

Epigenetic changes in B lymphocytes associated with house dust mite allergic asthma

Marien Pascual,^{1,2} Masako Suzuki,² Maria Isidoro-Garcia,³ Juana Padrón,³ Terrence Turner,² Felix Lorente,^{1,4} Ignacio Dávila^{1,4} and John M. Greally^{2,*}

¹Department of Allergy; University Hospital of Salamanca; ²Department of Genetics (Computational Genetics) and Center for Epigenomics; Albert Einstein College of Medicine; Bronx, NY USA; ³Department of Clinical Biochemistry; University Hospital of Salamanca; ⁴Department of Pediatrics; University of Salamanca; Salamanca, Spain

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Abbreviations: PBMCs, peripheral blood mononuclear cells; IgE, immunoglobulin E; NSAID, non-steroidal anti-inflammatory drug; IL, interleukin; RA, retinoic acid; HELP, HpaII-tiny fragment enrichment by ligation-mediated PCR; SEM, standard error of the mean; AERD, aspirin-exacerbated respiratory disease; IPA, ingenuity pathway analysis; FACS, fluorescent activated cell sorting; FDR, false discovery rate

Although there is no doubt about the influence of the genetic background in the onset of the allergic diseases, Epigenome-Wide Association Studies are needed to elucidate the possible relationship between allergic diseases and epigenomic dysregulation. In this study we aimed to analyze the epigenetic patterns, in terms of DNA methylation, of three well-characterized populations of house dust mite allergic subjects, aspirin-intolerant asthmatics and controls. As a first, genome-wide phase, we used the HELP assay to study the methylation patterns in CD19⁺ B lymphocytes in these populations, and found that there are reproducible epigenetic differences at limited numbers of loci distinguishing the groups, corroborated by bisulphite MassArray in a second validation phase of an expanded 40 subject group. These validated epigenetic changes occur at loci characterized as important for the immune response. One such locus is a new candidate gene, *CYP26A1*, which shows differential methylation patterns and expression levels between groups. Our results suggest that epigenomic dysregulation may contribute to the susceptibility to allergic diseases, showing for the first time differences in DNA methylation between allergic and non-allergic healthy subjects, both globally and at specific loci. These observations indicate that the epigenome may offer new pathophysiological insights and therapeutic targets in atopic diseases.

Introduction

There is significant evidence for genetic predisposition in the development of allergy and other related diseases. Many susceptibility genes have been identified as robust candidates in different populations and variants of these diseases.¹ However, the failure to explain the pathogenesis of these variants by using only genetic approaches highlights the multi-factorial nature of the disease.² In this sense, asthma and allergy can be defined as complex diseases, manifesting pleiotropy and likely to be genetically heterogeneous.

During the last decades we have witnessed a progressive rise in the prevalence of the allergic diseases, particularly in westernized countries.^{3,4} This is unlikely to be consistent with a model of expansion of genetic susceptibility in multiple populations, prompting the exploration of other factors that could be influencing this increase. Data have started to become available showing a connection between disease susceptibility and environmental exposure during intrauterine life or early childhood, potentially

mediated through epigenetic modifications, giving rise to the developmental origins of health and disease hypothesis.^{5,6} In response to an environmental challenge, these modifications allow an adaptive change modifying gene expression and the phenotype.²

Epigenetics may be defined as the study of any stable and heritable but reversible change in gene expression or cellular phenotype that occurs without changes in the genotype. One well-studied molecular mediator is the methylation of DNA, a process that occurs in mammals predominantly in cytosines followed by guanines (CpG dinucleotides).^{7,8} To date, several studies have shown the connection between aberrant patterns of DNA methylation and a variety of diseases like cancer.⁹⁻¹¹

Several environmental conditions have been linked with the development of allergic diseases.¹² The hygiene hypothesis focuses on early contact with older siblings and higher hygiene standards as risk factors to develop allergic diseases.¹³ Environmental tobacco exposure has been shown to predispose allergic sensitization in mice¹⁴ and not only maternal but also grandmaternal

