IDENTIFICATION OF HUB GENES AND NEUTROPHIL REGULATORY PATHWAY IN PRIMARY ANTIPHOSPHOLIPID SYNDROME

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MASTER OF SCIENCE

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by

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CANDIDATE'S DECLARATION

I Palak hereby certify that the work which is being presented in the thesis entitled "**Identification of hub genes and neutrophil regulatory pathway In Primary antiphospholipid syndrome"** in partial fulfillment of the requirements for the award of Master of Science in Biotechnology, submitted in the Department of Biotechnology , Delhi Technological University is an authentic record of my own work carried out during the period from 2024 to 2026 under the supervision of Prof Yasha Hasija.

The matter presented in the thesis has not been submitted by me for the award of any other degree of this or any other Institute.

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Certified that **Ajit Gautam** (enroll) has carried out their search work presented in this thesis entitled "**Identification of hub genes and neutrophil regulatory pathway In Primary antiphospholipid syndrome**" for the award of **Master of Science in Biotechnology** from Department of Biotechnology, Delhi Technological University, Delhi, under my supervision. The thesis embodies results of original work, and studies are carried out by the student himself/herself and the contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University/Institution.

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Ajit Gautam

Abstract

PAPS is a autoimmune disorder characterized by presence of aPL, which are main cause of repeated thrombotic complications. Earlier Antiphospholipid Syndrome (APS) was mainly detected in Individuals with pre-existing autoimmune pathologies such rheumatoid arthritis or systemic lupus erythematosus. One of the key mechanisms for thrombosis in PAPS is reported to be NETosis. This study focuses on identification of key hub genes and constructing a microRNA (miRNA)–messenger RNA (mRNA) in PAPS, primarily focusing on Neutrophil cells. Differentially expressed mRNA (DE-mRNA) in patients were taken from previous studies and systemically screened using differential gene expression data of GSE102215 dataset from GEO. A Total 2428 DEGs were obtained of which 955 were overexpressed and 1473 underexpressed, among the DEGs 2034 were found to be protein coding. The PPI network analysis disclosed top 10 consensus hub genes (STAT2, DDX58, MX1, IFIT3, IFIH1, ISG15, IRF7, EIF2AK2, STAT1, IFI35) identified by Cytoscape software using Maximal Clique Centrality (MCC) and Degree to rank nodes in the PPI interactions based on local topological features. The pathway and functional enrichment have done using Enrichr. 5 DE-miRNAs were obtained from published literature and their targets gene were identified via TarBase v9.0, out of 9782 miRNA target genes 1209 overlap with DEGs. A network between miRNA and hub genes was constructed which demonstrated most hub genes are regulated by hsa-mirna-146. In conclusion, several hub genes linked to PAPS were identified. New information on the underlying mechanisms of PAPS is provided by miRNA-mRNA network regulatory network that may be pertinent to pathogenesis of PAPS.

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CHAPTER 1

INTRODUCTION

1.1 Background

PAPS is an autoimmune disorder known for high risk of recurrent thrombosis and Obstetric complications and is characterized by the presence of aPL [1]. While PAPS was historically investigated as secondary disorder in patients who already suffered from disorders like rheumatoid arthritis or systemic lupus erythematosus, through molecular profiling it was confirmed that PAPS is a standalone entity with its own unique biomarkers and pathogenesis [2]. PAPS is a major cause of acquired thrombosis, known for its ability to promote thrombotic events in both venous and arterial circulatory pathways. Studies show that about 9% to 10% APS patients die within 10-year time frame [3]. PAPS remains clinically relevant due to recurrent thrombotic events, pregnancy loss and complication affecting multiple organ systems.

1.1 About Primary Antiphospholipid Syndrome

Primary antiphospholipid syndrome (PAPS) is an autoantibody mediated thrombo-inflammatory autoimmune disorder, primarily anti-β2-glycoprotein. Recent studies have highlighted the key role of interferon signalling pathway and the innate immune response in disease progression by disruption of anticoagulant pathways, complement activation and innate immune cell activation [4]. Two-hit hypothesis is a foundational model for thrombotic PAPS, presence of aPL signifies the first hit and created a pro-thrombotic environment, second hit is triggered by events such as infection and pregnancy that leads to blood clot formation [4]. During the first hit multiple cells are primed including neutrophil which emerged as one of the key players in PAPS related thrombosis [5]. Neutrophils contribute to clot formation through the release of extracellular

chromatin structures, commonly referred to as NETs (neutrophil extracellular traps). Nets provides a scaffold for the fibrin and platelets to aggregate, turning localized immune response into a thrombotic crisis.

1.3 Research gap

Even though we know neutrophils are one of the main causes, we still lack a comprehensive understanding of the molecular interaction and regulatory networks involved. The molecular complexity is further increased by microRNA (miRNA) which act as a regulator for gene expression, in many immune disease has-mir-146a is known to inhibit or enhance the inflammatory signalling [6]. Although certain genes and pathways have been implicated, there is a lack of integration of extensive transcriptomic data to pinpoint essential regulatory genes that coordinate these processes.

- There are no integrated transcriptomic studies specifically addressing neutrophil-related gene expression in PAPS.
- Identification of limited key regulatory hub genes associated with PAPS pathogenesis.
- Poor knowledge of miRNA-mediated regulation of inflammatory and immune signaling pathways in PAPS.
- Limited exploration of interferon signalling pathways and their role in neutrophil activation.
- No miRNA–hub gene interaction networks in PAPS studies.
- Very few bioinformatics studies combine together DEG analysis, PPI networks, hub gene analysis and miRNA interactions.

1.4 Objectives

This study aims to give meaning full insight on pathogenesis mechanism of PAPS and identify potential biomarkers and therapeutic target for further research.

- To retrieve and analyse complete genome of 24 strains of Alcaligenes faecalis
- Genome annotation for identification of coding sequence and functional genes.
- To identify and compare Antibiotic resistance genes with 24 strains using database.
- To perform pan genome analysis to identify core and accessory genes.

