DNA methylation and childhood asthma in the inner city

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Background: Epigenetic marks are heritable, influenced by the environment, direct the maturation of T lymphocytes, and in mice enhance the development of allergic airway disease. Thus it is important to define epigenetic alterations in asthmatic populations.
Objective: We hypothesize that epigenetic alterations in circulating PBMCs are associated with allergic asthma.
Methods: We compared DNA methylation patterns and gene expression in inner-city children with persistent atopic asthma versus healthy control subjects by using DNA and RNA from PBMCs. Results were validated in an independent population of asthmatic patients.
Results: Comparing asthmatic patients (n = 97) with control subjects (n = 97), we identified 81 regions that were differentially methylated. Several immune genes were hypomethylated in asthma, including *IL13*, *RUNX3*, and specific genes relevant to T lymphocytes (*TIGIT*). Among asthmatic patients, 11 differentially methylated regions were associated with higher serum IgE concentrations, and 16 were associated with percent predicted FEV_1. Hypomethylated and hypermethylated regions were associated with increased and decreased gene expression, respectively ($P < 6 \times 10^{-12}$ for asthma and $P < .01$ for IgE). We further explored the relationship between DNA methylation and gene expression using an integrative analysis and identified additional candidates relevant to asthma (*IL4* and *ST2*). Methylation marks involved in T-cell maturation *(RUNX3)*, T_H2 immunity *(IL4)*, and oxidative stress (catalase) were validated in an independent asthmatic cohort of children living in the inner city.
Conclusions: Our results demonstrate that DNA methylation marks in specific gene loci are associated with asthma and suggest that epigenetic changes might play a role in establishing the immune phenotype associated with asthma. (J Allergy Clin Immunol 2015;136:69-80.)

Key words: *DNA methylation, atopic asthma, epigenetics, T_H2 immunity, inner city*

Asthma can be inherited and is affected by environmental exposures. To date, genome-wide linkage and association studies have identified more than 100 asthma-associated gene variants.[1,2] However, sequence variants explain less than 10% of the risk of having asthma,[3] and several studies have shown that the risk of transmission of atopic disease from an affected mother is approximately 4 times higher than that from an affected father.[4] Because epigenetic marks are also heritable and can account for parent-of-origin patterns of inheritance,[5] it is logical to speculate that epigenetic marks play a role in the transmission of asthma.[6] Furthermore, although it is well established that asthma risk and severity are affected by specific environmental exposures, the

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Abbreviations used
- DMR: Differentially methylated region
- NK: Natural killer
- PC: Principal component
- PCA: Principal components analysis
- PEER: Probabilistic estimation of expression residuals
- RUNX3: Runt-related transcription factor 3
- WTA: Whole transcriptome–amplified

epigenome can be altered by many of these environmental exposures, and these environmentally induced epigenomic changes often lead to rapid and persistent changes in gene expression.[7] For example, *in utero* exposure to tobacco smoke is associated with childhood asthma, and this exposure can modify gene expression through DNA methylation.[8,9] In mice we have demonstrated that *in utero* supplementation with methyl donors alters locus-specific DNA methylation and predisposes mice to allergic airway disease by directing the differentiation of T lymphocytes, skewing toward a T_H2 phenotype.[10] Importantly, epigenetic mechanisms have been shown to specifically affect the expression of transcription factors involved in the development of mature T lymphocytes (T_H1, T_H2, and regulatory T cells),[11-13] providing a potential mechanism that links heritability, the environment, immune biology, and asthma.[14,15] In candidate gene studies DNA methylation has been shown to be related to childhood asthma in peripheral blood cells,[16,17] buccal cells,[18] or nasal epithelia.[19] Thus epigenetic marks represent logical biological changes to pursue when considering the cause and pathogenesis of asthma.

Because black children and families living in poverty are at particularly high risk of asthma,[20] we have investigated the relationship between asthma and DNA methylation in African American children residing in the inner city. We hypothesize that epigenetic marks in circulating PBMCs are associated with allergic asthma.

