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Transcription profiling identifies genes involved in severe asthma

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ABSTRACT

Severe asthma is a chronic respiratory disease with heterogeneous symptoms. This study aimed to determine the gene expression pattern and pathways related to severe asthma and subsequently identify potential predictor for steroid-resistant asthma. Peripheral blood B lymphocytes were isolated from subjects with severe steroid-resistant (n=7) and severe steroid-dependent (n=7) asthma. Total RNA was extracted from the B lymphocytes and subjected to microarray experiment. Data were analyzed using GeneSpring GX software for differential gene expression analysis and gene set enrichment analysis (GSEA) was used to analyze disease pathways. The prediction model was generated using Prophet software and real-time polymerase chain reaction (PCR) was performed to validate the microarray gene expression. 307 genes were differentially expressed between both groups with $p<0.001$ using unpaired t-test. Six genes were selected as steroid-resistant predictor based on a particular selection criteria. Class predictors were identified with a predictive accuracy of 93%. This study has provided a better insight into the expression pattern and pathways of severe asthma and provided potential prognosis biomarkers to discriminate between severe steroid-resistant and steroid-sensitive asthma.

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INTRODUCTION

Asthma is a respiratory disease that is characterized by symptoms such as a cough, airway hyperresponsiveness, shortness of breath, wheezing and mucus hypersecretion. It was estimated that out of all asthmatics, 5 to 10% suffer from severe asthma (Chung *et al.,* 2014). Severe asthma is being regarded as a heterogeneous disease due to its variable endotypes which involve innate immune activation, type 1 and type 17 immune responses, neutrophilic and/or eosinophilic inflammation as well

as different underlying molecular mechanisms (Hansbro *et al.,* 2017).

Previous studies have suggested that the cellular inflammatory events which had been found in mild to moderate asthma such as the notable roles of Th2 lymphocytes, eosinophils and basophils at the inflammatory sites, are not feature characteristics in patients with severe asthma (Djukanovic, 2000; Wenzel, 2005). Conversely, severe asthma is predominant with neutrophils inflammation (Wenzel *et al.,* 1997). Some patients with severe steroid-resistant asthma were reported to possess weak glucocorticoid receptor (GR) to glucocorticoid response element (GRE) interactions due to impaired nuclear translocation of GR (Ito *et al.,* 2006; Adcock *et al.,* 2008). Overactivation of nuclear activator protein-1 (AP-1) and nuclear factor kappa light chain enhancer of activated B cells (NF-κB), elevated expression of c-Jun N-terminal kinase (JNK) and human proto-oncogene c-Fos in the nucleus may intervene GR:GRE binding affinity (Adcock *et al.,* 2008, Ito *et al.,* 2006, Loke *et al.,* 2006). However, many severe steroid-resistant asthmatics were reported to have a normal nuclear translocation of GR and undisturbed GR: GRE binding affinity (Adcock *et al.,* 2008, Barnes, 2004). This suggests dissimilarity in response to corticosteroid within the severe asthma group which may involve other inflammatory pathways or expression pattern compared to patients with steroid-responsive asthma.

As a polygenic disorder, no single gene has yet been identified that can explain the underlying molecular mechanism of severe asthma disease (Melen and Pershagen, 2012). Therefore, to search for disease-related gene expression profiles, oligonucleotide microarrays have been used. Although the diagnostic value of the expression of each gene alone is regarded as minimal, high throughput gene analysis may increase the understanding of gene network, predictability and help in exploring potential biomarkers.

In this study, we sought to identify the gene expression profiles of peripheral blood B lymphocytes in patients with severe steroid-resistant and steroid-responsive asthma. In addition, we subsequently determine the gene signature that was associated with the differentiation of severe steroid-resistant from severe steroid-responsive asthma.

MATERIALS AND METHODS

Subjects

This study was approved by the Ethics and Research committee from the Faculty of Medicine, Universiti Kebangsaan Malaysia (UKM). Following ethical clearance, subjects were recruited from the Respiratory Clinic at UKM Medical Centre (UKMMC) who visited the clinic for monthly appointments. 50ml of peripheral blood was withdrawn upon receiving inform consent from patients and diagnosed by the respiratory experts as having bronchial hyperreactivity through positive bronchodilator reversibility test (≥15%). Each one of the asthmatics were classified into two different asthma phenotypes; severe steroid-dependent and severe steroid-resistant. Subjects were classified as atopic when the results are positive upon skin prick test, high total serum IgE level or having other allergic diseases like eczema, conjunctivitis and rhinitis.

Isolation of B Lymphocytes

Peripheral blood in the heparinized tubes were processed immediately upon withdrawal and peripheral blood mononuclear cells (PBMC) were separated by gradient centrifugation. B cells were isolated out from the PBMC using the CD19 EasySep® Positive Selection kit (Stemcell Technologies Inc., Canada). B cells purity were assessed using FACS Calib (Fluorescent Activated Cell Sorter, Beckton Deckinson, USA).

