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IDENTIFICATION OF DYSREGULATED GENES IN IDIOPATHIC INFLAMMATORY MYOPATHY mRNA DATA USING COMPUTATIONAL METHODS

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ABSTRACT

The current study was carried out to identify the key genes and their mechanism underlying the myositis. The integrated analysis of expression profiling. *In silco* was conducted to find the differentially expressed genes (DEGs) in IIM. Moreover, functional gene annotation, pathway enrichment analysis, construction of protein-protein interaction (PPI) network and identification of small molecule were applied for exploring the potential biological roles of DEGs in IIM.

Overall 204 DEGs were found in IIM patients compared using healthy controls. The analysis of DEG resulted in alteration of different metabolic and regulatory processes which are distributed as 88 biological processes, 26 molecular functions and 33 cellular component using DAVID online tool. KEGG was used for pathway enrichment analysis for identified DEGs after applying P value (<0.05) and gene count (>2) as threshold. Protein-protein interactions network was constructed and analyzed using STRING and Cytoscape tools and total 15 Hub proteins were identified with minimum 50 and maximum 93 degrees. Finally, Connectivity map (CMap) analysis was performed to identify 20 small molecules capable of altering the expression of gene in IIM. Study concluded the valuable information regarding the pathogenesis mechanism of myositis and suggesting a few compounds as a drug against IIM.

Keywords: idiopathic inflammatory myopathy, computational analysis

Abbreviations: differentially expressed genes (DEGs); protein-protein interaction (PPI); idiopathic inflammatory myopathy (IIM); polymyositis (PM), dermatomyositis (DM), juvenile idiopathic inflammatory myopathy (JIIM), inclusion body myositis (IBM), Necrotizing autoimmune myopathy (NAM) and Orbital Myositis (OM); creatinine kinase (CK), alanine transaminase (ALT), aspartate aminotransferase (AST), aldolase, lactate dehydrogenase (LD); Magnetic Resonance Imaging (MRI), electromyography (EMG); Interstitial lung disease (ILD)

INTRODUCTION

The idiopathic inflammatory myopathy (IIM) is an autoimmune diseases categorized by progressive proximal muscle weakness collectively refers to as myositis. IIM patient complains many symptoms including inflammation, weakness, soreness and mononuclear cell infiltrates in muscle tissue **(**Mammen, 2010; Love *et al.*, 1991**)**. It is subdivided histologically and clinically into 6 subtypes called polymyositis (PM), dermatomyositis (DM), juvenile idiopathic inflammatory myopathy (JIIM), inclusion body myositis (IBM), Necrotizing autoimmune myopathy (NAM) and Orbital Myositis (OM) (Greenberg, 2013; Oldroyd *et al.*, 2017; Khan *et al.*, 2017). Polymyositis is a T cell Orbital–mediated disorder against muscle, mostly found in children and adults between the age group of 5 to 15 and 40 to 60 years old respectively (Jakubaszek *et al.*, 2015). The elevated serum muscle enzymes and different autoantibodies against signal recognition particle (SRP), β-Hydroxy β-methylglutaryl-CoA reductase (HMG-COA reductase) and aminoacyl-tRNA synthetases have been detected in the patient of polymyositis. On muscle biopsy patient showed perifascicular atrophy inflammation or necrosis and the lack of dermatomyositis rashes which differentiate PM from DM (Mammen, 2016). Dermatomyositis (DM) is cutaneous inflammation which influences the skin muscle and tissues spreading into lungs and joints (Greenberg, 2013). It is also allied with a bigger risk of cancer mostly in ovarian, lungs, pancreatic, abdominal, colorectal cancer and non-Hodgkin lymphoma (Hill *et al.*, 2001**)**. Children and adult are mostly affected with it but it commonly occur more in woman like polymyositis. The pathology of DM comprise vasculitis and perifascicular atrophy. The inflammation cells are mostly B cells (Chen *et al.*, 2016). IBM is a spasmodic disorder and mostly occurs in male over the age of 50 years. The significant clinical feature of IBM is the presence of distal and proximal weakness in limbs, finger flexuous, ankle dorsiflexion, asymmetric muscle involvement (not seen in PM or DM), Mild facial weakness and dysphagia (Dimachkie *et al.*, 2013; Hilton-Jones, 2003).

