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Naresh Rambabu[1,#], Mano Joseph Mathew[2,#], Srini V. Kaveri[1], Jagadeesh Bayry[1,3,*]

[1]Institut National de la Santé et de la Recherche Médicale, Centre de Recherche des Cordeliers, Sorbonne Université, Université de Paris, F-75006 Paris, France

[2]École d'ingénieur généraliste en informatique et technologies du numérique, 30- 32 Avenue de la République, F-94800 Villejuif, France

[3]Indian Institute of Technology Palakkad, Palakkad, 678623, India

[#] These authors contributed equally to this report

***Corresponding author:** E-mail address: bayry@iitpkd.ac.in **(**Jagadeesh Bayry)

Abstract

Intravenous immunoglobulin (IVIG) is used to treat several autoimmune and inflammatory diseases, but some patients are refractory to IVIG and require alternative treatments. Identifying a biomarker that could segregate IVIG responders from non-responders has been a subject of intense research. Unfortunately, previous transcriptomic studies aimed at addressing IVIG resistance have failed to predict a biomarker that could identify IVIG-non-responders. Therefore, we used a novel data mining technique on the publicly available transcriptomic data of Kawasaki disease (KD) patients treated with IVIG to identify potential biomarkers of IVIG response. By studying the boolean patterns hidden in the expression profiles of KD patients undergoing IVIG therapy, we have identified new metabolic pathways implicated in IVIG resistance in KD. These pathways could be used as biomarkers to segregate IVIG non-responders from responders prior to IVIG infusion. Also, boolean analysis of the transcriptomic data could be further extended to identify a universal biomarker that might predict IVIG response in other autoimmune diseases.

Keywords: IVIG, Biomarkers, Autoimmune diseases, Immune metabolism, Therapy, Transcriptome, Boolean patterns

Highlights:

- Not all autoimmune patients respond to intravenous immunoglobulin (IVIG) immunotherapy.
- Several studies have attempted to identify biomarkers of IVIG response.
- Specificity and/or sensitivity of identified biomarkers of IVIG response are the major issues.
- Boolean analysis of the transcriptomic data could identify novel biomarkers of IVIG response.
- Boolean approach identified several metabolic and signaling pathways implicated in IVIG resistance.

1. Introduction

Intravenous Immunoglobulin (IVIG) is a therapeutic normal human Immunoglobulin G (IgG) prepared from the pooled plasma of several thousand healthy donors. Although, initially used in the immunoglobulin (Ig) replacement therapy of primary immunodeficiency (PID) patients, currently high-dose (1-2g/kg) of IVIG is used for the treatment of diverse autoimmune and inflammatory diseases [1]. IVIG is used as a first line therapy in several autoimmune diseases such as Guillain-Barré syndrome (GBS), Chronic inflammatory demyelinating polyneuropathy (CIDP), Idiopathic thrombocytopenia purpura (ITP), Kawasaki disease (KD), Autoimmune blistering disease, Inflammatory myopathies and others [2-4]. The beneficial effects of IVIG are mediated via diverse mechanisms [5, 6].

Autoimmunity stems from the inability of immune system to differentiate self-antigens from the foreign antigens. Mounting aberrant immune response to self-antigens has been linked to over 80 inflammatory disorders, collectively known as autoimmune diseases [7]. IVIG is used as first line therapy for treating several autoimmune diseases. However, not all autoimmune patients respond to IVIG therapy. Resistance to IVIG therapy has been reported in many autoimmune diseases including KD, ITP, CIDP and GBS [8-12].

2. Biomarkers of IVIG response

Several studies have attempted to identify biomarkers of IVIG response in various autoimmune diseases. In accordance with the therapeutic use of IVIG in autoimmune and inflammatory diseases, most of these markers followed the trend of either inflammatory cells like platelet, lymphocyte and neutrophil counts or molecules that are the hall marks of inflammation such as alanine aminotransferase, matrix metalloproteinase-8 C-reactive protein, neutrophil-derived elastase, inflammatory cytokines and chemokines or their receptors (IL-6, IL-1β, TNF, G-CSF, CCR2), damage-associated molecular patterns like High mobility group box protein 1 (HMGB-1), S100 calcium-binding protein A8 (S100A8), S100A9 [13-19]. However, specificity and/or sensitivity of these markers are the major issues. Even genetic studies on polymorphism (like activating and inhibitory Fcγ receptors (FcγR), Phospholipase A2 Group VII) have been attempted to predict IVIG response [20-22]. But those findings were not reproducible in all the patient groups of given pathology.

