

Microarray-based Identification of Novel Biomarkers in Asthma

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ABSTRACT

Bronchial asthma is a complicated and diverse disorder affected by genetic and environmental factors. It is widely accepted that it is a Th2-type inflammation originating in lung and caused by inhalation of ubiquitous allergens. The complicated and diverse pathogenesis of this disease yet to be clarified. Functional genomics is the analysis of whole gene expression profiling under given condition, and microarray technology is now the most powerful tool for functional genomics. Several attempts to clarify the pathogenesis of bronchial asthma have been carried out using microarray technology, providing us some novel biomarkers for diagnosis, therapeutic targets or understanding pathogenic mechanisms of bronchial asthma. In this article, we review the outcomes of these analyses by the microarray approach as applied to this disease by focusing on the identification of novel biomarkers.

KEY WORDS

asthma, inflammation, interleukin-13, mast cells, microarray

INTRODUCTION

The incidence of allergic diseases has dramatically increased in recent decades, particularly in developed areas. It has been reported that, at present, up to half of the population in Japan suffers from bronchial asthma, atopic dermatitis, or allergic rhinitis.¹ The medical cost for treating such patients is huge and on the increase. Thus, it is important socially as well as medically to clarify the pathogenesis of allergic diseases and to establish more useful strategies to overcome allergic disorders. Recent advances in the technology of functional genomics, such as massive parallel gene expression profiling using microarrays, are revolutionizing the approaches to these complex scientific questions.²⁻⁴ Several trials to dissect the pathogenesis of bronchial asthma using microarray technology have been performed, providing some novel pathogenic mechanisms of bronchial asthma as well as the information of gene expression profiling. The information of gene expression profiling would be relevant for finding drug targets or biomarkers for bronchial asthma. This article describes the pathogenesis of bronchial asthma; some fundamental as-

pects of functional genomics, particularly validation and design of the microarray analysis; and lastly, the outcomes of microarray analyses applied to bronchial asthma.

PATHOGENESIS OF BRONCHIAL ASTHMA

Bronchial asthma is a complicated and diverse disorder affected by genetic and environmental factors; however, it is widely accepted that it is a Th2-type inflammation originating in lung caused by inhalation of ubiquitous allergens.^{5,6} High expression of Th2 cytokines such as IL-4, IL-5, IL-9, and IL-13 in the lesions is the cardinal feature of bronchial asthma.⁷⁻¹¹ Inhalation of allergens results in infiltration of Th2 cells, mast cells, and eosinophils into asthmatic airways, and Th2 cytokines together with other mediators released from these cells generate asthmatic phenotypes such as mucous hypersecretion, infiltration of inflammatory cells, and airway hyperresponsiveness (AHR). It is thought that a combination of genetic and environmental factors contributes to activation of Th2-type immune responses.¹²

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FUNDAMENTAL ASPECTS OF FUNCTIONAL GENOMICS

Functional genomics is defined as the analysis of whole gene expression of a cell, tissue, or organ under given conditions.^{2,13} The most common tools used to carry out gene expression include complementary DNA (cDNA) microarrays, oligonucleotide microarrays, and serial analysis of gene expression (SAGE).² Particularly, microarray technology is now the most powerful tool for functional genomics. Especially, oligonucleotide microarrays provide direct information about mRNA expression levels.¹⁴ The findings of functional genomics can be applied to various uses: (1) Identification of drug targets correlating with the pathogenesis of diseases. (2) Biomarker determination to find genes correlated with disease-subclass determination, disease stage, or prognosis. (3) Drug discovery process for assessment of drug actions and patient responses.^{2,15} Microarray technology has been applied to various diseases such as malignancies, neurological disorders, and cardiovascular diseases as well as bronchial asthma.¹⁶⁻¹⁸

Although microarray technology is powerful and relevant, we need to improve the accuracy and precision of each analysis.¹⁹ The many factors affecting the accuracy and precision of microarray analysis can be categorized into three groups: (1) Biological variation intrinsic to each cell, tissue, or organ and influenced by genetic or environmental factors. (2) Technical variation introduced during the extraction, labeling, and hybridization of samples. (3) Measurement error associated with reading fluorescent signals.³ The problems of these variations could be resolved, at least, in part, by the following three types of replications: (1) Biological replication, an experiment replicated by producing a new biological sample. (2) Technical replication, an experiment replicated by producing a new sample of labeled nucleic acids from the same biological samples. (3) Technical repetition, hybridization of the same labeled sample to another array.¹⁹ However, these replications have limitations to improve the accuracy and precision. Particularly, biological variation is an inevitable factor, irrespective of the handling or the assay system. This problem is apparent from the result that the correlation between samples obtained from individual inbred mice will be as low as 30%.³ To confirm microarray data and to make it more useful for the researchers' primary purposes, it is important to validate the data and design the experiments well.

