*Expression profiling of genes related to asthma exacerbations*

Condensed title: Asthma exacerbation and Microarray

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**Summary**

*Background:* Asthma is a chronic airway inflammatory disease, however, the molecular mechanisms that underlie asthma exacerbation are only partially understood.

*Objective:* To identify gene expression signatures that reflect the acute exacerbation of asthma, we examined the differential expression of genes during asthma exacerbation and stable condition by using microarray analysis.

*Methods:* The subjects were mite-sensitive asthmatic children and non-asthmatic control children. The children were divided into 4 groups (AE: asthma exacerbation, n = 12; SA: stable asthma, n = 11; IC: infected control, n = 6; and NC: non-infected control, n = 5). Total RNA was extracted from peripheral blood mononuclear cells and subjected to microarray analysis with Illumina Human Ref8 BeadChip arrays. Welch's *t*-test was performed to identify genes whose expression was altered during asthma exacerbation. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed on samples collected from 43 asthmatic children and 11 control children to verify the microarray results.

*Results:* The expression of 137/16 genes was significantly up/down-regulated during asthma exacerbation assessed by microarray analysis. Of the genes, 62 were also differentially expressed during upper respiratory infection. Many of the asthma

exacerbation related genes were involved in defense responses and responses to external stimuli, but these associations disappeared after excluding the infection-related genes. Quantitative real-time RT-PCR confirmed that the genes related (S100A8 and GAS6) and unrelated to infections (CD200 and RBP7) were differentially expressed during asthma exacerbation ($P < 0.01$).

*Conclusions* Previously unidentified immune responses during asthma exacerbation may provide further clarification of the molecular mechanisms underlying asthma.

Key words: asthma exacerbation, microarray, respiratory infection, gene ontology

## Introduction

Asthma is the most common chronic disorder in childhood, and asthma exacerbation is an important cause of childhood morbidity and hospitalization. The prevalence of childhood asthma in Japan is 5.1% among infants, 6.4% among children and 3.2% among adults, and 1.146 million patients received ongoing medical care for asthma in 1996 [1]. Costs for treatment of asthma have great financial impact on health care worldwide. It was reported that the number of disability-adjusted life years lost due to asthma worldwide is similar to that for diabetes, liver cirrhosis or schizophrenia [2].

Asthma is a chronic airway inflammatory disease caused by infiltration of lymphocytes, mast cells and eosinophils into the airways, and T-helper type 2 cytokines play crucial roles in orchestrating the inflammatory responses [3]. Factors that influence asthma exacerbation include infections, such as those by Rhinovirus, *Chlamydia* and *Mycoplasma* species, and exposure to sensitized allergens [4-6]. However, the molecular mechanisms that underlie asthma exacerbation are only partially understood. To understand the molecular basis of asthma exacerbation, it would be helpful to examine expression of genes during asthma exacerbation. The microarray technique permits simultaneous analysis of expression of many genes. Therefore, large-scale gene expression analysis by microarray may clarify the disease pathways for asthma.

Two types of tissues have been used for human microarray studies of asthma. One is airway epithelium cells from patients and healthy subjects. Laprise et al. [7] performed microarray studies with tissue obtained from bronchial biopsies of 4 asthmatic patients before and after inhaled corticosteroid therapy and from those of 4 healthy subjects. Seventy-nine genes were differentially expressed between asthmatic subjects and controls, including nitric oxide synthase 2A (NOS2A), glutathione peroxidase 3 (GPX3), arachidonate 15-lipoxygenase (ALOX15), cystatin C (CTSC) and chemokine (C-X3-C motif) receptor 1 (CX3CR1). Lilly et al. [8] reported that expression of 141 sequences was up-regulated and 8 sequences were down-regulated during segmental allergen challenge in 5 asthmatic subjects and identified asthma-associated sequences, including the interleukin (IL)-3, IL-4 and IL-5 receptor subunit genes. Nasal mucosal cells obtained from subjects with childhood asthma with stable and exacerbated conditions as well as nonasthmatic controls were also analyzed, and expression of a large number of immune-related genes was up-regulated in exacerbated asthmatics [9].

