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GENE REGULATORY NETWORK OF COVID-19 IN DRUG INDUCED VERO CELLS

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ABSTRACT: Covid-19 is a pandemic disease affecting hundreds of million people across the globe. Until recently, only a three quarter of the world population received the first dose of a Covid-19 vaccine. Novel therapeutics is still being investigated to fulfil the unmet needs in the healthcare system. Studies using co-expressed gene clusters and regulatory networks are less explored. Therefore, in the present study, RNA sequencing expression data was used to identify optimal co-expressed gene clusters and the regulating transcription factors and kinases. Viruses related human protein-protein interactions were integrated and built a gene regulatory network to analyse and reveal hubs genes, molecular mechanisms and cellular pathways of Covid-19 disease. Further, virtual screening of drugs targeting the regulatory genes unveiled mTOR inhibiting Everolimus as the best candidate for therapeutic investigations in Covid-19 disease.

Keywords: Cluster Analysis, Gene regulatory networks, RNA-Seq, SARS-CoV-2, Therapeutics.

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1. INTRODUCTION

Covid-19 is a pandemic disease first identified in 2019 from its causative agent SARS-CoV-2 [1]. Though the emergency phase of the disease is over, it is continuously spreading across the globe. It succumbed more than a seven million people and affected more than a half billion people around the world [WHO Coronavirus Disease Dashboard, May 2024]. To combat with the disease, several vaccines are being development to prevent infection and spread of Covid-19 [2,3]. Some of the

recommended vaccines for Covid-19 are AstraZeneca/Oxford vaccine, Johnson and Johnson, Moderna, Pfizer/BionTech, Sinopharm, Sinovac, COVAXIN, Covovax and Nuvaxovi [2,3]. However, only three quarter of the world population and nearly two quarter population of the low-income countries received at least single dose (https://ourworldindata.org/covid-vaccinations) [4] until recently. Several drugs are also being investigated for re-purposing in Covid-19 therapy [5-7]. Remdesivir (Veklury) is the first FDA approved drug for use in Covid-19 infected adults and paediatric patients. The drug interacts directly with viral RNA polymerase and prevents its multiplication [8]. Recently, drug combinations such as Remdesivir plus Baricitinib are shown to be superior in reducing recovery time for Covid-19 patients [9-11]. The other emergency use FDA approved drugs include the virus targeting monoclonal antibodies such as REGEN-COV, Sotrovimab and Bamlanivimab plus Etesevimab and immune modulators such as Baricitinib (Olumiant) and Actemra (Tocilizumab). Nevertheless, the development of novel therapeutics for Covid-19 is still significant in view of unmet needs in the healthcare system. Experimental and clinical studies are proved indispensable in gaining deep insights into the understanding of the SARS-CoV-2 and its pathology [12,13]. The transcriptomic analysis under infected and Remdesivir treated condition would determine the disease associated biochemical reactions, pathways and its therapeutic targets. Recent studies successfully revealed metabolic reprogramming and disease therapies [14]; however, the regulatory network of elicited gene transcription factors and kinases is less explored. Such a network would reveal unprecedented information about the disease and its regulatory relationships. Therefore, the present study identifies co-expressed genes clusters, their transcription factors and kinases. It builds a gene regulatory network to uncover functional associations and disease pathways. Further, it evaluates significant targets for drug re-purposing in Covid-19 therapy.

2. MATERIALS AND METHODS

In the present study, an expression profiling by high throughput RNA sequencing experiment, GSE165955, was retrieved from NCBI GEO for implementing the cluster analysis. The experiment was performed on Chlorocebus sabaeus Vero E6 cells with three samples each for control (mock-infected) cells, control plus Remdesivir treated cells, SARS-CoV-2 infected cells and SARS-CoV-2 infected plus Remdesivir treated cells [14]. Expression profiles (FPKM) of these samples were refined by excluding transcripts with no expression or gene symbol and grouped by cell-wise. Log two transformation, quantile normalization followed by optimal co-expressed gene clustering was performed using clust algorithm [15]. Transcription factors over-represented in co-expressed gene clusters were extracted from ARCHS4, ENCODE, GEO, Enrichr and TRRUST transcription factor databases [16-19] by employing R enrichR package [18] with a p-value <= 0.01. Kinases over-represented in the extracted transcription factors were obtained from ARCHS4, CMap [20] and GEO kinase databases by employing enrichR with a p-value <= 0.01. Experimentally verified, literature