- To study evolutionary relationship among strain through core genome phylogenetic tree construction
- To correlate genomics features with habitat and with resistance profile.

CHAPTER 2

LITERATURE REVIEW

2.1 Primary Antiphospholipid Syndrome (PAPS)

PAPS is associated primarily with thrombosis and pregnancy related complications. Historically, APS has been studied primarily as a secondary phenomenon related to autoimmune diseases such as SLE. studies, have demonstrated that PAPS is a separate clinical entity with distinct molecular and immunologic characteristics. The disease is strongly associated with aPLs including anti-β2-glycoprotein I antibodies. These autoantibodies cause damage to the endothelium, activate the platelets, and induce abnormal coagulation, which ultimately increases the risk of thrombus formation.

2.2 Pathophysiology and Two-Hit Hypothesis

One of the most accepted explanations for thrombosis in PAPS is the "two-hit hypothesis". In this theory, the first hit is the persistent presence of antiphospholipid antibodies establishing a prothrombotic state. Further triggers such as infection, oxidative stress, surgery or pregnancy then initiate thrombotic events. Studies show that in this process, several immune and inflammatory pathways are activated. PAPS patients experience vascular inflammation and thrombosis, which are caused by endothelial dysfunction, platelet aggregation, complement activation and inflammatory signalling.

2.3 Role of Neutrophils and NETosis in PAPS

In recent years, neutrophils have been becoming more widely accepted as important contributors to the pathogenesis of PAPS. NETs are web-like structures outside of the cell made of chromatin fibers, histones, and antimicrobial proteins. NET formation is a beneficial process during infections to trap pathogens. However, excess NETs release may contribute to thrombosis and inflammation. NETs act as a scaffold for platelet and deposition of fibrin to promote clots. Antiphospholipid antibodies have also been shown to directly activate neutrophils, leading to increased production of NETs and vascular injury. Therefore, neutrophil-mediated inflammatory responses are considered to be important in the progression of PAPS.

2.4 Interferon Signalling and Immune Activation

Interferon signalling pathways have been pointed out as an important driver of autoimmune and inflammatory diseases. Type I interferons are involved in regulating innate immune responses, inflammatory signaling and immune cell activation. In PAPS patients, 49% patients have reported an increased expression of interferon-stimulated genes, suggesting the involvement of interferon signalling in the pathogenesis of the disease. Interferon-induced activation of neutrophils further enhances inflammatory responses and NET formation, thus contributing to thrombosis. However, the precise molecular interaction of interferon signalling pathways with neutrophil-associated genes in PAPS remains poorly understood and warrants further investigation.

2.5 MicroRNAs and Gene Regulation in PAPS

miRNAs are involved in several processes such as immune regulation, inflammation, apoptosis and thrombosis. Several miRNAs have been reported in autoimmune diseases due to their involvement of inflammatory signaling pathways. Since one miRNA can regulate multiple genes simultaneously, the study of miRNA-gene interactions might be more suitable to gain insight into the molecular mechanisms of PAPS. Furthermore, miRNAs can serve as biomarkers and therapeutic targets in autoimmune diseases.

2.5 Need for the Present Study

Although several studies have investigated thrombosis and immune dysregulation in APS, there is still little information on the neutrophil-related molecular mechanisms specifically in PAPS. Additionally, few studies explored the regulatory relationship between miRNAs and hub genes related to interferon signaling and neutrophil activation. Thus, the present study aims to perform an in-silico transcriptomic analysis using the GSE102215 dataset to identify hub genes and analyze miRNA-gene interactions associated with PAPS. The results of this study may contribute to the understanding of PAPS molecular pathogenesis and to identify biomarkers and targets for future research.

CHAPTER 3

METHODOLOGY

3.1 Data Acquisition

Gene expression data of PAPS was retrieved from GEO database using the keywords “Primary antiphospholipid syndrome”, “Homo sapiens [porgn: txid9606]” and “RNA-seq” [7]. After systematic review RNA expression profile dataset GSE102215 was downloaded. The data set included 9 healthy cases and 9 PAPS cases. The RNA dataset is freely available online at NCBI GEO datasets, and 5 DE-miRNA dataset was obtained from previous studies0[8] .

3.2 Identification of DEGs

We used GEO2R analysis platform to look at the differences in gene expression between PAPS and healthy samples, Cutt-offs were applied and genes with P-adjusted value (P.adj) below 0.05 and log fold change (logFC) of > 1 and < -1 were taken as significant DEGs [9]. A volcano plot was used for significant DEGs visualization. DEGs were further curated to obtain 2034 DE-mRNA using Biomart which were used for downstream analysis.

3.3 Functional Characterization of Genes

To investigate the function of the identified DEGs, we carried out enrichment analysis utilizing Reactome and KEGG pathways [10]. Moreover, Gene ontology was done for all protein coding genes, which categorizes genes into (BP),

(CC), (MF) [11], [12]. The Enrichr web-based platform was used for all of the enrichment analysis [13], [14], [15].

3.4 Cluster Analysis of PPI Network

Module analysis was done with Molecular Complex Detection (MCODE), a plugin for Cytoscape, to find genes in the PPI network that are very closely related to each other [16]. The most significant cluster were selected for further analysis using Biological Process and pathway analysis to understand their biological relevance.

3.5 DE-miRNA Target Prediction and miRNA–mRNA Network

To identify possible regulatory mechanisms between miRNA and genes, investigations were conducted. We used TarBase v9.0 to find experimentally validated target genes of DE-miRNA. The resulting miRNA–hub gene pair was used to construct a regulatory network.

