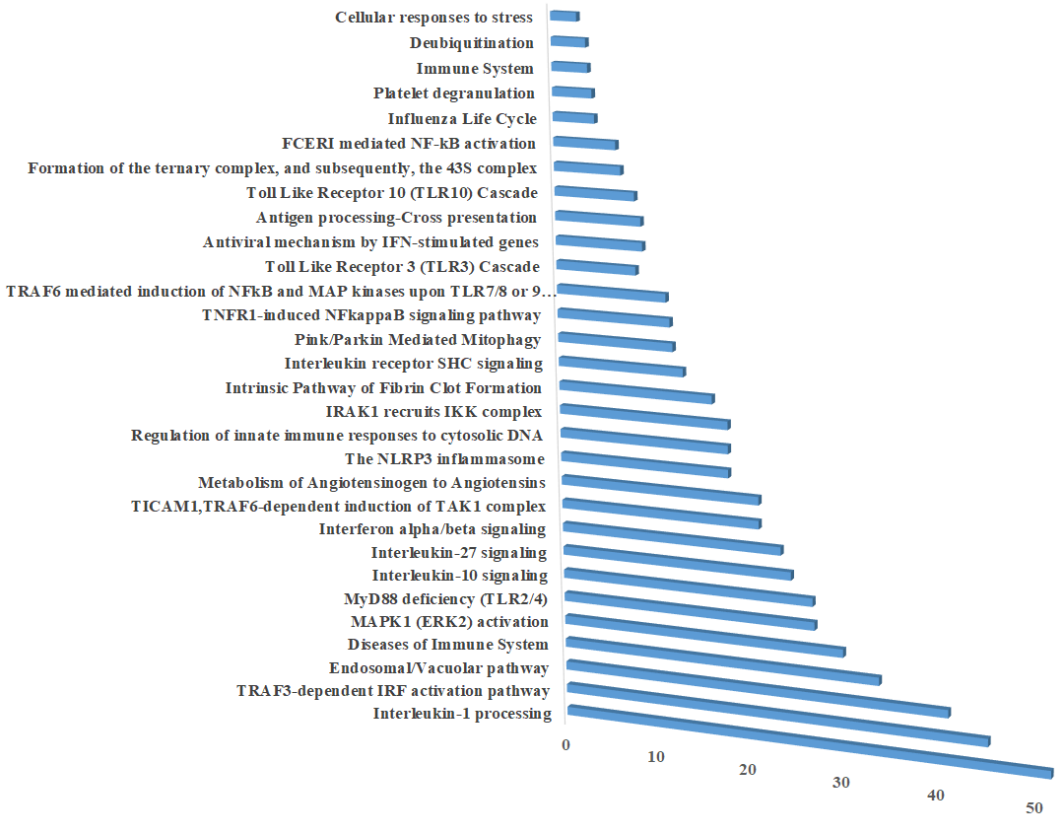

Figure 2. Bar plot illustrating the over-represented Reactome pathways in the COVID-19 genes. The width of bar plots is proportional to the number of genes in each pathway.

DISCUSSION

Collectively, the proteins encoded by 128 of the 149 COVID-19-associated genes are strongly connected in an interaction network, suggesting physical and functional associations among the corresponding proteins. Most of the 26 key genes in the network (Figure 1 and Table S1) encode components of the immune system (CD4, CD8A, HLA-B, IRF3, MYD88 etc.), including pro- and anti-inflammatory factors, such as chemokines and cytokines (CXCL8, CXCL10, IL1B, IL2, IL4, IL6, IL10, IL17A, IL18, IFNG) and the tumor necrosis factor (TNF). In many COVID-19 cases, exacerbated inflammatory responses ("cytokine storm") are observed, which result from the acute increase in the levels of circulating pro-inflammatory cytokines and chemokines, and their uncontrolled release, both at local and systemic levels[52-54]. Several studies, though, suggest that the human host's immune system is rather compromised upon SARS-CoV-2 infection and it is not capable of eliciting a sufficient immune response[55,56].

