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Brief Communication

Nasal Transcriptome and Epigenome Analysis Identifies the Pathogenic Features of Aspirin-Exacerbated Respiratory Disease

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ABSTRACT

Dysregulation of the arachidonic acid metabolic pathway is the most widely known pathomechanism of aspirin-exacerbated respiratory disease (AERD). This study aimed to perform integrative analysis of transcriptomic and epigenomic profiling with network analysis to determine the novel pathogenic features of AERD. Ten patients with asthma including 5 patients with AERD and another 5 patients with aspirin tolerant asthma (ATA) were enrolled. Nasal scraping was performed and nasal mucosa was used in omics profiling. Peripheral eosinophil counts, sputum eosinophil counts, fractional exhaled nitric oxide levels, and pulmonary function test results were evaluated. Differentially expressed genes (DEGs), differentially methylated probes (DMPs) and differentially correlated genes (DCGs) between patients with AERD and those with ATA were analyzed. Network analysis using ingenuity pathway analysis (IPA) was performed to determine the gene connection network and signaling pathways. In total, 1,736 DEGs, 1,401 DMPs, and 19 pairs for DCGs were identified. Among DCGs, genes related to vesicle transport (*e.g.*, *RAB3B* and *STX2*) and sphingolipid dysregulation (*e.g.*, *SMPD3*) were found to be hypo-methylated and up-regulated in AERD. Using the canonical pathway analysis of IPA with 78 asthma-related DEGs, signaling pathways of T helper cell differentiation/activation and Fcε receptor I were generated. Up-regulation of *RORγt* and *FcER1A* were noted in AERD. Gene expression levels of *RAB3B*, *SYNE1*, *STX2*, *SMPD3* and *RORγt* were significantly associated with sputum eosinophil counts. Quantitative real-time polymerase chain reaction was performed and mRNA expression levels of *STX2*, *SMPD3*, *RORγt*, and *FcER1A* were significantly higher in AERD compared to ATA. Distinct pathogenic features were identified by using integrative multi-omics data analysis in patients with AERD.

Keywords: Aspirin; hypersensitivity; asthma; transport vesicle; multiomics

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Disclosure
There are no financial or other issues that might lead to conflict of interest.

INTRODUCTION

Aspirin-exacerbated respiratory disease (AERD) is characterized by clinical features of persistent asthma, sinonasal inflammation with nasal polyposis, and hypersensitivity reactions to cyclooxygenase-1 inhibitors.[1] Patients with AERD are known to have more severe airway obstruction and frequent asthma exacerbation than those with aspirin tolerant asthma (ATA). A recent study has reported that the prevalence of AERD is 7% in adult asthmatics and 14% in severe asthmatics.[2]

Immunological pathophysiology of AERD is not fully understood; however, dysregulation of arachidonic acid metabolism is the most well-known pathophysiology.[3] Reduced levels of prostaglandin E2 as well as increased levels of prostaglandin D2 and leukotriene E4 play important roles in airway inflammation and bronchoconstriction in AERD.[4] In this context, leukotriene receptor antagonists are widely used in AERD treatment[5,6]; however, the levels of leukotriene E4 were reported to remain high, with increased eosinophil inflammation and impaired asthma control, in AERD patients despite the leukotriene receptor antagonist treatment.[7] To attenuate the activation of eosinophils, mast cells, and innate lymphoid cells in AERD pathogenesis, previous studies have suggested anti-IL4R (*e.g.*, dupilumab), anti-IL-5 (*e.g.*, mepolizumab or reslizumab), anti-IgE (*e.g.*, omalizumab), and anti-IL-33/TSLP antibody treatment in addition to inhaled corticosteroids with long-acting beta2-agonists in severe type 2 (T2) asthma.[1,8] Although these biologics are closely related to pathologic mediators in AERD, clinicians should consider the high medical cost and incomplete blockage of non-steroidal anti-inflammatory drug (NSAID)-induced reactions. In this pilot study, we performed RNA sequencing and methylation array using nasal scrapings from AERD patients and ATA patients to investigate the distinct omics signature and potential therapeutic targets of AERD.

MATERIALS AND METHODS

Study subjects and sample collection

Five patients with AERD and 5 patients with ATA were enrolled at Ajou University Hospital in Suwon, South Korea. AERD was defined as a recurrent clinical history (exacerbation of upper or lower respiratory reactions after ingestion of aspirin/NSAIDs) and/or a positive response to the lysine-aspirin bronchial provocation test. Inclusion/exclusion criteria and clinical parameters assessed at enrollment are presented in **Supplementary Data S1**.

Nasal scraping was performed with a pencil-shaped disposable nasal curette (Rhino-probe®; Arlington Scientific, Inc., Springville, UT, USA) for 3 times from the middle portion of either side of the inferior turbinate after 2 nasal lavages (10-mL saline each) to remove overlying mucus. All subjects gave written informed consent at the time of enrolment, and the study was approved by the Institutional Review Board of Ajou University Hospital (AJIRB-BMR-SUR-17-182).