Hansel et al. [10] performed microarray analysis of CD4+ T cells from patients with asthma. They identified 37 up-regulated and 3 down-regulated genes. Katsunuma et al. [11] performed differential display with T lymphocytes from patients with stable

and exacerbated childhood asthma and found that the genes encoding annexin II, IL-4, IL-5, interferon (IFN)-gamma, IL-12 receptor-beta, and integrin alpha 6 were expressed at higher levels during asthma exacerbation. Peripheral blood mononuclear cells (PBMCs) are easier to obtain and handle than bronchial epithelial cells. Lymphocytes, monocytes and dendritic cells in PBMCs are recognized as the sources of the cytokines related to allergic responses. Therefore, examining changes in expression of genes in PBMCs from asthmatic patients may provide clues regarding the disease pathway.

In the present study, we performed microarray analysis to identify changes in gene expression that reflect acute exacerbation of asthma and constructed a pathway of the molecular changes that occur during asthma exacerbation.

## Materials and methods

*Subjects*

Asthmatic patients were mite-sensitive asthmatic children who visited the pediatric clinic of Tsukuba Medical Center Hospital Tsukuba, Japan. Each patient was questioned regarding allergic symptoms and underwent a physical examination performed by a participating pediatrician. Asthma was diagnosed in patients according to the criteria of the US National Institutes of Health [3]. Asthmatic patients had to satisfy the following 2 criteria: (1) 2 or more episodes of wheezing and shortness of breath during the previous 12 months and (2) reversibility of the wheezing and dyspnea, either spontaneously or in response to bronchodilator treatment. Methacholine challenge testing was not done because of the young ages of the asthma patients; however, differential diagnosis of asthma in the affected children was made by participating pediatricians who had treated the children for more than 1 year, and they confirmed each diagnosis of asthma. Total and specific IgE titers for *Dermatophagoides farinae* were determined with the Pharmacia CAP System (Uppsala, Sweden). Children with asthma exacerbation who required hospitalization (asthma exacerbation (AE) group) and those with stable asthma without any exacerbation in at least the past 4 weeks (stable asthma (SA) group) were enrolled in the study. All of the patients in AE group

had episodes of progressive increasing coughing and wheezing. Responses to the initial bronchodilator treatment were not prompt or sustained for more than 3 h, and no improvement was observed more than 2 h after administration of intravenous steroids. All of them were treated with 1mg/kg, 4 times/day of methylprednisolone for 1-3 days (mean 2.3 ± 0.7 days) after hospitalization and with inhaled short acting beta-2 agonists as combination therapy until their symptoms disappeared. Symptom such as wheezing and dyspnea disappeared within a few days in all children, and maximum duration of hospitalization was 7 days (mean 3.9 ± 1.8 days). Severity of asthma exacerbation in these patients was moderate to severe according to the guidelines of the Global Initiative of Asthma (GINA, http://www.ginasthma.com). Exclusion criteria were (1) Fever ( >37.5 °C), (2) C-reactive protein (CRP) > 1.0 mg/ml, and (3) systemic steroid use prior to hospitalization. Children in SA group were independent from ones in AE group.

Control subjects without allergies or infections (non-infected control (NC) group) had to satisfy all of the following criteria: (1) no symptoms or history of allergic diseases, (2) no detectable dust mite-specific IgE antibody and (3) total serum IgE levels below the general population mean for their ages, (4) no symptoms of infection during past 4 weeks. Because asthma exacerbation is often associated with respiratory infection,

we recruited patients with respiratory tract infections and mild fever as infected controls (IC) group. Children were included in the IC group if they satisfied both the following criteria: (1) symptomatic respiratory infection (coughing and rhinorrhoea associated with mild fever (37.5-38 ºC), and (2) the absence of symptoms or history of any allergic diseases. A full verbal and written explanation of the study was provided, and written informed consent was obtained from the patient or his/her parents. This study was approved by the Ethics Committees of the University of Tsukuba and the Tsukuba Medical Center Hospital. The clinical characteristics of each group are shown in Table 1.

*RNA extraction*

Five milliliters of peripheral blood was taken from each patient. PBMCs were purified with Ficoll-Paque$^{TM}$ gradient (GE Healthcare, Piscataway, NJ). RNA was extracted from PBMCs with an RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions.

