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Allergy International

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Letter to the Editor

Gene expression analysis in airway-secreting extracellular vesicles upon house dust mite exposure

Dear Editor,

In recent years, extracellular vesicles (EVs), such as exosomes and microvesicles have been attracting attention as an information source for the development of new diagnostic and treatment methods. With regard to the history of exosomes, Johnstone RM *et al.* had discovered fine particles of approximately 50 nm in size released from sheep reticulocytes, which were later termed 'exosomes'.¹ Lotvall J *et al.* reported in 2007, approximately 30 years after the original discovery,¹ that RNA, such as microRNA (miRNA) and mRNA, is present in the exosomes and that these may be used for cell-to-cell transportation of miRNA. This report resulted in an immediate focus on exosome research.² Exosomes and microvesicles have various common features, e.g. they are both EVs, approximately 100 nm in size, covered by a lipid bilayer membrane, and contain proteins and RNA in the vesicles³; however, the production processes are significantly different. Exosomes are formed during the production of endolysosomes by the fusion of endosomes and lysosomes; on the other hand, microvesicles are released outside from the cells following budding from the cell membrane. Exosomes contain Testis-specific gene 10, Heat shock protein70 and tetraspanins, such as CD9, CD63 and CD81; furthermore, they contain proteins such as p53, epidermal growth factor and fibroblast growth factor. Because methods for the precise distinction between exosomes and microvesicles have not been established, they are often collectively termed EVs.³ In this article, EVs that are secreted into the airway are abbreviated as airway-secreting EVs (AEVs). Because information in the extracellular vesicles RNA (evRNA) contains intracellular information associated with the cellular response to the pathology of the recipient cells, it is the focus for a new source of biomarkers.⁴ Sampling of the airway mucosa and the lung tissue is difficult; therefore, evRNA is beneficial because useful information can be obtained non-invasively. In this study, we used a mouse model of asthma induced by the house dust mite (HDM) allergen. AEVs were collected after allergen exposure. By performing a comprehensive analysis of mRNA profiling within AEVs, we examined the usefulness of mRNA as pathological biomarkers of AEVs.

Our group has previously reported an experimental method for a mouse asthma model induced by HDM allergen.⁵ C57BL/6J mice

were administered either 2.0 mg/ml (100 µg/mouse) HDM extract (Greer Laboratories, Lenoir, NC, USA) dissolved in phosphate-buffered saline (PBS) or PBS alone (sham control) 3 times (day 1, 8 and 15). Each group included 4 mice. Seventy-two hours after final administration, bronchoalveolar lavage fluid (BALF) was collected (Fig. 1A). Isolation of AEVs from BALFs, was performed using ExoQuick Exosome Precipitation Solution (System Biosciences, Mountain View, CA, USA), according to the manufacturer's recommended protocol. Briefly, BALFs centrifuge at 4 °C (450 ×g, 10 min) to remove BAL cells and subsequently at 3000 ×g (15 min) to remove dead cells. The supernatants were filtered through 0.2-µm filters to remove particulate material larger than 200 nm and centrifuged at 3000 ×g for 15 min. After RNA preparation from AEVs, RNA libraries were prepared using the Ion Total RNA-Seq Kit v2 (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's recommended protocol. Sequencing of each sample was performed on the Ion Torrent PGM instrument using 318 chips (11,000,000 wells per chip) according to the protocol provided in the Total evRNA and Protein Isolation Kit (Invitrogen) with 500 flows (125 cycles). The expression profiles of AEVs mRNA were analysed by the Partek® Genomics Suite 6.5 software program (Partek, Munster, Germany). To identify the list of the genes upregulated by HDM allergen and signalling pathways possibly involved in their gene upregulation, we used Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems; <http://www.ingenuity.com>).

The mRNA profiling within AEVs after 72 h of final HDM allergen exposure was analysed using the Partek® Genomics Suite 6.5 software. The results are shown in a heat map (Fig. 1A) and a volcano plot (Fig. 1B). We observed that HDM allergen stimulation increased mRNA expression in 235 genes compared with the sham control. Further, we analysed the 235 genes using the IPA software; expression levels of these genes were significantly elevated ($p < 0.05$) and showed a 2-fold increase after 72 h of HDM stimulation compared with the sham control. We attempted to identify upstream regulatory molecules of the genes expressed in AEVs. Using IPA software analysis, we identified IL-4 (activation z-score 2.211, p value of overlap 1.38E-03) and IL-13 (activation z-score 2.351, p value of overlap 2.32E-04) as upstream regulator molecules of RNA in AEVs induced by HDM stimulation, with high confidence.

Th2 cytokines, such as IL-4 and IL-13, are critical factors in the development of allergic airway inflammation.^{6–8} In our experimental model, we observed IL-4 and IL-13 elevation in BALF (data not shown). Among the genes induced by HDM allergen,

Peer review under responsibility of Japanese Society of Allergy.

