International Journal of Innovative Research in Medical Science (IJIRMS)
Volume 02 Issue 09 September 2017, ISSN No. - 2455-8737
Available online at - www.ijirms.in

Open Access Journal | Research Article | DOI: 10.23958/ijirms/vol02-i09/21

EPO Signaling as a Predictive Marker of Disease Severity in RSV Infection

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Abstract

Respiratory syncytial virus (RSV) infection is common in children and often causes severe respiratory clinical complications. Therefore, the establishment of biomarkers that predict severe clinical outcome is required. We performed in silico analysis across 2 datasets with available information on gene expression and disease severity to identify predictive factors of RSV infection progression. First, we selected differentially expressed genes (DEGs) in the severe group. Second, we added the DEGs in pathway analysis to observe an alteration of pathway status in the severe group. This analysis revealed candidate genes that affect pathway status. Finally, we calculated the odds ratio of the candidate genes involved in disease severity to a severe clinical course. We found that erythropoietin (EPO), a glycoprotein hormone controlled by hypoxia-inducible factor (HIF)-1, is upregulated in children with severe disease. Furthermore, increased expression of BNIP3L and FECH, downstream genes regulated by EPO levels, are highly associated with a severe course of the disease in both datasets. We propose that EPO-driven downstream signaling, especially increased expression of BNIP3L and FECH, is a biomarker that defines disease severity and potential clinical complications in children with RSV infection.

Keywords: ***RSV infection, severity, biomarker, bioinformatics***

Introduction

RSV is one of the most common causes of lower respiratory tract infection (LRTI) in children under 5 years.[1] Most infected children with RSV recover spontaneously, although it often causes severe complications that require hospitalization.[2] In developed countries, the mortality of LRTI caused by RSV is 0.3%, while the mortality in developing countries is 2.1% in children under 5 years.[3] Because LRTI provoked by RSV often develops into a severe condition in a short period of time, the prediction of potential disease complications is important. Indeed, several predictive factors for disease severity, such as premature birth, congenital heart disease, and chronic lung disease, have been identified.[4] However, children without these known risk factors often end up hospitalized with severe complications.[5] This justifies the need for timely and

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accurate forms of diagnosis through molecular means that predict RSV LRTI disease evolution.

Using microarray and transcriptome analysis of peripheral blood, 2 groups have investigated the association of gene expression and disease severity in patients with RSV infection.[5,6] On the one hand, Mejias et al. identified the immune response pathway as a key factor following RSV infection. They observed that T cells, cytotoxic/NK cells, and plasma cell genes were underexpressed in children with severe RSV LRTI. On the other hand, Brand et al. focused on olfactomedin 4, the most altered gene when severe and mild complications were compared. Indeed, the increased expression of olfactomedin 4 is strongly associated with LRTI disease severity.[5] Our results are based on the reanalysis of these molecular datasets to identify prognostic biomarkers. We performed meta-analysis across these datasets to merge biomarkers that predict severe course of illness in children with RSV infection.

Materials and Methods

Dataset search

We searched the NCBI Gene Expression Omnibus (GEO) database from January 2013 to November 2016. The datasets that included information on disease severity and gene expression data post-RSV infection in children were downloaded from the GEO database.

Datasets

We used 2 datasets, GSE38900 and GSE69606, which include clinical information and transcriptome data from peripheral blood obtained at the early stage of RSV infection (at median time of 48 h post-admission in GSE38900 and within 24 h after first contact with the hospital in GSE69606; Table 1).These datasets exclude cases with co-infection of bacteria or virus, congenital heart disease, chronic lung disease, immunodeficiency, prematurity, and systemic steroid treatment within 2 weeks before presentation in GSE38900, and cases with corticosteroid use in the prior 48 hours, congenital heart disease, lung disease, and immunodeficiency in GSE69606. In GSE69606, data of the acute and recovery states are only included where we analyzed the samples in the acute stage.

Comparison of gene expression

For GSE38900, non-normalized data were log2-transformed. Gene expression was compared using an empirical Bayes/moderated t-statistic[7] in limma package in R. DEGs were defined as the false discovery rate (FDR) < 0.05 and P < 0.05 as well as the absolute difference between groups > 2-fold.

For GSE69606, background removal, quantile normalization, and probe set summarization for the raw data were performed using robust multichip analysis (RMA). The data were then log2-transformed. The procedure for the identification of DEGs was performed as described above.

Pathway analysis

The DEGs defined as described above were added into Ingenuity Pathways Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA). In the IPA analysis, the P value obtained by Fisher's exact test was used as a significant difference.