VALIDATION AND DESIGN OF MICROARRAY ANALYSIS

To confirm microarray data, particularly derived from oligonucleotide microarrays, there are two ways of validation, *in silico* analysis and laboratory-based analysis.²⁰ *In silico* analysis means comparison of

the microarray data with information available in the literature and databases. Laboratory-based analysis indicates independent experimental analysis of gene-expression using other methods. For the analysis of expression profiling at mRNA level, semi-quantitative reverse transcription PCR (RT-PCR), real-time RT-PCR, Northern blot, ribonuclease protection assay, or *in situ* hybridization can be used.²⁰ Particularly, the real-time RT-PCR method is superior in quantification of the samples and rapidity of the analysis, once it is established. It has been reported that the majority of array results were highly correlated with the results using real-time RT-PCR.^{15,20} On the other hand, the expression of approximately 7% of genes appeared significantly altered in opposite directions using the two methods,¹⁵ suggesting that a careful examination of the array data based on real-time RT-PCR is important. Following analysis at the mRNA level, analysis at the protein level using immunoblot or immunohistochemistry would be relevant for validation.²⁰ The coincidence ratio between transcription level and protein expression is unclear; however, it is estimated at less than 50%.²⁰ For example, it has been reported that microarray analysis showed that expression of the IL-2 receptor β chain was augmented in activated T cells compared to resting T cells at the transcription level, whereas its protein expression was invariable in both cells.²¹ Such a discrepancy may be caused by low sensitivity of the protein analysis or delayed kinetics of the protein expression. Mast cells are known to release abundant levels of proteases such as tryptase within 10 min after allergen stimulation and then they start to synthesize and release a variety of chemokines and cytokines at around 6 hours after the stimulation. These chemokines such as I-309 are expressed very high at mRNA levels but are relatively low at protein levels, especially measured at a prolonged culture period.²² Recently, these proteases are found to destroy various types of chemokines.²³ Therefore, to detect full protein levels of chemokines produced by mast cells, we should use specific protease inhibitors or wash the protease-rich medium at around 1 hr and then replace it with fresh culture medium.

APPLICATION OF MICROARRAY TECHNOLOGY TO BRONCHIAL ASTHMA

Because bronchial asthma is a complicated disorder, thus far, several trials to clarify its pathogenesis using microarray technology have been performed both *in vitro* and *in vivo*, providing us some novel pathogenic mechanisms of bronchial asthma as well as the information of gene expression profiling. Biomarkers newly found by using microarrays for asthma pathogenesis and potential diagnosis are summarized in Figure 1. Validation of the data and design of the experiments were carefully carried out in each analysis. The species of the samples are human, mouse, and