Majority of the studies on the quest for the biomarkers of IVIG response have been focused on KD patients. KD is an acute systemic vasculitis, which affect children under 5 years

old. Around 10-20% of KD patients are refractory to IVIG and are at high risk of developing coronary artery aneurysms [10]. Several prediction models such as Kobayashi, Egami, Kawamura, Sano and Formosa have been designed to address IVIG resistance in Japanese KD patients [23-27]. However, these models failed to predict IVIG resistance in other ethnicities, and also are not sensitive to predict IVIG resistance in other autoimmune diseases [28].

3. DNA Microarrays for the identification molecular biomarkers

One of the classical techniques used to identify molecular biomarker is microarray [29, 30], which provides a snapshot of all the biological processes taking place inside a cell. DNA microarray is used to study the transcriptional profiles associated with broad range of diseases to identify disease specific molecular biomarker(s) [31-35]. Unfortunately, the number of microarray datasets available in the public databases to study IVIG response is limited, thereby emphasizing the need to revisit these datasets and seek for hidden patterns using novel data mining techniques.

Several data analysis techniques are used to understand the biological meaning from the microarray data. Initially, numerical score based gene ranking was used to correlate a gene with a disease, thereby the gene that is highly expressed in a disease is considered to be implicated in then pathogenesis [36, 37]. Later, an enhanced version of such gene selection technique was used in clustering algorithms to identify huge list of genes exhibiting similar expression levels, comparing the expression levels of such genes with healthy subjects helps us to identify the genes associated with the disease [38]. Nowadays, sophisticated algorithms have been developed

to study complex transcriptional regulatory mechanisms such as co-expression, gene activation, and inhibition using microarray data [39]. Although, the current data mining techniques provide insights into complex gene regulatory mechanism, their ability in predicting biomarker(s), which could segregate IVIG non-responders from responders remain limited.

4. Boolean analysis of the transcriptomic data to identify novel biomarkers of IVIG response

In this study, we have used a novel boolean approach to segregate IVIG non-responders from responders, and identified that cellular metabolism in peripheral blood mononuclear cells plays a vital role in IVIG resistance. Using the GEO database, the expression profile of the data was extracted and normalized using the quantile method [40]. Log-transformation of the gene expression data was performed. Statistical analysis of gene expression data was based on the single-factor Analysis of variance (ANOVA). Sorted matrix (as a tab-delimited text file) was used for performing the heat map which was generated by selecting gene filtering parameters (FDR threshold < 0.05 and fold change threshold =1). Clustering was performed on the sorted matrix using TM4- MEV software (MultiExperiment Viewer, Dan- Farber Cancer Institute, Boston, MA) [41]. Data underwent Z- score normalization so that parameters with vastly different ranges could be compared directly. K-Mean clustering by using Pearson correlation as the distance metric was performed within parameters.

To identify the biomarkers that could distinguish IVIG responders from non-responders we studied the boolean behaviors in nearly 40,000 genes. The expression profile of the KD patients undergoing IVIG therapy was extracted from the GEO database (GSE18606). By

converting the microarray readout into 0's and 1's (>0 =1; <0=0), we were able to filter the genes, which exhibit switch like behavior (on/off) specific for the treatment condition (**Figure 1**). Later, we purified these genes that exhibit boolean behavior by manually removing the false positives with the aid of BART [42]. Using this approach, we were able to identify several genes exhibiting boolean behavior specific to IVIG responders and non-responders. Later, we performed enrichment analysis on these genes using EnrichR [43] and identified that cellular metabolism is implicated in IVIG resistance (**Table 1**).