Many diagnostic procedures are available for IIM detection e.g. blood test is used to measure the serum concentration of muscle enzymes i.e. creatinine kinase (CK), alanine transaminase (ALT), aspartate aminotransferase (AST), aldolase, lactate dehydrogenase (LD) and myositis autoantibody in patient. Furthermore, Magnetic Resonance Imaging (MRI), electromyography (EMG) and also muscle biopsy (as an invasive technique) are performed to differentiate among the types of inflammatory myopathies. Few Immunohistochemically staining procedures are also used such as major histocompatibility complex class 1 (MHC-1), T cells and macrophages often required to confirm symptoms of inflammation. For example, in case of Interstitial lung disease (ILD) pulmonary function test and high resolution computerized tomography lungs scanning are performed (Oldroyd *et al.*, 2017; Lundberg *et al.*, 2016).

A number of treatment and drugs are available for IIM treatment e.g. prednisolone and dexamethane, rituximab, abatacept, alemtuzumab, tocilizumab, anakinra, retinoids, calpeptin, and mizoribine and statin. Most of them reduce the muscles inflammation. Additionally, drugs like methotrexate, azathioprine, mycophenolate mofetil, tacrolimus, cyclophosphamide, rituximab, tocilizumab work as immunosuppressive (Carstens and Schmidt, 2014; Albayda *et al.*, 2012).

Advancement in bioinformatics method has proved significant prospective in biomedical research. These approaches reduce the investigation time and provide probabilistic and biologically significant results **(**Banwait *et al.*, 2015). In this study, bioinformatics methods are applied to explore the pathogenesis of myositis at molecular level and predicting the new therapeutic targets for myositis diseases**.**

MATERIALS AND METHODS

Data collection of microarray expression data

The gene expression profile (GSE48280) was downloaded from the website http://www.ncbi.nlm.nih.gov/geo/ (Gene Expression Omnibus database). All 19 IIM samples available were analyzed on the basis of GPL6244 [HuGene-1_0-st] Affymetrix Human Gene 1.0 ST Array [transcript (gene) version].

Identification of differentially expressed genes (DEGs)

The Linear Models package of R language (limma) was applied to analyze the microarray datasets (Hao *et al.*, 2017). There are differentially expressed genes were found discovered in IM patients compared to healthy individuals. The Benjamini and Hochberg procedure (False discovery rate -FDR) was applied for multiple testing modifications. FDR below 0.05 is considered as the DEGs threshold (Reiner-Benaim, 2007).

Gene ontology (GO) analysis of DEGs

DAVID (Database for Annotation, Visualization, and Integrated Discovery) was applied for the analysis of DEGs functions and classification of genes according to the Cellular component (CC), biological process and molecular function (Xiao and Yiqing, 2013).

Pathway enrichment analysis

The dysregulated biological pathways in IM were investigated in depth to find the changes at a functional level. Metabolic and non-metabolic pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) Database is applied as DAVID inputs for KEGG pathways enrichment analysis (Chen *et al.*, 2016; Xiao *et al.*, 2013**)**. A threshold of P value < 0.05 and gene count (>2) DEGs enclosed in a pathway were selected as the principles.

Protein-Protein Network Interactions

The Searching Tool (STRING; http://sting-db.org/) was selected for retrieval of Interacting Genes. This database is an online tool to estimate PPI info (Mering *et al.*, 2003). In the current study, the construction of PPI networks for the selected DEGs can be done using STRING database. The PPI networks were visualized and studied using the STRING database. The interactions (the default threshold > 0.4 in the STRING database) are selected to create the PPI network. As a final point, the obtained PPI networks are visualized from Cytoscape software (Vinayagam *et al.*, 2014). The topological structure of the network was evaluated and calculated the degree for each gene to select hub genes from PPI network (Xiao *et al.*, 2013). Finally, hub genes ware described in the network (degree ≥50).