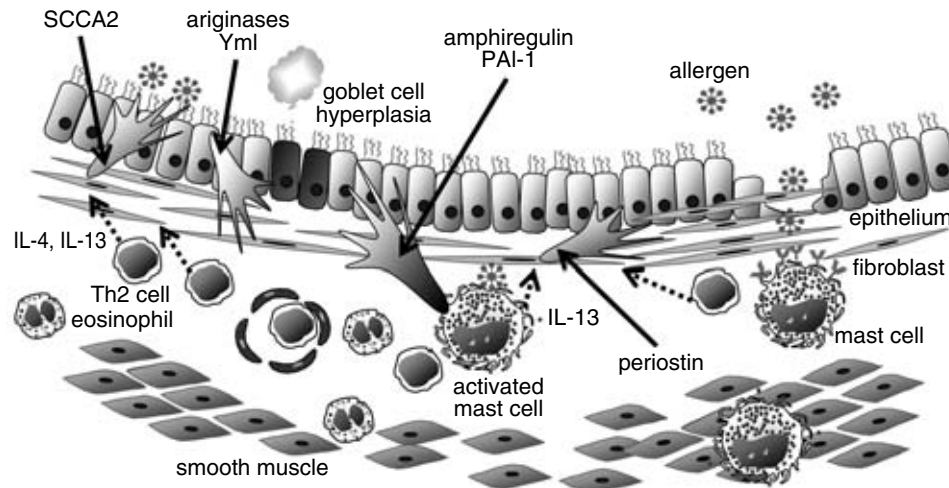


Fig. 1 The summary of novel biomarkers for asthma pathogenesis found using microarrays. SCCA2 is produced by lung fibroblasts of upon stimulation with IL-4 and IL-13 (ref. #25). It seems to inactivate the protease activity of mite allergens. Arginase I and arginase II are produced by airway epithelium with allergen stimulation (ref. #29). Yml and arginases are also produced by macrophages recruited into airways (ref. #46). They are considered to work on NO production and subepithelial fibrosis. PAI-1 (ref. #26) and amphiregulin (ref. #27, #28) are produced by activated mast cells and act on goblet cell hyperplasia (ref. #28) and subepithelial fibrosis (ref. #27). Periostin is produced by fibroblasts of asthmatics stimulated with IL-13 and acts on further fibroblast proliferation, *i.e.*, subepithelial fibrosis (ref. #46).

monkey. The human samples contain bronchial epithelial cells,^{24,25} smooth muscle cells,²⁴ and lung fibroblasts²⁴ stimulated with IL-13 (or IL-4 in case of epithelial cells),²⁵ and cultured human mast cells stimulated.²⁶⁻²⁸ The mouse samples are all lung tissues, analyzing ovalbumin- or *Aspergillus*-inducible asthmatic mice²⁹ or comparing a highly susceptible strain to allergen-induced AHR (A/J) versus a highly resistant strain (C3H/HeJ).³⁰ The monkey samples are lung tissues derived from *Ascaris*- or IL-4-inducible asthma.³¹ Interestingly, there is no direct comparison of the bronchial or lung tissues derived from asthma patients and normal donors. The analyses using human samples are restricted to *in vitro* experiments, and all *in vivo* experiments were performed using mice or monkeys. This might be due to the high biological variation among the samples. It is also possible to think that contamination even by a very small population of a different cell type having a certain highly expressed transcript may cause a virtual presence of the transcript in the whole population even where the major cell type lacks it. We have experienced that a virtual increase in several hundred kinds of transcripts was simply be caused by an increase in recruitment of eosinophils and other inflammatory cell types.³² Therefore, in order to avoid difficulty in interpreting the results, it is important to purify the target cell population as much as possible. However, mRNA is unstable, meaning that compli-

cated procedures for purification should be avoided.

IL-13-INDUCIBLE GENES IN NON-IMMUNE CELLS IN BRONCHIAL TISSUE

Lee *et al.*²⁴ and Yuyama *et al.*²⁵ designed experiments to analyze the effect of IL-13 on non-immune cells in bronchial tissue. IL-13 is a member of the Th 2-type cytokines, thought to have key roles in the pathogenesis of bronchial asthma.³³ Epithelial overexpression of an IL-13 transgene or administration of IL-13 in mice has shown that IL-13 induces asthma-like phenotypes independent of lymphocytes.³⁴⁻³⁷ Particularly, the IL-13 action on epithelial cells is crucial for generation of AHR and mucous production.³⁸ Furthermore, in human, the *IL13* variant Gln110Arg is genetically associated with bronchial asthma.^{39,40} Sheppard's group²⁴ listed a variety of genes induced by IL-13 in bronchial epithelial cells, smooth muscle cells, and lung fibroblasts, among which expression of some genes was validated by real-time RT-PCR, and so was expression of MCP-1 and a proinflammatory cytokine, IL-6, in fibroblasts by ELISA. MCP-1 is a chemokine recruiting lymphocytes, basophils, macrophages, and dendritic cells.⁴¹ Very few genes overlapped in expression profiling of three kinds of cells.⁴²

On the other hand, Yuyama *et al.*²⁵ identified the genes induced by either IL-13 or IL-4, another Th2-type cytokine sharing the receptor with IL-13, by the