5. Boolean approach sheds light on metabolic and signaling pathways specific to IVIG responders and non-responders

Based on the enrichment analysis, we identified that several metabolic and signaling pathways are implicated in IVIG resistance (**Figure 1**). We focused our analyses on the pathways that are linked to immune response. Also, to ensure accurate annotation, we selected the pathways with P values lesser than 0.009. In IVIG non-responders, interferon (IFN) regulatory factor 3 (IRF3)-mediated activation of type 1 IFN was turned on. Genes that increase myeloid cell number, and that promote pyrimidine metabolism, abnormal plasmacytoid dendritic cell physiology were also turned on (**Table 1**) (**Figure 1**). Of interest, though several genes (fifteen) were turned on in non-responder patients, these genes contribute to limited set of pathways. On the other hand, genes that modulate lymphocyte migration and Th17 response were switched off (**Table 1**). Interestingly, the genes that mediate palmitate biosynthesis were also switched off in IVIG non-responders (**Table 1**). Palmitate is a saturated fatty acid that acts as agonist for Toll-like receptor 4 (TLR4) receptor and promotes inflammation by activating macrophages and innate immune cells [44, 45]. Previous studies have highlighted the importance

of inflammation in IVIG resistance [25]. In IVIG responders, the genes that mediate mitochondrial calcium uptake and several aspects of mitochondria mediated adenosine triphosphate (ATP) production were switched off (**Table 1**). Interestingly, though the number genes switched off in responder patients with boolean behavior were small (six), these genes contribute to many pathways, particularly mitochondrial functions. The genes that mediate degranulation of mast cells were also switched off (**Table 1**). This suggest that suppression of mitochondrial function and inflammation plays a vital role in inducing immune homeostasis in IVIG responders.

6. Boolean behavior of genes could not be identified using conventional data analysis algorithms

There are several tools available to study the differentially expressed genes in a microarray dataset, but they are not developed to specifically study boolean behaviors in a microarray dataset. We performed data analysis on GSE18606 dataset using GEO2R and BART to study whether algorithms developed to study differentially expressed genes in a microarray dataset were able to shed light on boolean behavior, but both GEO2R and BART failed to index the genes exhibiting boolean behavior as differentially expressed. We also used MEV to performed K mean clustering, where we grouped the 40,000 genes into different clusters. We generated 25, 50, 75 and 1000 different clusters to see if clustering algorithm could group the genes that exhibit boolean behavior, but these genes were distributed across different clusters (**Table 2**).

7. Conclusion and perspectives

IVIG is used as first line therapy in several autoimmune and inflammatory diseases, but not all patients respond to IVIG treatment. Identification of biomarker that could segregate IVIG responders from non-responders remains a challenge. Patients who exhibit IVIG resistance need to undergo alternative therapy, thus identifying these patients in the early stages of disease will help clinicians to initiate an alternative treatment strategy. Microarray is a sophisticated technique that sheds lights on the complex processes occurring inside a cell and helps in identifying disease specific biomarkers. There are several software's available to understand the biological meaning hidden in the microarray data, each tool provides insights into the distinct biological processes taking place inside the cell. Despite these advancements in microarray technology, identifying a biomarker, which could segregate IVIG responders from non-responders remains a challenge. In this study we have used a novel data analysis technique, where we screened the genes that exhibit boolean or switch like behavior specific to IVIG responders and non-responders. Using this boolean approach we have identified that cellular metabolism plays a vital role in IVIG resistance. The KD patients who responded to IVIG therapy exhibited limited mitochondrial activity and low inflammation. Whereas, KD patients resistant to IVIG therapy had increased expression of genes that promote inflammation, abnormal regulation of Th17 response.

Taking into consideration the role of cellular metabolism in influencing the success of IVIG therapy, diagnostic assays could be developed to rapidly study these parameters in KD patients. This could help clinicians in identifying the IVIG non-responders at an early stage and to initiate alternative therapies to reduce the morbidity and associated therapeutic costs [46]. However, the role of cellular metabolism in the outcome of IVIG therapy in other autoimmune

diseases need to be studied to discover a universal biomarker. Currently, dimethyl fumarate, a small molecule targeting glycolytic pathway have been approved to treat multiple sclerosis [47-49]. Since mitochondria mediated ATP production was suppressed in IVIG responders, small molecules capable of selectively inhibiting mitochondrial function in immune cells could be used along with IVIG to treat IVIG non-responders.