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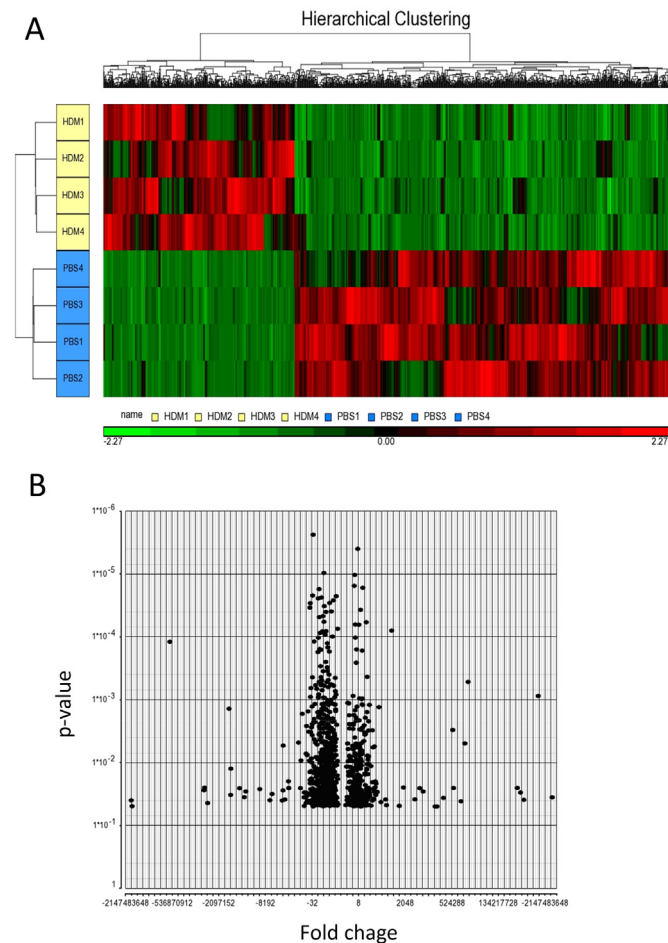


Fig. 1. Summary of genome-wide mRNA profiles of airway secreting extracellular vesicles (AEVs) in house dust mite (HDM)-treated or sham-control mice. **(A)** A hierarchical clustering analysis was performed on the basis of the expression patterns observed. Genes were linked together according to their expression levels (dendrogram, left panel). HDM-treated mice (yellow bar); sham control mice (blue bar). Gene-expression intensities were standardized between -2.0 (blue) and $+2.0$ (red). **(B)** Volcano plot showing gene expression levels in AEVs from HDM-treated vs. sham-control mice ($n = 4$).

the number of genes that were presumed to be located downstream of IL-4 and IL-13 were 13 and 10, respectively, according to IPA software analysis (Table 1). Of these, 6 genes (Cd40, Scgb3a1, Hsd3b2, Retnla, Oprm1 and Pfkp) were overlapped between IL-4 and IL-13. This might be because IL-4 and IL-13 bind to both α -chains of the IL-4 receptor.⁹ Although pathophysiological roles of these genes on allergic inflammation are largely unknown, an EV-included mRNA gene regulated by IL-13, CHI3L1, which encodes the YKL-40 protein, is associated with asthma severity.¹⁰ CD40 and Retnla have been reported to increase in murine asthma model.¹¹ In addition, the interaction between CD40 and its ligand is crucial for IL-12 production and effective humoral immunity such as IgE production.¹¹ These evidences suggest that the transport of mRNA genes regulated by IL-4 and IL-13 to recipient cells via EVs might be involved in the development of allergic immune responses.

Among the genes upregulated in AEVs by HDM allergen exposure, the presence of IL-33, which is one of the IL-4 regulated genes, has drawn our attention. IL-33 was increased 5.055-fold in AEVs after HDM allergen exposure. IL-33, which is released from epithelial cells, and other cytokines, i.e. thymic stromal

Table 1

The list of genes suspected to be regulated by IL-4 or IL-13.

IL-4 predicted to be activated. (z-score 1.896) Overlap p-value 4.80E-04	
Gene	Expression fold change
Retnla	411.113
Apoe	199.636
Ext1	17.220
Pydc4	11.168
Oprm1	7.879
Ccrn4l	7.271
Cmah	6.447
Pfkp	6.136
Il33	5.055
Cd40	5.040
Scgb3a1	4.296
Hsd3b2	3.716
Ablim1	2.710
IL-13 predicted to be activated. (z-score 2.351) Overlap p-value 2.30E-04	
Gene	Expression fold change
Retnla	411.113
Oprm1	7.879
Sprr2a2	7.262
Qsox1	6.878
Pfkp	6.136
Hdc	5.630
Cd40	5.040
Scgb3a1	4.296
Hsd3b2	3.716
Chi3l1	2.323

lymphopoietin (TSLP) and IL-25, that are produced from the airway and intestinal epithelium are collectively termed “epithelial derived cytokines”.^{12–14} IL-33 protein is generally present in the nucleus and is a transcriptional repressor. IL-33 is cleaved by activated caspase-3 and caspase-7 in cells undergoing apoptosis, the ability to bind to IL-33 receptors is lost.^{13,15} However, cells undergoing necrosis release full-length IL-33, which activates immune cells that express IL-33 receptors. Natural helper (NH) cells that secrete large amounts of Th2-type cytokines through the action of IL-33 have been discovered in the visceral fat tissue of mice. This demonstrates that IL-33 could induce allergic inflammation by direct stimulation of eosinophil, basophil and mast cells without activating Th2 cells.¹⁶ Thus, the function of IL-33 has attracted attention as a novel allergic inflammatory mechanism.^{17,18} It has been reported that among evRNA, mRNA transports RNA into EVs of recipient cells, resulting in protein expression. In this study, the identification of the presence of IL-33 gene in EVs suggests that expression of transmitted danger signal molecules by EVs in recipient cells could affect the immune response. In future, the role of immune regulatory molecules such as those in EVs requires to be elucidated.

In summary, we considered that the genetic information in EVs secreted in the respiratory tract contains molecular biological information associated with asthma pathogenesis and that genetic analysis of AEVs will be useful for pathological analysis. Moreover, the presence of genes that cause inflammation in EVs suggests that these genes may have a new immunomodulatory function through recipient cells. Because the role of AEVs in allergic inflammation remains unclear, further studies are required to elucidate the function.

Conflict of interest

The authors have no conflict of interest to declare.

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Received 1 February 2016

Received in revised form 26 March 2016

Accepted 8 April 2016

Available online xxx