mined and viruses related human protein-protein interactions (PPIs) were retrieved from VirusMINT database [21], and then filtered with intact-miscore minimum of 0.4 to obtain confident PPIs. These confident PPIs were further processed to include interactions only among transcription factors, kinases and co-expressed genes of each of the clusters. A gene regulatory network for each of the clusters was built from the confident PPIs by using R igraph package. Further, the networks of all the clusters were merged to get a complete gene regulatory network. Duplicated and isolated interactions were removed from the complete gene regulatory network and a core regulatory network was obtained. Topological properties of the core regulatory network were determined and the top five percent high degree nodes considering as hubs were obtained. Gene Ontology (GO) terms of biological processes, molecular functions and cellular components [22] for the nodes in core regulatory network were identified using enrichR with a p-value cut off of 0.01. FDA approved drugs targeting hubs and genes of pathways in cancer were extracted from DrugBank database [23]. Virtual screening of the drugs with 6M71 PDB structure of NSP12 protein (SARS-CoV-2 RNA polymerase II) as target was performed by using Autodock Vina [24].

3. RESULTS AND DISCUSSION

Co-expression clusters

The clust produced two distinct clusters of co-expressed genes, C0 and C1, which were exclusive, least dispersed and had stable biological annotations [15] (**Figure 1**). It was observed from the clusters that different regulation of co-expressing genes occurred in the infected samples while being stable in control and Remdesivir treated samples. Further, the SARS-CoV-2 genes, namely ORF1ab, S, ORF3a, E, ORF6, ORF7a, ORF7b, ORF8 and N were observed only in the second cluster (C1). It was observed that the number of co-expressed genes in C0 was higher than the number of co-expressing genes in C1. However, the number of transcription factors and kinases over-represented in co-expressed genes of C0 (730 and 1832 respectively) was less than that of the C1 (337 and 1599 respectively).

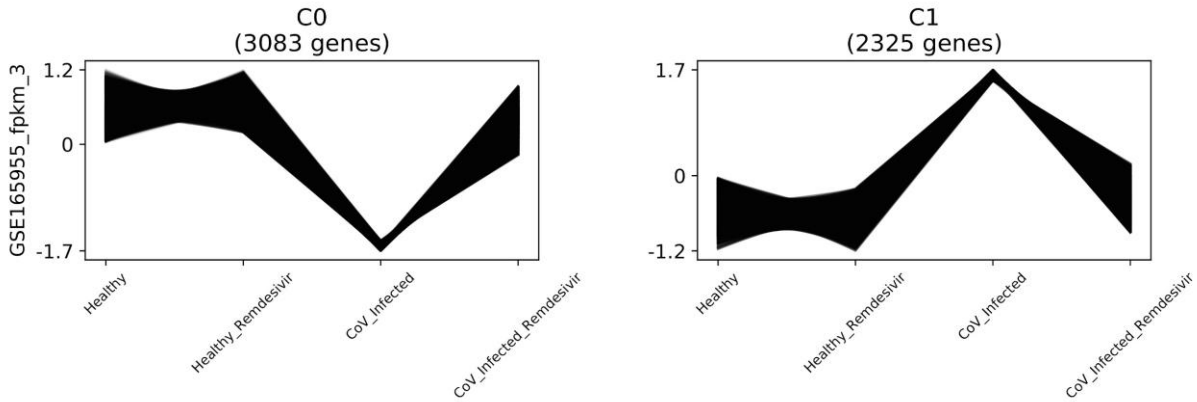

Figure 1. M-N plot of optimal co-expressed gene clusters. In cluster C0, the genes are negatively co-expressed only in the SARS-CoV-2 infected state while in cluster C1, the genes are positively co-expressed only in the SARS-CoV-2 infected state.