Identification of small molecules

The Connectivity Map (CMap) a database to assemble the entire transcriptome profile from cultured human cells and applies simple pattern-matching algorithms; both facilitate the detection of functional connections

between genes, diseases and drugs along with the transient feature of changes in common gene-expression (Xiao *et al.*, 2013; Yu *et al.*, 2017**)**. Using CMap, 204 DEGs were characterized into up-regulated and down-regulated genes. Furthermore, topmost ten up and down-regulated genes were chosen for enrichment analysis by GSEA (gene set enrichment analysis). The enrichment value lies between -1 to 1 (If the value is near to -1, small molecule will mimic normal status of cell and could be a good drug whereas value near to 1, small molecule will mimic the disease status).

RESULTS

DEGs in Myositis

The gene expression profile of 19 myositis including 5DM, 5PM, 5IBM and 5 normal controls were studied using the classical t-test in limma package (the cut-off is taken $P<0.05$). 204 differentially expressed genes were identified in IM.

Table 1. Clustering of DEGs on the basis of Biological Process:

GO Term	Gene Count	P-Value
innate immune response	23	1.90E-12
immune response	18	2.70E-08
type I interferon signaling pathway	17	2.30E-20
interferon-gamma-mediated signaling pathway	15	2.20E-16
defense response to virus	15	3.70E-11
inflammatory response	12	1.80E-04
antigen processing and presentation of peptide antigen via MHC class I	10	9.40E-13
cell adhesion	10	9.40E-03
complement activation	9	3.60E-07
antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent	8	5.50E-07
antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-independent	6	3.20E-09
complement activation	9	3.60E-07
antigen processing and presentation	7	4.10E-06
antigen processing and presentation of endogenous peptide antigen via MHC class I	4	1.60E-05
positive regulation of apoptotic cell clearance	4	1.60E-05
response to virus	8	2.30E-05
response to interferon-beta	4	3.70E-05
regulation of complement activation	5	8.00E-05
complement activation, classical pathway	7	1.20E-04
antigen processing and presentation of endogenous peptide antigen via MHC class I via ER pathway, TAP-independent	3	1.80E-04
negative regulation of viral genome replication	5	2.50E-04
regulation of immune response	8	4.70E-04
protection from natural killer cell mediated cytotoxicity	3	5.90E-04
maternal process involved in female pregnancy	4	9.20E-04
negative regulation of type I interferon production	4	1.60E-03
defense response	5	1.80E-03
response to interferon-alpha	3	2.60E-03
positive regulation of T cell mediated cytotoxicity	3	4.40E-03
positive regulation of type I interferon production	4	7.20E-03
protein deubiquitinating	5	7.30E-03
regulation of extrinsic apoptotic signaling pathway via death domain receptors	3	7.50E-03
positive regulation of I-kappa B kinase/NF-kappa B signaling	6	8.30E-03
negative regulation of viral entry into host cell	3	8.40E-03

Gene Ontology of DEGs

204 DEGs were analyzed for gene ontology analysis using DAVID online tool (with statistical cut-off of $p < 0.01$). These genes are classified according to the biological process, cellular component and molecular functions. Resultant significant enrichment of these genes are presented in Table1, 2 & 3.

Table 1 explain bunches of gene obtained when these significant enrichment of DEGS were categories conferring to biological process (P value=0.01). The gene count was much more high in 8 biological processes compare to the remaining processes. These process including innate immune response, immune response, type I interferon signaling pathway, interferon-gamma-mediated signaling pathway, defense response to virus , antigen processing and presentation of peptide antigen via MHC class I. as it was seen that most biological process disrupted in MHCI pathways which leads to severe myositis (Li *et al.*, 2009).