Omics data production and analysis

Detailed descriptions of RNA sequencing, methylation assay, and bioinformatic analysis are presented in **Supplementary Data S1**. Both RNA for RNA-seq and DNA for methylation assay were extracted from nasal mucosal tissues in the same sample. Library construction and sequencing for RNA-seq was performed according to the manufacturer's instructions. Differentially expressed genes (DEGs) were screened at a cutoff threshold of log2 (FC) ≥ |1|

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and *P* value < 0.05, and were selected according to aspirin intolerance after adjustment for atopic status. Methylation assays were scanned, and differentially methylated probes (DMPs) and regions were detected through the in-house python scripts using the numpy and scipy package[9] and ChAMP[10] at *P* value < 0.001. To perform analysis with as many combinations between DEG and differentially methylated regions (DMRs) as possible, the candidate genes for DEG and DMR were selected of *P* value rather than of adjusted *P* value. Spearman rank correlations were used to identify differentially correlated genes (DCGs) which show an inverse relationship between expression and methylation.

Pathway and network analyses

Ingenuity pathway analysis (IPA; QIAGEN, Redwood City, CA, USA) was used to understand the functional characteristics of DEGs of AERD.[11] IPA canonical pathway analysis was performed with DEGs to identify biological pathways that significantly affect mRNA expression in AERD (**Supplementary Data S1**). The significance of the association between our data set and the canonical pathways were measured as the number of molecules in each pathway that meets cutoff criteria (false discovery rate-adjusted *P* values < 0.05). According to the significance of association, the top 20 pathways were selected and specific signaling pathways were visualized. The genes that are up-regulated in AERD compared to ATA were shown in red, and genes that are down-regulated were shown in green. Depending on the degree of up/down regulation, the color intensity was expressed as dark or light.

Network analysis was performed using asthma-related DEGs to understand and predict interactions between these genes. Biological functions that were significantly associated with the genes in the core networks were identified by functional analysis on the Ingenuity's knowledge base. As our study was focused on asthma population, asthma-related terms were selected and used in further analyses.

Isolation of total RNA from the cells and real-time quantitative polymerase chain reaction (RT-qPCR)

Results of RNA sequencing and methylation array were verified by RT-qPCR in the second cohorts of AERD (n = 12) and ATA (n = 12). Total RNA was isolated from peripheral blood mononuclear cells (PBMCs) obtained in AERD and ATA patients using a Pure-Link RNA mini kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. With a special interest in T helper cells and mast cells in AERD pathogenesis, the levels of mRNA expression of *STX2*, *SMPD3*, *RORγt*, and *FcER1A* were analyzed as described in **Supplementary Data S1**.

RESULTS

Omics candidate and correlation for DCGs

Table 1 shows the clinical characteristics of the study subjects. Detailed results for candidates of each expression and methylation set, and DCGs are presented in **Supplementary Data S1** and **Supplementary Table S1**. Normalization and sample clustering was conducted to compare between the AERD and ATA groups (**Fig. 1A**, **Supplementary Table S2**). We found 1,736 DEGs: 832 were up-regulated and 904 were down-regulated in patients with AERD (**Fig. 1B and C**). Also, we found 1,401 DMPs: 13 with higher methylation and 1,388 with lower methylation in patients with AERD (**Fig. 2A**, **Supplementary Table S3**, **Supplementary Data S1**). In addition to the gene body regions, a large number of DMPs from AERD and ATA were frequently observed in the upstream regulatory region, such as transcription

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Table 1. Demographic data of the study subjects

Characteristics	AERD (n = 5)	ATA (n = 5)	*P* value
Age (yr)	55.2 ± 9.8	47.0 ± 7.3	0.310
Sex (female)	4 (80)	5 (100)	0.292
Atopy	2 (40)	2 (40)	1.000
Chronic rhinosinusitis	3 (60)	4 (80)	1.000
Total IgE (KU/L)	407.8 ± 484.7	250.2 ± 352.0	0.222
Sputum eosinophil (%)	74.3 ± 30.0	14.8 ± 18.8	0.032
Peripheral eosinophil count (/μL)	511.11 ± 317.98	800.00 ± 492.16	0.151
FeNO (ppb)	72.6 ± 52.4	46.6 ± 18.1	0.841
FEV1 (% Pred)	70.3 ± 14.3	77.4 ± 18.4	0.548
FEV1/FVC	70.4 ± 9.7	77.0 ± 11.1	0.421
MMEF (% Pred)	34.4 ± 9.8	43.6 ± 16.9	0.548
FEV1 % fall of after Lys-ASA BPT	23.65 ± 12.80	3.37 ± 4.52	0.083
PC20 of methacholine (mg/mL)	2.66 ± 3.32	4.97 ± 3.47	0.439
ACT score	19.6 ± 2.3	16.2 ± 5.5	0.289
SNOT22	32.0 ± 22.1	44.4 ± 27.5	0.401
Severe asthma	4 (80)	5 (100)	0.292