3.6 Data Visualization

All graphical representations were done using R studio, including volcano plot, enrichment plots and network visualization. The box plot for GSE102215 was obtained from GEO2R and Ven diagrams were created using Venny 2.1 a web-based tool [17], [18]

CHAPTER 4

RESULTS

4.1 Identification of Differntionally Expressed Genes

Differential expression analysis was conducted to compare the gene expression profiles of APS samples with those of healthy controls. Using the set parameters, a total of 2,428 genes displayed a difference expression profile compared to healthy control, of which 955 were overexpressed and 1473 underexpressed. Among the 2428 DEGs 2034 were confirmed to be DE-mRNA with the help of the Ensembl Biomart [19]. The boxplot demonstrated that the distribution was similar and all samples had the same median expression levels. There were no major batch effects or outliers, which means that normalization was successful and the data quality was of high, as showed in Fig. 1A. The 2428 genes in GSE102215 dataset were plotted, and the green and red dots represent the down and up-regulated genes, among these IL6 and C4BPA showed the highest fold change, as showed in Fig. 1B.

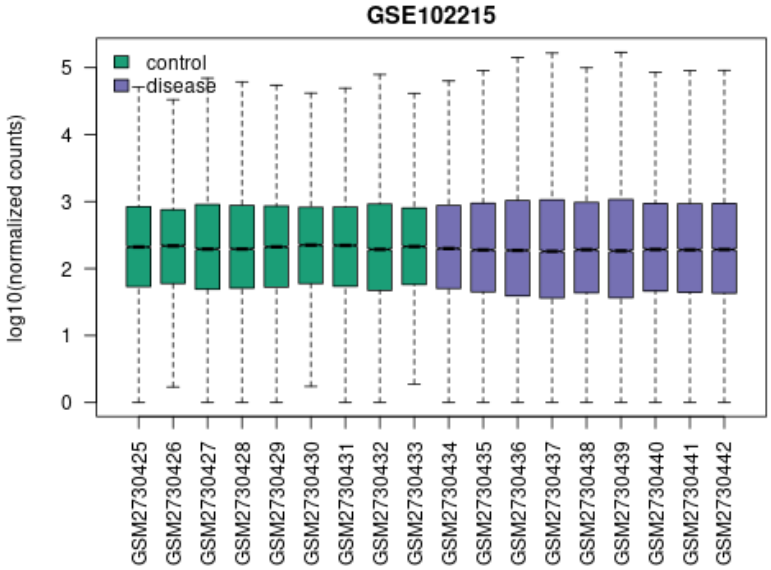

Fig.1. The value distribution of the chosen samples in GSE18606. The green boxes show normal samples, and the blue boxes show cases of PAPS. The black lines show the midpoint of each data set, and the arrangement of the data shows how standardized it is.

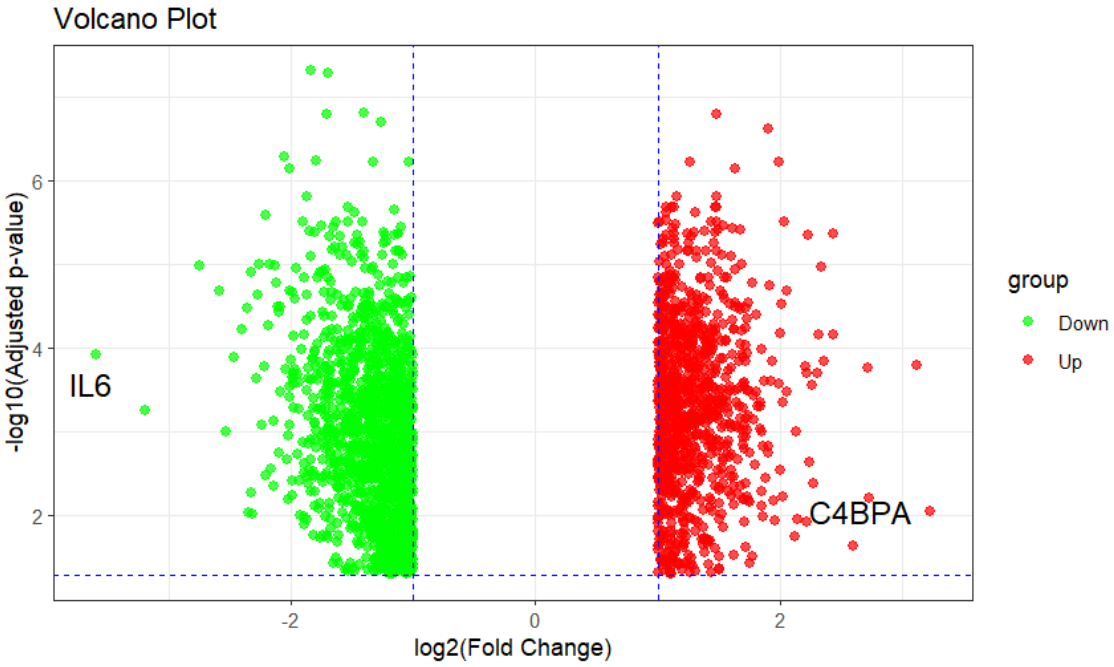

Fig. 2. The volcano plot of the previously determined DE-mRNAs in GSE18606. Volcano plot for finding DE-mRNAs. Red means upregulation, and green means downregulation.

4.2 Functional Profiling of DE-mRNA

Gene ontology (GO) BP revealed that these 2034 DE-mRNAs were markedly enriched in diverse functions, such as 'defence against virus' and 'cytokine mediated signalling' (Fig. 3A). "Ficolin-1-rich Granule" and Membrane processes were the highly enriched terms for GO CC analysis (Fig. 3C). MHC class 1 activity, Ubiquitin like protein ligases binding were among the enriched terms of GO MF analysis (Fig. 3B). For Reactome pathway analysis, top enriched terms were Immune system, Neutrophil dysregulation, Innate immune system, Interferon Alpha Beta signalling and Cytokine Signalling (Fig. 3D). Viral protein interaction, Antigen processing are among the enriched KEGG entries shown in (Fig. 3E).

