Transcriptome Analysis of Peripheral Blood Mononuclear Cells Response in Patients with Severe COVID-19 Reveals Crucial Genes Regulating Protein Ubiquitination

Background: We sought to further our understanding of the biological characteristics underlying severe COVID-19.

Material/Methods: RNA sequencing (RNA-Seq) analysis was used to evaluate peripheral blood mononuclear cells from 4 patients with severe COVID-19 and 4 healthy controls. We performed gene expression analyses to detect differentially expressed genes (DEGs). Enrichment analyses were performed to identify their molecular processes and signaling pathways, and the protein–protein interaction network was constructed to extract the core gene cluster. The investigation of protein-chemical interactions and regulatory signatures for specific regulatory checkpoints and powerful chemical agents was then conducted for these essential genes. Finally, we used single-cell RNA-Seq analysis from an online platform to show how these genes were expressed differently, depending on the kind of cell.

Results: A total of 268 DEGs were found. The biological process of protein ubiquitination was later discovered to be highly influenced by the core gene cluster (ITCH, TRIM21, RNF130, FBXO11, UBE2J1, and ASB16) at the transcriptome level. Six transcription factors, FNIC, FOXA1, YY1, GATA2, MET2A, and FOXC1, as well as miRNAs hsa-miR-1-3p and hsa-miR-27a-3p were identified. We found a potent chemical agent, copper sulfate, may regulate protein ubiquitination genes cooperatively, and the genes regulating protein ubiquitination could be expressed highly on the macrophages.

Conclusions: Taken together, we suggest that protein ubiquitination is a crucial functional process in patients with severe COVID-19. This study will give a deeper insight into biological characteristics and progression of COVID-19 and facilitate development of novel therapeutics, leading to significant advancements in the COVID-19 pandemic.

Keywords: COVID-19 • Transcriptome • Ubiquitination

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/937532
Background

COVID-19 is a respiratory condition caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which belongs to the Coronaviridae family and whose genome is composed of a single 30-kb strand of RNA [1]. As of August 2, 2022, there have been 583,238,204 confirmed COVID-19 cases, and it is reported that there have been 6,422,235 COVID-19-related deaths across 223 nations or regions [https://worldometers.info/coronavirus/]. The symptoms mainly manifest as inflammatory lesions in the lungs. The virus also damages the digestive system, nervous system, and cardiovascular systems of patients, and an infection could result in death due to multiple organ failure [2]. It was reported that 80% of patients were asymptomatic or had mild disease, while about 14% had severe disease, and 5% were in critical condition [3]. The grade of severity in COVID-19 was related to the age, sex, and other health conditions of the patient, such as the occurrence of chronic diseases, including diabetes and heart disease [4]. Therefore, it is particularly important to obtain knowledge regarding the treatment of patients with severe and critical disease, as such patients are more likely to deteriorate rapidly, resulting in cytokine (inflammatory) storms, respiratory distress, multi-organ dysfunction, and potential death [2].

Mutations always occur during the spread of viruses. Mutations were especially observed to occur in patients infected with the delta variant, which is highly infectious and has resulted in more severe illness in individuals after it originated in India in December 2020 [5]. Vaccination is reported to be the most effective method for the prevention of COVID-19 disease [6], since no dedicated antiviral drugs are available for the treatment of patients with severe COVID-19, and the use of drugs is limited to the management of symptoms and complications. Therefore, it is important to obtain insights into the characteristics and molecular mechanisms of biological reactions occurring in patients with COVID-19 in order to develop new and efficacious clinical therapeutic targets.

The SARS-CoV-2 genome contains 4 major structural proteins, namely the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins [7]. The S1 subunit region of the S protein facilitates the binding of the virus to the angiotensin- converting enzyme 2 receptor on the surface of human lung cells [8]. After the virus enters the cells, an immune response is generated by the host, and pattern recognition receptors, such as toll-like receptors that are present on antigen-presenting cells (APCs), receive signals [9].