Values are given as number (%) for categorical variables and as mean ± standard deviation for continuous variables. *P* values were analyzed by the Mann-Whitney test and Fisher's exact test.
AERD, aspirin-exacerbated respiratory disease; ATA, aspirin tolerant asthma; FeNO, fractional exhaled nitric oxide; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; MMEF, maximum midexpiratory flow; Lys-ASA BPT, lysine-aspirin bronchoprovocation test; PC20 of methacholine, provocative concentration causing 20% fall in forced expiratory volume in 1 second; SNOT22, 22-item Sinonasal Outcome Test.

start site, untranslated region, and 1st exon, according to the ratio of the region occupied by the whole genome (**Fig. 2B**). With DEGs and DMPs, 83 pairs for DEG and its CpG were selected (**Supplementary Table S4**). Finally, 19 pairs (19 DMPs and 18 DEGs) with negative correlations at $P < 0.05$ were selected (**Fig. 3**, **Table 2**).

Pathway analysis and gene connection network of AERD

Results of canonical pathway analysis using all 1,736 DEGs are shown in **Supplementary Fig. S1**. Among 78 asthma-related DEGs (**Supplementary Table S5**), 37 were found to be connected in the network (**Fig. 4A**, **Supplementary Table S6**). Based on this gene connection network, the top 20 enriched canonical pathways were discovered by using the IPA (**Supplementary Table S7**). Among these top 20 canonical pathways, signaling pathways of T helper cell differentiation/activation and Fcε receptor I were generated with a special interest in T helper cells and mast cells. Up-regulation of the *RORγt* and *FcER1A* were noted in patients with AERD (**Fig. 4B and C**).

Genes affecting airway eosinophilic inflammation

Logistic regression analysis was performed to evaluate whether the levels of specific gene expression affect airway eosinophilic inflammation adjusted for age and sex. Gene expression levels of *RAB3B*, *SYNE1*, *STX2*, *SMPD3*, and *RORγt* were significantly associated with sputum eosinophil counts (**Supplementary Table S8**).

Validation with RT-qPCR in the second cohort

The clinical characteristics of the second cohort are presented in **Supplementary Table S9**. The levels of mRNA expression of *STX2*, *SMPD3*, *RORγt*, and *FcER1A* were significantly higher in patients with AERD than in those with ATA ($P = 0.038$, $P < 0.001$, $P < 0.001$, and $P = 0.046$, respectively) (**Fig. 5**).

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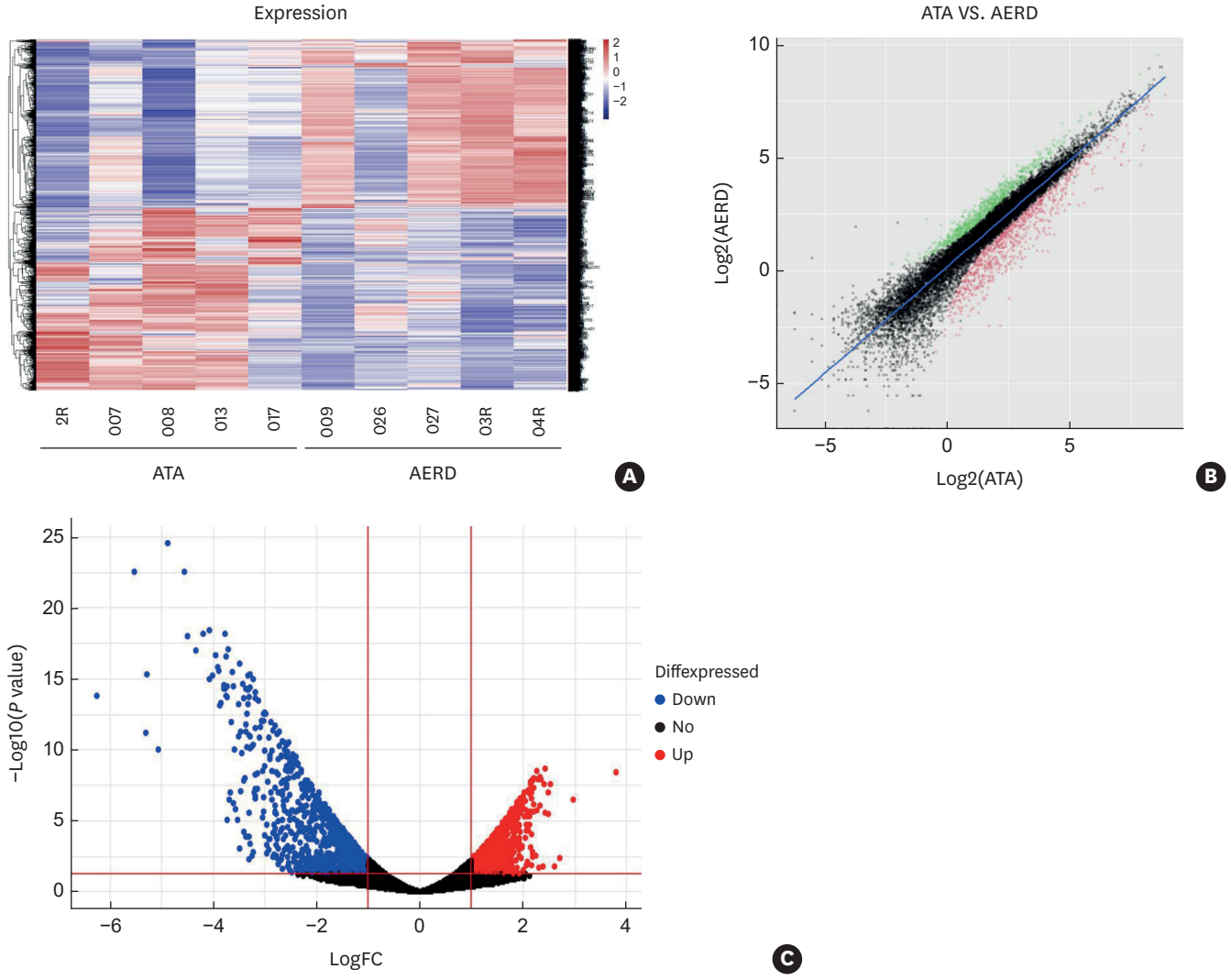

