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

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35
36 **Abstract**
37
38   Intravenous immunoglobulin (IVIG) is used to treat several autoimmune and

39 inflammatory diseases, but some patients are refractory to IVIG and require alternative

40 treatments. Identifying a biomarker that could segregate IVIG responders from non-responders

41 has been a subject of intense research. Unfortunately, previous transcriptomic studies aimed at

42 addressing IVIG resistance have failed to predict a biomarker that could identify IVIG-non-

43 responders. Therefore, we used a novel data mining technique on the publicly available

44 transcriptomic data of Kawasaki disease (KD) patients treated with IVIG to identify potential

45 biomarkers of IVIG response. By studying the boolean patterns hidden in the expression profiles

46 of KD patients undergoing IVIG therapy, we have identified new metabolic pathways implicated

47 in IVIG resistance in KD. These pathways could be used as biomarkers to segregate IVIG non-

48 responders from responders prior to IVIG infusion. Also, boolean analysis of the transcriptomic

49 data could be further extended to identify a universal biomarker that might predict IVIG response

50 in other autoimmune diseases.

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52

53 **Keywords:** IVIG, Biomarkers, Autoimmune diseases, Immune metabolism, Therapy,

54 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].

85   Autoimmunity stems from the inability of immune system to differentiate self-antigens

86   from the foreign antigens. Mounting aberrant immune response to self-antigens has been linked

87   to over 80 inflammatory disorders, collectively known as autoimmune diseases [7]. IVIG is used

88   as first line therapy for treating several autoimmune diseases. However, not all autoimmune

89   patients respond to IVIG therapy. Resistance to IVIG therapy has been reported in many

90   autoimmune diseases including KD, ITP, CIDP and GBS [8-12].

91

92   **2. Biomarkers of IVIG response**

93   Several studies have attempted to identify biomarkers of IVIG response in various

94   autoimmune diseases. In accordance with the therapeutic use of IVIG in autoimmune and

95   inflammatory diseases, most of these markers followed the trend of either inflammatory cells like

96   platelet, lymphocyte and neutrophil counts or molecules that are the hall marks of inflammation

97   such as alanine aminotransferase, matrix metalloproteinase-8 C-reactive protein, neutrophil-

98   derived elastase, inflammatory cytokines and chemokines or their receptors (IL-6, IL-1β, TNF,

99   G-CSF, CCR2), damage-associated molecular patterns like High mobility group box protein 1

100   (HMGB-1), S100 calcium-binding protein A8 (S100A8), S100A9 [13-19]. However, specificity

101   and/or sensitivity of these markers are the major issues. Even genetic studies on polymorphism

102   (like activating and inhibitory Fcγ receptors (FcγR), Phospholipase A2 Group VII) have been

103   attempted to predict IVIG response [20-22]. But those findings were not reproducible in all the

104   patient groups of given pathology.

105

106   Majority of the studies on the quest for the biomarkers of IVIG response have been

107   focused on KD patients. KD is an acute systemic vasculitis, which affect children under 5 years

108   old.   Around

109   10-20% of KD patients are refractory to IVIG and are at high risk of developing coronary artery

110   aneurysms [10]. Several prediction models such as Kobayashi, Egami, Kawamura, Sano and

111   Formosa have been designed to address IVIG resistance in Japanese KD patients [23-27].

112   However, these models failed to predict IVIG resistance in other ethnicities, and also are not

113   sensitive to predict IVIG resistance in other autoimmune diseases [28].

114

115   **3. DNA Microarrays for the identification molecular biomarkers**

116   One of the classical techniques used to identify molecular biomarker is microarray [29,

117   30], which provides a snapshot of all the biological processes taking place inside a cell. DNA

118   microarray is used to study the transcriptional profiles associated with broad range of diseases to

119   identify disease specific molecular biomarker(s) [31-35]. Unfortunately, the number of

120   microarray datasets available in the public databases to study IVIG response is limited, thereby

121   emphasizing the need to revisit these datasets and seek for hidden patterns using novel data

122   mining techniques.

123

124   Several data analysis techniques are used to understand the biological meaning from the

125   microarray data. Initially, numerical score based gene ranking was used to correlate a gene with

126   a disease, thereby the gene that is highly expressed in a disease is considered to be implicated in

127   then pathogenesis [36, 37]. Later, an enhanced version of such gene selection technique was used

128   in clustering algorithms to identify huge list of genes exhibiting similar expression levels,

129   comparing the expression levels of such genes with healthy subjects helps us to identify the

130   genes associated with the disease [38]. Nowadays, sophisticated algorithms have been developed

131   to study complex transcriptional regulatory mechanisms such as co-expression, gene activation,

132   and inhibition using microarray data [39]. Although, the current data mining techniques provide

133   insights into complex gene regulatory mechanism, their ability in predicting biomarker(s), which

134   could segregate IVIG non-responders from responders remain limited.