Post-translational modifications are chemical modifications necessary for precise regulation of protein properties and functions, which greatly increase the complexity and functional diversity of the proteome and ensure the dynamic cellular response to the external environment and intracellular factors [10]. Ubiquitination is a post-translational modification that is achieved through a coordinated and sequential enzymatic cascade. Ubiquitin is a 76-amino acid protein that is activated by the ubiquitin-activating (E1) enzyme in an ATP-dependent reaction. Ubiquitin is subsequently transferred to the active Cys residue of the ubiquitin-binding (E2) enzyme, and is then attached to the substrate mediated by the ubiquitin-binding (E3) enzyme. So far, 2 E1s, nearly 30 E2s, and more than 600 E3s have been found in humans [11]. Protein ubiquitination is an essential process in several eukaryotic biological processes, including cellular proteostasis, DNA repair, trafficking, immune functions, and antiviral responses [12,13]. Ubiquitination plays a key role in viral replication and affects the virulence of the influenza virus via the production of infectious virus particles in the cytoplasmic domain of influenza A virus membrane protein 2 [14]. It was reported that the ubiquitination of Beclin1 would inhibit autophagy and reduce MERS-coronavirus infections [15]. Ubiquitination has also been shown to affect the pathogenesis of acute lung injury and other lung diseases and to particularly affect the functioning of the alveolar epithelial barrier [16]. Siu et al. demonstrated that SARS-CoV open reading frame 3a (ORF3a) mediated the functioning of the NLRP3 inflammasome via TRAF3-dependent ubiquitination [17]. Furthermore, SARS-CoV-2 ORF7a usurps the ubiquitination system of the host and promotes the antagonism attributable to the interferon I response [18].

Itchy E3 ubiquitin protein ligase (ITCH) could play a role in innate immunity. The ITCH substrates would act as central participants for accelerating the degradation of c-Jun and JunB [19], and ubiquitinating PLC-γ1 and PKC-θ for T cell anergy [20]. In addition, ITCH might enhance the termination of antiviral responses via ubiquitination and degradation of MAVS [21]. Tripartite motif protein (TRIM21) is an E3 ligase that enhances target specificity during ubiquitination. In the pro-inflammatory environment, it would translocate to the nucleus rather than to the cytoplasmic environment [22]. Das et al suggested that TRIM21 interacts with the complex of IFI35 and Nmi in an uncontrolled anti-viral signaling environment [23]. Ring finger proteins (RNF) account a large class of potential ubiquitin ligases, and it is shown that RNF130 could negatively regulate T-cell receptor signaling in T cells infected with the human T-cell lymphotropic virus-I [24]. Christof et al suggested that the inactivation of F-box only protein 11 (FBXO11) as a tumor suppressor was related to the occurrence of lymphoproliferative pathologies [25]. It might be crucial for the release of PRC2-mediated repression throughout the maturity of the erythroid [25]. Ubiquitin-conjugating enzyme E2 J1 (UBE2J1) is an E2 (ubiquitin-conjugating) enzyme involved in processes involving endoplasmic reticulum-associated protein degradation on the endoplasmic reticulum, where it could degrade incorrectly folded proteins. UBE2J1 could regulate the interferon
pathway and degrade the promotion of infection with RNA viruses [26]. Most reports on ASB16 have primarily focused on lncRNA ASB16 antisense RNA 1, as it is recognized as an oncogene in several types of cancer, including gastric cancer [27], hepatocellular carcinoma, and glioma [28,29]. For instance, in gastric cancer, ASB16-AS1 would regulate the TRIM37 levels to induce the NF-κB pathway and promote tumor proliferation [27].

Copper has a powerful ability to neutralize infectious viruses, such as bronchitis virus, poliovirus, human immunodeficiency virus type 1 (HIV-1), and other enveloped or non-enveloped single- or double-stranded DNA and RNA viruses [30,31] because copper can damage the genomic DNA and RNA of the virus [32]. Moreover, Doremalen et al indicated that SARS-CoV-2 is very sensitive to copper [33]. In clinical practice, copper sulfate is frequently used locally as a fungicide, bactericide, and astringent [34].

In this study, we pre-processed raw data from the Gene Expression Omnibus (GEO) GSE167930 dataset and identified differentially expressed genes (DEGs) to investigate biological features and progression in patients with severe COVID-19. The functions and signaling pathways of these DEGs were identified to gain knowledge regarding their molecular processes. Then, we examined core gene clusters in patients with severe COVID-19 by performing protein–protein interaction (PPI) network analysis and determined the most significant gene cluster. Genes in this cluster were found to be involved in the biological process of protein ubiquitination. Furthermore, using these identified genes at regulatory checkpoints, we recognized microRNAs and transcription factors. Moreover, to specify the potent chemical agents responsible for protein ubiquitination in patients with severe COVID-19, we conducted an analysis of the protein-chemical interaction network. Finally, using data from a public dataset, we performed single-cell RNA-seq analysis to show how these genes are expressed differently in different cell types.