The NF-kB subunits, NFKB1 and RELA (Table S1), are tightly connected to several pro-inflammatory agents. NF-kB can modulate the host's immediate innate immune response to SARS-CoV-2 infection. In particular, SARS-CoV-2-mediated activation of NF-kB was found to induce the expression of the genes *IL1*, *IL2*, *IL6*, *IL8* and *TNF*[57]. Human miRNAs could attenuate the effects of the COVID-19-induced inflammatory cascade (e.g., organ injuries), by targeting and suppressing the gene coding for NFKB1[17].

The effector gene *OAS1* affects susceptibility to SARS-CoV-2 and has a protective effect against COVID-19 severity[58-60]. SARS-CoV-2 is suggested to bind and activate TLR4 (Toll-like receptor 4), a

sensor for innate immunity, leading to increased expression of ACE2, the major receptor for SARS-CoV-2 cell entry[61], thereby facilitating coronavirus entry and amplifying the inflammatory response[62,63]. CSF2 (colony-stimulating factor 2) was also found to be over-expressed in SARS-CoV-2-infected human lung epithelial cells[64].

Over-expression of the cytokine *IL6* has been reported to be highly associated with the mortality risk of COVID-19; IL6 also acts as a perpetrator of the SARS-CoV-2-induced cytokine storm[65-67]. However, *IL6* appears to be down-regulated in the blood of COVID-19 patients (Figure 3), consistent with the findings of a previous bioinformatics study by Özbek and colleagues (2023), wherein *IL6* was not found to be significantly dysregulated in diverse SARS-CoV-2-infected tissues[55]. This is probably due to the dual role of IL6 as a pro- and anti-inflammatory cytokine[68-69], as well as the diverse immune response patterns observed in COVID-19 patients[55,70]. The pleiotropic anti-inflammatory cytokine *IL4*[71] is also down-regulated in COVID-19 blood samples (Figure 3), in agreement with previous studies suggesting cytokine storm suppression during SARS-CoV-2 infection[55,56].

Increased expression of *TLR4* greatly affects heart failure in COVID-19 patients[63,72]. TLR4 is known to trigger the activation of pro-inflammatory cytokines[73], including TNF, which is also up-regulated in COVID-19 (Figure 3); increased expression of *TNF* was found to be a prognostic factor for mortality among COVID-19 patients with comorbidities and disease progression[74]. MYD88, which plays a central role in TLR/IL1R-mediated signalling in innate and adaptive immunity[11], is over-expressed in the COVID-19 group (Figure 3); *MYD88* polymorphisms were shown to be tightly associated with COVID-19 severity[75]. Finally, the expression level of the interferon-inducible gene *OAS1* is markedly higher in the SARS-CoV-2 infected patients, as compared to the healthy samples; this is in agreement with the findings of a recent study, wherein SARS-CoV-2 infection significantly increased the expression of *OAS1*[76].

The causal genes of diseases/disorders usually share common regulatory mechanisms in order to ensure their coordinated regulation. Similarly, disease-related miRNAs act in a cooperative manner so as to exert their regulatory effect upon their corresponding target genes[77-78]. In our study, all eight miRNAs that target the top-22 genes are associated in a pairwise fashion (Figure 4), suggesting participants in an intricate regulatory network, wherein these miRNAs act in a synergistic fashion to co-target and co-regulate the expression of COVID-19 genes.

There is evidence that several of those eight miRNAs are associated with SARS-CoV-2 and COVID-19. Vastrad and colleagues (2020) analyzed gene expression data from whole blood from human patients with COVID-19 and they found hsa-miR-5692a among those miRNAs that target genes that are up-regulated in COVID-19 samples as compared to healthy controls[79]. Moreover, hsa-miR-340-5p was down-regulated in the peripheral blood collected from COVID-19 patients[80]. According to Katopodis *et al.* (2022), hsa-miR-3121-3p and hsa-miR-570-3p could regulate the expression of mediators of the SARS-CoV-2 cell entry[81]. Also, hsa-miR-1276 was shown to have the capacity to bind viral RNA and to be modulated significantly in pneumocytes, suggesting a role in SARS-CoV-2 infection[82]. Bartoszewsk *et al.* (2020) suggested potential SARS-CoV-2-mediated modulation of host miRNAs, including hsa-miR-664a-3p, in order to perpetuate coronaviral persistence/replication and evade host immune defences[25].