*Correspondence to: John M. Greally; Email: john.greally@einstein.yu.edu
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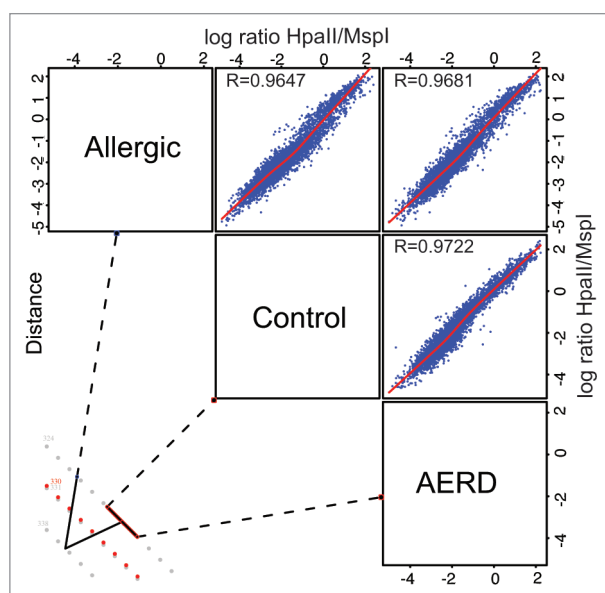


Figure 1. Unsupervised clustering and global pairwise (Pearson) correlations of normalized ratios from the average values of allergic (n = 3), control (n = 3) and AERD (n = 3), using Ward's minimum variance clustering and distances calculated as the Euclidean distance between average ratios. Pairwise correlations are shown in the upper right portion of the figure, where R values indicate the Pearson correlation for each pair.

smoking during pregnancy has been associated with higher predisposition to develop asthma during childhood.¹⁵ Exposure to high levels of airborne polycyclic aromatic hydrocarbons during pregnancy is another environmental condition that has been associated with childhood asthma.¹⁶

One of the main environmental contributors to epigenetic remodeling is diet.^{17,18} It has been shown that diets to be enriched in methyl donors during pregnancy enhance the severity of allergic disease in mice.¹⁹ This suggests a model of changing diet in westernized countries (where asthma and allergy are more prevalent) potentially influencing the development of allergy-related diseases.²⁰

With increasing attention being paid to early exposures and events leading to susceptibility to allergy or asthma,²¹ and with the implication of methyl donor-supplemented diets, which can influence DNA methylation levels,¹⁹ it becomes worthwhile to test whether epigenetic mechanisms could be playing a role in these disorders. The aim of this study was to explore whether aberrant DNA methylation occurs in B lymphocytes from allergic asthmatic, type I hypersensitive patients and compare these with the DNA methylation pattern from B cells extracted from patients diagnosed with bronchial asthma, nasal polyps and intolerance to aspirin (aspirin-exacerbated respiratory disease, AERD), a non-IgE form of hypersensitivity, and a control population characterized to have no history or laboratory evidence of allergy.

Results

Technical validation of cell sorting and HELP assays. In order to explore recurrent epigenetic variations in our subject cohorts,

we isolated peripheral CD19⁺ B-lymphocytes and used FACS to confirm the purity of the sorted cellular populations. Almost 90% of the cells were CD3⁺CD45⁺CD20⁺, which is an immunophenotype characteristic of human B cells, validating this isolation method.

To validate the technical performance of the HELP assay, six loci, two each representing constitutively hypomethylated (~10%), moderately methylated (50–60%) and hypermethylated (~90%) loci in both allergic and non-allergic control subjects were selected. Primers were designed for MassArray to cover both HpaII sites within the fragment tested by the HELP assay. The methylation values determined independently by HELP and MassArray showed an R correlation value of 0.90. This result confirms our ability to discriminate different methylation levels using the HELP assay (Sup. Fig. 1).

Non-allergic and allergic asthmatic subjects exhibit distinct global differences in DNA methylation. The high resolution HELP assay was used as an initial, genome-wide approach to identify whether any epigenetic signature can be found distinguishing the B lymphocytes from allergic asthmatic (n = 3), AERD (n = 3) and healthy subjects (n = 3), with two females and one male in each group. Both unsupervised and supervised clustering of the samples were performed. When we tested the group relationships by unsupervised clustering with global pairwise (Pearson) correlations, it was observed that the groups of non-allergic asthmatic aspirin-intolerant (AERD) and healthy subjects cluster together, whereas allergic asthmatics remain as a separate branch (Fig. 1).