Figure 1 depicts the sequential workflow of this study.

Material and Methods

Datasets, Data Pre-Processing, and Differential Expression Analysis

We used RNA-Seq datasets of 4 patients with severe COVID-19 and 4 healthy controls, from whom peripheral blood samples were gathered, from the GEO database of the National Center for Biotechnology Information. The GEO accession number of
the dataset was GSE167930, and it was already deposited for the earlier study our team published [35]. The patient information is provided in Table 1. To identify significant DEGs, the edgeR package by online OmicShare tools was used to analyze the RNA-Seq count dataset. The edgeR package operates on a read count table where the columns represent independent libraries and the rows represent genes. The counts show how many reads have been aligned to each gene overall. After the normalization process, the significant DEGs associated with severe COVID-19 were filtered if P<0.05 and |log2 FC| ≥1. The OmicShare tool, a free online platform for data analysis (https://www.omicshare.com/tools), was used to perform the differential expression analysis and create the graphics. The volcano plot was plotted with log2(FC) as the horizontal coordinate and -log10(P value) as the vertical coordinate, and points with significant differences were given different colors to show the significance of the difference between the severe COVID-19 and healthy control groups. The heatmap aggregated the gene expression abundance of 4 patients with severe COVID-19 and 4 healthy controls. After Z-score normalization of the rows, the progressive color was used to visualize the gene expression abundance and thus the pattern of variation of different genes in the samples.

**Enrichment Analysis**

*Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Enrichment Analysis*

To characterize biological mechanisms, gene ontology (GO) and functional enrichment (biological processes, cellular component, and molecular functions) analyses were performed, to analyze the DEGs in patients with severe COVID-19 using the database for annotation, visualization, and integrated discovery (DAVID) v6.8 (https://david.ncifcrf.gov). The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was utilized to identify signaling pathways that were significantly associated with COVID-19. A P value <0.05 was considered as the cutoff criterion for quantifying the top-listed annotations and pathways. A scatter plot was performed to visualize the significant GO and KEGG pathways. In the plots, the Y-axis labels denoted the names of the pathways, and the X-axis labels reflected the rich factor. The rich factor was determined by dividing the number of genes in the target gene set that are members of a particular pathway by the number of these genes in the background gene set. The size of the bubble denoted the number of pathway-associated genes in the target gene collection, and the color of the bubble represented the P value.

**Gene Set Enrichment Analysis**

Gene set enrichment analysis (GSEA) was used to analyze the genomic information of each sample according to its degree of differential expression. GSEA identified whether the preset gene set was enriched at the top or bottom of the sequencing table by sequencing the genes by the degree of differential expression between the severe COVID-19 and healthy control groups. Since the analysis examines genome expression rather than the expression of specific genes, it can take into account more subtle expression changes. To obtain insight into the signaling and metabolic molecules involved in biological pathways, all the genetic information regarding patients with
Severe COVID-19 and healthy controls were uploaded onto GSEA software, and analysis was performed using the Reactome pathway database. A standard metric was applied based on a normalized enrichment score (NES) >1, P value <0.05, and FDR (q-value) <0.05, and the top list was ranked by the NES. The enrichment analysis and figures mentioned above were performed using the OmicShare tools.

**PPI Network Analysis and Gene Cluster Identification**

To construct the PPI network interaction diagram, the DEGs identified in severe COVID-19 samples were uploaded to STRING. Then, the results obtained using STRING were uploaded into Cytoscape v.3.7.1., and the molecular complex detection (MCODE) plug-in of Cytoscape was used for cluster analysis. Next, the genes in the cluster with the highest scores were imported into DAVID to determine the biological processes in which this gene cluster participated significantly. Subsequently, genes identified to be involved in the most significant biological process were imported into the NetworkAnalyst tool for further analysis.

**Regulatory Signatures Network Analysis**

To detect the transcriptional signatures and post-transcriptional regulatory signatures of genes involved in protein ubiquitination, the interacting network of transcription factors and miRNAs were identified using the NetworkAnalyst tool. The JASPAR database was used for transcription factors, and the Tarbase database was used for miRNAs.