Fig. 1. Gene expression signatures in patients with AERD and those with ATA. (A) Heatmap; (B) Scatter plot; (C) Volcano plot for differentially expressed genes. AERD, aspirin-exacerbated respiratory disease; ATA, aspirin tolerant asthma.

DISCUSSION

This is the first study to demonstrate the transcriptomic and epigenomic profiles of patients with AERD compared to those with ATA. To discover the distinct signature of AERD, integrated analysis of transcriptomes and epigenomes was performed with the nasal tissues from asthmatics. First, we conducted RNA sequencing and methylation array to obtain DEG and DMP candidates. DEG and DMP that are adjacent to the corresponding gene pairs and negatively correlated each other were selected as the final DCG of AERD, distinct genes that are regulated by methylation. Among these DCGs, we found the genes related to vesicle transport (*STX2* and *RAB3B*) and sphingolipid regulation (*SMPD3*). In addition to discovering DCG, gene connection network and pathway analyses were performed to investigate the relationships between these genes and the novel pathways involved in AERD. Among the top 20 canonical pathways, genes related to T helper cell/mast cell activation (up-regulated *RORγt*, down-regulated *TGF-β*, and up-regulated *FCER1A*) were identified. In addition, the mRNA expression levels of these genes were significantly associated with sputum eosinophil

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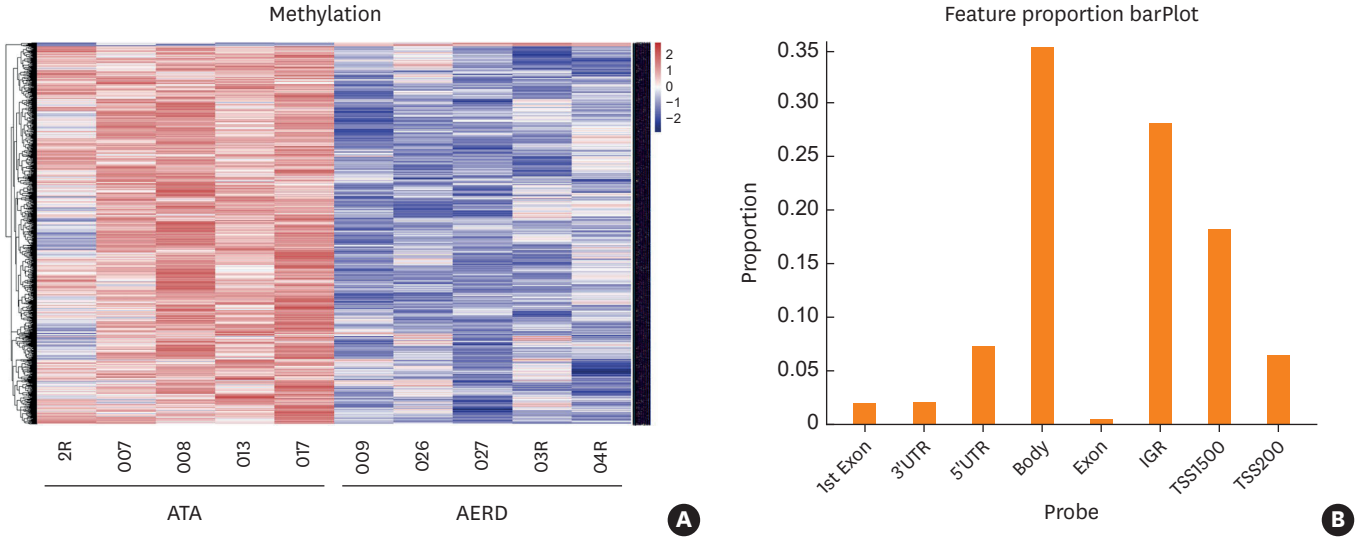

Fig. 2. Genome-wide methylation profiling of differentially methylated regions. (A) Pheatmap of EPIC (top 1,000 variable CpGs) according to aspirin intolerance. (B) Genomic distribution of DNA methylation changes in AERD compared to ATA by genomic features.
1st Exon, first exon region; UTR, untranslated region; Body, gene body; IGR, intergenic region; TSS200, region from transcription start site to –200 nt upstream of transcription start site, TSS1500, –200 to –1500 nt upstream of transcription start site, AERD, aspirin-exacerbated respiratory disease; ATA, aspirin tolerant asthma.