To represent these group relationships in another way, we generated a Q-Q plot from the p-value calculated by a Student's t-Test (Fig. 2). The Q-Q plot enabled us to see any deviation from the null distribution (False Discovery Rate, FDR = 10%) of the expected p-value distribution. Only when the allergic asthmatics were compared with the other two groups were we able to detect an enrichment of significant values and a deviation from the null distribution. We therefore combined the control and AERD groups as a single, "non-allergic" group for comparison with the allergic subject cohort for a supervised two-dimensional hierarchical cluster illustrated by a heat map. A total of 451 differentially-methylated loci distinguishing the B cells from the non-allergic and allergic groups with a p-value < 0.05 calculated by a Student's t-Test and an absolute difference of log₂(HpaII/MspI) ratio >1.5 were chosen for this analysis, and are represented by a heat map (Fig. 3).

Ingenuity Pathway Analysis (IPA, www.ingenuity.com) was used to explore the genes differentially methylated between the control and the allergic groups. All genes showing different shifts in methylation between control and dust allergic groups were selected. These gene-specific methylation changes were defined as occurring within the annotated gene body or the promoter, defined as ±1,000 bp from the transcription start site. This analysis showed that the majority of the genes that were differentially methylated were related to immune responses such as antigen presentation pathways (p-value = 2.6 e-3), IL-4 signaling (p-value = 2.2 e-3) and vitamin D receptor and retinoic X receptor signaling (VDR/RXR signaling) (p-value = 2.5 e-3) (Fig. 4 and Sup. Table 1).

The promoter region of *CYP26A1* is significantly hypermethylated in allergic asthmatic subjects. Promoter regions showing adjacent changes in HELP assay data with the most consistent differences (absolute $\log_2$ ratio (HpaII/MspI) >1.5 and p-value < 0.05 by Student's t-Test between allergic and control groups) were analyzed. After identifying a number of potential candidates (Sup. Table 2), we focused on the promoter region of *CYP26A1*, a gene encoding a protein involved in retinoic acid clearance, a pathway defined as statistically significant in the Ingenuity Pathway Analysis and that appeared to be significant differentially methylated by HELP assay. Five pairs of bisulphite MassArray primers along the promoter of the gene were designed (Sup. Table 3) for this second, quantitative phase of analysis in B CD19⁺ cells from all 40 subjects.

The average HELP values for each set of allergic (n = 3) (black) and control (n = 3) subjects (red) is shown in Figure 5A, a visualization using the UCSC Genome Browser of a ~5 kb region at *CYP26A1*. In this representation, values below the zero line represent relative hypermethylation and those above the line relative hypomethylation. These HELP assay differences were confirmed in a larger population (n = 40, half allergic asthmatic patients and half control subjects) by bisulphite conversion of B cell DNA followed by PCR amplification and MassArray.

The average of percentage methylation for each CG dinucleotide site within each group, along with the SEM, is available in Supplemental Table 4. Consistent with our HELP data, the allergic patients show a relative hypermethylation when compared with the healthy subjects as shown in Figure 5B. Of the 25 CG dinucleotides tested, 17 could be individually assessed and the remaining 8 were in such close proximity with other CG that measurements represented averages of the multiple adjacent CGs. Out of the 25 CG measurements obtained, 8 were statistically different (p-value < 0.05, as calculated by a Student's t-Test) (Sup. Table 4).

Decreased expression levels of *CYP26A1* in allergic asthmatic subjects correlate with the hypermethylation of its promoter. To characterize this finding further, we then investigated whether these differences in DNA methylation along the promoter were correlated with differences in gene expression. Expression analysis was performed in triplicate using RNA from CD19⁺ purified B cells from a separate validation group of three allergic patients and three controls. Higher methylation levels in allergic patients correlated with a two-fold decrease of expression of the gene compared to healthy control subjects (p-value = 0.03) (Fig. 5C).

Discussion

B lymphocytes are key players in adaptive and humoral responses and have a role in allergy through the production of specific IgE, a hallmark of type I hypersensitive responses and characteristic of our allergic patient population. In addition, the balance between Th1 and Th2 cells in allergic diseases is biased towards an exaggerated Th2 cell response. This CD4⁺ T lymphocyte subpopulation appears soon after encountering an allergen in a previous