Gene regulatory networks

The PPIs obtained from VirusMINT database were already known involved in viral infections and were validated by different experimental techniques such as affinity chromatography, co-immunoprecipitation, foot-printing, pull down or hybrid assays and recorded in multiple research articles. Gene regulatory network of the confident PPIs of each cluster was built as discussed in the methods section and a core gene regulatory network containing transcription factors, kinases and the co-expressed genes was extracted and visualized as shown in the **Figure 2** below. The network contained 2380 interactions among 792 nodes. Further, there were 20 fully connected components where nodes interact among themselves. The hub nodes in the network with high degree were transcription factors such as TP53 (42), JUN (31), HDAC2 (28), SF3A2 (21) and SMAD4 (15), kinases such as EGFR (36), SRC (28), ERBB2 (20) and ERBB3 (20) and co-expressed gene products such as DLG4 (24) and YWAZ (15).

Figure 2. A gene regulatory network in Covid-19. Gray coloured nodes are transcription factors and light blue coloured nodes are kinases. Positively and negatively co-expressed gene nodes in SARS-CoV-2 infection are coloured in a palette of maroon and orange colours respectively. The intensity of the colours is proportional to their absolute co-expression value.

Functional annotations

GO terms of the nodes in the core regulatory network revealed that the genes were mostly the constituents of membrane and non-membrane bound organelles. These genes were associated with positive or negative transcriptional regulation of genes by RNA polymerase. Further, they were involved in cellular protein modifications and phosphorylations. It was observed that transcription regulatory sequence specific DNA binding and protein serine/threonine and tyrosine phosphorylations were their mode of actions as seen in **Figure 3**. Disease pathways associated with the genes in the network were recorder. It was observed that the genes were significantly related to the diseases such as human cancer, hepatitis and viral infections.