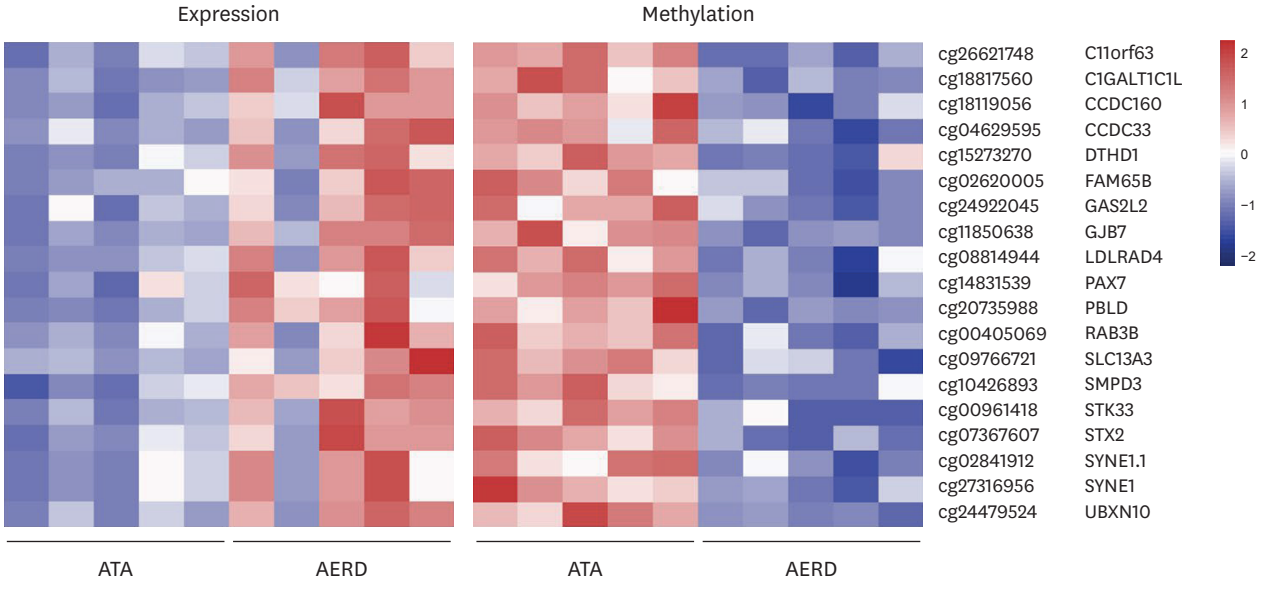

Fig. 3. Heatmap of differentially correlated gene pairs having an inverse correlation between expression and methylation. Left and right are heatmaps for differentially expressed genes and differentially methylated probes, respectively, which are correlated with each other.
AERD, aspirin-exacerbated respiratory disease; ATA, aspirin tolerant asthma.

counts. Collectively, we identified the distinct transcriptomic and epigenomic signature of AERD, which may contribute to the pathogenesis of eosinophilic inflammation in AERD.

The most important cells involved in the pathogenesis of AERD are eosinophils and mast cells.[12] Preformed mediators in intracellular granules are released to the outside of the cells via multiple steps of membrane fusion.[13] Most of the membrane fusion events are mediated by soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors proteins (*e.g.*, syntaxin or vesicle-associated membrane protein).[14] In the present study, we found the

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Table 2. Top 19 candidate differentially correlated genes significantly different between AERD and ATA