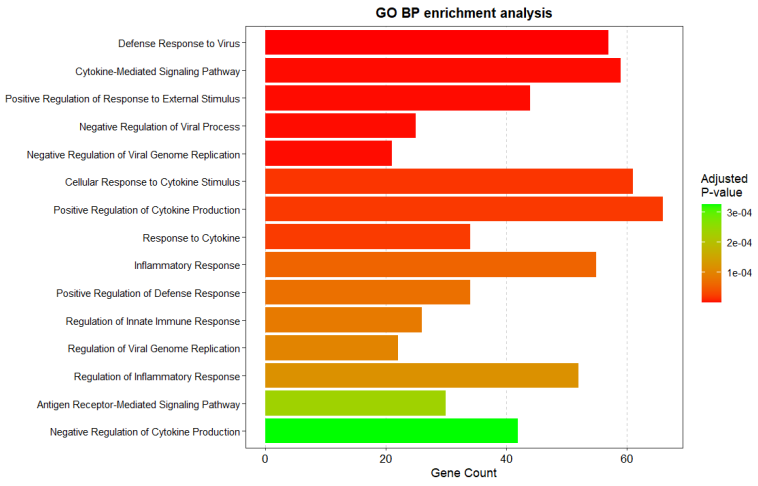

Fig. 3. (A) The enrichr database conducted GO BP functional annotation analysis and of these 2034 DE-mRNAs.

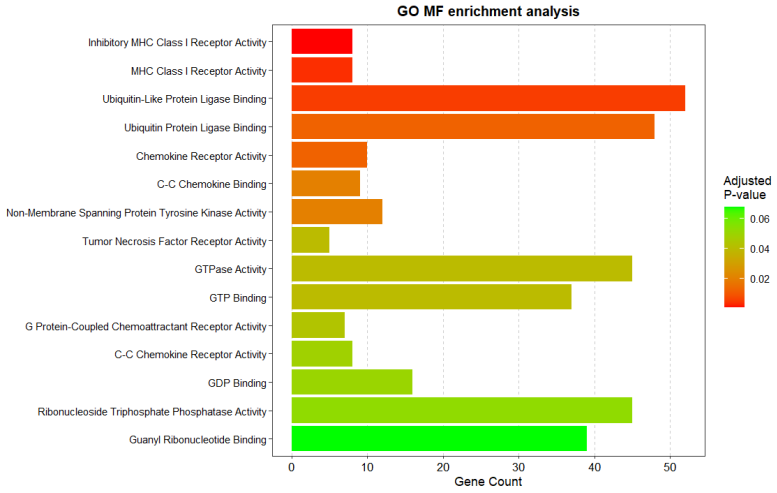

Fig. 3. (B) The enrichr database conducted GO MF functional annotation analysis and of these 2034 DE-mRNAs.

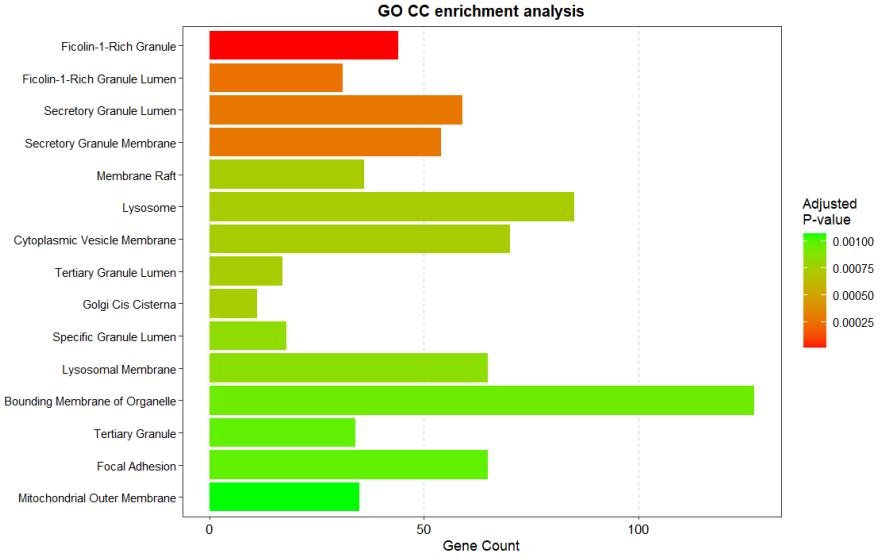

Fig. 3. (C) The enrichr database conducted GO CC functional annotation analysis and of these 2034 DE-mRNAs.

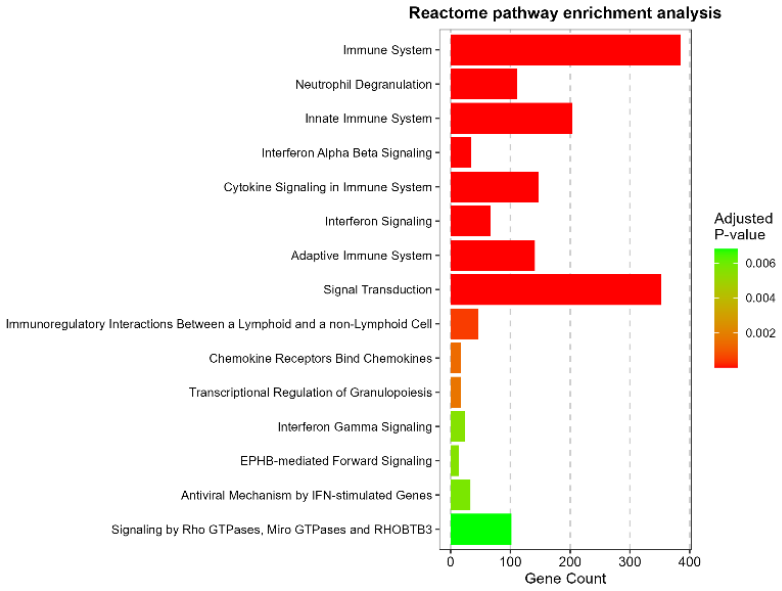

Fig. 3. (D) Reactome pathway analysis of these 2034 DE-mRNAs of these 2034 DE-mRNAs.