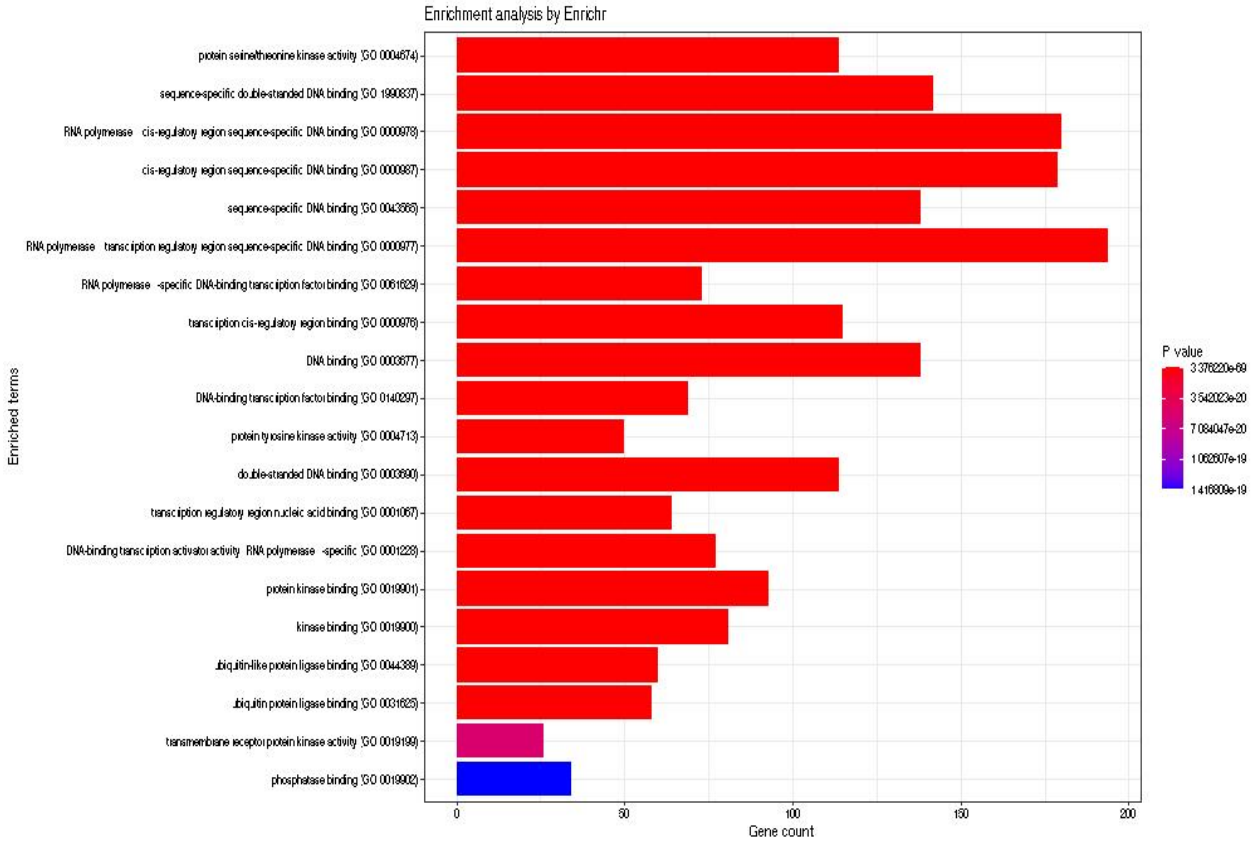

Figure 3. Enrichment analysis of core genes. The genes in the core regulatory network are enriched in biological process, molecular function and cellular component terms of GO.

Potential drug targets

Top ten ligands with the highest binding affinities were considered as potential candidates in Covid-19 therapy and is shown in the **Table 1**.

Table 1. List of top ten approved candidates for NSP12 protein.

Generic Name	Binding Energy (kcal/ mol)	Target Gene	Properties
Everolimus	-10.8	MTOR	Kinase
Temsirolimus	-10.7	MTOR	Kinase

Vinblastine	-10.5	JUN	Transcription factor
Sulfasalazine	-9.5	PPARG, CHUK	Kinase
Loperamide	-9.2	CALM1	Co-expressed Gene
Adapalene	-8.9	JUN	Transcription factor
Adapalene	-8.9	RARA, RXRA, RXRB, RXRG	Kinase
Ingenol mebutate	-8.6	PRKACA, PRKCA	Kinase
Ripretinib	-8.5	KIT, PDGFRA, PDGFRB	Kinase
Flunarizine	-8.1	CALM1	Co-expressed Gene
Bosutinib	-8.1	CDK2	Kinase
Imatinib	-8.1	ABL1, KIT, NTRK1, PDGFRA, PDGFRB	Kinase

From the table, it is clear that the drugs such as Everolimus and Temsirolimus which target mTOR gene product have the best binding affinities. Everolimus targeting mTOR had the best binding affinity making two polar contacts each with TYR32 and GLN773 and one polar contact each with LYS47 and THR710 alpha helical amino acids of the 6M71. Binding of Everolimus with 6M71 PDB structure can be visualized in the **Figure 5**.

