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Invited review article

Does epigenetics play a role in human asthma?

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ABSTRACT

Asthma and other allergic diseases are among the most prevalent chronic non-communicable diseases of childhood. According to the World Health Organization, asthma affects >7.0 million children under 18 in the United States, with an economic burden that is estimated to exceed that of tuberculosis and HIV/AIDS combined. Despite much research, the natural history of asthma and its pathogenesis are still in many ways elusive. This review discusses our current understanding of the role epigenetic processes play in asthma pathogenesis, focusing on genome-wide, population-based studies.

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Introduction

Asthma and other allergic diseases are among the most prevalent chronic non-communicable diseases of childhood.^{1,2} According to the World Health Organization, asthma affects >7.0 million children under 18 in the United States, with an economic burden that is estimated to exceed that of tuberculosis and HIV/AIDS combined.³

Despite much research, the natural history of asthma and its pathogenesis are still in many ways elusive. While epidemiologic studies indicate that the disease begins in the pre-school years even when chronic symptoms appear in early adulthood,^{4,5} firm diagnostic criteria to distinguish children who will wheeze transiently during early life lower respiratory illnesses from children who will wheeze persistently, and then develop asthma, are still lacking. Yet, such criteria are urgently needed, because at least at present asthma can be treated but not cured, and therefore the focus must be on prevention. A number of asthma predictive algorithms have been proposed and refined over time,^{6–8} but their sensitivity, specificity and predictive value remain suboptimal. Interestingly, these tools rely on family history and the child's clinical

characteristics in early life but do not incorporate variables that can be measured already at birth.

Allergy and asthma epigenetics comes of age

It is in this context that the potential role of epigenetics in regulating the susceptibility to and the severity of asthma and allergic disease is drawing more and more attention, as shown by a continuous and steep rise in the number of publications.⁹ Remarkably, certain years saw reviews outnumber primary research papers – a pattern that points to an unusual level of expectation and interest. Such interest conceivably stems from multiple considerations. Epigenetic processes, environmental stimuli and developmental programs are connected by strong functional links. That asthma has a strong environmental component was clearly illustrated by seminal epidemiologic studies that revealed major differences in asthma prevalence among countries with more or less Westernized life styles,^{2,10} while the critical role of early life exposures as determinants of asthma during adulthood has been repeatedly emphasized.² To the extent that epigenetic mechanisms faithfully and sensitively transduce environmental signals and preside over the time-dependent unfolding of developmental differentiation programs, their involvement in asthma and allergy is both possible and probable. Another critical reason behind the rising interest in asthma and allergy epigenetics is the

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inability of genome-wide association studies (GWAS) to account for more than a limited proportion of the total phenotypic variance in asthma¹¹ even though the disease is well known to have a strong genetic component.² Current studies on rare variants (as opposed to the common ones that GWAS typically interrogate) will probably improve the situation only marginally. The realization that genetics cannot “explain” asthma and allergic disease has shifted interest towards potential alternative sources of phenotypic variance, primarily the environment and development, both of which are intertwined with epigenetic events. At the same time, interrogating the epigenome, particularly the methylome, has become increasingly feasible and can be pursued as a tool to assess the contribution of epigenetic mechanisms to allergic disease pathogenesis.

Studying epigenetics

Epigenetics studies heritable changes in gene activity that are independent of alterations in the underlying DNA sequence.^{12–14} Among epigenetic modifications, studies in human asthma and allergic diseases have primarily focused on DNA methylation, a process with intimate albeit complex connections with the regulation of gene expression. DNA methylation is a robust epigenetic mark, and user-friendly, quantitative methods to extensively survey the methylome are now widely available and are replacing candidate gene studies. These genome-wide methods rely on straightforward assays and streamlined analytical pipelines, and require DNA rather than chromatin isolation procedures.¹⁵ Therefore DNA methylation studies, unlike the more challenging analyses of post-translational histone modifications, are flourishing and virtually represent the totality of the epigenetic studies performed in human populations with asthma and allergy.