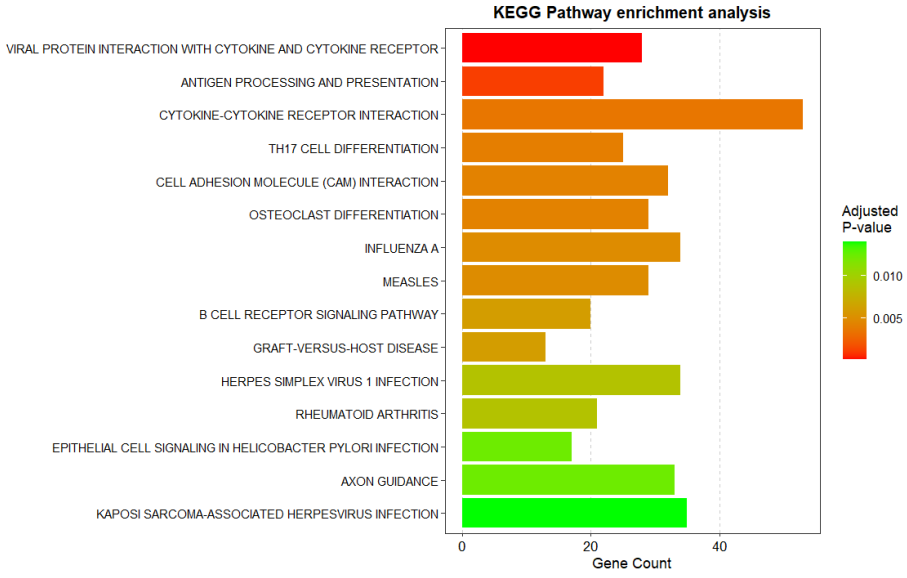

Fig. 3. (E) KEGG pathway analysis of these 2034 DE-mRNAs of these 2034 DE-mRNAs.

4.3 Protein interaction network establishment and hub genes

An interaction network was built using STRING and imported to Cytoscape to look at how the identified genes interact with each other. The network consisted 2020 nodes and 4033 edges. Using the CytoHubba plugin, a topological analysis was done to find the top 20 genes in the network. IFIT3 was ranked first using MCC method, followed by RSAD2, ISIG15, IFIT1, MX1, IFIH1, IFIT2, EIF2AK2, OASL, OAS3, OAS2, DDX58, USP18, IFI35, HERC5, IRF7, STAT1, MX2, STAT2, IFI6. STAT1 was ranked first via Degree with a score of 84 indicating high interactions, STAT1 is followed by IL6, CD8A, DDX58, IFIH1, IRF7, GRB2, JUN, FCGR3A, UBC, CCL5, EIF2AK2, ISG15, TYROBP, BCL2, IFIT3, IFI35, FCGR3B, MX1. These 40 genes were screened and 10 common genes among MCC and Degree were characterised as hub genes (Fig. 4A). A network of hub genes was constructed to show the interactions between top genes (Fig. 4B). KEGG analysis of these genes revealed involvement in various viral disease (Fig. 4C). A Reactome pathway analysis revealed the key role of Interferon signalling and cytokine signalling in PAPS (Fig. 4D).

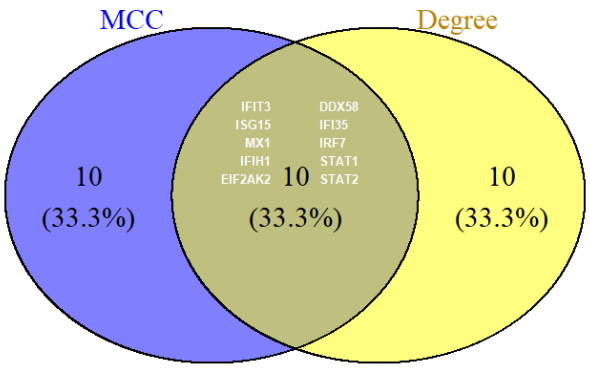

Fig. 4. (A) Ven diagram representing 10 common genes between MCC and Degree top 20

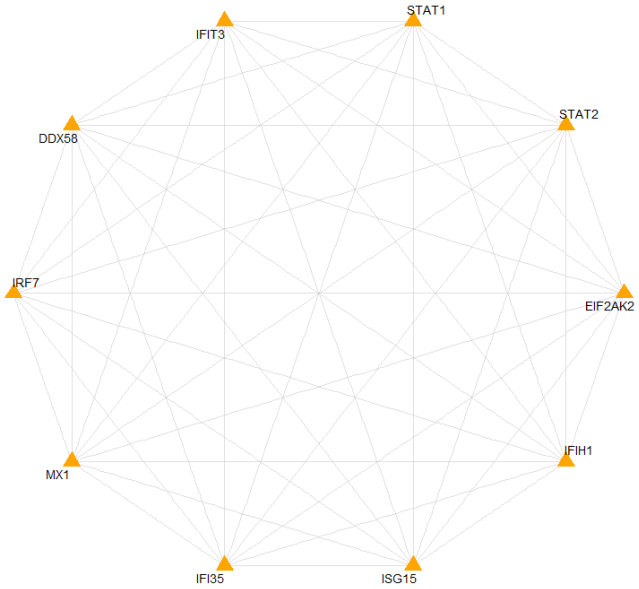

Fig. 4. (B) PPI network of hub genes.