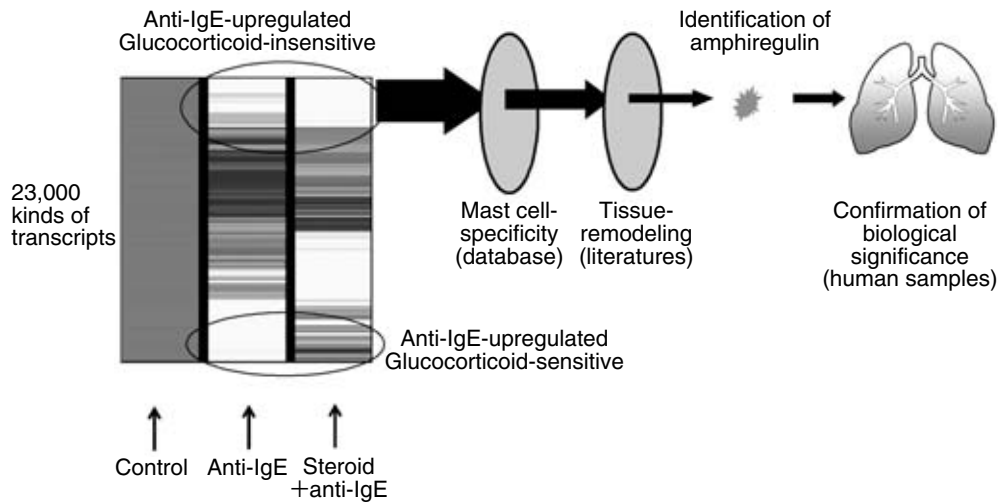


Fig. 2 The strategy to identify amphiregulin as a glucocorticoid-resistant mast cell-specific molecule. In references #28 and #29, the authors used GeneChip® (Affymetrix) to find an anti-IgE-upregulated and glucocorticoid-resistant gene cluster. Then, amphiregulin was selected as one of target molecules by filtering with mast cell specificity and descriptions regarding tissue remodeling. Finally, biological significance was confirmed by demonstrating the selected expression in lung mast cells obtained from asthmatics.

microarray approach, and they validated expression of 12 genes among the identified genes induced by both IL-13 and IL-4 by real-time RT-PCR.²⁵ They furthermore compared expression of 12 genes with the expression profiling of the samples derived from bronchial biopsies from atopic asthma patients, finding that expression of four genes-*SERPINB3*, *SERPINB4*, *KAL-1*, and *MAP17*-was up-regulated or present in the asthma samples. They also found these proteins and confirmed that the squamous cell carcinoma antigen 2 (SCCA2) coded by the *SERPINB4* gene, a member of the ovalbumin serpin family, inhibited the cysteine proteinase activity of a major mite allergen, Der p 1.⁴³ These results indicated that SCCA2 produced by IL-13 or IL-4 in bronchial epithelial cells has a protective role against an extrinsic proteinase activity. Among 12 genes, induction of IL-13R α 2 by IL-13 or IL-4 was confirmed by immunostaining.⁴⁴ It has been shown that IL-13R α 2 acts as a decoy receptor, inhibiting the IL-13 signal^{37,38,44} indicating that there is a negative feed-back regulation for the IL-13 signal in bronchial epithelial cells by induction of IL-13R α 2.

Takayama *et al.*⁴⁵ recently found that periostin, an extracellular matrix protein possessing four fasciclin I domains in its middle, is induced by IL-4 or IL-13 equally, independent from TGF- β in lung fibroblasts. Periostin co-localized with other extracellular matrix proteins involved in subepithelial fibrosis in both asthma patients and asthma-induced wild-type mice, but not in either ovalbumin-inhaled IL-4 or IL-13 knockout mice, which showed less fibrosis. Periostin

had an ability to bind to fibronectin, tenascin-C, collagen V, and periostin itself. These findings suggest that IL-4 and/or IL-13 would contribute to subepithelial fibrosis of bronchial asthma in a TGF- β -independent pathway by inducing periostin, and this pathway would co-operate with the TGF- β -dependent pathway.

INDUCIBLE GENES IN ACTIVATED HUMAN MAST CELLS

Using microarray technology, Cho and collaborators²⁶ tried to identify inducible genes in a human mast cell line, HMC-1, activated by phorbol ester and calcium ionophore, finding that expression of the plasminogen activator inhibitor type-1 (PAI-1) was up-regulated. They confirmed by ELISA that activated HMC-1 cells and primary cultured human mast cells secreted PAI-1. PAI-1 inhibits the plasminogen activator converting plasminogen to plasmin, which enhances proteolytic degradation of the extracellular matrix. These results indicated that activated mast cells could play an important role in airway remodeling by secreting PAI-1. Wang *et al.*²⁷ and Okumura *et al.*²⁸ used Oligonucleotide microarrays to examine the up-regulated genes expressed by activated mast cells. These two groups separately identified amphiregulin as a transcript that is markedly increased following aggregation of Fc ϵ RI. Amphiregulin induced tissue remodeling, *i.e.*, proliferation of lung fibroblasts and marked induction of MUC5AC transcripts in a human respiratory epithelial cell line. Both groups showed that amphiregulin-positive mast

cells were increased in the airways of asthmatics. These investigators employed culture-derived human mast cells for microarray as the first screening and they confirmed the *in vivo* expression and function of this up-regulated gene. Figure 2 illustrate the typical strategy for identifying a novel biomarker, amphiregulin in this case, by using microarrays as coincidentally employed in the two independent studies.^{27,28}