**Protein–Chemical Interaction Network Analysis**

To identify potential antiviral agents, the protein-chemical interaction network was identified using the Comparative Toxicogenomics Database in the NetworkAnalyst tool. Cytoscape was used to visualize this interaction network.

**Single-Cell RNA-Seq Analysis**

We used single-cell RNA-Seq analysis on a free online database platform named "Large-scale single-cell analysis reveals critical immune characteristics of COVID-19 patients" (http://covid19.cancer-pku.cn/#/differential-expression) to show the cell type-specific expression of the genes regulating protein ubiquitination in this study. Cell types were allocated based on their canonical markers using t-distributed Stochastic Neighbor Embedding (t-SNE) for dimensionality reduction and clustering the cells. All figures were gathered from this web resource.

**Results**

**Sample Information Processing and Screening of DEGs**

To identify the characteristics of severe COVID-19 in this study, we analyzed the RNA-Seq count dataset using the edger package and filtered the DEGs if the P value was <0.05 and |log2 FC| ≥1; we obtained a count dataset of 10 894 genes and 268 DEGs (Figure 2A). The gene cluster in each patient with severe COVID-19 and healthy controls is shown on the heatmap (Figure 2B).
**Figure 3.** Gene Ontology and KEGG enrichment analysis of 268 differentially expressed genes (DEGs) in severe COVID-19. The top 10 terms in (A) molecular function, (B) cellular component, and (C) biological process category are presented. The total of 8 significant pathways involved in (D) KEGG enrichment are shown using a scatter plot. Colors represent the \( P \) values and sizes of the spots represent the counts of genes.
Enrichment Analysis

The GO project is a resource with extensive computable knowledge that focuses on the functions of genes and their products. Here, we performed GO enrichment analysis for the 268 DEGs identified in patients with severe COVID-19 with regard to 3 categories: biological process, cellular component, and molecular function. Figure 3 shows the top 10 terms in each category. For molecular function (Figure 3A), the most significant term was “amide transmembrane transporter activity”; and the “intracellular part” was mostly enriched in the cellular component (Figure 3B). Then, the “isopentenyl diphosphate biosynthetic process” and “mevalonate pathway” played a significant role in the biological process (Figure 3C). To demonstrate the biochemical processes in patients with severe COVID-19, pathway analysis was performed using the KEGG and Reactome databases. As shown in Figure 3D, there were 8 significant pathways in KEGG, including “terpenoid backbone biosynthesis”, “fanconi anemia pathway”, and “endoctyosis”. Figure 4 shows the top 3 pathways in the Reactome database, which were identified using GESA software. We observed that 17, 21, and 19 genes were enriched significantly in “insulin receptor recycling” (Figure 4A), “latent infection of homo sapiens with Mycobacterium tuberculosis” (Figure 4B), and “transferrin endocytosis and recycling” (Figure 4C).
Construction of the PPI Network and Further Excavation of Gene Clusters Involved in the Protein Ubiquitination Biological Process

To acquire insights into the network of reactions attributable to DEGs in severe COVID-19 samples, 268 DEGs were uploaded to the STRING database. We observed that 261 nodes and 307 edges were obtained with PPI enrichment at a $P$ value of 0.01. Figure 5 shows this data file, which was obtained using Cytoscape. To further identify the core genes playing key roles, MCODE was used to obtain the network data. Table 2 presents the 9 identified gene clusters. The genes in the cluster with the highest score (ANAPC1, TRIM21, ASB16, RNF130, FBXO11, UBE2J1, FBXO41, and ITCH) were further selected for biological process enrichment analysis to determine which pathway was most involved in the process. The highest statistical significance was observed for the biological process “protein ubiquitination” with 6 genes, ITCH, TRIM21, RNF130, FBXO11, UBE2J1, and ASB16, based on the $P$ values and FDR (Table 3). These 6 critical genes involved in protein ubiquitination were analyzed further.