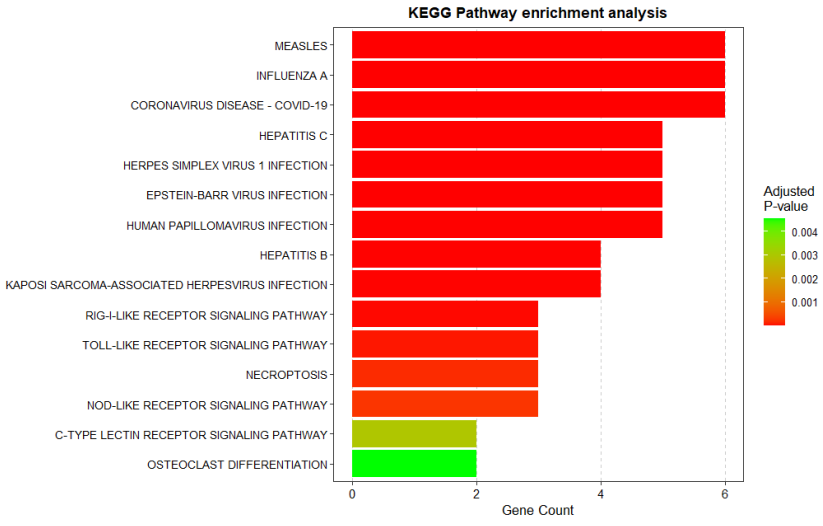

Fig. 4. (C) KEGG pathway enrichment of 10 hub genes.

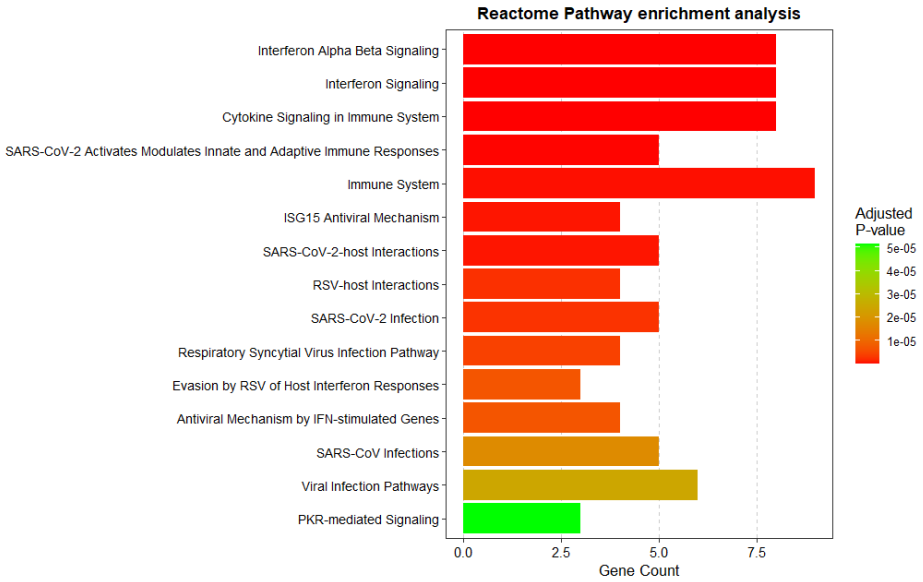

Fig. 4. (D) Reactome pathway enrichment of 10 hub genes.

4.4 Cluster analysis by MCODE

To learn more about how the protein–protein interaction network is set up internally, the MCODE algorithm was used to find modules. This method made it possible to find gene clusters that are very interconnected, which usually indicates involvement in similar biological processes. The top two clusters were taken for enrichment analysis to find. Cluster 1 has 27 nodes and 327 edges and using KEGG pathway analysis cluster showed enrichment in Hepatitis C Influenza A and Measles pathways (Fig. 5B). GO BP enriched terms included viral and interferon signalling pathways. Cluster 2 has 20 nodes and 128 edges and is enriched in inflammatory response, chemotaxis and dendritic cell migration as shown in GO BP anaylsis (Fig. 5C). KEGG pathway enrichment top terms were Viral processes, TNF signalling pathway etc. (Fig. 5D).

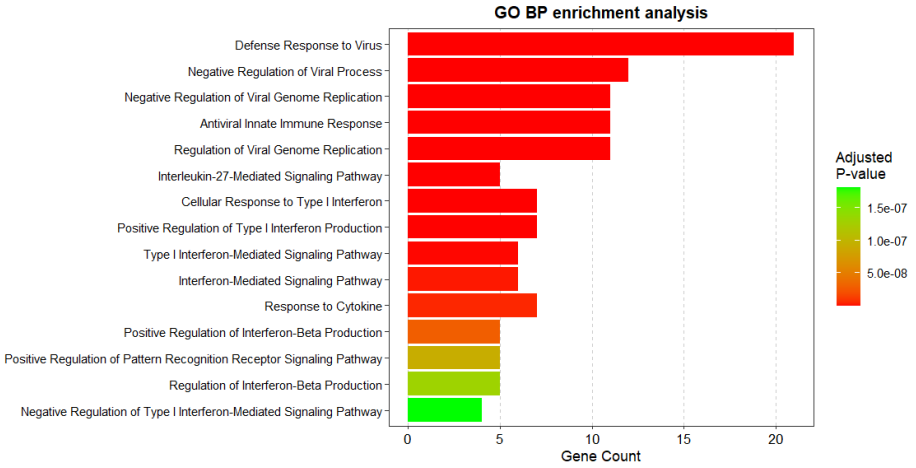

Fig. 5. MCODE algorithm was used to find out functional module inside the PPI network of 2034 DE-mRNA. **(A)** GO biological process of Cluster 1.