We used Illumina BeadArray with single-color array (Illumina, San Diego, CA) as a microarray platform. For Illumina BeadArray assay, cRNA was synthesized with an Illumina® RNA Amplification Kit (Ambion, Austin, TX) per the manufacturer's

instructions. In brief, 500 ng of total RNA from PBMCs were reverse transcribed to synthesize first- and second-strand cDNA, purified with spin columns and then *in vitro* transcribed to synthesize biotin-labeled cRNA. A total of 750 ng biotin-labeled cRNA was hybridized to each Illumina Human Ref8 BeadChip arrays (Illumina) at 55 °C for 18 h. The hybridized BeadChip was washed and labeled with streptavidin-Cy3 (GE Healthcare) and then scanned with the Illumina BeadStation 500 System (Illumina). The scanned image was imported into BeadStudio software (Illumina) for analysis. Twenty-two thousand transcripts representing 8 whole-genome samples can be analyzed on a single BeadChip. We included at least 1 technical replicates (i.e., the same cRNA samples) for each BeadChip. The correlation coefficient for identical RNAs was 0.993 to 0.998 ($r^2$) in the present study.

*Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)*

PBMCs from children of each group were purified by Ficoll-Paque$^{TM}$ gradient (GE Healthcare). Total RNA was extracted from PBMCs with an RNeasy Kit (Qiagen). Quantitative real-time RT-PCR was performed with the TaqMan Universal Master Mix and Assay-on-Demand Gene Expression Kit (Applied Biosystems, Foster City, CA) per the manufacturer's instructions. The endogenous control GAPDH was used to normalize

the sample with the $\Delta\Delta C_T$ method for relative quantification with SDS software 2.2 (Applied Biosystems).

*Molecular network and gene ontology (GO) analysis*

GO analysis was performed with the Database for Annotation, Visualization and Integrated Discovery (DAVID 2.0, http://david.abcc.ncifcrf.gov/) [12]. DAVID 2.0 assigns genes of interests to GO: Biological Process categories of the Gene Ontology Consortium (www.geneontology.org) and identifies overrepresented categories for the genes of interest within the biologic process system.

To investigate whether differentially expressed genes belong to specific pathways or networks, we used IPA version 6.0 software (Ingenuity™ Systems, Mountain View, CA). This software is web-based and allows identification of biologic networks relevant to each researcher's experiment. A data set containing the gene identifiers and the corresponding expression values was uploaded into the Ingenuity Pathways Knowledge Base. These uploaded genes (referred to as focus genes) were then used as the starting points for generating biologic networks, and the network was constructed to be enriched with the genes of interest. IPA also determines a statistical score for each network according to the fit of the network to the set of focus genes. The

score is the negative log of *P* and denotes the likelihood of the focus genes in the network being found together due to chance.

*Statistical Methods*

For microarray analysis, background-corrected values for each probe on the BeadChip array were extracted using BeadStudio version 1.5.1.3 (Illumina). Detection *P* values were computed from the background model characterized by the chance that the target sequence signal was distinguishable from the negative controls on the same chip. This is based on the average of negative control genes and is called the method of background normalization by Illumina. The extracted values were exported to the software GeneSpring version 7.3.1 (Silicon Genetics, Redwood, CA), and per chip and per gene normalization were performed. The statistical significance of the microarray data was calculated using the Welch t-test, and multiple tests were corrected by the Benjamini and Hochberg false discovery rate [13]. An analysis of covariance (ANCOVA) using the proportions of lymphocyte, basophil, and monocyte counts as the covariate and a random sampling procedure was performed using the R version 2.7.0 (http://www.r-project.org/). The sample size calculation was calculated with the samr package for the R language (http://www-stat.stanford.edu/~tibs/SAM/) [14]. Statistical

1 significance of real-time RT-PCR was calculated with Mann-Whitney U test.
2 Significance was defined as $P < 0.05$.
3

## Results

Our data processing strategy is outlined in Figure. We first identified transcripts that were expressed by at least 20 of the 34 samples on Human Ref8 BeadChip arrays with the detection *P* values < 0.01. Among the 12,073 expressed transcripts, those satisfying all of following criteria were selected as up/down-regulated transcripts in the microarray analysis : (1) more than 2-fold increase/decrease on average and (2) transcripts showing statistically significant differences between asthma exacerbation and stable status ($q$ < 0.05). The power of the study using sample sizes 12 vs 12 was approximately 0.6 with the following assumptions; mean difference of 2.5-fold for AE vs SA; total number of genes, 10,000; and hypothesized number of truly non-null genes, 200. The power was approximately 0.8 with a mean difference of 3.0-fold (supplementary Figure 1). Therefore, our sample size was sufficient for the detection of genes having moderate differences in expression, but it may not be enough for genes displaying small difference in expression.