No.	CpG	Gene	Chromosome	Position	deltaBeta	*P* value	Spearman correlation *P* value	Coefficient	Methylation status	CpG strand
1	cg00405069	*RAB3B*	1	52455390	0.093931056	0.000365814	0.01592	−0.75758	Hypo	R
2	cg00961418	*STK33*	11	8616172	0.100100845	0.000407352	0.00446	−0.84242	Hypo	F
3	cg02620005	*FAM65B*	6	24858932	0.078997582	0.000935402	0.01367	−0.7697	Hypo	F
4	cg02841912	*SYNE1*	6	152955983	0.147620605	0.000848519	0.04403	−0.66061	Hypo	R
5	cg04629595	*CCDC33*	15	74592566	0.07859813	0.000967707	0.01841	−0.74545	Hypo	R
6	cg07367607	*STX2*	12	131314725	0.088207183	8.41E-5	0.01165	−0.78182	Hypo	F
7	cg08814944	*LDLRAD4*	18	13245910	0.168579157	0.000619824	0.00556	−0.8303	Hypo	F
8	cg09766721	*SLC13A3*	20	45237014	0.250931029	0.000379822	0.03509	−0.68485	Hypo	R
9	cg10426893	*SMPD3*	16	68482821	0.068515315	0.000878768	0.00198	−0.87879	Hypo	F
10	cg11850638	*GJB7*	6	88032210	0.038727513	0.000283272	0.03938	−0.67273	Hypo	R
11	cg14831539	*PAX7*	1	18988324	0.041727136	0.000411022	0.03114	−0.69697	Hypo	R
12	cg15273270	*DTHD1*	4	36324571	0.15612112	0.000540358	0.02117	−0.73333	Hypo	F
13	cg18119056	*CCDC160*	X	133370772	0.135241043	0.000763094	0.01165	−0.78182	Hypo	R
14	cg18817560	*C1GALT1C1L*	2	43903689	0.079273105	0.000547077	0.02117	−0.73333	Hypo	F
15	cg20735988	*PBLD*	10	70062188	0.06438048	0.000699214	0.02751	−0.70909	Hypo	R
16	cg24479524	*UBXN10*	1	20512796	0.035058005	0.000335534	0.01165	−0.78182	Hypo	F
17	cg24922045	*GAS2L2*	17	34079987	0.070053651	0.000604509	0.01367	−0.7697	Hypo	R
18	cg26621748	*C11orf63*	11	122753109	0.100435567	8.57E-6	0.04403	−0.66061	Hypo	F
19	cg27316956	*SYNE1*	6	152958899	0.118130546	0.000881494	0.00088	−0.90303	Hypo	R

P values and coefficients were calculated by the spearman correlation test. "Hypo" in "Methylation status" column represent hypo-methylated, "F" and "R" in "CpG strand" column mean forward (+) and reverse (−) strands, respectively.
AERD, aspirin-exacerbated respiratory disease; ATA, aspirin tolerant asthma.

STX2 gene was differentially correlated between patients with AERD and those with ATA. In addition, Rab proteins also participate in intracellular vesicle trafficking and release of granules.[15,16] A previous study has reported that single-nucleotide polymorphisms on the *RAB1A* gene are associated with the risk of AERD and the responsiveness of airways to aspirin.[17] Consistent with these previous studies, we observed that the *RAB3B* gene was a more significant DCG in patients with AERD compared to those with ATA. Furthermore, sputum eosinophil counts were significantly associated with the mRNA expression levels of the *STX2* and *RAB3B* genes. Taken together, genes related to vesicle transport were significantly hypo-methylated and up-regulated in patients with AERD, which may promote eosinophilic inflammation in a target tissue.

Dysregulation of sphingolipids in AERD and severe asthma has been reported in several studies.[6,18-21] Increased levels of serine palmitoyl transferase, long-chain base subunit 2,[20] and *SMPD1*[22] have been suggested to induce ceramide increase and to augment eosinophilic inflammation in AERD.[20,22] In this context, we found that the *SMPD3* gene was hypo-methylated and up-regulated in patients with AERD compared to those with ATA. This finding implies that sphingolipids may play a role in the pathogenesis of AERD, which is supported by previous study results.

T2 inflammatory responses are immunologically essential in patients with AERD. Th2 and Th17 inflammation can affect each other, resulting in the augmentation of T2 immune responses.[23-25] Inhibition of RORγt, a regulator of Th17 differentiation, diminished Th17 and Th2 immune responses in an animal model of allergic asthma.[24] TGF-β plays an important role in the development of regulatory T (Treg) cells. The defective regulatory function of Treg cells has been noticed in pollen-allergic subjects.[26] Based on these previous studies, up-regulated *RORγt* and down-regulated *TGF-β* may play a role in the augmentation of T2 inflammation in patients with AERD.