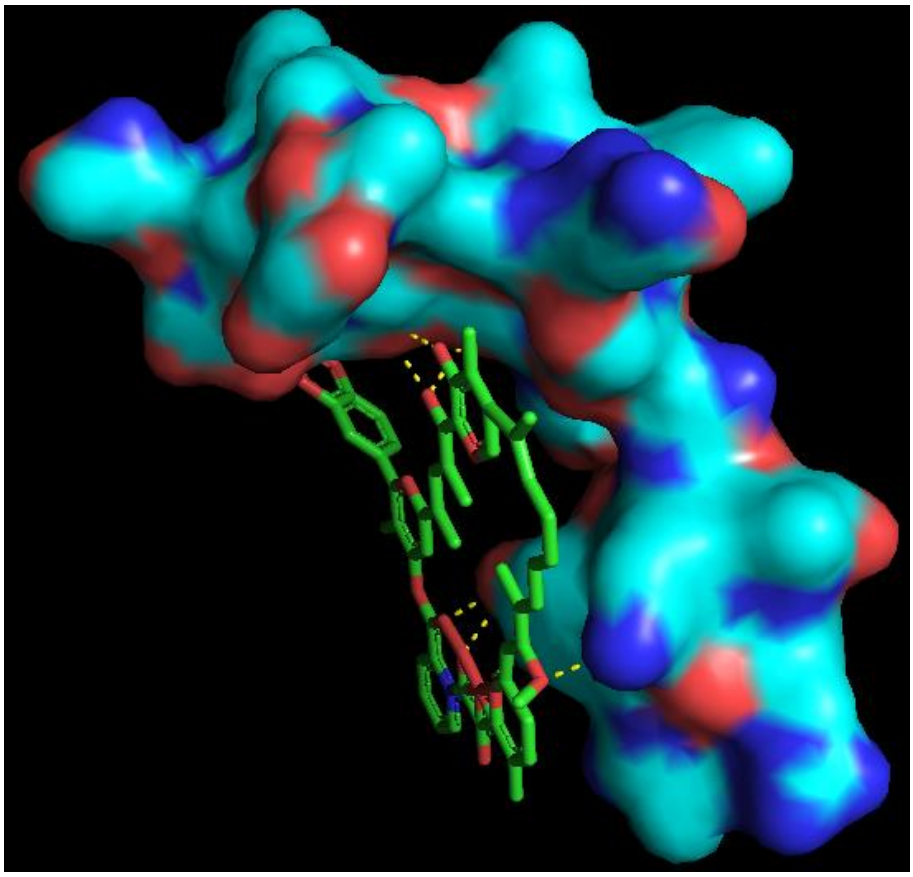

Figure 5. Drug-gene interaction of Everolimus. Yellow coloured dashed lines indicate the polar contacts of Everolimus ligand with the 6M71 target protein structure.

Sirolimus, with a pro-drug Temsirolimus, has the similar role as Everolimus [25,26]. Imatinib is another drug acts by inhibiting tyrosine kinases [27] such as ABL1, KIT, NTRK1, PDGFRA and PDGFRB present in the core gene regulatory network. Both Sirolimus and Imatinib are actively being tested for Covid-19 treatment [28,29], Everolimus and other potential drugs of this study may need further investigating for novel therapeutic intervention strategies against SARS-CoV-2 infection and disease.

Cluster2hub

Cluster2hub, a GitHub tool was developed to identify hubs from RNA-seq gene expression data. It is an open source, user friend tool available at https://github.com/DHAMMAPALB/cluster2hub. It can be used to automatically detect clusters and enrich transcription factors and kinases in co-expressing genes of each of the clusters. It can also build a gene regulatory network for each cluster and identify functional annotations and pathways. Further, a user defined top interacting nodes can be extracted and potential drug candidates are revealed from the drug-target interactions database.

DISCUSSION

Remdesivir was the first FDA approved drug for emergency use in the treatment of Covid-19. Cluster analysis of the high-throughput sequencing experiment consisting of Remdesivir treated controls and infected Vero cell samples produced distinctly regulated optimal co-expressed genes. Further, the existence of viral genes in the co-expression gene clusters suggested coordinated up-regulation of the host and SARS-CoV-2 genes in Covid-19 infection. Co-expressed genes were observed to have different regulatory relationships and were significant enriched with several transcription factors and the related kinases. Gene regulatory network involving transcription factors, kinases and co-expressed gene products provided invaluable information in understanding the biological processes, molecular mechanisms and cellular pathways of Covid-19 disease. Further, the high interacting nodes could server as potential targets in therapeutic inventions for Covid-19 disease. Everolimus is a derivative of Rapamycin (Sirolimus) and it was suggested to inhibit SARS-CoV-2 infection using an mTOR inhibition with an unknown mechanism [25]. In the core gene regulatory network, mTOR is a transcription factor that interacts with four different genes, viz. YWHAZ, BCL2L1, MTMR3 and AKT1. YWHAZ had the negative co-expression value while BCL2L1 and MTMR3 had the positive co-expression value suggesting that the mTOR's ability to differentially regulate many genes. Its interplay with AKT1 kinase, a hub gene, implies its co-ordination with several transcription factors and kinases for down-stream gene regulations. Further, recent studies highlighted the merit of exploring mTOR inhibitors for Covid-19 therapy due to its promising effects observed in patients with severe Influenza A (H1N1) pneumonia and acute respiratory failure [30,31,32].

4. CONCLUSION

The present study revealed Everolimus as the potential targets for SARS-CoV-2 RNA polymerase II and also emphasized on the utility of mTOR inhibitors for Covid-19 related pharmaceutical research.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals or humans were used for the studies that are based on this research.

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

None declared.

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