Table 2. Clusters of DEGs on the basis of Cellular Component.

GO Term	Genes Count	P-value
Integral component of luminal side of endoplasmic reticulum membrane	9	2.5E-11
MHC class I protein complex	6	8.6E-9
Extracellular exosome	47	3.6E-8
Blood micro particle	11	1.7E-7
ER to Golgi transport vesicle membrane	7	2.1E-6
Phagocytic vesicle membrane	7	4.4E-6
Membrane	36	5.4E-6
Extracellular space	25	3.8E-5
TAP complex	3	1.6E-4
Cell surface	14	1.7E-4
Cytosol	42	2.8E-4
Focal adhesion	11	6.0E-4
Membrane raft	8	8.0E-4

Table 2 clarify the bunches of gene obtained when these significant enrichment of DEGS were categories conferring to cellular components (P value<0.001) and according to threshold P value and gene count most of the DEGs are found in extracellular exosome formation, in cytosol, membrane, extracellular space and cell surface. Whereas few DEGs were observed in cellular component associated with immunity e.g. MHC class I protein complex, TAP complex and phagocytic vesicle membrane.

Table 3. Clustering of DEGs based on the Basis of Molecular Function.

GO TERM	Count	P-value
Peptide antigen binding	7	6.10E-08
TAP1 binding	4	1.80E-06
TAP2 binding	3	1.80E-04
TAP binding	3	1.80E-04
Peptide antigen-transporting ATPase activity	3	1.80E-04
Protein binding	88	4.00E-04
Complement binding	3	1.60E-03
Complement component C1q binding	3	2.00E-03
Beta-2-microglobulin binding	3	3.10E-03
Serine-type endopeptidase activity	8	3.60E-03
Integrin binding	5	8.80E-03
MHC class I protein binding	3	9.20E-03

Table 3 clarify the bunches of gene obtained when these significant enrichment of DEGS were categories conferring to molecular function ($p < 0.01$).The study shown that the changes in gene expression affects mainly protein binding, serine-type endopeptidase activity and peptide antigen binding.

Pathway enrichment analysis

A total of 17 pathways were found on the basis of selected criteria. The most significant enriched pathways were Antigen processing and presentation with a P value = 3.00E-11), Herpes simplex infection with a P value = 3.20E-06, Phagosome with P value = 5.70E-06, Staphylococcus aureus infection with P value = 1.50E-08 and Complement and coagulation cascades with P value=1.80E-06 (Table 4).

Table 4. KEGG enrichment of dysregulated DEGs in IM.

KEGG Term	Count	P-Value	Benjamini
Antigen processing and presentation	12	3.00E-11	3.00E-09
Staphylococcus aureus infection	9	1.50E-08	7.50E-07
Complement and coagulation cascades	8	1.80E-06	6.10E-05
Herpes simplex infection	11	3.20E-06	7.90E-05
Phagosome	10	5.70E-06	1.10E-04
Pertussis	7	4.20E-05	6.90E-04
Viral myocarditis	6	1.20E-04	1.70E-03
Graft-versus-host disease	5	1.70E-04	2.20E-03
Allograft rejection	5	2.70E-04	3.00E-03
Type I diabetes mellitus	5	4.50E-04	4.40E-03
Autoimmune thyroid disease	5	1.00E-03	9.10E-03
Cell adhesion molecules (CAMs)	7	1.40E-03	1.10E-02
Systemic lupus erythematosus	6	5.90E-03	4.40E-02
Influenza A	6	1.70E-02	1.10E-01
HTLV-I infection	7	2.30E-02	1.40E-01
Endocytosis	7	2.40E-02	1.40E-01
Epstein-Barr virus infection	6	2.40E-02	1.30E-01

Table 5. Recognized hub genes in the PPI network.