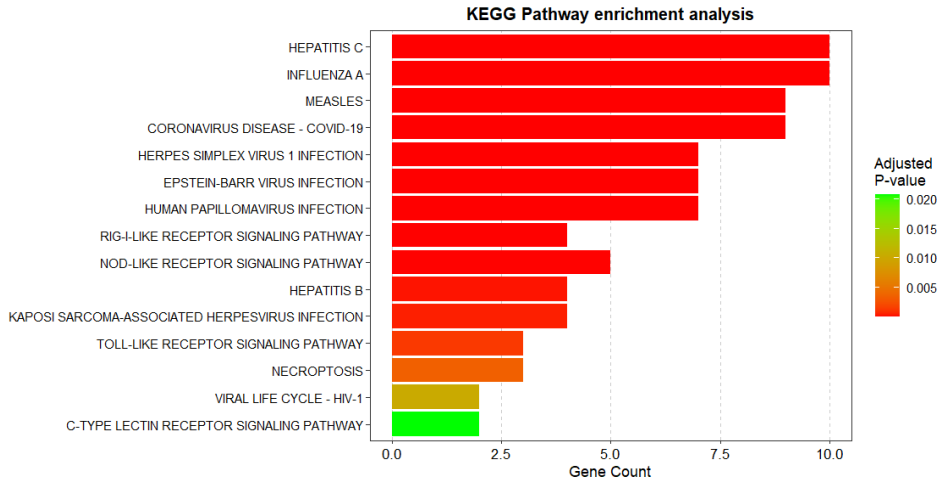

Fig. 5. (B) KEGG pathway enrichment analysis of Cluster 1.

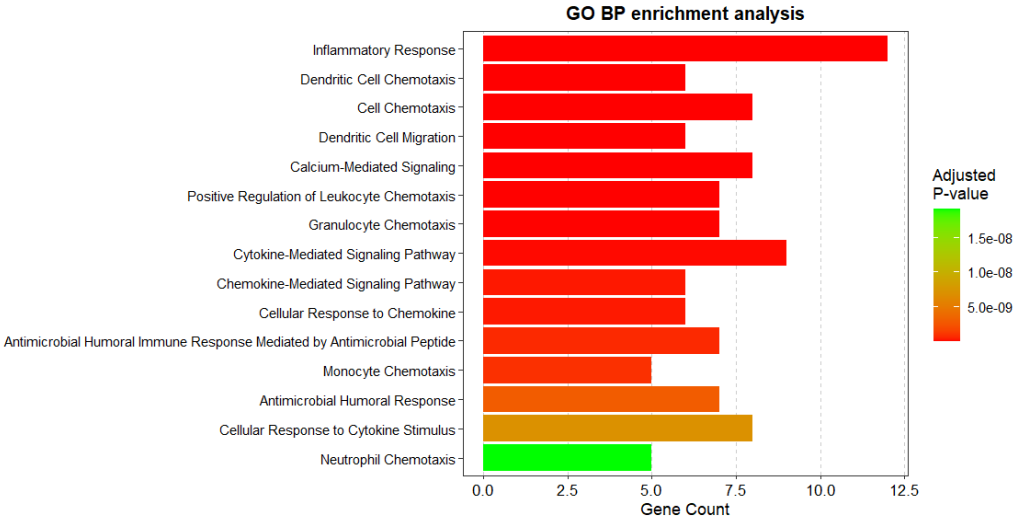

Fig. 5. (C) GO biological process of Cluster 2.

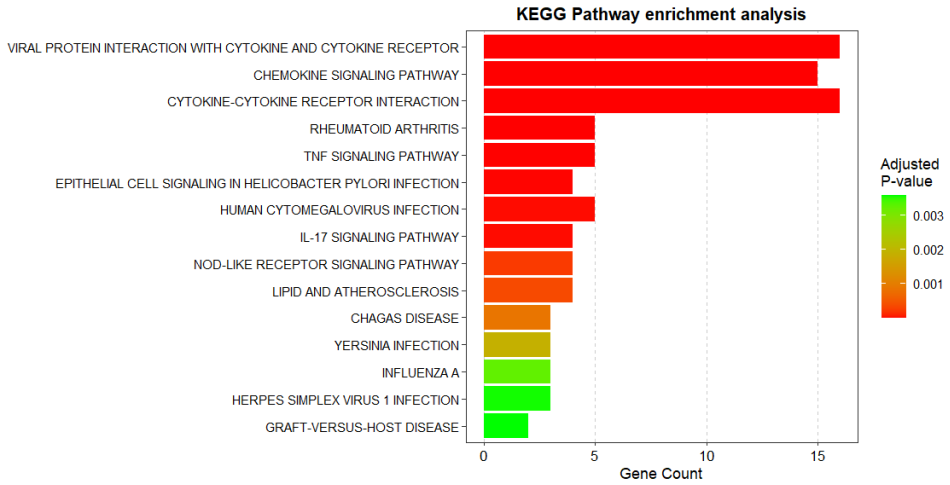

Fig. 5. (B) KEGG pathway enrichment analysis of Cluster 1.

4.5 Constructing miRNA-hub gene network

DE-miRNA target was obtained from experimentally validated database that included 5 downregulated miRNAs in PAPS neutrophil cells (has-miR-155-5p, has-miR-222-3p, has-miR-125a-5p, has-miR-125b-5p, hsa-miR-146a-5p) [8]. Predicted targets for 5 DE-miRNAs were 9782 of which 1209 overlapped with DE-miRNA (Fig. 6A). A hub gene-miRNA network was constructed, of 10 hub genes 7 are regulated by 3 DE-miRNAs. hsa-miR-146a-5p was observed to regulated 5 hub genes (Fig. 6B).