RNA Extraction

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Germany). Purity and integrity of the RNA were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., USA). Total RNA with RIN (RNA integrity number) of more than 8.0 was chosen. Low quality of RNA was excluded.

cDNA Synthesis, Labelling, Hybridization and Detection

For each sample, 50 to 500ng of total RNA was reverse transcribed and amplified using Illumina TotalPrep RNA Amplification kit (Ambion Inc., USA). Amplified RNA (aRNA) was labelled with biotin and hybridized onto the HumanRef-8 Expression BeadChip (Illumina Inc., USA) for 16 to 20 hours in the Illumina oven. Slides were washed, dried and scanned in the BeadArray Reader (Illumina Inc., USA). The HumanRef-8 Expression BeadChip is an oligonucleotide array which contains 24,355 known gene probes.

Statistical Analysis

Data were imported into BeadStudio software (Illumina Inc., USA). Background intensities were subtracted from feature intensities to get a high confidence level estimation towards hybridized probes. Output file contained the mean value of pixel intensities which represent the expression signal of every gene in the samples, the detection score, the standard deviation for each bead and performance score control. Linearized raw data values were exported into GeneSpring GX10.0.02 (Agilent Technologies Inc., USA) in a tab-delimited

text format for subsequent analysis. Signal intensity values were further processed involving level determination, log transformation, quantile normalization, and baseline transformation. Normalized data were filtered by 90% flag present and corrected for multiple testing correction by using Benjamini Hochberg (False Discovery Rate) with $p<0.05$. Unpaired Student's T-test was used to screen differentially expressed genes followed by hierarchical clustering using similarity metric Pearson centred and average linkage as linkage rule. Principle component analysis (PCA) was done to reduce the dimensionality of data and changed into a dominant profile or entities.

Class Prediction

After a robust gene selection, a prediction model was generated onto the training cohort with the known class using a web-based tool called Prophet. The class prediction model was then applied towards the testing cohort to test whether this small group of genes can predict the asthma phenotype in the test cohort.

Gene Set Enrichment Analysis (GSEA)

GSEA from the Pathway Studio 6.0 (Ariadne Genomics, USA) was applied to the filtered microarray data by choosing Kolmogorov Smirnov statistic and False Discovery Rate (FDR) ≤0.25. Gene sets from the microarray data were compared with the pathway and ontologies existed in the software database such as Ariadne ontology; and gene ontology: biological process, molecular function and cellular component.

Gene Validation using Real-Time PCR

Three genes were selected to validate the microarray results using eight samples. Reverse transcription was performed using 200ng of total RNA and Quantitect Reverse Transcription kit (Qiagen, Canada). The real-time PCR reaction was prepared by adding the SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc., USA) onto the cDNA template and run on CFX96™ real-time PCR system (Bio-Rad Laboratories, Inc., USA). All analyses were performed in triplicate, and relative RNA levels were determined using beta-actin (*ACTB*) and glyceraldehyde 3-phosphate (*GAPDH*) as an internal control.

RESULTS AND DISCUSSION

Study Subjects

Samples from 14 asthmatics were used in the microarray experiment taking into account the quantity and quality of the RNA. Female subjects made up the majority with 71% as opposed to male subjects. For severe steroid-dependent group (n=7), the subjects' age range is between 22 to 60 years with a mean value of 50.3±11.9 years, while for severe steroid-resistant (n=7), the age range is between 31 to 65 years with a mean value of 51.6±11.9 years. Malay represented the highest among all race with 42%, followed by Chinese and Indian, both 28%. The characteristics of the study subjects are listed in Table 1.

From the 50ml of peripheral blood withdrawn, CD19+ B cells were successfully purified by >90% when analyzed using flow cytometry. Sufficient RNA was obtained, and all total RNA had met subsequent strict quality control measure by choosing the only RNA with RIN more than eight.

Differential Gene Expression and GSEA Analysis

Data were normalized and filtered by flag present with at least 50% out of total samples where 12,943 filtered genes appeared. Unpaired T-test was applied to the filtered genes by comparing the severe sensitive and resistant steroid group. Based on the statistical analysis, 307 genes were differentially expressed between the two groups with $p<0.001$. Unsupervised hierarchical clustering was done following statistical analysis and successfully divided the samples into its respective phenotype (Figure 1).

GSEA was applied towards data which had been normalized and filtered by flag present. Genes found in the significant (FDR≤0.25) gene sets by GSEA were compared with significant genes from different phenotype. An FDR of 25% indicates that the result is likely valid three out of four times, which is reasonable in the setting of exploratory discovery. Using a more stringent FDR cutoff may lead to an overlooking of potentially significant results (Subramanian *et al.,* 2005). Only five genes were downregulated out of 307 statistically significant genes in severe steroid-resistant compared to severe steroid-dependent group. Pathways that significantly expressed genes in steroid response are listed in Table 2.