Name	Total interactions With protein	Degree
OAS2	40	93
GBP2	24	59
HERC5	22	50
HLA-A	33	79
HLA-B	33	79
HLA-C	29	69
HLA-E	27	62
HLA-F	26	60
ICAM1	23	52
IFI27	22	50
IFI35	26	60
IFIT2	31	73
MX2	31	73
TRIM22	22	50
SP110	28	63

Protein-protein interaction network

PPI network of the Differentially Expressed Gene in IM were built through STRING database, which contains 100 nodes (proteins) and 373 edges (protein-protein associations) with an average node degree: 8.65 and PPI enrichment P value is < 1.0e-16. Nodes degree were visualized using Cytoscape and the DEGs with a degree >50 are shown in Table 5. Degree indicates hub protein (Highly connected nodes). The hub proteins with the highest degree were OAS2 (degree=93), HLA-A, HLA-B (degree= 79), IFIT2 and MX2 (degree=73).

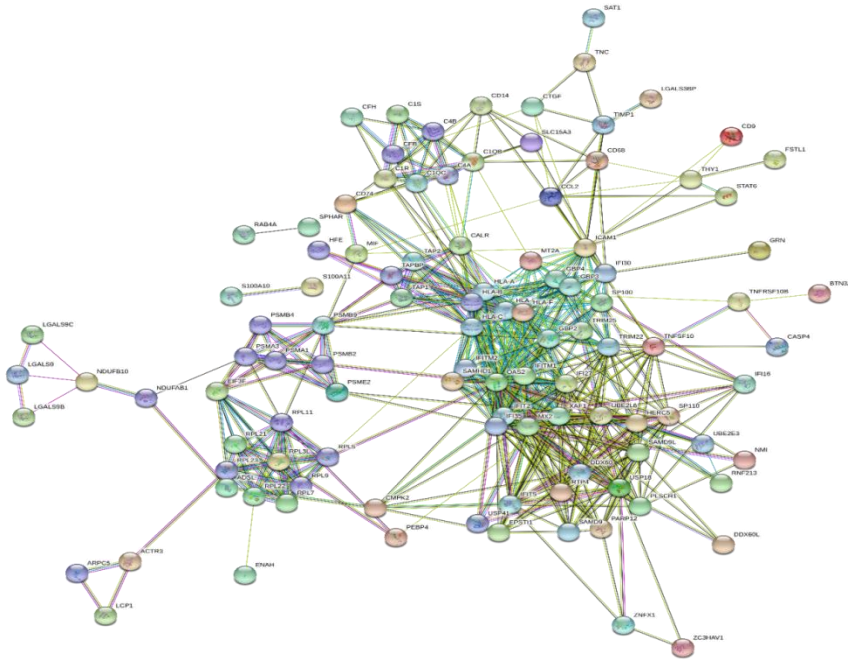

Fig. 1. Protein-Protein Interaction Network.

Table 6. List of small molecule that can emulate the normal cells status.

Rank	CMap name	Enrichment
1	Tanespimycin	-0.389
2	Prestwick-642	-0.902
3	Thioperamide	-0.824
4	Roxarsone	0.851
5	Monensin	-0.706
6	Clonidine	0.823
7	Lomustine	-0.822
8	Oxytetracycline	0.9
9	Cefalexin	0.755
10	Amoxapine	0.727
11	Fipexide	0.876
12	Pentetic acid	-0.713
13	N6-methyladenosine	0.779
14	Prestwick-967	0.776
15	Rolipram	0.771
16	Minocycline	-0.694
17	Cloperastine	0.644
18	Cefixime	-0.764
19	Atovaquone	0.849
20	3-acetamidocoumarin	-0.76

Identification of related active small molecules

To identify the related active small molecules against the IM, we carried out computational bioinformatics analysis of DEGs using CMap database. The data were analyzed according to permutated result and enrichment score which was based on Xiao and Yiqing algorithm (enrichment values are between -1 to 1, which indicates that if enrichment value of small molecule is near -1 so it can reverse the abnormal status of IM and selected as best therapeutic target and if it is near 1 then it cannot be effected for IM treatment). This analysis identified 1309 small molecules having enrichment values from -1 to 1, from which above 20 are shown in Table 6. It was concluded that Prestwick-642(enrichment = -0.902), thioperamide (enrichment = -0.824) and lomustine (enrichment = -0.822) were having enrichment values near to -1 and could possibly reverse the status of IM.