Statistical analysis

Odds ratio including multivariate analysis calculated by logistic regression analysis was performed using the generalized linear model (GLM) package in R with the family as the binominal.

Results

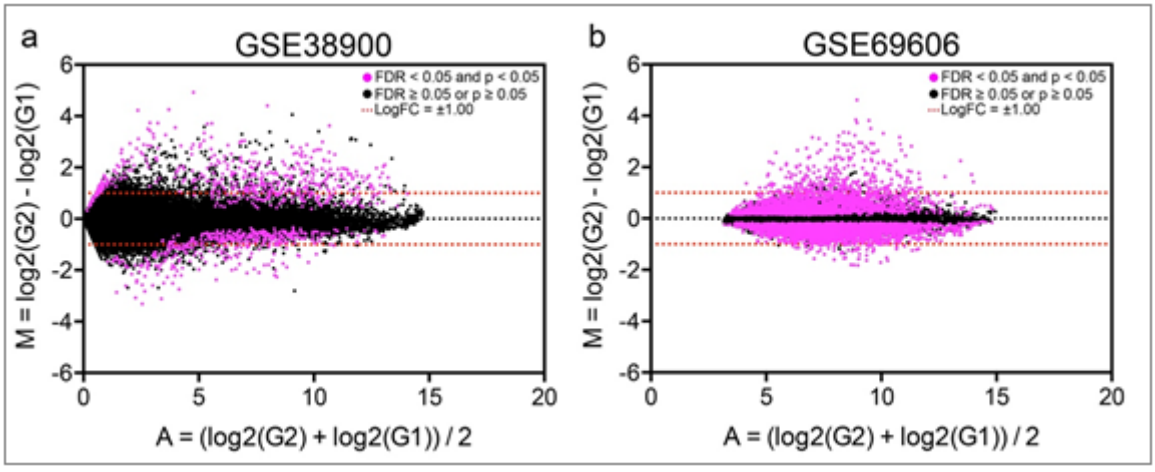

Figure 1: Gene expression profile

MA plot shows the comparison of gene expression in the severe and non-severe groups in GSE38900 (a) and GSE69606 (b). Pink dots are genes with FDR < 0.05 and P value < 0.05. The pink dots located off-center over the red line (LogFC = 1) are defined as DEGs.

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Table 1: Baseline characteristics

		GSE38900	GSE69606
Clinical course	healthy	5	0
	mild	8	9
	moderate	9	9
	severe	7	8
Age (months)	median (range)	3.97 (2.67-6.13)	NA
Gender	male	2 (40%)	NA
	female	3 (60%)	

NA: Not available

Table 2: Top 25 DEGs in GSE69606

Upregulated	Fold difference	Downregulated	Fold difference
OLFM4	100.9	**GZMH**	-6.2
MMP8	46.8	**FGFBP2**	-6.1
CAECAM8	41.5	**LGALS2**	-6.0
CA1	33.2	**GNLY**	-5.5
CHI3L1	28.7	**KLRF1**	-5.1
SELENBP1	28.1	KLRC2	-4.9
DEFA4	27.4	TGFBR3	-4.7
EPB42	25.3	**GZMK**	-4.7
LTF	23.7	ALYREF	-4.7
CRISP3	22.5	**KLRD1**	-4.6
CEACAM6	19.6	**GZMB**	-4.5
BPI	19.1	YME1L1	-4.2
ARG1	18.9	**FCER1A**	-4.0
AHSP	18.6	**SPON2**	-3.9
ANXA3	18.4	RGS1	-3.9
GYPA	18.0	AKR1C3	-3.7
ELEANE	17.9	XCL2	-3.6
MPO	17.9	**ITPKB**	-3.6
HP	17.5	**SH2D1B**	-3.6
PRTN3	16.2	**KLRB1**	-3.4
SNCA	15.4	IGHG3	-3.4
TNS1	14.7	CD160	-3.4
MS4A3	14.5	**PRF1**	-3.4
LCN2	14.0	ADGRG1	-3.4
SLC4A1	13.9	PTGDR	-3.3

Bold font indicates genes that were also identified in the original analysis.