Regulatory Signatures and Protein-Chemical Interactions Between Genes Involved in Protein Ubiquitination

To identify the more substantial mechanisms at the transcriptional level of genes involved in the protein ubiquitination

Figure 5. A network of protein–protein interactions (PPI) was built using STRING and Cytoscape. Different colors are used to denote the various clusters that MCODE examined. The PPI network has 261 nodes and 307 edges.
networks of regulatory sig
atures (transcription factors and miRNAs) were constructed. Figure 6 shows that a total of 26 transcription factors might influence genes ASB16, UBE2J1, TRIM21, ITCH, and FBXO11 and regulate protein ubiquitination during severe COVID-19. Six transcription factors, FNIC, FOXA1, YY1, GATA2, MET2A, and FOXC1, linked more than 2 genes. Among these miRNAs, hsa-mir-1-3p and hsa-mir-27a-3p were also found to significantly exert regulation on over 5 of the genes involved in protein ubiquitination. The miRNA hsa-mir-1-3p was connected with ITCH, TRIM21, RNF130, FBXO11, UBE2J1, and ASB16 (Figure 7). We also identified 64 chemical agents related to ubiquitination that could be used for identifying potential antiviral agents; among them, 21 chemical agents were associated with more than 2 genes. Copper sulfate was found to significantly correlate with 5 genes, ITCH, TRIM21, RNF130, FBXO11, and ASB16 (Figure 8).

| Cluster | Score | Nodes | Edges | Node IDs |
|---------|-------|-------|-------|----------|
| 1       | 7     | 8     | 28    | ANAPC1, TRIM21, ASB16, RNF130, FBXO11, UBE2J1, ITCH |
| 2       | 4     | 5     | 10    | RPS18, RPS28, WDR3, RPP21, UTP20 |
| 3       | 4     | 5     | 10    | HCAR2, PCP2, TAS2R19, TAS2R31, TAS2R46 |
| 4       | 4     | 10    | 21    | AGFG1, RAB3D, RAB3A, RAB22A, RABGGTA, FDPS, MVB, PMVK, IPP, MVK |
| 5       | 2     | 3     | 3     | ITGAL, SELPG, SELP |
| 6       | 1.667 | 3     | 3     | TTC30B, DYNC2H1, TTC30A |
| 7       | 2     | 3     | 3     | CHMP2B, MVB12B, MVB12A |
| 8       | 2     | 3     | 3     | ACTR3C, ARPC1A, ARPC2 |
| 9       | 1.667 | 4     | 4     | ABCB6, TAP1, SLC46A1, ABCB7 |

Table 3. Biological processes enrichment analysis of genes in cluster 1.

| Term                                                        | Genes                                      | P-value   | FDR        |
|-------------------------------------------------------------|--------------------------------------------|-----------|------------|
| Protein ubiquitination                                      | ITCH, TRIM21, RNF130, FBXO11, UBE2J1, ASB16 | 0.026×10^{-6} | 0.0138×10^{-4} |
| Negative regulation of NF-kappaB transcription factor activity | ITCH, TRIM21                             | 0.025     | 0.67       |
| Protein ubiquitination involved in ubiquitin-dependent protein catabolic process | ITCH, ANAPC1                               | 0.053     | 0.85       |
| Ubiquitin-dependent protein catabolic process               | ITCH, FBXO11                               | 0.063     | 0.85       |

Single-Cell RNA-Seq Analysis

The expression of the 6 genes (ITCH, TRIM21, RNF130, UBE2J1, FBXO11, and ASB16) regulating protein ubiquitination in patients with severe COVID-19 was evaluated using an online single-cell RNA-Seq platform. Eight cell types were identified, including CD8 T cells, CD4 T cells, B cells, monocyte, natural killer cells, macrophages, neutrophils, and dendritic cells (Figure 9A). Figure 9B shows the integration of ITCH, TRIM21, RNF130, UBE2J1, FBXO11, and ASB16 expression across different cells, and they were obviously enriched most significantly in macrophages, with a mean of 0.306. Among these genes, as shown in Figure 9C, ITCH was mostly enriched in macrophages, with a mean of 0.234; TRIM21 was expressed highly in macrophages, with a mean of 0.157; RNF130 was also mostly enriched in macrophages, with a mean of 0.874; UBE2J1 was also highly expressed in macrophages, with a mean of 0.547; FBXO11 was highly enriched in macrophages, with a mean of 0.092, while ASB16 was enriched rarely in all cell types and it was expressed mostly in CD8 T cells, with a mean...
Subsequently, Figure 9D shows the scatter plots of all the cells with ITCH, TRIM21, RNF130, UBE2J1, FBXO11, and ASB16 expression, and Figure 9E shows the scatter plots of macrophages. The above results indicated that the genes regulating protein ubiquitination in patients with severe COVID-19 could be expressed on the macrophages.