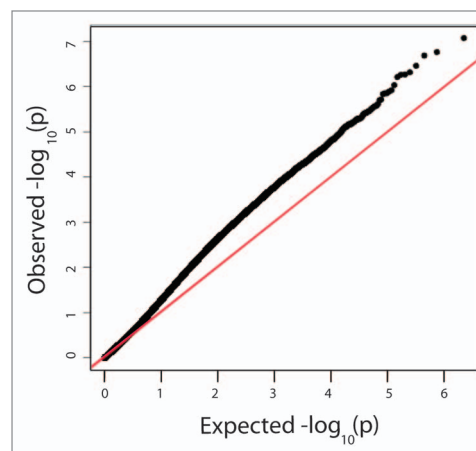


Figure 2. Q-Q plot of p-values calculated as the result of comparing the allergic group against the other two groups combined. The observed $-\log_{10}$ p-value is represented in the y-axis, while the expected $-\log_{10}$ p-value is represented along the x-axis. The red line indicates the theoretical normal distribution (no difference between populations). In this case when we compare the allergics to the combined control group, the observed p-values are greater than expected under the null hypothesis with an FDR = 10%.

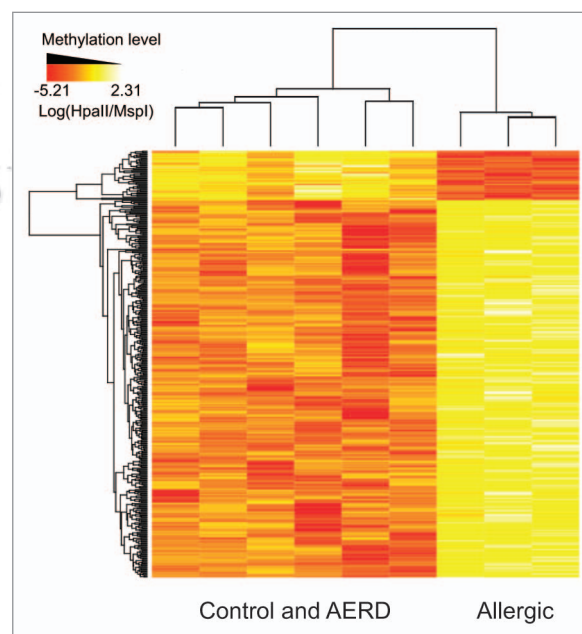


Figure 3. Two-dimensional hierarchical supervised clustering of genes differentially methylated (p-value < 0.05 and an absolute difference in $\log_2$ Ratio >1.5) between allergic patients and the combined control group, illustrated by a heatmap. Cases are represented in columns; probe sets are represented in rows.

sensitized subject. Th1/Th2 cell subsets are mutually counterbalanced²² and apart from their differences in cytokine production, it has been shown that they also differ in their epigenetic marks (reviewed in ref. 23). Since epigenetic patterns vary between different cell types, and the Th1/Th2 cell count is expected to be different between allergic and non-allergic subjects, we avoided

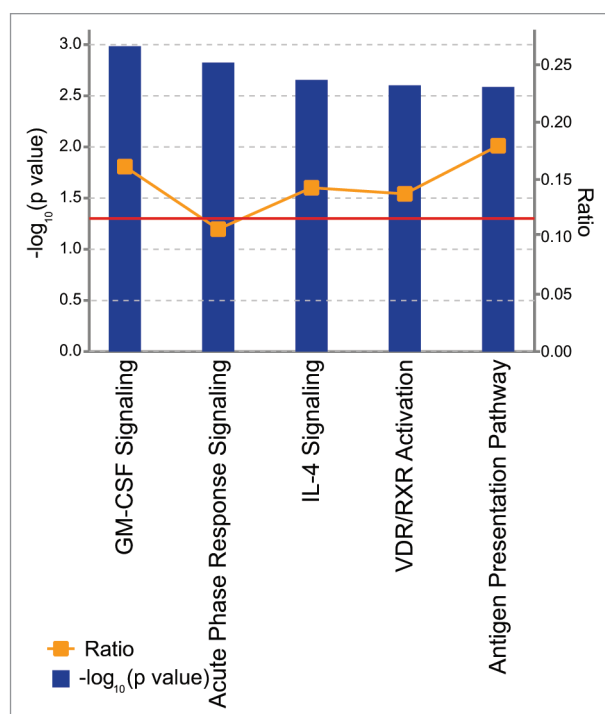


Figure 4. Ingenuity Pathway Analysis of genes that are differentially methylated between allergic and control groups. The p-value is calculated with the right-tailed Student's t-Test and represented as the inverse logarithm of the value for each case. The red line shows the threshold for a p-value of 0.05. The ratio is calculated dividing the number of genes that show differential methylation for each pathway by the total of genes related to that particular pathway. The several highest-ranked pathways are shown, demonstrating the epigenetic dysregulation to be targeted primarily to genes involved in antigen presentation and IL-4 signaling.