A total of 158 transcripts were up-regulated, and 31 transcripts were down-regulated 2-fold (in total, 189 transcripts); further, the change in the expression of 153 (137/16 = up/down-regulated) transcripts was statistically significant ($q$ < 0.05). Because asthma exacerbation is often associated with respiratory infection, we also

analyzed the genes that were differentially expressed during upper respiratory infection. Of the 153-asthma exacerbation-related genes, nearly half (62 genes) were differentially expressed with $q$ values < 0.05. The genes that were up- and down-regulated in PBMCs during asthma exacerbation and upper respiratory infections are listed in Table 2 ($q$ < 0.001) and supplementary Table 1 (>2-fold changed transcripts).

Because the statistical power of the NC vs IC comparison was approximately half of that obtained by comparing of the AE vs SA, the head-to-head comparison of the $q$ values was not valid. In order to verify the results in Table 2 and supplementary Table 1, we randomly selected 6 samples each from the AE and SA groups 10 times and performed the Welch's $t$-test (supplementary Table 2). The results obtained were generally similar, and approximately two-third of 153 asthma exacerbation-related transcripts showed trends for significance (number of unadjusted $P$ values < 0.05, average 100, range 92–106). Repetition of the procedure for 100 times also showed similar results (number of unadjusted $P$ values < 0.05, average 99, range 88–111), suggesting that particular samples of the dataset are unlikely to influence the overall results. However, the results obtained using 6 AE vs. 6 SA samples were not as significant as those obtained using 12 AE vs. 11 SA samples because the $P$ values were influenced by the number of samples in the test.

There was an inter-group variability in the monocyte cell composition in PBMC ($P < 0.05$, Table 1), and in order to incorporate the cell composition as a covariate, we performed ANCOVA analysis using 189 transcripts that were 2-fold upregulated or downregulated along with the cell composition data obtained from the microarray experiment (supplementary Table 1). The results obtained from the ANCOVA analysis did not differ considerably from those obtained without the incorporating cell composition data.

GO analysis revealed that the genes which were up- and down-regulated during asthma exacerbation could be categorized into those involved in defense responses and responses to external stimuli ($q < 0.05$). However, these associations disappeared after excluding the 62 infection-related genes ($q > 0.05$, Table 3).

Pathway analysis was used to identify networks of genes that interact functionally with each other. The results of pathway analysis using 91 asthma exacerbation-related genes that are not associated with respiratory infections are shown in Table 4 and supplementary Figures 2–5. Four networks were identified for asthma exacerbation. The IPA software generates a large global molecular network based on hundreds of thousands of curated direct and indirect physical and functional interactions between orthologous mammalian genes from the published, peer-reviewed content in

the Ingenuity Knowledge Base. Several highly connected genes were identified in these networks, indicating that they are hub genes.

We performed quantitative real-time RT-PCR to verify the results of microarray analysis. Several transcripts of the same genes were included in the microarray analysis, and some showed a discordant expression pattern (i.e. one transcript variant showed up/down regulation in the asthma exacerbation samples, but the others showed the opposite pattern of expression); therefore, we chose genes with a consistent pattern of expression or small genes with a single transcript in the microarray analysis. The results of quantitative real-time RT-PCR results of the selected genes confirmed that the expression of these genes was elevated during asthma exacerbation ($P < 0.01$, Table 5). The differences in the expressions of the CD200 and Retinol binding protein 7 (RBP7) genes between the IC and NC groups were not statistically significant ($P > 0.05$), while S100 calcium-binding protein A9 (S100A9) and growth arrest-specific 6 (GAS6) were highly up-regulated in the IC group ($P < 0.01$) relative to the expressions in the NC group.

## Discussion

In the present study, we performed microarray analysis to identify genes related to asthma exacerbation in children, and identified 153 up/down-regulated genes during asthma exacerbations.

Up/down-regulated genes in patients who required hospitalization for asthma exacerbation were involved in defense responses and nearly half the asthma exacerbation-related genes were differentially expressed during upper respiratory infection, in spite of excluding children with fever and elevated CRP. These results suggest that most asthma exacerbations are related to infections, or that asthma exacerbation and respiratory infections share a common underlying mechanism.

