Short communications

Basophil-expressing genes related with the efficacy of sublingual immunotherapy

Running title: Basophil DPF2 expression may determine SLIT efficacy

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ABSTRACT

Basophils have been implicated not only in allergy pathogenesis, but also in the efficacy of allergen immunotherapy (AIT). We recently conducted a clinical study of sublingual immunotherapy (SLIT) in patients with Japanese cedar pollinosis using a purified cedar allergen. Patients were stratified into the high-responder (HR) and non-responder (NR) groups, and comprehensive microarray analysis was performed on peripheral basophils in the patients from both groups. A total of 153 genes were found to be differentially expressed in the HR and NR patients. The majority of the differentially expressed genes were intracellular molecules and showed higher expression levels in HR patients than NR patients. The mRNA expression levels of the gene encoding D4, zinc, and double plant homeodomain (PHD) fingers family 2 (DPF2) were found to be significantly correlated with copy number variation (CNV). Genetic variation in the DPF2 gene and its expression in basophils is a candidate determinant for SLIT efficacy.

Key words: apoptosis, copy number variation, D4, zinc, and double plant homeodomain fingers family 2, microarray analysis
Introduction

Various sublingual immunotherapy (SLIT) drugs for treating allergic rhinitis have recently been launched in Japan. They are attractive because of the high efficacy and potential to provide a radical treatment. However, regardless of its high efficacy, SLIT does not improve disease conditions in a certain proportion of patients.

We previously compared NR patients with those whose conditions had substantially improved upon SLIT (high responder; HR), and demonstrated the potential determinants of SLIT efficacy. Patients with high correlations among serum Th1/Th2 cytokines tended to respond well to SLIT. As major sources of Th1/Th2 cytokines are CD4+ T cells, the contribution of taste receptors on CD4+ T cells to the SLIT efficacy was suggested.

Basophils have recently served as targets for allergen immunotherapy (AIT). Basophils produce IL-4 by various stimuli, including IgE-dependent allergen recognition. The proportions of basophils expressing C-type lectin receptors and FcγRII were reduced and increased, respectively, by subcutaneous immunotherapy (SCIT). Epicutaneous immunotherapy (EPIT) for peanut allergy patients downregulated CD63 expression in basophils, along with increased peanut-specific IgG4 and IgG4/IgE ratios and decreased Th2 cytokine production. Herein, we performed comparative microarray mRNA expression analysis of basophils collected from HR and NR patients to elucidate the basophil-dependent mechanisms in SLIT efficacy.
Materials and Methods

SLIT on Japanese cedar pollinosis (JCP)

The study design, patient recruitment, cedar pollen extract administration, and clinical efficacy evaluation were performed as described in a recent study\(^1\). The study included 202 adult JCP patients presenting symptoms of allergic rhinitis from February to April for at least three consecutive years. The patients tested positive for the skin-test and IgE against cedar pollen allergen. The study was registered in the University Hospital Medical Information Network Clinical Trials Registry Database (UMIN000016532) and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Experimental procedures were approved by the ethical committee of all participant institutions. All patients provided informed written consent before participation.

All subjects were administered with a cedar pollen allergen as described previously\(^1\). From one drop of 2 Japanese allergy units (JAU)/ml, the amount of allergen was gradually increased until it reached up to 20 drops of 2,000 JAU/ml as the maintenance dose at 5 weeks, then continued for 2 years.

Nasal symptoms of patients from February 1st to April 30th through 4 pollen seasons were investigated following the Japanese guidelines for allergic rhinitis. The patients recorded their symptoms using an allergy diary as previously described\(^1\). The quality of life (QOL) of the patients were determined thrice, once at the end of February, in the middle of March, and in the middle of April each year, using the Japan Rhinoconjunctivitis Quality of Life Questionnaire No.1 (JRQLQ No1). At the end of the each pollen season, the efficacy of SLIT was evaluated based on the description in the allergy diary and JRQLQ No1\(^1\).
Cell preparation and microarray analysis

Blood samples were collected twice immediately before the start and the end of allergen administration. Basophils were purified as CD123^+CD11c^{low}BDCA-1^{−}BDCA-4^{−}HLA-DR^{−} cells from peripheral blood as previously described\(^1\). Genomic DNA (gDNA) of the samples were extracted from blood cells.

Microarray analysis for basophils was performed as previously described\(^6\). Comparative genome-wide transcriptome analysis was performed using the GeneChip Human Gene 1.0 ST Array Platform (Thermo Fisher Scientific, Waltham, MA). A total of 17,743 out of the 33,297 probe sets were analyzed for transcripts associated with specific functionally annotated sites in the genome sequences. The results were normalized as z-scores using the equation \(z = \frac{v - \mu}{\sigma}\), where \(v\) is the raw value for a subject, \(\mu\) is the mean value for all HR and NR patients, and \(\sigma\) is the associated standard deviation in the population. The log2 ratios of the averaged data were calculated between the HR and NR groups.