Table 3: Upstream prediction

Upstream		GSE38900	GSE69606
HIPK2	Activation z-score	2.236	2.641
	Overlap *P* value	4.82E-04	6.69E-09
	DEG	**ANK1**, **FECH**, **KLF1**, SPTB, TFR2	ALAS2, **ANK1**, BCL2L1, **FECH**, GATA1, HBE1, HBZ, **KLF1**, SLC25A37, SLC4A1
EPO	Activation z-score	2.562	2.998
	Overlap *P* value	5.49E-04	1.08E-13
	DEG	**ANK1**, **BLVRB**, **BNIP3L**, **CA1**, CREG1, FASLG, **FECH**, **GYPA**, **KLF1**, **RHAG**	ABCA13, ALAS2, **ANK1**, BCL2L1, **BLVRB**, **BNIP3L**, **CA1**, CA2, CHIT1, **FECH**, GATA1, GSPT1, **GYPA**, HBG2, **KLF1**, MPO, MYB, NFE2, PTCD4, **RHAG**, SELP, SLC4A1, STRADB, TAL1

Bold font indicates differentially expressed genes observed commonly across 2 datasets.

DOI: 10.23958/ijirms/vol02-i09/21

Table 4: Odds ratio of DEGs regulated by EPO

	GSE38900			GSE69600		
	OR	**95% CI**	***P* value**	**OR**	**95% CI**	***P* value**
ANK1	2.67E+09	1.65E-206-NA	0.996	50.999	5.412-1308.526	*0.003
BLVRB	11.666	1.691-117.768	*0.019	2.83E+09	1.55E-172-NA	0.996
BNIP3L	10.624	1.664-97.330	*0.019	24.000	3.293-288.442	**0.004
CA1	21.333	2.249-511.040	*0.017	6.94E+08	1.42E-122-NA	0.994
FECH	14.999	2.214-149.770	**0.009	13.333	1.952-133.770	*0.013
GYPA	2.67E+09	1.65E-206-NA	0.996	28.333	3.205-663.926	**0.008
KLF1	0.450	0.053-2.767	0.409	50.999	5.412-1308.526	**0.003
RHAG	2.67E+09	1.65E-206-NA	0.996	5.20E+08	1.44E-153-NA	0.995

OR: Odds ratio
NA: Not applicable
CI: Confidence interval

Table 5: Multivariate analysis of candidate gene expression in clinical course

	Crude			Adjusted		
	OR	**95% CI**	***P* value**	**OR**	**95% CI**	***P* value**
BNIP3L	10.625	1.664-97.330	*0.019	9.435	1.300-105.796	*0.038
Age	0.878	0.524-1.142	0.478	0.963	0.523-1.308	0.846
Gender	0.562	0.090-3.175	0.514	0.640	0.076-5.344	0.670
FECH	15.000	2.214-149.770	**0.009	14.219	1.880-173.534	*0.017
Age	0.878	0.524-1.142	0.478	0.962	0.516-1.301	0.846
Gender	0.562	0.090-3.175	0.514	0.486	0.047-4.247	0.511

OR: Odds ratio
Age was evaluated as a continuous variable.

Gene expression alteration

The cohorts were divided into 2 groups, severe or non-severe (mild and moderate), and the gene expression pattern was compared between these groups (Table 1). In GSE38900, 234 genes were differentially expressed (144 upregulated and 121 downregulated in the severe group) (Figure 1a). In GSE69606, 289 DEGs were identified (231 upregulated and 47 downregulated in the severe group) (Figure 1b). Although the grouping criteria had a modest difference in GSE69606 (severe vs. mild in the original analysis, whereas severe vs. non-severe [moderate and mild] in this study), surprisingly, the DEGs in our study are almost identical to the result of the original analysis (Table 2).

Pathway analysis

The DEGs in each dataset were independently introduced into IPA to identify pathways up- or downregulated. We focused on upstream regulator analysis based on overlapping P values and activation z-scores. Among upstream regulators that altered its activation status in each dataset, only 2 upstream regulators overlapped across the sets. EPO: P = 5.49-04E in GSE38900 and P = 1.08E-13 in GSE69606. HIPK2 (homeodomain-interacting protein kinase 2): P = 4.82-04E in GSE38900 and P = 6.69E-09 in GSE69606 (Table 3). Both pathways are upregulated in the severe group. EPO: z = 2.562 in GSE38900 and z = 2.998 in GSE69606; HIPK2: z = 2.236 in GSE38900 and z = 2.2641 in GSE69606 (Table 3).