There are a number of limitations to the present study. First, we used PBMCs, which are a heterogeneous population of cells. It is possible that the differential expression of genes observed in this study merely reflects changes in the distributions of various cell populations. However, such changes do not occur without changes in intracellular signal transduction and gene expression [15]. ANCOVA analysis using cell composition data revealed that the results did not differ considerably from those obtained without incorporating the cell composition data. Also, we retrieved the expression data of the transcripts in T cells, B cells, natural killer cells, monocytes and

dendritic cells from the GNF SymAtlas database (http://wombat.gnf.org/SymAtlas/), and the expressions of these transcripts were not restricted to particular populations of cells (data not shown). These results suggest that the changes that take place in transcription during asthma exacerbations are less likely to be greatly influenced by the changes in particular populations of cells.

Second, since the sample size of the NC vs IC groups was small, the power to detect the infection-related genes may be not sufficient. GO analysis (Table 3) showed that infection-related gene functions such as defense responses and response to external stimuli did not show any association after the 62 infection-related genes were removed, suggesting that we had excluded some of the infection-related genes during analysis. However, we cannot exclude the possibility that true infection-related genes were also included in the remaining 91 transcripts.

Third, we did not test for the presence of viruses in the samples collected from the AE and IC groups. Asthma exacerbation is often associated with not only viral respiratory infection [16] but also other pathogens such as *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* [17]. In the present study, we used samples from patients with respiratory tract infections and identified the asthma-exacerbation-related genes that were not associated with respiratory infection, i.e. nearly half the total number of

asthma exacerbation-related genes.

CD200 is type 1a transmenbrane protein expressed by thymocytes, activated T cells, B cells, dendritic cells, endothelial cells and neurons [18]. CD200/CD200R interactions transmit inhibitory signals to cells of the haematopoetic lineage, and these interactions are reported to play various roles in immune responses such as the induction of regulatory T cells [19] and tumor suppression [20]. A recent study revealed that the CD200 fusion protein significantly reduced the severity of experimental arthritis in mice and the expression of genes encoding proinflammatory cytokines such as tumor necrosis factor, IL-1B and IL-10 [21]. Our results showed that CD200 expression was significantly reduced during asthma exacerbation and that this reduction was not due to infection, suggesting that CD200 could be used for the treatment of patients with asthma exacerbation. RBP7 belongs to the fatty acid binding protein family and binds all-trans-, 13-cis-, and 9-cis-retinol with relatively high affinity [22]. RBP7 is expressed in various tissues, and it has been shown that human peroxisome proliferator-activated receptor alpha (PPARalpha) can regulate the hepatic RBP7 level in response to synthetic PPAR ligands [23]. PPARalpha and PPARgamma have been shown to exert potent anti-inflammatory effects that are primarily mediated through the modulation of pro-inflammatory gene expression and inflammatory cell functions [24], and these were

highly connected in the network analysis (Table 4). The role of RBP7 in immune reactions has not yet been examined, and a further study is needed to elucidate the role of RBP7 for immune responses.

In conclusion, the present study identified the genes related to asthma exacerbation, and half of these genes were differentially regulated during respiratory infection. We identified the asthma-exacerbation related genes that were not associated with infection and some of these results were validated using RT-PCR. Although the regulation and function of these genes in asthma are mostly unknown, these gene signatures for asthma exacerbation provide further clarification of the molecular mechanisms that underlie asthma.

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## Figure legends

**Figure** Gene expression profiling strategy and general work flow. Human-Ref8 Expression Bead CHIP (Illumina) were used to identify genes differentially expressed during asthma exacerbation. * AE/SA was calculated by dividing the gene expression levels in asthma exacerbation (AE) by those in the stable condition (SA). IC: infeced controls. NC: non-infected controls.