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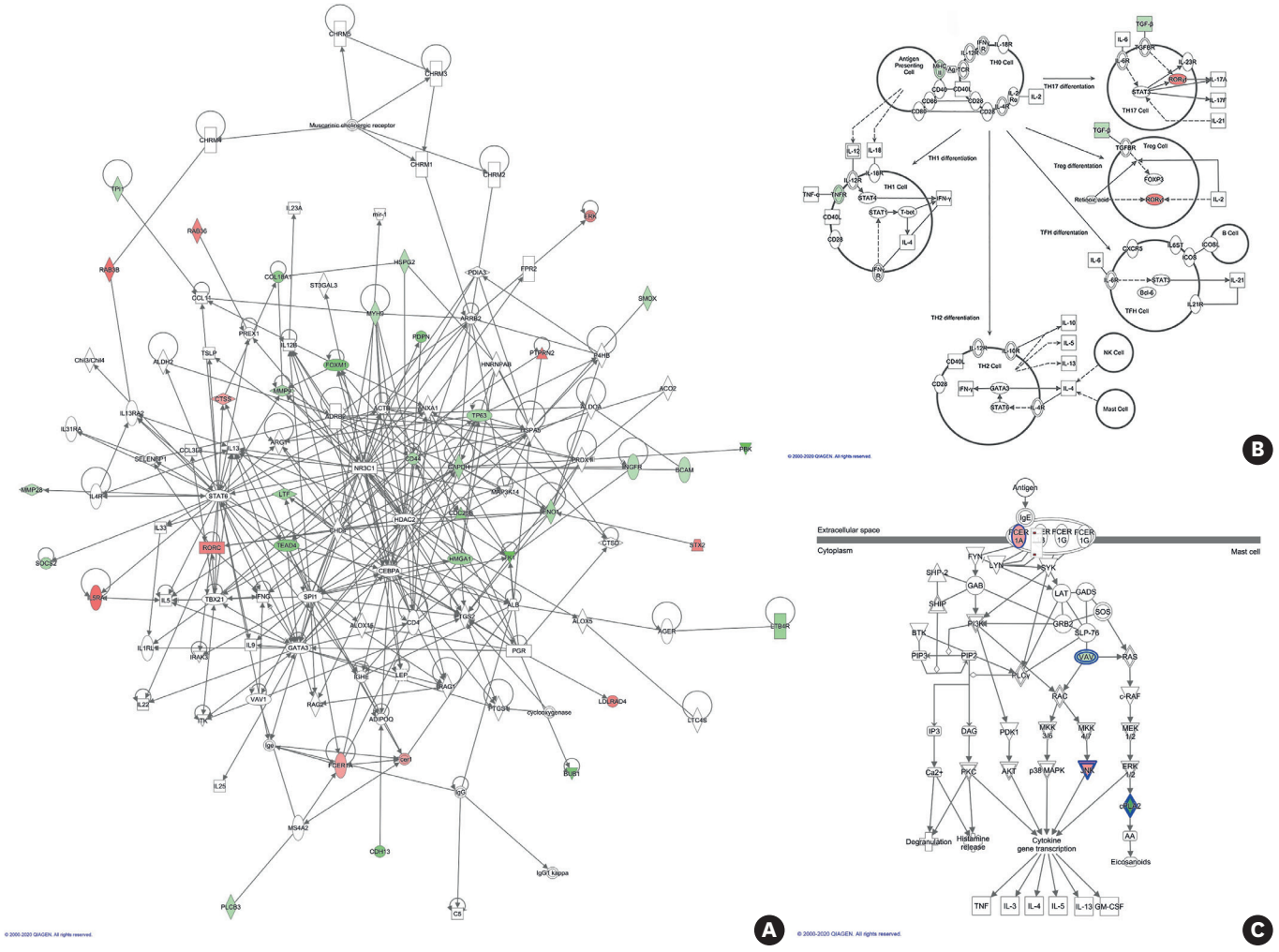

Fig. 4. Gene connection and signaling pathways of AERD. (A) Gene connection network of AERD. Up- (red) or down- (green) regulated genes are highlighted. (B) Signaling pathway associated with T helper cell differentiation/activation (C) Fc Epsilon RI signaling pathway.
AERD, aspirin-exacerbated respiratory disease.

Mast cell activation as well as release of cysteinyl leukotrienes and prostaglandin D2 is an essential component of AERD pathogenesis. Treatment with omalizumab, a monoclonal anti-IgE antibody that disrupts the FcεRI: IgE complex and decreases FcεRI expression, has been reported to successfully reduce the levels of leukotrienes and eosinophils in AERD patients.[8] In the present study, up-regulation of the *FCER1A* gene expression was observed, suggesting the role of FcεRI in AERD pathogenesis, which is consistent with the results of previous studies.

This study has some limitations. First, nasal tissues were used to profile the transcriptome and epigenome. Expression profiling with bronchial airway epithelial cells may have higher statistical power[27]; however, it is difficult to perform invasive bronchoscopy for collecting target tissues in actual clinical practice. Strong correlations between bronchial and nasal airway gene expression profiles have been reported in previous studies.[27,28] Therefore, nasal cytology of nasal curette specimens, a simple and non-invasive procedure, can be a good alternative.[29,30] Secondly, this study had no controls. Thirdly, the number of the study subjects