135

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

137   **response**

138   In this study, we have used a novel boolean approach to segregate IVIG non-responders

139   from responders, and identified that cellular metabolism in peripheral blood mononuclear cells

140   plays a vital role in IVIG resistance. Using the GEO database, the expression profile of the data

141   was extracted and normalized using the quantile method [40]. Log-transformation of the gene

142   expression data was performed. Statistical analysis of gene expression data was based on the

143   single-factor Analysis of variance (ANOVA). Sorted matrix (as a tab-delimited text file) was

144   used for performing the heat map which was generated by selecting gene filtering parameters

145   (FDR threshold < 0.05 and fold change threshold =1). Clustering was performed on the sorted

146   matrix using TM4- MEV software (MultiExperiment Viewer, Dan- Farber Cancer Institute,

147   Boston, MA) [41]. Data underwent Z- score normalization so that parameters with vastly

148   different ranges could be compared directly. K-Mean clustering by using Pearson correlation as

149   the distance metric was performed within parameters.

150

151   To identify the biomarkers that could distinguish IVIG responders from non-responders

152   we studied the boolean behaviors in nearly 40,000 genes. The expression profile of the KD

153   patients undergoing IVIG therapy was extracted from the GEO database (GSE18606). By

154   converting the microarray readout into 0's and 1's ($>0 =1; <0=0$), we were able to filter the

155   genes, which exhibit switch like behavior (on/off) specific for the treatment condition (**Figure**

156   **1**). Later, we purified these genes that exhibit boolean behavior by manually removing the false

157   positives with the aid of BART [42]. Using this approach, we were able to identify several genes

158   exhibiting boolean behavior specific to IVIG responders and non-responders. Later, we

159   performed enrichment analysis on these genes using EnrichR [43] and identified that cellular

160   metabolism is implicated in IVIG resistance (**Table 1**).

161

162   **5. Boolean approach sheds light on metabolic and signaling pathways specific to IVIG**

163   **responders and non-responders**

164   Based on the enrichment analysis, we identified that several metabolic and signaling

165   pathways are implicated in IVIG resistance (**Figure 1**). We focused our analyses on the

166   pathways that are linked to immune response. Also, to ensure accurate annotation, we selected

167   the pathways with P values lesser than 0.009. In IVIG non-responders, interferon (IFN)

168   regulatory factor 3 (IRF3)-mediated activation of type 1 IFN was turned on. Genes that increase

169   myeloid cell number, and that promote pyrimidine metabolism, abnormal plasmacytoid dendritic

170   cell physiology were also turned on (**Table 1**) (**Figure 1**). Of interest, though several genes

171   (fifteen) were turned on in non-responder patients, these genes contribute to limited set of

172   pathways. On the other hand, genes that modulate lymphocyte migration and Th17 response

173   were switched off (**Table 1**). Interestingly, the genes that mediate palmitate biosynthesis were

174   also switched off in IVIG non-responders (**Table 1**). Palmitate is a saturated fatty acid that acts

175   as agonist for Toll-like receptor 4 (TLR4) receptor and promotes inflammation by activating

176   macrophages and innate immune cells [44, 45]. Previous studies have highlighted the importance

177 of inflammation in IVIG resistance [25]. In IVIG responders, the genes that mediate

178 mitochondrial calcium uptake and several aspects of mitochondria mediated adenosine

179 triphosphate (ATP) production were switched off (**Table 1**). Interestingly, though the number

180 genes switched off in responder patients with boolean behavior were small (six), these genes

181 contribute to many pathways, particularly mitochondrial functions. The genes that mediate

182 degranulation of mast cells were also switched off (**Table 1**). This suggest that suppression of

183 mitochondrial function and inflammation plays a vital role in inducing immune homeostasis in

184 IVIG responders.

185

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

187 **algorithms**

188 There are several tools available to study the differentially expressed genes in a

189 microarray dataset, but they are not developed to specifically study boolean behaviors in a

190 microarray dataset. We performed data analysis on GSE18606 dataset using GEO2R and BART

191 to study whether algorithms developed to study differentially expressed genes in a microarray

192 dataset were able to shed light on boolean behavior, but both GEO2R and BART failed to index

193 the genes exhibiting boolean behavior as differentially expressed. We also used MEV to

194 performed K mean clustering, where we grouped the 40,000 genes into different clusters. We

195 generated 25, 50, 75 and 1000 different clusters to see if clustering algorithm could group the

196 genes that exhibit boolean behavior, but these genes were distributed across different clusters

197 (**Table 2**).