DNA methylation occurs at cytosines in individual CpG dinucleotides and in clusters of CpG sites called CpG islands. The relationship between DNA methylation and gene expression is context-dependent. Typically, promoter methylation promotes gene silencing, whereas an unmethylated promoter is necessary but not sufficient for gene expression and the relevant gene is said to be poised for expression. However, methylation in gene bodies is often associated with high gene transcription.^{15,16}

Current techniques to study DNA methylation rely on bisulfite conversion of DNA, a reaction in which unmethylated cytosines are converted to thymines and only methylated cytosines are preserved as such in the sequence. While the decreasing cost and higher quality of next-generation sequencing are improving research in many areas of genomics, whole-genome bisulfite sequencing is still challenging because the reduced complexity of the DNA sequence that results from the bisulfite conversion process makes it difficult to unambiguously map 100–200 bp-long reads to the genome. Therefore, targeted (gene- and/or pathway-specific) bisulfite sequencing or genome-wide microarrays are still widely used. Currently, the most popular platform for genome-wide DNA methylation profiling is the 450k Illumina Human Methylation BeadChip that interrogates approximately 450,000 CpG sites throughout the genome. This platform surveys individual CpG sites and its output can be intuitively understood as the percentage of DNA methylation at each site.¹⁷ The Illumina 450k array was designed to cover 99% of RefSeq genes with a global average of 17.2 probes per gene region, 96% of CpG islands, 92% of CpG shores, and 86% of CpG shelves.¹⁷ Differences in DNA methylation are detected using two types of Infinium probes on bisulfite-converted DNA. Infinium I probes are designed in pairs (one against the methylated locus and the other against the unmethylated locus). In contrast, Infinium II probes are designed to bind both the methylated and the unmethylated locus. The methylation state is detected upon a single base extension and a distinct signal is given from labeled

nucleotides.¹⁷ It is noteworthy that despite their popularity, these arrays are unable to discriminate 5-methylcytosine from 5-hydroxymethylcytosine,¹⁸ an epigenetic modification that occurs primarily in the brain and embryonic stem cells.

Most genome-wide studies performed in asthma and allergy have targeted DNA methylation in peripheral blood cells, and only a few have surveyed airway cells or tissues. Indeed, although the lung is a major target organ in asthma and allergy, obtaining lung tissue can be problematic in adults and is virtually impossible in children. On the other hand, because immune alterations accompany and often precede a clinical diagnosis of allergic diseases and asthma,^{2,19–22} information from peripheral blood immune cells may still be relevant to disease pathogenesis.

Epigenetic alterations in concurrent allergic disease

So far, most if not all genome-wide epigenetic studies in allergy and asthma sought to identify DNA methylation signatures in patients with *concurrent* disease. Thus a recent study surveyed associations between serum IgE concentrations and DNA methylation in 95 nuclear pedigrees from individuals in their twenties. Replicated associations between IgE and low methylation were found at 36 loci. Genes annotated to these loci encode known eosinophil products, and also implicate phospholipid inflammatory mediators, specific transcription factors and mitochondrial proteins.²³ Another recent study compared DNA methylation patterns and gene expression in 6–12 years old inner-city children with persistent atopic asthma versus healthy control subjects. Results were validated in an independent population of asthmatic patients. Eighty-one differentially methylated regions were identified. Several immune genes were hypomethylated in asthma, including IL13, RUNX3, and TIGIT. Among asthmatic patients, 11 differentially methylated regions were associated with higher serum IgE concentrations, and 16 were associated with percent predicted FEV₁. 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.²⁴

Food allergy was the phenotype analyzed in 11–15 month old children using a supervised learning approach to discover a 96-CpG signature that distinguished food-allergic and food-sensitized individuals as well as food-allergic and non-allergic infants.²⁵ The authors also showed that their methylation signature outperformed both egg- and peanut-specific serum IgE levels as a predictor of clinical allergy. Of note, food allergy status was correctly predicted in a replication cohort of 48 individuals with an accuracy of 79.2%.

A growing challenge with epigenetic epidemiology is that a vast amount of data is generated and new statistical techniques are necessary to make sense of it. This is because of small-n-large-p (few observations relative to the number of predictors) and because traditional methods are not optimized for identifying complex biological processes. Two recent studies relied on Random Forest, a machine learning algorithm used for classification that can handle the data issues discussed above.²⁶ A forest composed of classification trees is grown using randomly selected bootstrap samples of the data to form training and testing sets of study participants. At each node within each tree, the training set is partitioned into different classes with the split determined by a subset of randomly chosen predictors. These two levels of randomness, random selection of training/testing sets and random testing of predictors, allow the random forest to produce robust classification predictions. Once the forest is grown using the training sets, the observations in the testing sets are classified via the forest and misclassification rates can be used to evaluate the accuracy of the forest.²⁶ Of note, it is likely that methylation changes at a

combination of CpG sites could influence disease states. Random Forest allows for the identification of multiple interacting predictors and identifies which of these are most important without imposing a structure or model on the way that it takes place.