The limitations of this study are that the differential expression status of the predicted miRNAs, and their corresponding pairwise interactions, have not been validated experimentally in the context of COVID-19.

In conclusion, in this study, by applying an integrative bioinformatics pipeline and stringent criteria, we discovered eight functionally related miRNAs that potentially co-target and co-regulate pivotal COVID-19-associated genes. This panel of miRNAs could represent potential signature components for COVID-19, which can be taken into consideration in the clinical setting for updating and complementing the current biomarkers towards improving the accuracy of diagnosing SARS-CoV-2-infected patients.

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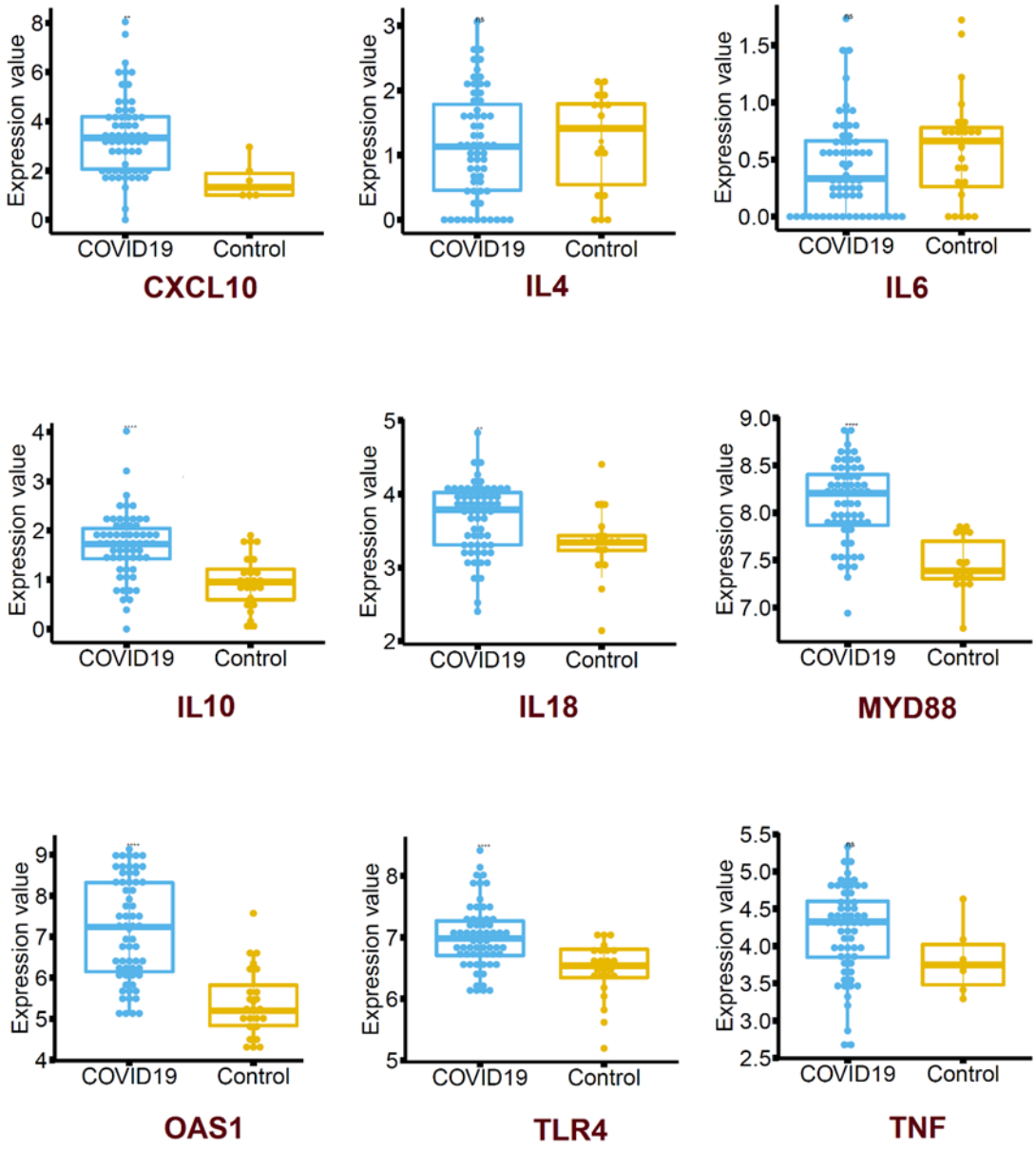