the potential for these confounding effects by focusing our study on CD19⁺ B lymphocytes as a cell type less likely to be subject to population variability. In order to avoid any epigenetic variation due to current presence or absence of allergen stimulation in this population of cells, we chose patients with house dust mite allergy, an allergen permanently present in the environment. Moreover, a very well-defined population diagnosed with aspirin intolerance, asthma and nasal polyps was included. This asthmatic population without allergies or abnormal IgE antibody responses served as a supporting comparison group for the IgE-producing asthmatics.

For this study, a two-stage design was used, a genome-wide approach in a subset of individuals, (n = 9) and validation of candidate loci in a larger (n = 40) group of subjects. The microarray-based high-resolution HELP assay²⁴ was performed, confirming its technical performance using a number of loci predicted to have a range of methylation values, demonstrating a strong correlation (R = 0.90) between the genome-wide and quantitative single locus data (Sup. Fig. 1). This allowed us to continue with the analysis of the global patterns and specific loci at which dysregulation of cytosine methylation was suspected. The goal of the study was not to be comprehensive in terms of identifying all cytosine methylation changes in the genome, but to test whether such changes

were occurring at all, with the identification of loci only where the most substantial and consistent changes are occurring.

After a strict process of quality control and normalization,²⁵ both unsupervised and supervised clustering of the samples were performed. The sample-to-sample relationships were tested by unsupervised clustering and global pairwise correlations, and illustrated as a dendrogram (Fig. 1). The clustering of the control group and the AERD group could point to similar epigenetic pattern between these groups distinguishing them from the allergic subjects, possibly due to epigenetic organization distinctive to IgE-activated B cells. This observation indicates for the first time that the differences in DNA methylation patterns in CD19⁺ B lymphocytes were more concordant between control subjects and AERD than when compared with allergic subjects, in whose B lymphocytes there presumably includes a more substantial subset that is IgE-producing.

Supervised analysis of our data allows the representation of the results in complementary ways. The plot (Fig. 2) reinforces the results shown in the dendrogram, pointing out that the strongest differences are between the allergic and the combined “non-allergic” group. A supervised two-dimensional hierarchical cluster, illustrated by a heat map, revealed the differential patterns of global DNA methylation in 451 differentially-methylated loci chosen based on a p value threshold of 0.05 (Fig. 3). As illustrated, it can be observed that the allergic group has a general tendency towards hypomethylation (yellow) with a few loci gaining methylation (red) when compared with the control and AERD groups. The moderate changes in the degree of methylation are consistent with the epigenetic dysregulation affecting a specific subset of the B cell population, which may be the major contributors to the allergic phenotype.

Ingenuity Pathway Analysis software was used to explore the genes closest to the most strongly differentially-methylated loci between the allergic and control groups. This analysis showed that the majority of the genes that are differentially-methylated in allergic subjects and controls have functions related to immune responses such as antigen presentation pathways and IL-4 signaling (see Fig. 4 and Sup. Table 1). The vitamin D receptor/retinoic acid receptor (VDR/RXR) activation (p-value = 2.5 E-03) was one of the highest-scoring signal canonical pathways generated from IPA, while the same loci tested for metabolic pathways showed retinol metabolism (p-value = 6.26 E-04, data not shown) to be strongly represented. To our knowledge, this is the first evidence of DNA methylation changes in B lymphocytes directly affecting pathways related to immune signaling and thus potentially influencing disease pathogenesis.

As the basis for relating methylation with transcriptional changes, we identified the loci with multiple significant methylation changes reported by the HELP assay located in promoters of genes related to the over-represented pathways identified using IPA. From the potential candidates, we focused on the promoter region of *CYP26A1*, a gene coding for a protein involved in retinoic acid clearance²⁶ that has been shown to be dynamically regulated by RA.²⁷

The methylation levels were tested in a larger cohort of B lymphocyte samples (n = 40) using bisulphite conversion of