Discussion

SARS-CoV-2 has continued to remain a threat worldwide. Almost 10% to 20% of patients infected with SARS-CoV-2 progress to severe COVID-19 with pneumonia, respiratory failure, and potentially fatal situations, although most individuals infected with SARS-CoV-2 are usually asymptomatic or have mild symptoms in Wuhan, China [36]. Hence, it is important to understand the characteristics and molecular mechanisms of the biological reactions occurring in patients with severe COVID-19 thoroughly, for the development of biomarkers and therapeutic targets. In this study, we identified a network-based framework that helped us gain insight into the gene expression patterns in peripheral blood samples obtained from patients with severe COVID-19. RNA-Seq is considered significantly important in biomedical research. We used this method to identify 268 significant DEGs in patients with severe COVID-19. To characterize molecular processes, these DEGs were uploaded into the GO, KEGG, and Reactome databases to identify gene functions and signaling pathways, and PPI network analysis was performed. To focus on the core gene clusters, we detected 6 genes (ITCH, TRIM21, RNF130, FBXO11, UBE2J1, and ASB16) involved in the protein ubiquitination process that played a key role in patients with severe COVID-19.

Patients with severe COVID-19 were more likely to have underlying comorbidities, such as diabetes (7.3%), cancer (5.6%), hypertension (6.0%), and a higher fatality rate, according to the American College of Cardiology clinical bulletin [2,37]. Yang et al revealed that the most distinctive comorbidities of the critically ill adult pneumonia patients with SARS-CoV-2, who were admitted to the intensive care unit, were cerebrovascular diseases (22%) and diabetes (22%) [38]. Interestingly, in our study, we identified 17 genes that were significantly enriched in the insulin receptor recycling pathway that played a key role in regulating diabetes. This could be considered as
Evidence indicating the relationship between diabetes and severe COVID-19 and might be related to insulin receptor recycling. It has been reported that infection with Mycobacterium tuberculosis, which is also a risk factor for SARS-CoV-2, could lead to worsened respiratory symptoms [39]. The results of the enrichment analysis performed using the Reactome database revealed that the pathway of latent infection of Homo sapiens with Mycobacterium tuberculosis was involved in the development of severe COVID-19.

The MCODE plugin of Cytoscape is widely used for finding highly interconnected regions in a PPI network based on topology. Using this algorithm, 1 cluster, including 8 genes (ANAPC1, TRIM21, ASB16, RNF130, FBXO11, UBE2J1, FBXO41, and ITCH) was considered as the core gene cluster that performed important functions in patients with severe COVID-19. Then, using biological process enrichment analysis, we found that 6 genes (ITCH, TRIM21, RNF130, FBXO11, UBE2J1, and ASB16) from this cluster were highly significant in the protein ubiquitination process, which showed that ubiquitination played a role in the pathogenesis of severe COVID-19.

Figure 7. Interaction network between genes involved in protein ubiquitination and targeted miRNAs. Genes are shown in blue; miRNAs are shown in pink; miRNAs targeting more than 2 genes simultaneously are shown in green; miRNAs targeting 5 genes simultaneously are shown in yellow. There are 134 nodes and 187 edges.
Figure 8. Protein–chemical interaction network. Of a total of 70 nodes, genes are colored in yellow, chemical agents are colored in blue, chemical agents that target more than 2 genes at once are shown in green, and chemical agents that target 5 genes at once are shown in red. The interaction network has 64 nodes and 96 edges.

A major role in the biological processes at the mRNA level during severe COVID-19 in the present study. As mentioned before, the ubiquitination process would regulate immune functions and antiviral responses and also affect the pathogenesis of acute lung injury [12,13]. Therefore, it is understandable that ubiquitination would affect the severity of SARS-CoV-2 infections. Zhang et al identified 4 important proteins (USP5, IQGAP1, TRIM28, and Hsp90) that participate in ubiquitination-based host immune response modulation [40]. In our study, the core genes ITCH, TRIM21, RNF130, FBXO11, UBE2J1, and ASB16 were shown to play essential roles in the ubiquitination process in the transcriptomic profile of patients with severe COVID-19.