Declaration of Competing Interest :

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Figure Legend

Figure 1. Genes and the major pathways that exhibit switch like behavior (on/off) in Kawasaki disease patients specific for the IVIG treatment condition. Several genes in IVIG resistant and responder Kawasaki disease patients display switch like behavior (on/off). The cluster number of those genes are highlighted on the right side of the figure. The key pathways that are associated with those genes are also listed.

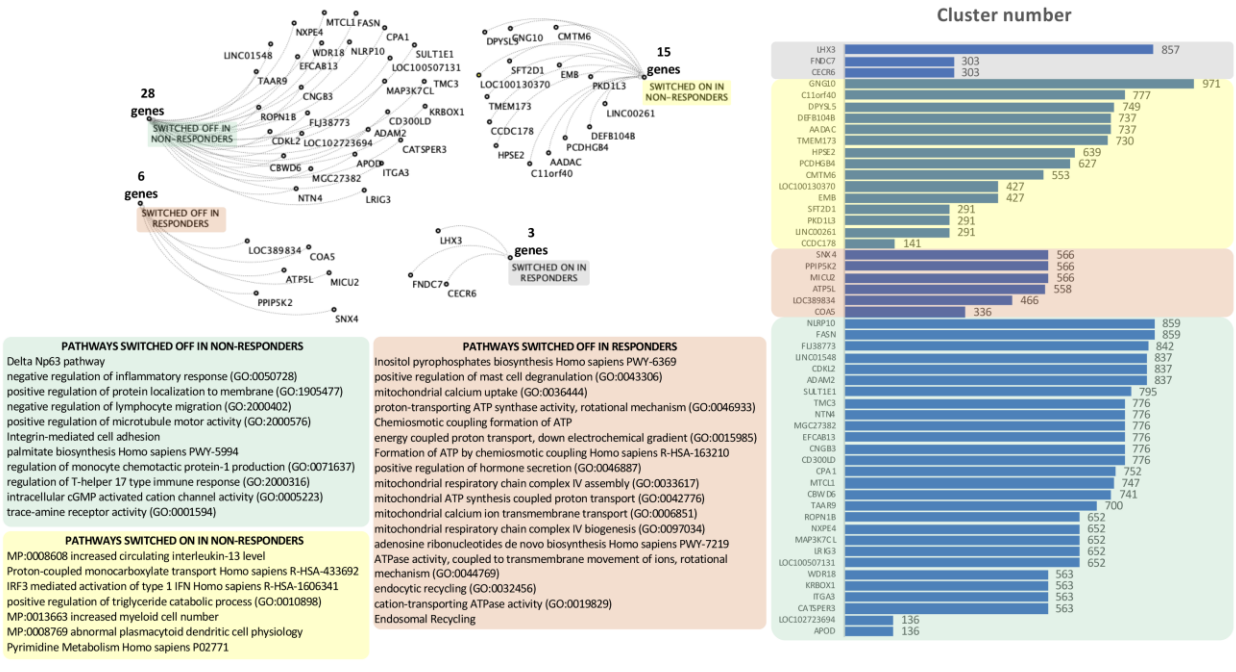

Table 1: Major pathways specific to IVIG responders and non-responders in Kawasaki disease

Main pathways that are switched off in non-responder patients

Name	P-value	Adjusted p-value	Odds Ratio	Combined score	Source
Delta Np63 pathway	0,001965	1	30,4	189,43	Bioplanet 2019
Negative regulation of inflammatory response (GO:0050728)	0,005315	1	18,32	95,92	GO biological process 2018
Positive regulation of protein localization to membrane (GO:1905477)	0,007787	1	15,04	73,01	GO biological process 2018
Negative regulation of lymphocyte migration (GO:2000402)	0,008372	1	119,05	569,39	GO biological process 2018

Positive regulation of microtubule motor activity (GO:2000576)	0,008372	1	119,05	569,39	GO biological process 2018
Integrin-mediated cell adhesion	0,008596	1	14,29	67,95	Bioplanet 2019
Palmitate biosynthesis Homo sapiens PWY-5994	0,00976	1	102,04	472,39	HumanCyc 2016
Regulation of monocyte chemotactic protein-1 production (GO:0071637)	0,00976	1	102,04	472,39	GO biological process 2018
Regulation of T-helper 17 type immune response (GO:2000316)	0,00976	1	102,04	472,39	GO biological process 2018
Intracellular cGMP activated cation channel activity (GO:0005223)	0,00976	1	102,04	472,39	GO molecular function 2018
Trace-amine receptor activity (GO:0001594)	0,00976	1	102,04	472,39	GO molecular function 2018