Table 1. Characteristics of samples

Table 1. Characteristics of samples

| | AE* (n=12) Mean (range) | SA* (n=11) Mean (range) | IC* (n=6) Mean (range) | NC* (n=5) Mean (range) | *P* value** Total cell count | *P* value$ Propotion in PBMC |
|---|---|---|---|---|---|---|
| Age (year) | 7.5 (3-14) | 7.0 (3-12) | 3.8 (3-6) | 5.8 (3-13) | 0.877 | |
| No. of male/female | 4/8 | 5/6 | 3/3 | 2/3 | | |
| Classification of asthma severity (moderate/severe) | 7/5 | 6/5 | | | | |
| Severity of asthma exacerbation (moderate/severe) | 2/10 | | | | | |
| Whole blood cell count (/μl) | 11,650 (7,800-14,700) | 9,536 (6,500-16,000) | 9,700 (4,300-13,200) | 8,060 (4,900-9,500) | 0.021 | |
| Lymphocytes | 2,449 (1,176-4,305) | 3,893 (2,492-8,480) | 2,208 (1,204-3,288) | 3,770 (1,936-5,605) | 0.006 | 0.084 |
| Eosinophils | 614 (0-1,575) | 765 (405-988) | 100 (0-181) | 119 (95-142) | 0.407 | - |
| Monocytes | 423 (0-775) | 404 (198-658) | 624 (387-792) | 282 (279-285) | 0.9 | 0.028 |
| Basophils | 32 (0-82) | 76 (0-376) | 19 (0-43) | 57 (20-95) | 0.2 | 0.564 |
| CRP (mg/dl) | 0.3 (0.0-0.9) | 0.0 (0.0-0.1) | 0.8 (0.5-1.2) | 0.0 | 0.059 | |
| Total serum IgE (IU/ml) | 1,225 (49-5,000) | 1,341 (507-2,600) | | | 0.237 | |
| *Dermatophagoides farinae*-specific IgE (UA/ml) | 74.8 (1.2-100) | 96.5 (65.2-100) | | | 0.242 | |

*AE: Asthma exacerbation, SA: Stable asthma, IC: Infected control, NC: Non-infected control

** *P* values were calculated by comparing cell counts between AE and SA groups

$*P* values were calculated by comparing proportions in PBMC between AE and SA groups. Proportions of lymphocytes, monocytes and basophils were calculated with dividing them by the sum of lymphocytes, monocytes and basophils.

Table 2. Asthma exacerbation related genes

| Gene Name | Description | Fold change* | *q* value AE vs SA** | *q* value IC vs NC** | Accession$ |
|---|---|---|---|---|---|
| Up-regulation | | | | | |
| SVIL | Supervillin | 3.3 | 0.00041 | 0.442 | NM_003174 |
| CAST | Calpastatin | 3.2 | 0.00040 | 0.729 | NM_001750 |
| CREB5 | cAMP responsive element binding protein | 3.1 | 0.00069 | 0.001 | NM_001011666 |
| CSDE1 | Cold shock domain containing E1 | 2.9 | 0.00019 | 0.746 | NM_001007553 |
| LATS2 | LATS, large tumor suppressor, homolog 2 | 2.8 | 0.00040 | 0.330 | NM_014572 |
| DYSF | Dysferlin, limb girdle muscular dystrophy | 2.7 | 0.00082 | 0.011 | NM_003494 |
| ECGF1 | Endothelial cell growth factor 1 | 2.7 | 0.00082 | 0.046 | NM_001953 |
| NFIX | Nuclear factor I/X | 2.7 | 0.00013 | 0.034 | NM_002501 |
| FLJ14107 | Hypothetical protein FLJ14107 | 2.5 | 0.00023 | 0.796 | NM_025026 |
| BCL3 | B-cell CLL/lymphoma 3 | 2.4 | 0.00034 | 0.050 | NM_005178 |
| RBP7 | Retinol binding protein 7 | 2.4 | 0.00013 | 0.746 | NM_052960 |
| PRAM1 | PML-RARA regulated adaptor molecule 1 | 2.4 | 0.00023 | 0.931 | NM_032152 |
| ALDH3B1 | B1, transcript variant 2 | 2.3 | 0.00035 | 0.977 | NM_001030010 |
| S100A9 | S100 calcium binding protein A9 | 2.3 | 0.00017 | 0.001 | NM_002965 |
| C1orf24 | Chromosome 1 open reading frame 24 | 2.3 | 0.00065 | 0.320 | NM_022083 |
| PTPRE | Protein tyrosine phosphatase, receptor | 2.2 | 0.00036 | 0.070 | NM_130435 |
| LOC196463 | Hypothetical protein LOC196463 | 2.2 | 0.00013 | 0.039 | NM_173542 |
| ECE1 | Endothelin converting enzyme 1 | 2.2 | 0.00013 | 0.461 | NM_001397 |
| GRINA | Glutamate receptor, ionotropic, N-methyl D-asparate-associated protein 1 | 2.2 | 0.00013 | 0.115 | NM_000837 |
| IBRDC3 | IBR domain containing 3 | 2.2 | 0.00013 | 0.184 | NM_153341 |
| GAS6 | Growth arrest-specific 6 | 2.1 | 0.00065 | 0.003 | NM_000820 |
| UBTD1 | Ubiquitin domain containing 1 | 2.1 | 0.00034 | 0.495 | NM_024954 |
| PHLDA1 | Pleckstrin homology-like domain, family A, member 1 | 2.1 | 0.00013 | 0.076 | NM_007350 |
| TAPBP | TAP binding protein | 2.1 | 0.00023 | 0.273 | NM_172208 |
| EPB49 | Erythrocyte membrane protein band 4.9 | 2.1 | 0.00065 | 0.148 | NM_001978 |
| DKFZp761E198 | DKFZp761E198 protein | 2.0 | 0.00041 | 0.004 | NM_138368 |
| CSF3R | Colony stimulating factor 3 receptor | 2.0 | 0.00019 | 0.208 | NM_172313 |
| IGF2BP2 | Insulin-like growth factor 2 mRNA binding protein 2 | 2.0 | 0.00013 | 0.934 | NM_006548 |
| C14orf58 | Chromosome 14 open reading frame 58 | 2.0 | 0.00075 | 0.009 | NM_017791 |
| LIMK2 | LIM domain kinase 2 | 2.0 | 0.00065 | 0.977 | NM_001031801 |
| Down-regulation | | | | | |
| TIPRL | TIP41, TOR signalling pathway regulator- | 0.5 | 0.00050 | 0.796 | NM_152902 |
| CD200 | CD200 antigen | 0.5 | 0.00036 | 0.746 | NM_001004196 |
| FLJ21742 | Hypothetical protein FLJ21742 | 0.5 | 0.00013 | 0.653 | NM_032207 |
| FBLN5 | Fibulin 5 | 0.4 | 0.00065 | 0.094 | NM_006329 |