Interestingly, GSEA analysis results exhibited downregulation of a few pathways that are involved in inflammation such as T cell and mast cell activation as well as cytoskeleton regulation in severe steroid-resistant compared to severe steroid-dependent asthma. This finding was similar to a study by Miranda et al. (2004) who reported that late-onset asthma group was not associated with allergy compared to early-onset asthma. Severe late-onset asthma reveals low numbers of CD3+ T lymphocytes and mast cells in the airway tissue biopsy when compared to severe early-onset asthma. No obvious inflammation was found at the airway of endobronchial biopsy and thin subepithelial basement membrane was displayed,

Table 1: Characteristic of the study subjects

Parameter	Severe steroid-dependent (n=7)	Severe steroid-resistant (n=7)	p-value
Age (year ± SD; range)	50.3 ± 11.9 (22-60)	51.6 ± 11.9 (31-65)	-
Gender			0.575
Male	2	2	
Female	5	5	
Race			0.96
Malay	4	2	
Chinese	2	2	
Indian	1	3	
Atopic	4	3	0.261
Non-atopic	3	4	
Daily medication (inhaled corticosteroid)	>800µg or <800µg + LABA*	>800µg or <800µg + LABA	-
Have symptom during blood withdrawal	No	Yes	-

*LABA = long-acting beta agonist

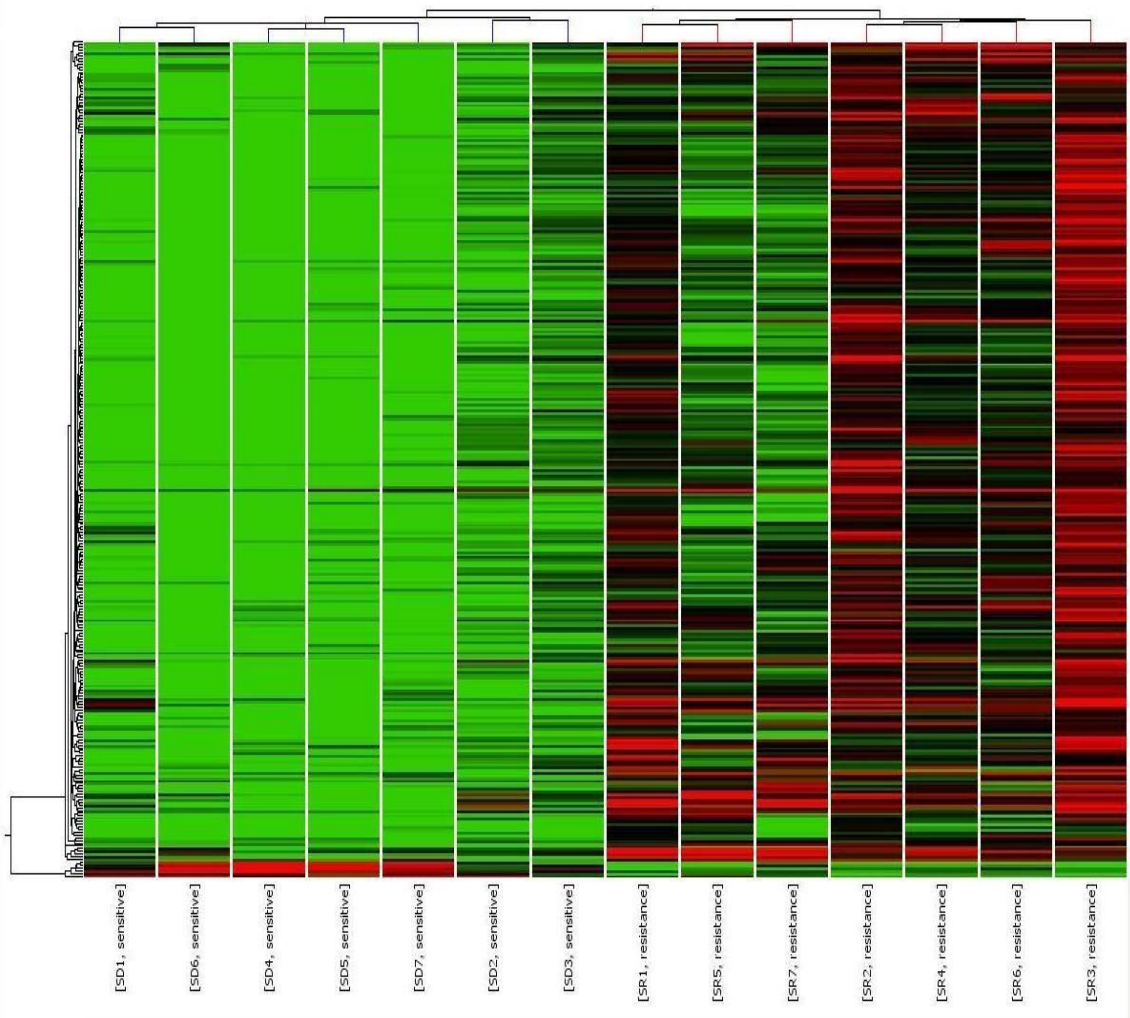

Figure 1: Hierarchical clustering of 307 differentially expressed genes in severe steroid-dependent and severe steroid-resistant asthma (Unpaired T-test: p<0.001)