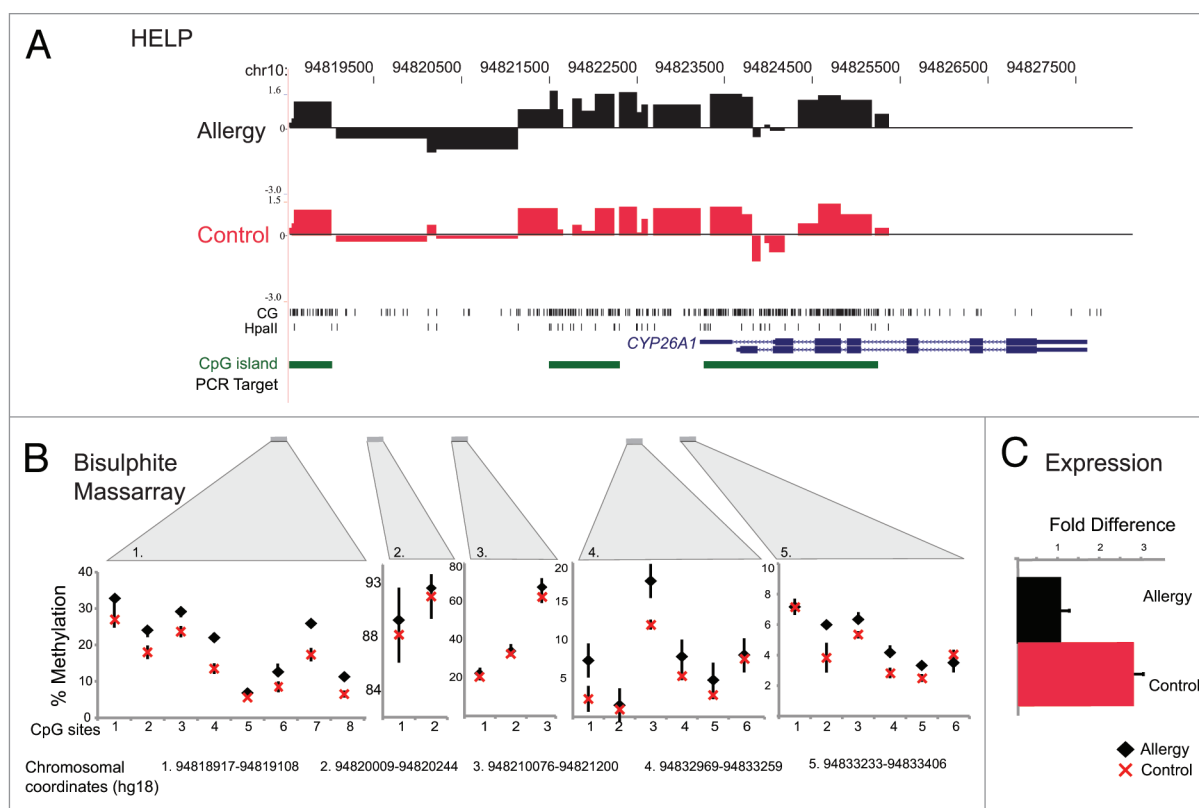


Figure 5. (A) Visualization of HELP data (averaged for each set of 3 samples) in a ~5 kb region using the UCSC Genome Browser. Allergy (black) and control (red) tracks are shown, where deflections below the zero line represent increased methylation and deflections above the line represent relative hypomethylation. The locus shown is the differentially methylated region in the promoter of *CYP26A1*. (B) Methylation percentage and SEM (standard error of the mean) of CGs from six loci along the promoter in 20 allergic (black) and 20 control (red) subjects. Consistent with our HELP data, the allergic patients show a relative hypermethylation when compared with the healthy subjects. (C) The decreased expression of the *CYP26A1* gene in patients correlates with the increase in DNA promoter methylation.

DNA followed by PCR amplification and MassArray along the promoter. Consistent with our HELP data for this locus, the promoter of this gene was more methylated in allergic subjects than in healthy individuals (see Fig. 5A and B). To characterize this finding further, we tested whether the relatively modest differences in DNA methylation within the *CYP26A1* promoter region were associated with differences in gene expression in B cells from allergy patients and healthy control subjects. The higher methylation levels in allergic patients correlated with a two-fold decrease in *CYP26A1* expression levels compared to healthy control subjects (Fig. 5C). Even this relatively small number of patients shows differences in *CYP26A1* expression, serving as an indicator that even the intermediate changes in DNA methylation can be associated with changes in gene expression.