METHODS

Study population

Our study population consisted of inner-city children aged 6 to 12 years with atopy and persistent asthma (cases, n = 97) and without atopy or asthma (healthy control subjects, n = 97) recruited by 6 sites of the Inner-City Asthma Consortium from census tracts that contain at least 20% of households living at less than the US government–defined poverty level.[21] All subjects reported being African American, Hispanic with Dominican/Haitian background, or both. The validation population consisted of 101 African Americans between 6 and 12 years of age with atopic asthma collected by the Inner-City Asthma Consortium independent of the primary study population. Please refer to the Methods section in this article's Online Repository at www.jacionline.org for more information on selection of the study population.

DNA methylation and gene expression data collection

DNA methylation in PBMCs was measured on Illumina's Infinium Human Methylation 450k BeadChip (Illumina, San Diego, Calif) and validated by using pyrosequencing with custom-designed primers (see Table E1 in this article's Online Repository at www.jacionline.org). Gene expression was assessed on Nimblegen Human Gene Expression arrays (12x135k). Please refer to the Methods section in this article's Online Repository at www.jacionline.org for details of the protocols used for data collection.

Data quality controls

We first examined the quality control figures created by using the minfi R package for the 450k data.[22] We observed a strong bimodal distribution of methylation values in the 450k data, as previously observed.[23] Further data quality for Illumina 450k and expression arrays was assessed by using principal components analysis (PCA). The principal components were examined for correlation with all clinical/demographic and laboratory data to identify observable batch effects or covariates that explained variation (see Table E2 in this article's Online Repository at www.jacionline.org).[24] As a result of PCA, we removed one outlier sample from the 450k methylation data, whereas no outlier samples were identified in expression arrays. Although no laboratory variables were significantly correlated with principal components in the methylation data set, we included 4 laboratory variables in the expression analysis (labeling, date and concentration of whole transcriptome–amplified (WTA) RNA, and hybridization date) because of their significant correlation with principal components (PCs). Of all demographic and clinical data, age and sex are strongly associated with the top PCs in both methylation and expression data sets. Although race/ethnicity is not associated with the top PCs, we included these covariates in the statistical model because of their known associations with DNA methylation.[25]

In addition to PCA, probabilistic estimation of expression residuals (PEER) factors[26] were estimated to account for unknown batch effects. The number of PEER factors included in downstream analyses was 1 for 450k and 5 for gene expression. The number of PEER factors was based on the inflection point seen on visual inspection of the weighting of the inferred factors.[26]

Overview of statistical analyses

The goal of our analyses was to determine whether DNA methylation and gene expression changes are associated with asthma, IgE levels, and percent predicted FEV_1. Cases and control subjects were used to determine whether methylation marks were associated with asthma, whereas the other 2 outcomes (IgE level and FEV_1) were analyzed only within asthmatic patients. We first identified differentially methylated regions (DMRs) associated with atopic asthma. For asthma-associated DMRs, we explored whether the DMRs were influenced by the cell composition of PBMCs. Next, we explored the relationship between PBMC gene expression and asthma, IgE levels, and percent predicted FEV_1 using the same approach outlined above. Lastly, we investigated the relationship of DNA methylation to gene expression in PBMCs. DNA methylation–gene expression relationships were examined by using (1) plots of gene expression versus DMRs and analysis of enrichment for inverse correlations of DMRs and gene expression and (2) integrated analysis of single-probe DNA methylation and gene expression. The second approach focused on single methylation probes as opposed to DMRs because of the limitation of statistical methodologies; all available methods for integrative analysis are limited to analysis of 1 methylation and 1 expression probe in statistical models.

Our rationale for inclusion of DMRs in this analysis is 4-fold: (1) identification of regions as opposed to single CpGs is conceptually consistent with what is known about DNA methylation patterns in the human genome[27]; (2) DMRs increase power to detect associations[28,29]; (3) DMRs allow for analysis of all probes on the array, which, in turn, facilitates finding regions of interest; and (4) DMR analysis has been used successfully in patients with other diseases.[30-32] The alternative to DMR analysis is to remove between 20,000 and 140,000 probes from the analysis depending on the required frequency and distance of a variant in the African American population from the CpG motif. It is known that single nucleotide polymorphisms contained in the Infinium probes can affect binding and result in spurious methylation measurements[33]; however, our combined *P* value method[34] requires 2 or more adjacent probes and, as such, minimized this effect in the methylation analysis.

Statistical analyses of DNA methylation data

Data from the methylation array were normalized with the SWAN method[35] contained in the R package minfi,[36] and the normalized M-values were used in all downstream analyses. β values (which range from 0-1) of

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