Green denotes upregulated gene and red denotes downregulated gene pattern of expression. Each row denotes a distribution of expression patterns across phenotypic clusters. Similar colours denote similar patterns of expression, whereas different colours denote different patterns of expression. SD = severe steroid-dependent; SR = severe steroid-resistant.

Table 2: List of significant differentially expressed genes found in the GSEA pathway enriched in steroid response asthma (FDR≤0.25)

GSEA significant gene set	Gene set p-value	Statistically significant gene list
Mast cell activation	<0.00001	*PIK3R1, PLCB3, RASGEF1B, LOC340156, MAP2K6, CAMK2B, ALOX12B, TSLP, CADPS, RPH3AL, EXOC7, SYT8, EXOC*
T cell activation	<0.00001	*PLCB3, PIK3R1, RASGEF1B, LOC340156, CAMK2B, CADPS, RPH3AL, EXOC7, SYT8, EXOC5, ALOX12B, TSLP, TES, MYOZ1, UTRN, ASB4*
Actin cytoskeleton regulation	<0.00001	*FG18, ITGA2, GNG13, PIK3R1, RAPGEF3, MAP2K6, TES, MYOZ1, UTRN*
G-protein coupled receptors signalling	<0.00001	*GNAS, ADRA1D, OR9A4,*
Drug response	<0.00001	*GNAS, MAP2K6*

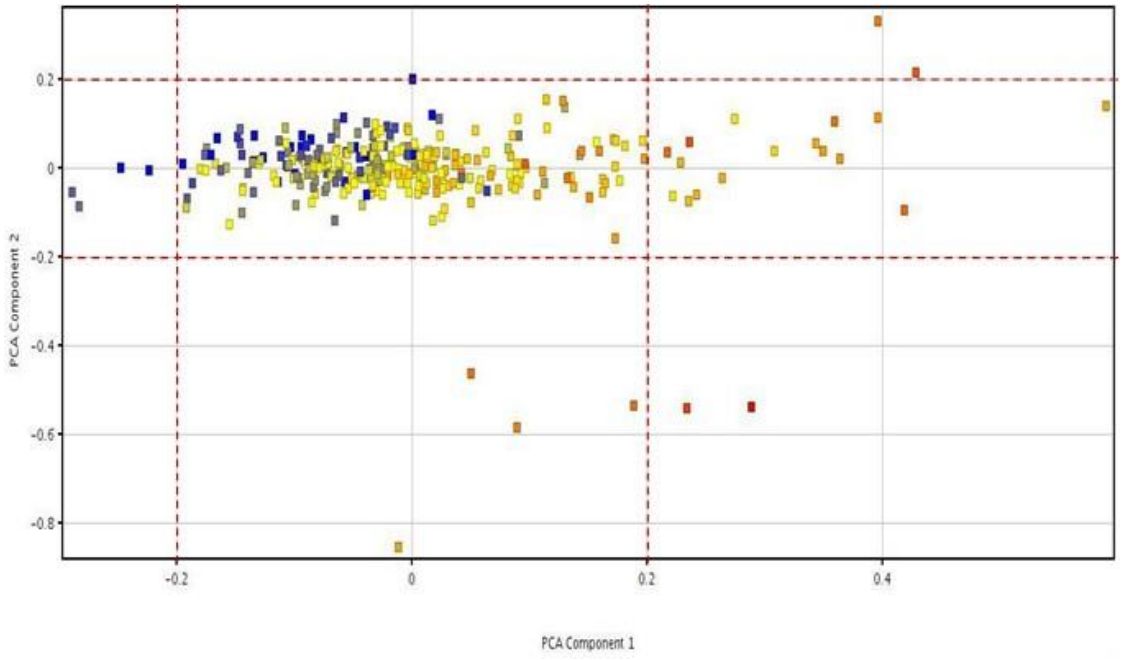

Figure 2: PCA analysis towards significant differentially expressed genes for steroid response with correlation metric cut-off at <-0.2 and >0.2 as indicated by the dashed line. Each dot represents the differentially expressed gene.