It was reported that the phosphorylation of the ITCH regulatory process controls the balance of T-helper (TH) 2 cytokine production by negatively regulating JUNB turnover [41]. Importantly, it was reported that the Th1/Th2 balance in COVID-19 has been linked to the outcome of the disease [42]. In addition, TRIM21 could provide the last line of defense for invading viruses by...
Figure 9. Single cell RNA-seq analysis of COVID-19 patients from online database platform. (A) Eight-cell types were identified by the cell markers; cells were clustered by the tSNE method. (B) Violin plot of the integration of ITCH, TRIM21, RNF130, UBE2J1, FBXO11, and ASB16 expression across different cells. (C) Violin plot of ITCH, TRIM21, RNF130, UBE2J1, FBXO11, and ASB16 expression across different cells, respectively. (D) Scatter plots of all the cells with ITCH, TRIM21, RNF130, UBE2J1, FBXO11, and ASB16 expression. (E) Scatter plots of macrophages with ITCH, TRIM21, RNF130, UBE2J1, FBXO11, and ASB16 expression.

acting as a sensor that intercepts antibody-coated viruses that escape extracellular neutralization and break through the cell membrane [43]. Consequently, once in contact with the Fc of the virus-bound antibody, TRIM21 triggers a coordinated effector and signaling response that prevents viral replication while inducing an antiviral cellular state; this may be in part the result of its different expression in patients with severe COVID-19. Therefore, ITCH and TRIM21 were considered to play a role in the termination of antiviral responses, which might explain why extremely severe conditions are observed in patients with COVID-19. Then, it is worth mentioning that researchers have demonstrated the participation of RNF130 in the cellular process of ubiquitin-like ligase activity in A549 cells infected with SARS-CoV-2 by the proteomic approach [44].
special concern is that the inactivation of FBXO11 contributes to increased levels of BCL6 [25]. Interestingly, Kaneko et al observed a striking reduction in Bcl-6+ germinal center B cells in spleens of acute SARS-CoV-2-infected patients [45], which might explain the limited durability of antibody responses in severe COVID-19. It has been reported that UBE2J1 regulates the interferon pathway negatively by bringing about RFX3 ubiquitination and degradation for the promotion of infection with RNA viruses [26]. Furthermore, type I and III interferons are innate cytokines that are important in the first-line defense against viruses, including SARS-CoV-2 [46]. In gastric cancer, ASB16 could induce the NF-kB pathway to promote tumor proliferation [27]. Kircheis et al showed that the NF-kB pathway might be a potential target for the treatments of patients at a critical stage of COVID-19 [47]. Overall, these genes play an important role in regulating COVID-19 pathogenesis: UBE2J1 may serve as the first line of defense of SARS-CoV-2. ITCCH might regulate the Th1/Th2 balance, FBXO11 could contribute to the antibody responses, ASB16 might induce the NF-kB pathway, and TRIM21 may provide the last line of defense to prevent SARS-CoV-2 replication.