* The fold change was determined by calculating the ratio of global normalized signals from the PBMCs of AE group to those from the PBMCs of SA group.

**AE: Asthma exacerbation, SA: Stable asthma, IC: Infected control, NC: Non-infected control

$ GenBank accession numbers.

Among the 153 asthma exacerbation-related genes, 34 genes with q < 0.001 are listed in this table.

**Table 3. GO analysis for asthma exacerbation-related genes**

| Gene function | All genes (n=153) *P* value (*q* value)* | Infection unrelated genes (n=91) *P* value (*q* value)* |
|---|---|---|
| defense response | $5.1 \times 10^{-10}$ ($2.7 \times 10^{-6}$) | N/A[$] |
| response to wounding | $8.2 \times 10^{-10}$ ($2.0 \times 10^{-6}$) | 0.014 (1.0) |
| inflammatory response | $1.1 \times 10^{-8}$ ($2.0 \times 10^{-5}$) | 0.16 (1.0) |
| response to external stimulus | $2.1 \times 10^{-8}$ ($2.7 \times 10^{-5}$) | 0.027 (1.0) |
| response to stress | $7.8 \times 10^{-6}$ ($5.9 \times 10^{-3}$) | 0.12 (1.0) |

*Exact probability of randomly sampling a given number of genes (up/down-regulated gene during asthma exacerbation) and observing a specific number that belongs to the gene functions. *P* values were corrected by the Benjamini and Hochberg false discovery rate, and expressed as *q* value.

[$]*P* and *q* values were not available because the gene function was not significant with regard to genes that were not related to the infection.