Table 3: Character of 6 genes chose for class prediction analysis based on p-value (>0.001); fold change (>1.4) and expressed in the steroid response group

Symbol	p-value	Fold change	Gene expression (steroid-resistant)
ALS2CR14	9.46 E-04	1.85	increase
FLJ10490	4.71 E-04	1.45	increase
PSIP1	3.94 E-07	1.44	increase
RARRES2	1.39 E-04	1.44	increase
FAM63B	8.84 E-04	1.43	increase
SLC38A6	4.82 E-04	1.41	increase

Table 4: Percentage of accuracy, sensitivity and specificity of a class predictor for steroid-dependent asthma using three different algorithms

Model	Accuracy (%)	Positive prediction value (%)	Sensitivity	Specificity (%)
DLDA	93	88	100	86
PAMR	93	88	100	86
KNN (n=2,3,4,5)	93	88	100	86

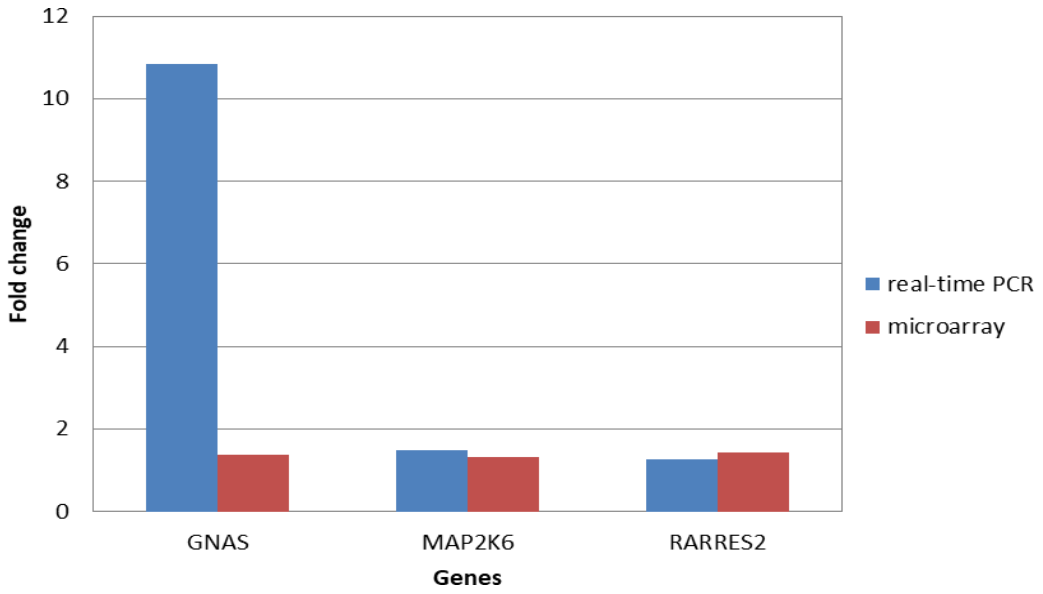

Figure 3: Validations of microarray data by using real-time PCR method indicate that *RARRES2*, *MAP2K6* and *GNAS* genes showed a similar direction of upregulation in the severe steroid-resistant asthma

different from other asthma biopsies that showed subepithelial basement membrane thickening (Miranda *et al.,* 2004). Another study reported that IL-4 and IL-13 mRNA and protein level of inflammatory cells acquired from bronchial alveolar lavage samples were lower in severe compared to milder asthma patient regardless of early or late onset asthma (Wenzel *et al.,* 2004).

Actin cytoskeleton regulation and lymphocytes activation were downregulated in severe steroid-resistant asthma, and one of the genes that is involved in both pathways is testis derived transcript (3 LIM domains) (*TES*). Previously, *TES* was reported to have lower expression in most of the cancer cell line even in the primary cell tumours like ovarian cancer, breast cancer, uterus sarcoma cancer (Sarti *et al.,* 2005) and hematopoietic cancer (Tatarelli *et al.,* 2000). Studies have shown that its gene expression was influenced by methylation at the CpG *TES* (Tatarelli *et al.,* 2000; Tobias *et al.,* 2001). Another research on breast invasive ductal carcinoma T47D cells transfected with *TES* displayed anti-proliferative effects (Garvalov *et al.,* 2003) and uncover activation of extrinsic and intrinsic apoptotic pathways (Sarti *et al.,* 2005). Therefore, unidentified atopic inflammation reaction in the severe late-onset asthma reported previously may be due to B cells apoptosis resulting in a diminished number of T cells and mast cells activation which later decreases the production of IgE. In this present study, expression of *TES* was reduced in B cells of steroid-dependent asthma which may be the subsequent effect of unmethylated *TES* DNA promoter leading to B cells apoptosis.