Key pathways that are switched off in responder patients

Name	**P-value**	**Adjusted p-value**	**Odds Ratio**	**Combined score**	**Source**
Inositol pyrophosphates biosynthesis Homo sapiens PWY-6369	0,002098	0,319	476,19	2936,47	HumanCyc 2016
Positive regulation of mast cell degranulation (GO:0043306)	0,002098	1	476,19	2936,47	GO biological process 2018
Mitochondrial calcium uptake (GO:0036444)	0,002697	1	370,37	2190,93	GO biological process 2018
Proton-transporting ATP synthase activity, rotational mechanism (GO:0046933)	0,003296	1	303,03	1731,85	GO molecular function 2018

Chemiosmotic coupling formation of ATP	0,003894	1	256,41	1422,64	Bioplanet 2019
Energy coupled proton transport, down electrochemical gradient (GO:0015985)	0,003894	1	256,41	1422,64	GO biological process 2018
Formation of ATP by chemiosmotic coupling Homo sapiens R-HSA-163210	0,004791	1	208,33	1112,71	Reactome 2016
Positive regulation of hormone secretion (GO:0046887)	0,00509	1	196,08	1035,4	GO biological process 2018
Mitochondrial respiratory chain complex IV assembly (GO:0033617)	0,005986	1	166,67	853,06	GO biological process 2018
Mitochondrial ATP synthesis coupled proton transport (GO:0042776)	0,006284	1	158,73	804,72	GO biological process 2018
Mitochondrial calcium ion transmembrane transport (GO:0006851)	0,006284	1	158,73	804,72	GO biological process 2018
Mitochondrial respiratory chain complex IV biogenesis (GO:0097034)	0,006284	1	158,73	804,72	GO biological process 2018
Adenosine ribonucleotides de novo biosynthesis Homo sapiens PWY-7219	0,007477	0,5683	133,33	652,78	HumanCyc 2017
ATPase activity, coupled to transmembrane movement of ions, rotational mechanism (GO:0044769)	0,007477	1	133,33	652,78	GO molecular function 2018
Endocytic recycling (GO:0032456)	0,008074	1	123,46	594,96	GO biological process 2018
Cation-transporting ATPase activity (GO:0019829)	0,00867	1	114,94	545,74	GO molecular function 2018
Endosomal Recycling	0,009563	1	104,17	484,36	Elsevier pathway collection

Important pathways that are switched on in non-responder patients

Name	P-value	Adjusted p-value	Odds Ratio	Combined score	Source
MP:0008608 increased circulating interleukin-13 level	0,003745	1	266,67	1489,98	MGI mammalian phenotype level 4 2019
Proton-coupled monocarboxylate transport Homo sapiens R-HSA-433692	0,004492	1	222,22	1201,21	Reactome 2016
IRF3 mediated activation of type 1 IFN Homo sapiens R-HSA-1606341	0,004492	1	222,22	1201,21	Reactome 2016
Positive regulation of triglyceride catabolic process (GO:0010898)	0,005239	1	190,48	1000,31	GO biological process 2018
MP:0013663 increased myeloid cell number	0,005985	1	166,67	853,08	MGI mammalian phenotype level 4 2019
MP:0008769 abnormal plasmacytoid dendritic cell physiology	0,006731	1	148,15	740,89	MGI mammalian phenotype level 4 2019
Pyrimidine Metabolism Homo sapiens P02771	0,007476	0,8373	133,33	652,8	Panther 2016

407
408

Table 2: General characteristics and parameters of K-Medians Clustering

Clusters	KMC mode	Iterations	Converged	Pearson Correlation
1000	Calculated Means	24	TRUE	TRUE
25	Calculated Means	100	TRUE	TRUE
50	Calculated Means	69	TRUE	TRUE
75	Calculated Means	66	TRUE	TRUE