Table 4. Gene networks identified by pathway analysis

| Network ID | Genes in network | Score* | Detected genes** | Top functions | Hub genes§ |
|---|---|---|---|---|---|
| 1 | Akt, BAIAP2, CAST, CD200, CSDE1, CSF3R, CTDSP1, ERK, FBLN5, HDAC9, Histone h3, Jnk, Mapk, MBP, Nfat, NFAT5, NFkB, NFKBIZ, PAK2, PDGF BB, PHLDA1, Pkc(s), PPHLN1, PPM1B, PRAM-1, PTGDR, PTPRC, PTPRE, Rac, RNASE2, RNF19B, S100P, TFPI, TGM2, TNFRSF10D | 56 | 25 | Cellular Assembly and Organization, Nervous System Development and Function, Cell-To-Cell Signaling and Interaction | Histone h3, Jnk, Mapk, Nfat5, NFkB, |
| 2 | APOA1, ARHGAP11A, C19ORF44, CLPB, DDX3Y, DHX8, DSC2, EWSR1, F12, FAM129A, FYTTD1, GOLIM4, HNF4A, HOXA9, HP, KRR1, L-triiodothyronine, LILRA3, LIMS1, MINA, MRPS18B, MYC, NBPF3, NBR1, PARVB, PF4V1, PTGDS, RMND5B, RPL41, SF1, SHBG, SLC25A19, TCIRG1, TCN1, ZNHIT3 | 31 | 16 | Cancer, Cell Morphology, Hematological Disease | EWSR1, HNF4A |
| 3 | AGT, AKAP9, Ca2+, CACNG5, CCR9, DLG4, EPB49, FCN2, GHRL, GPD2, IGF2BP2, LATS2, MAP2K4, MAP3K14, MAPK3, MKK3/4/6, NLRP12, NMDA Receptor, NRCAM, PPARγ ligand-PPARγ-Retinoic acid-RARα, PPARG, PVALB, retinoic acid, RNASE, S100A12, SLC8A3, SPIN1, SSH1, SSH2, SSH3, TAOK1, VNN1, YWHAA, YWHAB, YWHAE | 29 | 15 | Gene Expression, Cancer, Immune and Lymphatic System Development and Function | AGT, MAP2K4, PPARG, YWHAB |
| 4 | ARF4, ASPM, ATRX, CDKN1A, COL16A1, dihydrotestosterone, ECE1, GNB4, HIST1H4C, KLF7, LIMK2, LOC100129193, LYVE1, MBOAT5, MSMB, NFKBIA, NKIRAS2, NNMT, PDZK1IP1, PLXNC1, PPARA, RBP7, S100A11, SERPINB10, SLC1A4, SVIL, TAPBP, Tgf beta receptor, TGFB1, TGFBR2, TGFBRAP1, TNF, TPM4, Type I / Type II receptor, YTHDC1 | 26 | 14 | Cancer, Cellular Function and Maintenance, Reproductive System Disease | PPARA, TGFB1, TGFBR2, TNF |

* Score is based on Fisher exact test and the score > 3 corresponds to $P < 0.001$.

** Pathway analysis was constructed using the 91 asthma exacerbation-related genes that were not associated with the infection. Among the network genes, the genes detected in our microarray analysis are underlined.

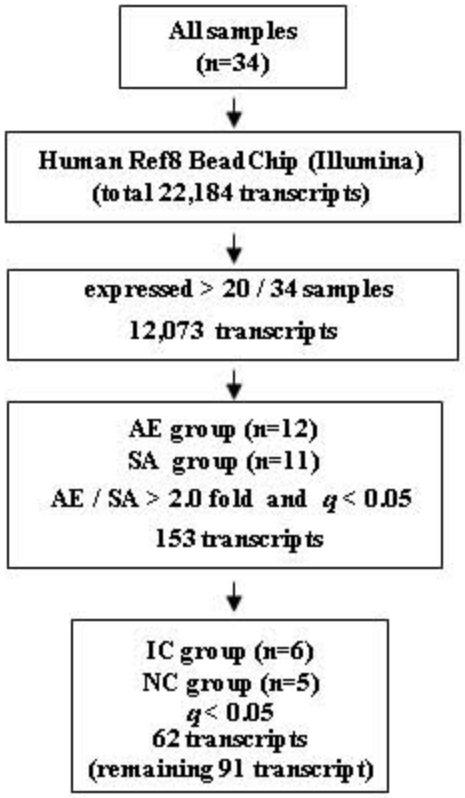
§ Genes that were highly connected (>5 connections) in the network.

Table 5. Result of quantitative RT-PCR

| Gene Name | Fold change* | *P* values** |
|---|---|---|
| S100A9 | 2.4 | <0.0001 |
| GAS6 | 1.8 | 0.0003 |
| RBP7 | 1.5 | 0.0003 |
| CD200 | 0.5 | 0.0003 |

* The fold change was determined by dividing mean relative quantification values of AE group by those of SA group.

**P values were calculated with Mann-Whitney U test


All samples
(n=34)
Human Ref8 Bead Chip (Illumina)
(total 22,184 transcripts)
expressed > 20 / 34 samples
12,073 transcripts
AE group (n=12)
SA group (n=11)
AE / SA > 2.0 fold and q < 0.05
153 transcripts
IC group (n=6)
NC group (n=5)
q < 0.05
62 transcripts
(remaining 91 transcript)