Mast cell activation pathway also decreased in severe steroid-resistant asthma which might be caused by *PIK3RI* gene as one of the regulatory subunit (p85α) of IA class PI3K family. P85α regulatory subunit is the most highly expressed regulatory subunit such as p55α, p50α, p85β and p85γ (Doi *et al.,* 2008). The study has also reported that PI3K is the negative regulator of IgE production (Doi *et al.,* 2008). An experiment conducted on B lymphocytes of unimmunized p85α$^{-/-}$ mice revealed the increased degree of IgE binding towards B cells surface via CD23 compared to wild type. Serum IgE production was also significantly elevated in mice without p85α (Doi *et al.,* 2008). Two different mechanisms involved in the negative regulation of IgE production have been suggested. First, suppression of class switch recombination (CSR) mechanism which is important for the antibody isotype switching. High expression of *IP3K* induced the expression of differentiation-2 (ID2) inhibitor during the cross-linking of antigen and B cell receptor (BCR). ID2 then drive the restriction of activation-induced cytidine deaminase (AID) and CSR (Doi *et al.,* 2008). The second mechanism implicates IgE regulation at protein synthesis level where inhibition of IgE secretion in concentrated cell culture experienced an adverse effect when *PI3K* was suppressed although the amount of IgE mRNA was not decreased (Doi *et al.,* 2008). Activation of mast cells is triggered by the formation of the complex between allergen and allergen-specific IgE which bind onto high-affinity FcεRI receptor. The diminished of mast cell activation pathway in steroid-resistant asthma is most likely caused by increased expression of *PIK3RI* which is the negative regulator of IgE production.

Class Prediction

Few approaches have been used to reduce the number of genes for class prediction. 307 genes for steroid response were filtered by p-value, fold change (more than 1.4) or falls in the PCA correlation matrix. PCA is usually used to show data and structure analysis, but in some introduction level, it also can be utilized in reducing the probe dimension. PCA was applied towards the 307 genes based on the steroid response component. 29 genes were found to be strongly involved in the steroid response based on the PCA matrix correlation cut-off point and gene isolation at <-0.2 and 0.2> (Figure 2). Six genes were selected as they fulfilled the criteria of gene selection (Table 3).

Prediction model was built based on the six genes signature of expression measurement for training set using Prophet (http://www.gepas.org). Comparisons were done between a few class prediction algorithms including diagonal linear discriminant analysis (DLDA), nearest shrunken centroid (PAMR) dan k-nearest neighbours (KNN). Four samples of severe steroid-dependent and four samples of severe steroid-resistant asthma were used as a training set, and 14 asthma samples with known steroid response were used as test set. Gene with the low error rate in the confusion metric were chosen as the predictor. Four genes: *PSIP1*, *RARRES2*, *SLC38A6* and *FLJ10490* are able to predict the difference between severe steroid-dependent and resistant group with low error rate through KNN and PAMR model. The sensitivity and specificity of the predictor are shown in Table 4.

Retinoic acid receptor responder 2 (*RARRES2*) encodes chemerin chemotactic protein which trigger the chemotaxis via ChemR23 receptor. ChemR23 is a G-coupled protein receptor that is linked to chemokine receptor expressed specifically on dendritic cells and macrophages (Wittamer *et al.,* 2003) as well as human natural killer cell receptor (Parolini *et al.,* 2007). Most of the chemokine and chemoattractant molecules usually attract various groups of leukocyte subpopulation (Murphy *et al.,* 2000; Banchereau *et al.,* 2001).

ChemR23 receptor also increased its RNA expression in the lung macrophages that experienced OVA-induced inflammation, as well as positive expression in the epithelium bronchial (Oostendorp *et al.,* 2004). T cells, monocytes and bone marrow CD34+ precursor also generally exhibited upregulation of ChemR23 expression following activation (Migeotte *et al.,* 2002). Cross-link between IgE and FcεRI mediated by antigen on the mast cells surface secrete tryptase enzyme which is a strong activator of chemerin where it rapidly activates the prochemerin (inactive form of chemerin). Mice without ChemR23 receptor on its IgE-dependent passive acute anaphylaxis dermal showed decline numbers of leukocytes on the dermis (Zabel *et al.,* 2008).

Previous studies reported that PC4 and SFRS1 interacting protein-1 (*PSIP1*) gene are more involved in atopic dermatitis (AD) (Watanabe *et al.,* 2011; Sugiura *et al.,* 2007; Ayaki *et al.,* 2002). AD is an inflammatory disease closely related to asthma where patients also experience hypersensitivity towards environmental antigen and elevated level of serum IgE. Children with AD most likely will develop asthma in their life compared to children without AD (Cooper, 1994; Beltrani, 1999). Ochs and colleagues (2000) also reported that *PSIP1* specific IgE autoantibody was detected on AD patients using western blotting.

Real-Time PCR

Microarray gene data validation was conducted using real-time PCR. *RARRES2*, *GNAS* and *MAP2K6* genes were chosen for validation of steroid response (Figure 3). *ACTB* and *GAPDH* were the housekeeping genes used as internal control. All the validated genes showed a similar pattern with the microarray data which further confirmed the expression profiles of the selected genes in the different phenotype of asthma.