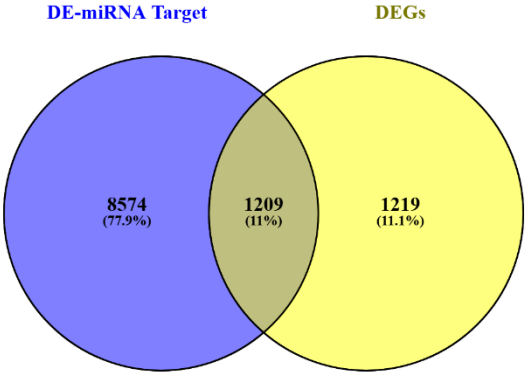

Fig. 6. (A) TarBase v9.0 was used to predict the DE-miRNA target. Predicted targets for DE-miRNA and their target DEGs

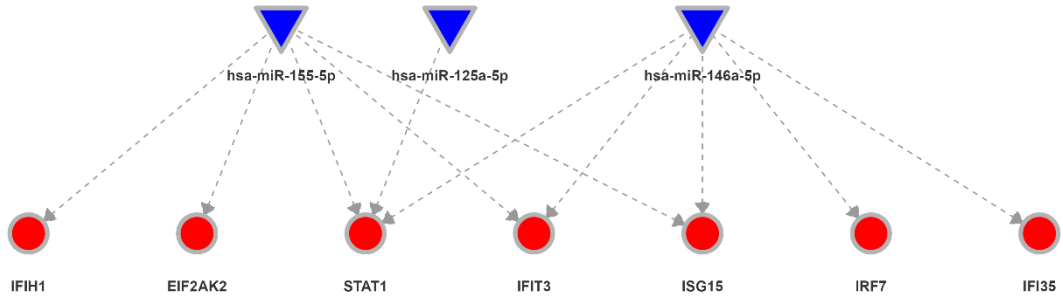

Fig. 6. (B) DE-miRNA–hub gene interaction

CHAPTER 5

DISCUSSION

People suffering from PAPS experience recurrent thrombosis and pregnancy complications, Heparin and Warfarin are some most widely used clinical medicine to counteract venous and arterial thrombosis in PAPS patients. Aspirin is a commonly used blood thinner used to prevent blood clots in high-risk obstetric PAPS patents [20]. However, these medicines come with high risk for bleeding. Warfarin is a teratogenic medicine that can cause birth defects which forces pregnant women to take heparin shots daily [21]. Although current therapeutic strategies can reduce the thrombosis by about 30%, there remain lack of comprehensive transcriptomics integration and limited understanding of interaction network. Our study focuses on understanding underlaying interactions through bioinformatics approach. By combining differential expression analysis, PPI network construction and enrichment analysis we identified novel genes that are associated with PAPS pathogenesis and diagnosis. With this study we have obtained 10 key hub genes in PPI network though MCC and Degree topological method, overlap between these strengthened the reliability of these genes. Type I interferon signalling is one of the key drivers of PAPS pathogenesis with 49% of the patient's exhibiting overexpression of type 1 interferon signalling genes [22]. Among these genes IRF7 and STAT1 are known to be master regulators of type 1 IFN-signalling, in addition IFIH1, DDX58 and STAT2 amplify interferon response and cause inflammation [23].

Amidst 10 hub genes only 7 (STAT1, IFIT3, IFIH1, ISG15, IRF7, EIF2AK2, IFI35) were regulated by DE-miRNA, all 7 genes are upregulated showing positive correlation with downregulated miRNA. 3 of the 5 miRNAs are involved in hub gene regulation and among them hsa-miR-155-5p and hsa-miR-146a-5p shown to be prime regulator targeting 5 genes each. In PAPS patients these miRNA are known to be upregulated in monocytes, however in neutrophil cells it is found to be downregulated [24]. Downregulation of these 2 miRNA in neutrophils appears to be directly related with pro thrombotic events in PAPS patients.

Gene enrichment GO CC of all 2034 DE-mRNA shows high level of Ficolin-1 rich granules, these are secretory granules in human neutrophil cells and some monocytes. Upon release Ficolin-1 promote lectin complement pathway, cytokine release and interaction with antiphospholipid antibodies [22]. Important markers of NET release, such as neutrophil elastase (NE), citrullinated histone H3 (Cit-H3), and peptidylarginine deiminase 4 (PAD4), are positively linked with complement activation [25]. Reactome pathway shows strong enrichment in innate immune system and neutrophil dysregulation suggesting PAPS is driven by innate immune activation and neutrophil mediated inflammatory and thrombotic processes. GO MF enrichment shows many DE-mRNAs are involved in ubiquitin-protein ligase binding, which emphasizes their involvement in inflammatory and NETosis. Wheantiphospholipid antibodies (aPL) bind to neutrophils, the TLR4 pathway is activated. A particular ligase known as TRAF6 binds ubiquitin chains to signalling proteins in this pathway. The outcome is that this "ubiquitin tag" functions as a scaffold to attract the kinases (such as IRAK4) required to initiate the oxidative burst (ROS generation), which causes the neutrophil to release its DNA and create a NET [26], [27].

Cluster analysis of highly interconnected genes within PPI network was done using MCODE to understand the core regulatory genes. Cluster 1 was enriched in Antiviral, interferon type 1 signalling and Cytokine response as shown in GO BP, strengthening our analysis further. Cluster 2 showed primary association with inflammatory response and chemotaxis, including leucocyte migration, dendritic and neutrophil chemotaxis. The division of modules suggests that PAPS pathogenesis may involve parallel but connected biological processes, combining the recruitment of inflammatory cells with antiviral immune activation. These results were supported by functional enrichment analysis. The KEGG pathway analysis focused on chemokine signalling, cytokine–cytokine receptor interactions, and processes associated with viral infections, such as COVID-19 and influenza. These pathways demonstrate the effectiveness of conserved antiviral immune responses rather than a real infection. Additionally, immune system functions, neutrophil degranulation, and interferon signalling were found to be considerably enriched by Reactome pathway analysis. This implies that innate immune responses are cooperating.

CONCLUSION

In conclusion, this study helped us in identifying hub genes associated with PAPS, and a miRNA-hub gene network to understand the regulatory pathway. The enrichment analysis points towards interferon signalling and immune activation strengthening the hub genes as regulators of PAPS neutrophil cells. Our works points out how neutrophil-mediated inflammatory responses and INF signalling contribute to progression of the disease and provided new insights to PAPS molecular mechanism highlighting potential targets for treatment and diagnosis. However, only one GEO set was used for this analysis and we understand that this represents as a limitation to our current study. To validate these results and put them into therapeutic and diagnostic applications, more clinical research and experimental validations are needed.

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