CYP26A1 belongs to a large and ubiquitous family of proteins known as the cytochromes P450 and has been recognized to have the major role in the clearance of retinoic acid (RA)^{26,27} the active metabolite of Vitamin A. Retinoic acid is known to be an important regulator in the immune system, and it has been related to the susceptibility to allergic diseases.²⁸ Low retinol concentrations in serum have been correlated with the development of atopic manifestations²⁹ and have been described in

asthmatic subjects compared to healthy controls.³⁰ On the other hand, murine models have shown that low vitamin A diets hinder the development of Th2 responses in ovalbumin challenged mice, whereas high levels of vitamin A intake in the same animals showed a shift towards Th2 with an aggravation of allergic symptoms³¹ and upregulation of IgE in serum.³²

Although the specific role of RA in the onset and development of atopic diseases remains unresolved, it appears to be clear that an imbalance in the RA metabolism has an impact on the immune system (reviewed in ref. 33). We have identified for the first time the dysregulation of DNA methylation patterns in purified CD19⁺ B lymphocyte populations from house dust mite-allergic patients compared with non-allergic subjects. These changes occur at multiple loci throughout the genome, with differences in methylation in genes involved in processes critical for immune responses. We have shown global and local evidence for selective targeting of epigenetic differences to genes involved in RA signaling pathways in these house dust mite allergic patients and focused on *CYP26A1*, a gene involved in retinoic acid catabolism but not previously associated with allergic diseases. We validated the relative hypermethylation state at the promoter of this locus in allergic patients that correlates with a decrease in the expression of the gene.

The main limitation in the present study is the limited population sample size for the genome-wide approach (9 subjects), constraining our power to detect methylation changes comprehensively. Further analyses in an expanded cohort of subjects are needed to characterize our findings more robustly. The limitation of the current study is likely to be a lack of comprehensiveness of the full extent of the changes throughout the genome, with many loci remaining undetected. The moderate degrees of change of methylation are consistent with the epigenetic dysregulation affecting only a specific subset of the B cell population. Defining the nature of this subset will be an interesting further avenue of exploration. We also demonstrate the value of an epigenome-wide approach that allow us to circumvent any a priori assumptions about genes involved in susceptibility to atopic diseases. These new insights in epigenetic regulation and particularly in the retinoic acid metabolism, a diet supplement whose intake has an impact on the immune system³² and that varies as a result of differences in the dietary intake,³⁴ could be a new venue of research that will bring a better understanding of disease pathogenesis and that could lead to the development of innovative therapies.

Materials and Methods

Subjects. A total of 43 unrelated Caucasian individuals participated in this study; all of them recruited by the Allergy Department of University Hospital of Salamanca (Spain). The study followed the recommendations of the Hospital Ethics Committee and written informed consent was obtained in all cases. Subjects were enrolled as controls when meeting all the following strict criteria: (i) absence of symptoms or history of asthma or other pulmonary diseases; (ii) no symptoms or history of allergy; (iii) negative skin prick tests to a battery of common aeroallergens and (iv) absence of familiar history of asthma or allergic diseases. Allergic asthmatic patients were enrolled if they had a previous diagnosis of house dust mite allergic asthma, with serum IgE levels >100 kU/L and a positive skin prick tests and specific IgE to *Dermatophagoides pteronyssinus*. In addition we also included ASA Triad patients diagnosed following EPOS guidelines.³⁵ The groups were age (Controls and AERD: 49.10 ± 3.67; Allergics: 41.13 ± 3.72) and sex matched (Controls and AERD: 14 women, 9 men; Allergics: 14 women, 7 men).

Skin prick tests were performed according to the recommendations of the European Academy of Allergology and Clinical Immunology (EAACI), using a battery of aeroallergens (ALK-Abello, Madrid, Spain) previously described in reference 36. Antihistamines were discontinued before skin testing according to published guidelines. Saline was used as negative control and histamine 10 mg/ml was used as positive control. Skin tests were considered positive if at least one allergen elicited a wheal reaction of more than 3 mm in diameter after subtraction of the negative control. Total serum and specific IgE levels were measured by a fluoroenzyme immunoassay with the Pharmacia Cap System® (Pharmacia, Uppsala, Sweden), following the instructions of the manufacturer.

Isolation of B lymphocytes. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque PLUS (GE

Healthcare Bio-Sciences, Sweden) density gradient (1,500 rpm, 30 minutes, room temperature). CD19⁺ B lymphocytes were then extracted from the buffy coat using an immuno-magnetic approach provided by Invitrogen (Invitrogen, Carlsbad, California USA) and following the manufacturer's protocol, in which the cells were obtained by positive selection.