CONCLUSION

This study had shown the involvement of a few specific pathways in each of the severe subphenotypes of asthma. Overall, the association of actin cytoskeleton regulation, cytokine and chemoattractant secretion, and inflammatory cells activation played important roles in the contribution of peripheral blood B lymphocytes towards severe asthma phenotypes.

As a recommendation, clinical validation study is needed to further confirm the robustness of the genes identified which has the ability to predict steroid response status. Gene knockout or RNA interference (RNAi) methods might also be used to see which pathway will be affected by the inhibition of predictor gene.

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REFERENCES

Adcock, I. M., Ford, P. A., Bhavsar, P., Ahmad, T. and Chung, K. F. 2008. Steroid resistance in asthma: Mechanisms and treatment options. Current Allergy and Asthma Reports, 8, 171-178.

Ayaki, M., Ohoguru, N., Azuma, N., Majima, Y., Yata. K., Ibarika, N., Singh, D. P., Ko, V. and Shinohara, T.

2002. Detection of cytotoxic anti-LEDGF autoantibodies in atopic dermatitis. *Autoimmunity*, 35(5), 319-327.

Banchereau, J., Schuler-Thurner, B., Paluck, A. K. and Schuler, G. 2001. Dendritic cells as vectors for therapy. *Cell*, 106(3), 271-274.

Barnes, P. J. 2014. Corticosteroid resistance in airway disease. *Proceedings of the American Thoracic Society*, 1, 264-268.

Beltrani, V. S. The clinical spectrum of atopic dermatitis. 1999. *Journal of Allergy and Clinical Immunology*, 104(3 Pt 2), S87-98.

Chung, K. F., Wenzel, S. E., Brozek, J. L., Bush, A., Castro, M., Sterk, P. J., Adcock, I. M., Bateman, E. D., Bel, E. H., Bleecker, E. R., Boulet, L., Brightling, C., Chanez, P., Dahlen, S., Djukanovic, R., Frey, U., Gaga, M., Gibson, P., Hamid, Q., Jajour, N. N., Mauad, T., Sorkness, R. L. and Teague, W. G. 2014. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *European Respiratory Journal*, 43(2), 343–373.

Cooper, K. D. 1994. Atopic dermatitis: recent trends in pathogenesis and therapy. *Journal of Investigative Dermatology*, 102(1), 128-137.

Djukanović, R. 2000. Asthma: A disease of inflammation and repair. *Journal of Allergy and Clinical Immunology*, 105(2 Pt 2), S522-526.

Doi, T., Obayashi, K., Kadowaki, T., Fujii, H. and Koyasu, S. 2008. PI3K is a negative regulator of IgE production. *International Immunology*, 20(4), 499-508.

Garvalov, B. K., Higgins, T. E., Sutherland, J. D., Zettl, M., Scaplehorn, N., Köcher, T., Piddini, E., Griffiths, G. and Way, M. 2003. The conformational state of Tes regulates its zyxin-dependent recruitment to focal adhesions. *Journal of Cell Biology*, 161(1), 33-39.

Hansbro, P. M., Kim, R.Y., Starkey, M. R., Donovan, C., Dua, K., Mayall, J. R., Liu, G., Hansbro, N. G., Simpson, J. L., Wood, L. G., Hirota, J. A., Knight, D. A., Foster, P. S. and Horvat, J. C. 2017. Mechanisms and treatments for severe, steroid-resistant allergic airway disease and asthma. *Immunology Review*, 278(1), 41-62.

Ito, K., Chung, K. F. and Adcock, I. M. 2006. Update on glucocorticoid action and resistance. *Journal of Allergy and Clinical Immunology*, 117, 522-543.

Loke, T. K., Mallett, K. H., Ratoff, J., O'Connor, B. J., Ying, S., Meng, Q., Soh, C., Lee, T. H. and Corrigan, C. J. 2006. Systemic glucocorticoid reduces bronchial mucosal activation of activator protein 1 components in glucocorticoid-sensitive but not glucocorticoid-resistant asthmatic patients. *Journal of Allergy and Clinical Immunology*, 118, 368-375.

Melen, E. and Pershagen, G. 2012. Pathophysiology of asthma: lessons from genetic research with particular focus on severe asthma. *Journal of Internal Medicine*, 272, 108-120.

Migeotte, I., Franssen, J. D., Goriely, S., Willems, F. and Parmentier, M. 2002. Distribution and regulation of expression of the putative human chemokine receptor HCR in leukocyte populations. *European Journal of Immunology*, 32(2), 494-501.

Miranda, C., Busacker, A., Balzar, S., Trudeau, J. and Wenzel, S. E. 2004. Distinguishing severe asthma phenotypes: role of age at onset and eosinophilic inflammation. *Journal of Allergy and Clinical Immunology*, 113(1), 101-108.