Disease severity responsive genes

Among the predicted upstream regulators of the DEGs, we focused on DEGs observed across the 2 datasets. The number of DEGs that overlapped across the datasets is 3 in the HIPK2 and 8 in the EPO pathways (Table 3). Interestingly, all 3 DEGs in the HIPK2 pathway were also observed in the EPO pathway as DEGs (Table 3). We next investigated the effect of expression of DEGs on disease severity. We calculated an odds ratio of all the DEGs when the expression was above median (Table 4). This analysis revealed 4 genes in GSE38900 and 5 genes in GSE69606 as disease severity-related genes with significant P values (Table 4). Among those genes, BNIP3L and FECH showed the highest significance across the datasets. In the previous report, age influences immune response that is essential for disease severity. Therefore, we performed a multivariate analysis to account for other factors such as age and gender in GSE38900. In this analysis, BNIP3L and FECH remained significant (Table 5).

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Discussion

In this study, we performed in silico analysis to identify biomarkers that predict a severe course of RSV LRTI. The unbiased screening revealed the relationship between the upregulation of EPO and HIPK2 transcription factor with disease severity. EPO binds to its receptor (EPOR) on erythroid progenitors to stimulate their proliferation and differentiation into hemoglobin-containing erythrocytes.[8] HIPK2, a serine/threonine-protein kinase, is involved in p53-mediated apoptosis and cell cycle regulation.[9] Thus, these pathways play roles in distinct systems; however, DEGs that form the HIPK2 pathway in this study are completely overlapped with the genes that form the EPO pathway (Table 3). This indicates that the EPO pathway includes more DEGs compared to the HIPK2 pathway and this prompted us to focus on the EPO pathway in disease severity.

Much evidence has revealed that EPO is induced by hypoxia-inducible factor 1 (HIF1) in a hypoxic state.[10,11] HIF1 is composed of 2 subunits, HIF1α and HIF1β .[12] In normoxic conditions, HIF1α is under ubiquitination dependent proteolysis. Once cells are under hypoxic conditions, HIF1α is stabilized and translocates into the nucleus. Then, HIF1α forms a heterodimer with HIF1β and activates transcription of HIF1-regulated genes such as EPO.[11,10] Different studies have revealed that bacterial infection also activates HIF1 independently of hypoxia.[13] RSV infection also causes hypoxia-independent activation of HIF1α.[14] These lines of evidence might provide a clue to understand the mechanism by which EPO activation is involved in the disease severity in RSV infection.

Among the downstream genes of the EPO pathway, increased expression of BNIP3L and FECH are related to disease severity, suggesting that these molecules might play a central role in disease complications and final clinical outcomes. BNIP3L is a member of the Bcl-2 family that regulates apoptosis through the mitochondrial pathway.[15] FECH, also known as ferrochelatase, is involved in the heme biosynthetic pathway and is required for the insertion of iron into protoporphyrin to form the heme group.[16] Both HIF1α and EPO induce BNIP3L and FECH expression.[17,18] HIF1α-driven BNIP3L induction promotes cell death in certain tissues such as neurons. In contrast, BNIP3L induction does not induce cell death in fibroblasts and tumor cells.[19] A recent report revealed that treatment with antioxidants such as N-acetylcysteine and butylated hydroxyanisole inhibits cell death caused by HIF1α-driven induction of BNIP3L. Also, small molecule inhibitors of HIF1 such as dihydroxybenzoate (DHB) or dimethyloxalylglycine (DMOG) also suppress HIF1α-BNIP3L-dependent neuronal cell death.[20] These facts suggest that treatment with antioxidants or HIF1 inhibitors can be effective for a severe course of RSV infection.

Although further clinical work is required, validation of these findings may enable BNIP3L and FECH expression profiling to become a valuable tool for identifying high-risk LRTI patients after RSV infection and those who are eligible for adjuvant therapeutic interventions.

Conclusion

Upregulation of EPO-driven signaling, especially increased expression of BNIP3L and FECH, can be biomarkers that define disease severity and potential clinical complications in children with RSV LRTI.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research (C), Grants-in-Aid for Young Scientists (B), and a St. Marianna University Grant. We thank all the members of staff at department of pharmacogenomics for discussions.

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DOI: 10.23958/ijirms/vol02-i09/21

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Abbreviations

RSV: respiratory syncytial virus
EPO: erythropoietin
LRTI: lower respiratory tract infection
HIF: hypoxia-inducible factor
GEO: Gene Expression Omnibus
DEG: differentially expressed gene
RMA: robust multichip analysis
GLM: generalized linear model
EPOR: erythropoietin receptor
HIPK2: homeodomain-interacting protein kinase 2
DHB: dihydroxybenzoate
DMOG: dimethyloxalylglycine

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