Copy number variation (CNV) analysis

CNV analysis was performed as previously described\(^2\). The reaction products were analyzed using the Agilent SurePrint G3 Human CNV MicroArray Kit, 2×400K, using the Japanese male HapMap sample NA19000 and UCSC Human Genome build hg19. Probes matching single gDNA sites extracted 2,959 CNV regions. Among them, CNV regions with \(\geq 3\) probes and log2 ratio \(\geq 0.25\) were identified using ADM-2 algorithms.

Statistical analysis

Data of mRNA analyses were presented in heat maps. Linear regression analysis was performed for
correlations between $DPF2$ CNV and mRNA levels.
Results

Twenty-five HR and NR JCP patients were selected from patients who received 2-year SLIT as described previously\(^1\). From basophils of both groups post-SLIT, RNA was subjected for the microarray analysis. The peripheral basophil population was unaffected by SLIT, and no difference in the population was observed between HR and NR patients, as described\(^1\). However, we identified 153 differentially expressed genes between HR and NR patients (Fig. 1A). The majority genes were more strongly expressed in HR than NR patients; only 12 genes showed higher expression in NR. There were many genes encoded intracellular molecules, such as transcription factors, enzymes, and interacting proteins. Approximately 30 small nuclear RNAs were identified (Fig. 1B).

We next investigated the relationship between their expression levels and corresponding CNV values. We observed a significant correlation in only one gene/CNV pair, namely, the D4, zinc and double plant homeodomain (PHD) fingers family 2 (DPF2) (Fig. 2). A significant correlation between the gene expression and CNV in the *DPF2* gene was similarly seen in HR, NR, and HR plus NR groups.
Discussion

The number of differentially expressed genes in basophils (153 genes) identified in this study was larger than that (56 genes) previously reported in CD4+ T cells. We previously evaluated the significance of genes differentially expressed in CD4+ T cells of HR and NR patients by analyzing their associations with CNVs. Since CNV is generated by multiplication and deletion of DNA segments ranging in size from $10^3$ to $10^6$ nucleotides that often contains a whole gene with its regulatory region, CNVs directly affect gene expression levels in many cases. A significant correlation between the expression and CNV was observed only in the DPF2 gene. DPF2, an ubiquitously expressed d4-protein family member, is multifunctional. DPF2 is suggested to influence gene transcription, noncanonical NF-κB pathway, chromatin organization, and protein-protein interactions via its PHD domain. The involvement of DPF2 in apoptosis is consistent with our previous findings indicating the contribution of the apoptotic pathway in basophils to SLIT efficacy. Further investigations into the role of DPF2 in basophil function are required.

The significant correlation between the expression and CNV in the DPF2 gene was observed to be similar across HR, NR, and HR plus NR groups. These results are different form our previous findings, in which the substantial correlation between TAS2R43 gene expression in CD4+ T cells and its CNV was observed in HR but not NR patients. The reason for these contrasting correlation patterns is unclear, although distinct mechanisms may be involved. Based on the correlated and uncorrelated patterns in HR and NR patients, respectively, observed in the TAS2R43 genes, a factor that regulates TAS2R43 expression in CD4+ T cells other than its CNV is likely to exist in NR patients. Contrastingly, CNV in the DPF2 gene that affects its expression levels in basophils of both HR and NR patients could act as the direct determinant of SLIT efficacy.
Conclusively, we identified genes differentially expressed in the basophils of HR and NR patients. DPF2 expression was found to be correlated with its CNV. Further studies comparing HR patients before and after SLIT, along with functional studies on basophils are required to elucidate the mechanisms underlying SLIT efficacy.
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Conflict of Interest: The authors declare no conflict of interest to declare.
1 References


Figure Legends

Figure 1. Differential mRNA expression in the basophils of HR and NR patients. Genome-wide transcriptome analysis of basophils from HRs and NRs was performed as described in the Methods. Log2 ratios of the average expression levels in basophils between HR and NR patients after SLIT (Post) are indicated with red (HR > NR) and green (HR < NR). The genes whose difference in the log2 ratio between HRs and NRs was >0.15 before and after treatment are listed (A). The classification and percentage of identified genes are shown (B).

Figure 2. Correlation between expression and CNV in the DPF2 gene. The correlations between DPF2 mRNA expression and CNV in the HR plus NR (ALL; left panel), HR (center), and NR (right) groups before SLIT were examined. Normality of the distributions was confirmed by performing the D’Agostino-Pearson omnibus normality test.
In the context of CNV analysis, the correlation coefficients ($r$) and p-values ($p$) are as follows:

- **ALL**: $r = 0.498$, $p = 0.0006$
- **HR**: $r = 0.546$, $p = 0.0085$
- **NR**: $r = 0.688$, $p = 0.0004$