Murphy, P. M., Baggiolini, M., Charo, I. F., Hébert, C. A., Horuk, R., Matsushima, K., Miller, L. H., Oppenheim. J.J. and Power, C. A. 2000. International Union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacology Review*, 52(1), 145-176.

Ochs, R. L., Muro, Y., Si, Y., Ge, H., Chan, E. K. and Tan, E. M. 2000. Autoantibodies to DFS 70 kd/transcription coactivator p75 in atopic dermatitis and other conditions. *Journal of Allergy and Clinical Immunology*, 105(6 Pt 1):1211-1220.

Oostendorp, J., Hylkema, M. N., Luinge, M., Geerlings, M., Meurs, H., Timens, W., Zaagsma, J., Postma, D. S., Boddeke, H. W. and Biber, K. 2004. Localization and enhanced mRNA expression of the orphan chemokine receptor L-CCR in the lung in a murine model of ovalbumin-induced airway inflammation. *Journal of Histochemistry & Cytochemistry*, 52(3), 401-410.

Parolini, S., Santoro, A., Marcenaro, E., Luini, W., Massardi, L., Facchetti, F., Communi, D., Parmentier, M., Majorana, A., Sironi, M., Tabellini, G., Moretta, A. and Sozzani, S. 2007. The role of chemerin in the colocalization of NK and dendritic cell subsets into inflamed tissues. *Blood*, 109(9), 3625-3632.

Sarti, M., Sevignani, C., George, Calin, A., Aqeilan, R., Shimizu, M., Pentimalli, F., Picchio, M. C., Godwin, A., Rosenberg, A., Drusco, A., NegrinI, M., and Croce, C. M. 2005. Adenoviral Transduction of *TESTIN* Gene into Breast and Uterine Cancer Cell Lines Promotes Apoptosis and Tumor Reduction *In vivo*. *Clinical Cancer Research*, 11(2), 806-813.

Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., Paulovich, A., Pomeroy, S. L., Golub, T. R., Lander, E. S.

and Mesirov, J. P. 2005. *Proceedings of the National Academy of Sciences of the United States of America*, 102(43), 15545-15550.

Sugiura, K., Muro, Y., Nishizawa, Y., Okamoto, M., Shinohara, T., Tomita, Y. and Usukura, J. 2007. LEDGF/DFS70, a major autoantigen of atopic dermatitis, is a component of keratohyalin granules. *Journal of Investigative Dermatology*, 127(1), 75-80.

Tatarelli, C., Linnenbach, A. and Croce, C. M. 2000. Characterisation of the Human *TESTIN* Gene Localized in the FRA7G Region at 7q31.2. *Genomics*, 68(1), 1-12.

Tobias, E. S., Hurlstone, A. F. L., MacKenzie, E., McFarlane, R. and Black, D. M. 2001. The *TES* gene at 7q31.1 is methylated in tumours and encodes a novel growth-suppressing LIM domain protein. *Oncogene*, 20(22), 2844-2853.

Watanabe, K., Muro, Y., Sugiura, K. and Tomita, Y. 2011. IgE and IgG(4) autoantibodies against DFS70/LEDGF in atopic dermatitis. *Autoimmunity*, 44(6), 511-519.

Wenzel, S. 2005. Severe asthma in adults. *American Journal of Respiratory and Critical Care Medicine*, 172(2), 149-160.

Wenzel, S. E., Balzar, S., Chu, H. W. and Cundall, M. 2004. The phenotype of steroid-dependent severe asthma is not associated with increased lung levels of TH-2 cytokine. Presented at the 25th Collegium Internationale Allergologicum Symposium, Bornholm, Denmark

Wenzel, S., Szefler, S., Leung, D., Sloan, S., Rex, M. and Martin, R. 1997. Bronchoscopic evaluation of severe asthma persistent inflammation associated with high dose glucocorticoids. *American Journal of Respiratory and Critical Care Medicine*, 156(3), 737-743.

Wittamer, V., Franssen, J. D., Vulcano, M., Mirjolet, J. F., Le Poul, E., Migeotte, I., Brézillon, S., Tyldesley, R., Blanpain, C., Detheux, M., Mantovani, A., Sozzani, S., Vassart, G., Parmentier, M. and Communi, D. 2003. Specific recruitment of antigen-presenting cells by chemerin, a novel processed ligand from human inflammatory fluids. *Journal of Experimental Medicine*, 198(7), 977-985.

Zabel, B. A., Nakae, S., Zúñiga, L., Kim, J. Y., Ohyama, T., Alt, C., Pan, J., Suto, H., Soler, D., Allen, S. J., Handel, T. M., Song, C. H., Galli, S. J. and Butcher, E. C. 2008. Mast cell-expressed orphan receptor CCRL2 binds chemerin and is required for optimal induction of IgE-mediated passive cutaneous anaphylaxis. *The Journal of Experimental Medicine*, 205(10), 2207-2220.