DISCUSSION

Myositis is a critical clinical problem as it is a rare disease of muscle with various histological and pathological symptoms. Therefore there is an urgent need to understand the mechanism underlying myositis for preventive measure and develop new therapeutic targets for the treatment of diseases (Lundberg, 2016). In current study, bioinformatics approaches were applied to investigate the molecular mechanism of myositis through identifying the total 204 DEGs. These DEGs are significant to understand the myositis mechanism and to develop novel diagnostic biomarkers for IM.

The result of GO cluster study shown that these Differentially Expressed Genes are mostly involved in immune system, immune diseases, membrane transport, and primary immune deficiency and infection diseases and previous study have been shown that overexpression of MHC molecule on the cell surface of muscle which result in the production of autoantibodies leads to the inflammation of muscle (Suárez-Calvet *et al.*, 2014**)**.

The dysregulated genes in IM were significantly enriched in 12 KEGG pathways and antigen processing & presentation was observed to be the most significant pathways in the development of IM. Twelve genes were involved in antigen processing, namely CD74 molecule(CD74), lysosomal thiol reductase(IFI30) , TAP binding protein(TAPBP), calreticulin (CALR), major histocompatibility complex A(HLA-A), class I, major histocompatibility complex, class I B (HLA-B), major histocompatibility complex, class I, C (HLA-C), major histocompatibility complex, class I E(HLA-E),major histocompatibility complex, class I F(HLA-F), proteasome activator subunit 2(PSME2), transporter 1, ATP binding cassette subfamily B member (TAP1), transporter 1, ATP binding cassette subfamily B member (TAP1)

PPI network was constructed based on 386 nodes and 755 edges using String database. Constructed PPI network was visualized in Cytoscape. Hub proteins have additional multifarious interactions related to other genes, showing their significant roles in basic mechanisms of disease. (Li *et al.*, 2009). Therefore, identification of hub genes might help in the development of beneficial methodologies for the treatment of IM patients. 15 hub proteins were identified namely 2'-5'-oligoadenylate synthase 2 (OAS2), guanylate binding protein 2 (GBP2), HECT and RLD domain containing E3 ubiquitin protein ligase 5 (HERC5), Major Histocompatibility Complex, Class I, A (HLA-A), major histocompatibility complex, class I, B (HLA-B), major histocompatibility complex, class I, C (HLA-C), major histocompatibility complex, class I, E (HLA-E),major histocompatibility complex, class I, F (HLA-F), intercellular adhesion molecule 1 (ICAM1), interferon alpha inducible protein 27 (IFI27), interferon induced protein 35 (IFI35),interferon induced protein with tetratricopeptide repeats 2 (IFIT2),MX dynamin like GTPase 2 (MX2),tripartite motif containing 22 (TRIM22) and SP110 nuclear body protein (SP110). The maximum degree in the construction of PPI networks was OAS2 gene (degree, 93). Our study revealed that the protein-protein interactions between dysregulated DEGs could play vital roles in succession and progress of myopathies through those molecular and biological progressions.

CMap analysis of the DEGs identified a set of small molecules that can reverse the status of IM and help to imitate the normal cell status. For example Prestwick-642, thioperamide and lomustine molecules have a key role for developing new therapeutic drugs for treating IM. Although, clinical trials on animals is very necessary to find out the effectiveness of these drugs on IM. Advance investigation of these small molecules need to be carried out.

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