INDUCIBLE GENES IN LUNG TISSUES DERIVED FROM OVALBUMIN-OR *ASPERGILLUS* - INDUCIBLE ASTHMATIC MICE

Zimmermann and collaborators tried to identify inducible genes in the lung tissues derived from ovalbumin- or *Aspergillus*-inducible asthmatic mice.²⁹ Ovalbumin and *Aspergillus* induced 496 and 527 genes, respectively, among 12,422 genes, and only 291 genes overlapped among the identified genes, indicating that there exist pathogenic differences related to the nature of the allergen and/or the immunization route. Among the overlapping genes, expression of arginase I, arginase II, and cationic amino acid transporter 2 (CAT) was significantly augmented. These three molecules are involved in uptake and metabolism of arginine. The precise role of these molecules in the pathogenesis of bronchial asthma is unclear. However, because arginine is metabolized to nitric oxide (NO) by NO synthase, induction of arginase I, arginase II, and CAT may affect the NO synthesis. Alternatively, induction of these molecules may enhance collagen synthesis. Furthermore, it turned out that the cells predominantly expressing arginase are macrophages, and that expression of arginase I and arginase II is enhanced by IL-4 and IL-13, abundantly expressed in the lung tissues of asthmatic mice. These results are consistent with the microarray data demonstrating that thioglycolate-elicited macrophages stimulated by IL-4 showed up-regulation of expression of arginase as well as Ym1.⁴⁶

A SUSCEPTIBILITY FACTOR TO ALLERGEN-INDUCED AHR

It is known that A/J mice and C3H/HeJ mice are highly susceptible and highly resistant to allergen-inducing AHR, respectively. Karp and collaborators designed a unique experiment using microarray technology to try to identify a susceptibility factor to dissect this difference.³⁰ They found that 227 genes exhibited great change in expression among 7350 genes, and paid an attention to C5 among the listed genes, because C5 was situated near a locus correlated with allergen-induced bronchial hyperresponsiveness. It turned out that A/J mice, but not C3H/HeJ mice, have a 2-bp deletion in a 5' exon of the C5 gene that renders them deficient in C5 mRNA and protein production. Furthermore, they found that C5 induced IL-12 production in monocytes. These re-

sults indicated that C5 is involved in determining susceptibility to bronchial asthma by inducing IL-12 production, which counterbalances the Th2-type immune responses.

ASCARIS - OR IL-4-INDUCIBLE GENES IN LUNG TISSUES DERIVED FROM ASTHMATIC MONKEYS

A DNAX group tried to identify *Ascaris* - or IL-4-inducible genes in lung tissues derived from asthmatic monkeys by the microarray approach.³¹ They found that 169 cDNAs among 40,000 changed their expression levels in either pair-wise comparison. Expression of some listed genes was validated by real-time RT-PCR, and several chemokines-such as MCP-1, MCP-3, and eotaxin-showed significant changes in expression. MCP-1, MCP-3, and eotaxin are known to recruit Th2 cells, eosinophils, basophils, macrophages, and dendritic cells.^{40,47} The result concerning high expression of MCP-1 overlapped the result by Lee and collaborators.²⁴

CONCLUSIONS

In summary, the microarray analyses that have been performed so far have provided us not only the information about gene expression profiling, but also some novel pathogenic mechanisms of bronchial asthma. Most of the postulated novel mechanisms are correlated with enhancement of the Th2-type inflammation, or the effector phases of two Th2 cytokines, IL-4 and IL-13. It is important to make more efforts to identify the molecules involved in the pathogenesis of bronchial asthma. After discovery of all player molecules in asthma pathology in the future, the information about the gene expression profiling based on functional genomics will be applied to understand the network of these molecules. Furthermore, such an application of functional genomics would be relevant also to clarify the pathogenesis of various diseases other than bronchial asthma.

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