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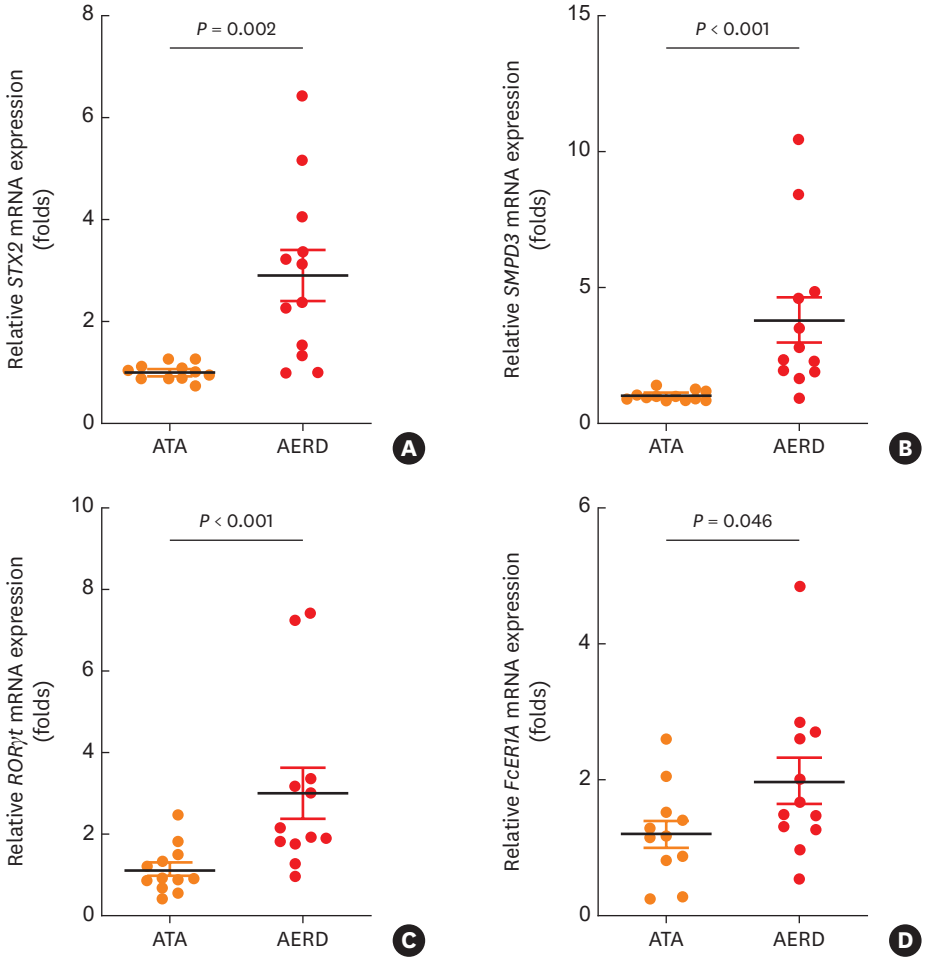

Fig. 5. Real-time quantitative polymerase chain reaction in the second cohorts. Comparison of *STX2* (A), *SMPD3* (B), *RORγt* (C) and *FcER1A* (D) mRNA expression in peripheral blood mononuclear cells collected from patients with ATA (n = 12) and AERD (n = 12). Data are presented as fold changes compared with the ATA. Data are represented as means ± standard deviation. *P* values were analyzed by the Mann-Whitney test.
AERD, aspirin-exacerbated respiratory disease; ATA, aspirin tolerant asthma.

was too small and only one female patient was included. Due to these limitations, it may be difficult to generalize our results to other populations. Thus, we confirmed results of RNA sequencing and methylation array by traditional RT-qPCR in separate cohorts. Since PBMCs were used in the second cohort analysis, our results may not completely represent the nasal tissue omics signature. Further studies with a larger sample size are needed to confirm our results. In this study, we attempted to iteratively apply machine learning approaches to overcome the limitations (**Supplementary Tables S10** and **S11**, **Supplementary Fig. S2**).

In conclusion, our results demonstrated distinct omics signatures related to vesicle transport, sphingolipid regulation, and T helper cell/mast cell activation in AERD, which may play an important role in the pathogenesis of AERD. Based on these pilot omics study results, further functional *in vitro*/*in vivo* studies regulating these relevant pathway and molecules are warranted to suggest the potential therapeutic role.

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SUPPLEMENTARY MATERIALS

Supplementary Data S1
Methods and results

Click here to view

Supplementary Table S1
Trimming statistics on demultiplexed reads by sample

Click here to view

Supplementary Table S2
Differentially expressed genes between patients with aspirin-exacerbated respiratory disease and aspirin tolerant asthma

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Supplementary Table S3
Differentially methylated region between patients with aspirin-exacerbated respiratory disease and aspirin tolerant asthma

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Supplementary Table S4
Differentially correlated genes between patients with aspirin-exacerbated respiratory disease and aspirin tolerant asthma

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Supplementary Table S5
Asthma-related differentially expressed genes between patients with aspirin-exacerbated respiratory disease and aspirin tolerant asthma

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Supplementary Table S6

Asthma-related differentially expressed genes which are connected in the network

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Supplementary Table S7

Canonical pathways of transcriptome data for asthma-related terms

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Supplementary Table S8

Gene expressions predicting the sputum eosinophil count

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Supplementary Table S9

Clinical characteristics of validation cohort

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Supplementary Table S10

Classification report with average for each precision, recall, and F1 score

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Supplementary Table S11

Scores from multiple classifiers and their parameter option combinations on expression set of differentially correlated genes

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Supplementary Fig. S1

Top 10 canonical pathway (A) and heatmap results of disease and functions (B) in aspirin-exacerbated respiratory disease transcriptome data compared to aspirin tolerant asthma. (A) Genes are ranked by the negative log of the *P* value of the enrichment score. The color scheme is based on Z scores, with activation in orange and inhibition in blue. (B) The color scheme is presented based on Z scores, with activation in orange, inhibition in blue, and undetermined directionality in gray.

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Supplementary Fig. S2

ROC curve of the logistic regression, random forest and SVM classifiers for each gene expression set (A) and methylation feature set from differentially correlated gene candidates (B). ROC curves for each of gene expression (A) and DNA methylation signatures (B) were made with reference to the Supplementary Table S10.

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Supplementary References

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