Flow cytometry. For fluorescent staining, ~2 × 10⁶ cells were incubated shielded from light at room temperature for 15 minutes. The panel of antibodies used was CD3-FITC, CD45-APC and CD20-PE (Becton Dickinson Bioscience, San Jose, California USA). We proceeded immediately to fluorescence-activated cell sorting, using a FACScalibur (Becton Dickinson Bioscience, San Jose, California, USA) with PAINT-A-GATE-PROTM and CellQuest® software for the immunophenotypic analysis (Becton Dickinson Bioscience, San Jose, California, USA).

HELP assay. The microarray-based high-resolution HpaII tiny fragment Enrichment by Ligation-mediated PCR (HELP) assay³⁷ was performed in a total of nine match-age samples: three controls, three patients diagnosed with aspirin-exacerbated respiratory disease (AERD) and three patients with house dust mite allergic asthma. After high molecular weight DNA isolation, two separate digestion reactions were set up for each sample using either HpaII or MspI until completion, then purified by phenol/chloroform and ethanol precipitation. The digested DNA was then ligated to a mixture of dual adaptors that served as a priming site for a ligation-mediated PCR amplification.

To determine the proportion of methylated (generated in MspI representation only) fragments versus the unmethylated fragments (MspI and HpaII representations), the samples were labeled using 9 mer random primers, conjugated with Cy5 for HpaII and Cy3 for MspI as previously described in reference 38. Both HpaII and MspI representations for each sample were co-hybridized on a customized 2.1 million feature Roche-NimbleGen microarray, with coverage of 1.32 million unique sites in the human genome, representing 98.5% of CpG islands and 91.1% of RefSeq promoters at the Roche-NimbleGen Service Laboratory (Roche Applied Science, Iceland).

After hybridization and image acquisition the quality of the hybridization was tested. A quality control of HpaII and MspI hybridizations and quantile normalization were performed as previously described in reference 25 (density plots shown in **Sup. Fig. 2**). Categorization of methylation states was defined using a HpaII/MspI ratio threshold of zero, defining hypomethylated loci with a positive log₂ ratio value, whereas more methylated loci had a negative value.

Bisulphite conversion and PCR massarray. Sodium bisulphite conversion of B lymphocyte genomic DNA from 20 allergic and 20 control individuals was performed using the EZ DNA Methylation Kit (Zymo Research, Orange, California USA) using the standard manufacturer's protocol before conducting PCR. The primers were designed with MethPrimer (<http://urogene.org/methprimer>) and then checked for specificity using BiSearch (<http://bisearch.enzim.hu/>) and adapted for MassArray analysis. All the amplicons had a range size between 200–400 bp

and contained at least one HpaII site (Sup. Table 3). PCRs were performed in 25 µl volume reactions using the Roche FastStart High Fidelity kit. The analysis of the single CpG methylation was performed using MassArray (Sequenom, Inc., San Diego, California, USA) as described in reference 39.

All samples were analyzed in duplicate, the mean methylation value of all sites was averaged for each sample and SEM was calculated. A T-Test was then performed to evaluate differences that were statistically significant.

RNA extraction and expression analysis. Total RNA was isolated from purified CD19⁺ B lymphocytes using Trizol (Invitrogen, Carlsbad, California, USA). Due to the limited amount of RNA, a T7 linear amplification followed by cDNA synthesis was performed. Quantitative PCRs were performed on 50 ng of each sample in triplicate using LightCycler 480 SYBR Green Master Mix (Roche Applied Science, Indianapolis, USA) with a LightCycler 480 96 well plate format. Primer sequences for analysis of the target genes were designed using the Invitrogen software (Sup. Table 5). The 2^{-ΔΔC_t} (Livak) method⁴⁰ was used to

calculate the relative expression level of transcripts normalized to GAPDH. Expression analysis was performed in triplicate.

Data analysis. Dendrograms were plotted using the function within the HELP package in Bioconductor, as previously published in reference 25. Heat maps were plotted using the heatmap function in R while Q-Q plots used the qq function (<http://www.stephenturner.us/qqman.r>).

The original data for this study were submitted to GEO and will be publicly available once the manuscript is published. Follow this link to view the record of the data: www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=jvmjpecmkwkkcnw&acc=GSE26080

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Note

Supplemental materials can be found at: www.landesbioscience.com/journals/epigenetics/article/16061

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