Furthermore, to obtain more information regarding the mechanisms for ubiquitination development in patients progressing to severe COVID-19, transcription factors and miRNAs were detected at the post-transcription level. In this regard, we identified 26 transcription factors, including FNIC, FOXA1, YY1, GATA2, MET2A, and FOXC1 transcripts, from more than 2 genes. Surprisingly, this result was in accordance with those observed after the regulatory network analysis of lung epithelial cells in COVID-19 by Islam et al, in which significantly important transcription factors, such as FOXC1, GATA2, YY1, FOXL1, and NFKB1, were identified [48]. YY1 is known to be involved in viral diseases, such as those involving activation of HIV-1 replication [49], and promotion of oncogenic HPV [50]. GATA2 could regulate severe pulmonary alveolar proteinosis and hematologic disorders [51]. Interestingly, Wang et al reported that GATA2 was upregulated with the decrease of lymphoid progenitors and the increase of immature myeloid progenitors of the bone marrow in patients with severe COVID-19 [52]. This might indicate that GATA2 could be a key transcription factor in patients with severe disease and may be involved in the relationship between the increase in lymphopoenia observed with an increase in myelopoesis. Several miRNAs were identified at the post-transcriptional level. Among these, mir-27a-3p and hsa-miR-1-3p were targeted by 5 genes. Mir-27a-3p, which regulated M2macrophage polarization [53], also functions via a negative-feedback mechanism in inhibiting lung fibrosis [54]. Then, hsa-miR-1-3p, which expressed differently in small cell lung cancer [55], was considered to contribute to the pathology and progression in idiopathic pulmonary arterial hypertension [56]. Also, mir-17-5p [57], mir-98-5p [58], and mir-146a-5p [59] were considered to be associated with cancers. In addition, some of these miRNAs, such as miR-22-3p, which was involved in human umbilical cord blood-derived mesenchymal stem cell development [60], and miR-146a-5p, which regulated LPS-induced apoptosis and inflammation [61], played a role in acute lung injury. In addition, miR-17-5p and miR-142-5p were detected in the peripheral blood of COVID-19 patients [62]. Finally, several chemical agents showed potent therapeutic action and enabled us to identify potential antagonists to COVID-19. For example, quercetin exhibits antiviral and anti-inflammatory functions upon the entry of the influenza A virus (IAV). Furthermore, the chemical component cyclosporine demonstrates the ability to inhibit the replication of RNA viruses, including SARS-CoV and MERS-CoV [63]. Importantly, the report listing the therapeutic options against 2019-nCoV that was published last year included cyclosporine [64]. Surprisingly, copper sulfate was found to be significantly correlated with 5 genes, ITCCH, TRIM21, RNF130, FBXO11, and ASB16, for protein ubiquitination in patients with severe COVID-19. Hongrui et al demonstrated that copper sulfate treatment can induce pulmonary fibrosis through the activation of epithelial-mesenchymal transition induced by the TGF-β1/Smad and MAPK pathways [65], which indicated copper sulfate can cause chronic pulmonary inflammation. We can look forward to further research on the positive or negative roles that copper sulfate plays in the acute and severe pulmonary inflammation stage, such as with severe COVID-19. Lastly, using an online single-cell RNA-Seq database platform, we evaluated the expression of the genes ITCCH, TRIM21, RNF130, UBE2J1, ASB16, and FBXO11 to search for the location of these genes controlling protein ubiquitination, and the results validated the gene expression was highly enriched in macrophages. Macrophages are plastic immune cells necessary for maintaining homeostasis and controlling inflammation, and Lin et al hypothesized that the ubiquitination status regulated by ITCCH contributes to macrophage polarization [66]. Therefore, the results in our study indicated that ubiquitination in macrophages might be a potential risk route of severe COVID-19 infection. However, because we used the bioinformatics evidence through an independent single-cell RNA-Seq dataset online, additional histological techniques will be helpful to validate these findings and strengthen our conclusion. After all, there were some limitations to this study. It would be much more precise to validate the transcriptomics data by proteomics analysis of the same cohort; however, the samples used in this experiment were already used up. Furthermore, we searched for other published papers on whole transcriptome sequencing of peripheral blood from patients with COVID-19. By calculating the raw data in the research of Overmyer et al, we found ITCCH, TRIM21, RNF130, UBE21, ASB16, and FBXO11 were all altered at the mRNA level [67], and the supplementary material of Li et al showed that the changes of ITCCH, TRIM21, RNF130, and FBXO11 were significant at the transcriptome level [68], which could indirectly prove the plausibility of our results.
Conclusions

In the present study, we aimed to provide insight into the potential pathogenic processes in patients with severe COVID-19 that might facilitate the development of novel and effective clinical treatment targets. We first determined 268 DEGs in patients with severe COVID-19. Using these DEGs, we identified key molecular processes and signaling pathways. Then, based on the PPI network, we further identified the most significant gene cluster, which was found to be involved in protein ubiquitination. The 6 genes ITCCH, TRIM21, RNF130, FBXO11, UBE2J1, and ASB16 were identified to be involved in protein ubiquitination at the transcriptome level. Furthermore, the transcription factors FNIC, FOXA1, YY1, GATA2, MET2A, and FOXC1 and the miRNAs hsa-miR-1-3p and hsa-miR-27a-3p regulated the greatest number of genes involved in protein ubiquitination. Subsequently, we found that copper sulfate may be a potent chemical agent for protein ubiquitination biological processes in severe COVID-19. Finally, macrophages might be the major localization for genes regulating protein ubiquitination in patients with severe COVID-19. Overall, protein ubiquitination could play a role in crucial functions, and our findings will contribute to the knowledge regarding the biological properties in severe COVID-19 progression and contribute to the creation of new therapeutic approaches that will help us make progress against the pandemic.

Ethics Statement

This study was approved by the Ethics Committee of Nanfang Hospital, Southern Medical University (approval no: NFEC-2020-033) and the Ethics Committees from the collaborated centers.

Declaration of Figures’ Authenticity

All figures submitted have been created by the authors, who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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