Figure 3. Differential gene expression analysis between COVID-19 (blue) and healthy control (golden brown) samples.

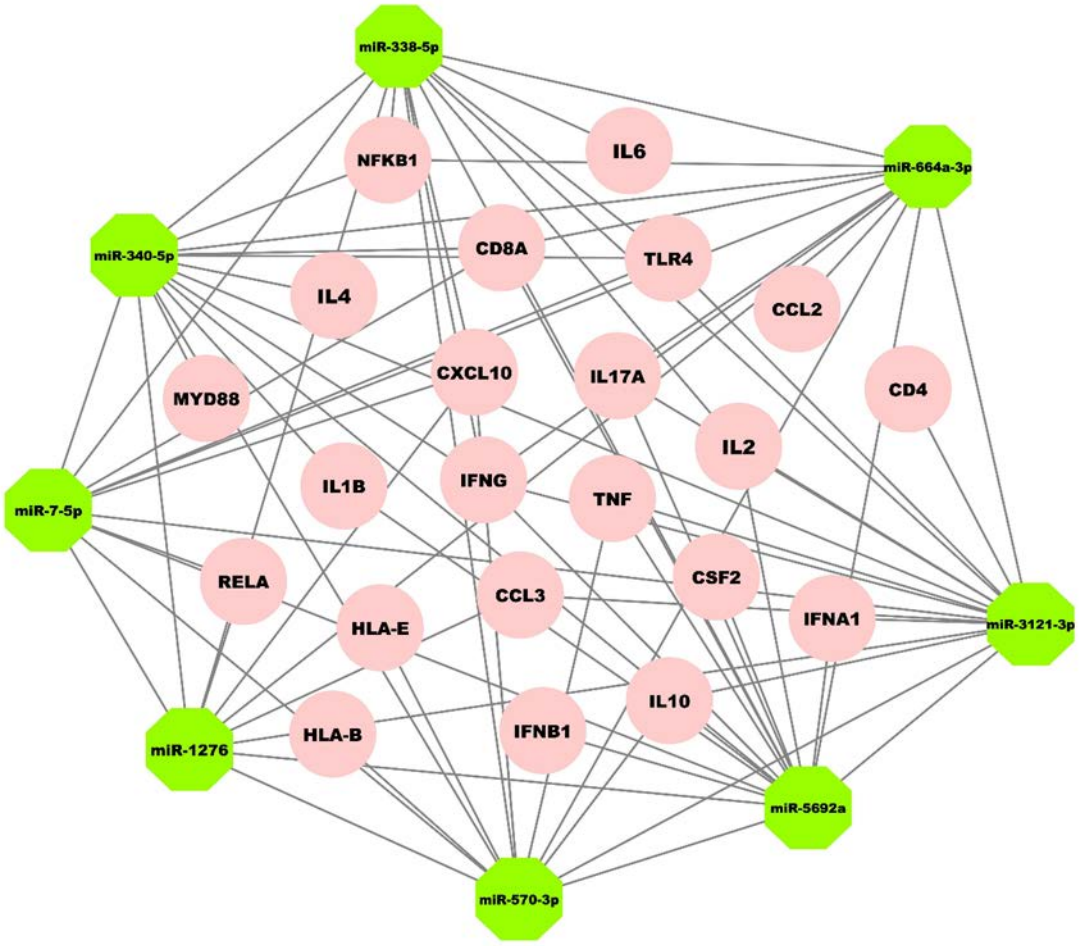

Figure 4. MiRNA-target gene network in COVID-19. The miRNAs are represented by polygons, and the miRNA target genes are denoted by circles.

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