198

199 **7. Conclusion and perspectives**

200   IVIG is used as first line therapy in several autoimmune and inflammatory diseases, but

201   not all patients respond to IVIG treatment. Identification of biomarker that could segregate IVIG

202   responders from non-responders remains a challenge. Patients who exhibit IVIG resistance need

203   to undergo alternative therapy, thus identifying these patients in the early stages of disease will

204   help clinicians to initiate an alternative treatment strategy. Microarray is a sophisticated

205   technique that sheds lights on the complex processes occurring inside a cell and helps in

206   identifying disease specific biomarkers. There are several software's available to understand the

207   biological meaning hidden in the microarray data, each tool provides insights into the distinct

208   biological processes taking place inside the cell. Despite these advancements in microarray

209   technology, identifying a biomarker, which could segregate IVIG responders from non-

210   responders remains a challenge. In this study we have used a novel data analysis technique,

211   where we screened the genes that exhibit boolean or switch like behavior specific to IVIG

212   responders and non-responders. Using this boolean approach we have identified that cellular

213   metabolism plays a vital role in IVIG resistance. The KD patients who responded to IVIG

214   therapy exhibited limited mitochondrial activity and low inflammation. Whereas, KD patients

215   resistant to IVIG therapy had increased expression of genes that promote inflammation,

216   abnormal regulation of Th17 response.

217

218   Taking into consideration the role of cellular metabolism in influencing the success of

219   IVIG therapy, diagnostic assays could be developed to rapidly study these parameters in KD

220   patients. This could help clinicians in identifying the IVIG non-responders at an early stage and

221   to initiate alternative therapies to reduce the morbidity and associated therapeutic costs [46].

222   However, the role of cellular metabolism in the outcome of IVIG therapy in other autoimmune

223 diseases need to be studied to discover a universal biomarker. Currently, dimethyl fumarate, a

224 small molecule targeting glycolytic pathway have been approved to treat multiple sclerosis [47-

225 49]. Since mitochondria mediated ATP production was suppressed in IVIG responders, small

226 molecules capable of selectively inhibiting mitochondrial function in immune cells could be used

227 along with IVIG to treat IVIG non-responders.

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230 **Declaration of Competing Interest :**

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380

381   **Figure Legend**

382

383   **Figure 1. Genes and the major pathways that exhibit switch like behavior (on/off) in**

384   **Kawasaki disease patients specific for the IVIG treatment condition.** Several genes in IVIG

385   resistant and responder Kawasaki disease patients display switch like behavior (on/off). The

386   cluster number of those genes are highlighted on the right side of the figure. The key pathways
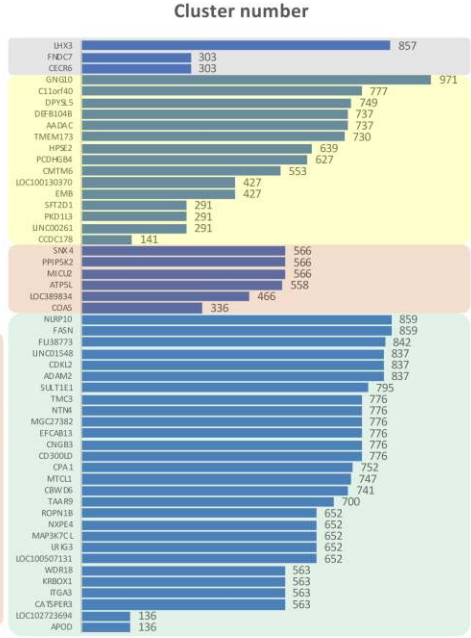
387   that are associated with those genes are also listed.

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

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396
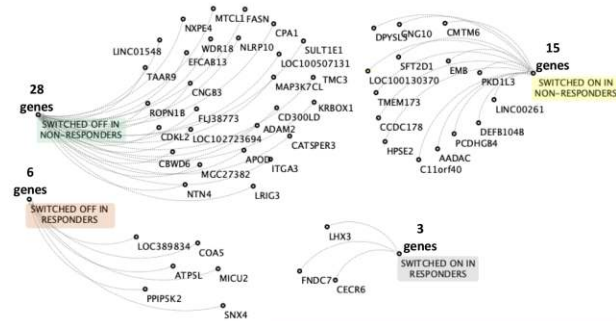
397 *Main pathways that are switched off in non-responder patients*

398

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

Name	P-value	Adjusted p-value	Odds Ratio	Combined score	Source
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

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402 *Key pathways that are switched off in responder patients*
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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

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405 *Important pathways that are switched on in non-responder patients*

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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

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

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