A recent genome-wide study used the Random Forest approach to examine the association of DNA methylation with eczema in the first generation of the Isle of Wight birth cohort at age 18 years, seeking confirmation in cord blood from the second generation of the same cohort. This work identified 88 CpGs associated with eczema at age 18 in the first generation. In the second generation, about half of these CpGs showed the same direction of association with eczema risk. Two CpGs in novel putative eczema genes (*PROZ* and *NEU1*) emerged from this analysis.²⁷ In a subsequent study focused on atopy and high serum IgE, in stage 1, 62 sites were selected, of which 22 were associated with atopy in stage 2 (*P*-value range 6.5E–9 to 1.4E–5) and 12 associated with high IgE levels (*P*-value range 1.1E–5 to 7.1E–4) at the Bonferroni adjusted alpha (0.05/62 = 0.0008). Of the 19 available sites, 13 were replicated. The 13 replicated sites were investigated for functional annotation in DAVID and for individual biological relevance via literature review. Functional annotation of the 10 genes (*ZFPM1*, *PRG2*, *KIAA0494*, *EPX*, *ATL3*, *LSM14B*, *COPA*, *ZNF862*, *ASCC1*, and *PVALB*) associated with the 13 replicated CpG sites revealed three statistically significant annotations: polymorphism, eosinophil, and asthma. The most interesting of these findings involved two genes (*EPX* and *PRG2*) in the KEGG pathway for asthma (Benjamini *P*-value = 0.00056) and associated with the eosinophils annotation (Benjamini *P*-value = 0.0087).²⁸

Epigenetic predictors of allergic disease

Despite their potential clinical relevance, the interpretation of studies focused on concurrent asthma or allergy is problematic because it is impossible to determine whether a given alteration associated with disease is a cause or a consequence of that disease. A study design in which the epigenome is surveyed to discover predictors of disease in early life or even at birth, that is, *prior* to the emergence of disease symptoms, is therefore preferable whenever feasible. This design is particularly compelling because epidemiologic studies indicate that asthma begins in the pre-school years even when chronic symptoms do not appear until early adulthood.^{4,5}

A recent study relied on a high-coverage platform to search for DNA methylation signatures predictive of childhood asthma in cord blood mononuclear cells from 36 children (18 non-asthmatics, 18 asthmatics by age 2–9 years) enrolled in the Tucson Infant Immune Study, an unselected birth cohort closely monitored for asthma for over a decade. Cord blood cells were found to harbor 589 differentially methylated regions associated with childhood asthma. Network and upstream regulator analysis showed that a subset of these regions mapped to genes that cluster in immunoregulatory and pro-inflammatory pathways.²⁹ The identification of epigenetic signatures *at birth* implies that there is an epigenetic component to disease pathogenesis, and suggests that the genes harboring differential methylation contribute to placing the child on a trajectory to disease.

This possibility is supported by another interesting study that examined the methylome of children with or without food sensitization at two time points (birth and 12 months). A supervised learning approach led to the identification of a novel 92-CpG signature in CD4⁺ T cells that distinguished children who developed clinical food allergy by age 12 months, and was enriched in genes encoding MAP kinase signaling molecules. Importantly, this signature was stable from birth until 12 months of age, suggesting that the children bearing that signature were on an epigenetic path to disease already at birth.³⁰

An integrated approach to the study of asthma epigenetics

Relying on a novel design that integrates epigenomics and transcriptomics with *in vitro* and *ex vivo* cellular models, a recent study explored the hypothesis that IL-13, the only Th2 cytokine sufficient to induce experimental asthma^{31,32} and a major player in human asthma,³³ promotes airway disease through epigenetically-mediated events. In particular, this study assessed whether IL-13 exposure alters DNA methylation patterns in the airways, targets specific epigenetic pathways, and induces long-lasting changes. An initial screening for IL-13-dependent DNA methylation changes was performed in cultured primary airway epithelial cells within a short window after *in vitro* exposure. Targets were then validated in lung tissues from asthmatics to identify IL-13-responsive CpGs that also differ between asthmatic and non-asthmatic subjects. Importantly, this epigenetic signature was characterized with respect to the transcript abundance of nearby genes.³⁴

This integrated approach proved quite effective. Epigenetic modifications at thousands of CpG sites were found to occur after a single 24 h *in vitro* exposure to IL-13. Interestingly, IL-13-responsive CpG sites were enriched near genes that have been previously associated with asthma. Moreover, a significant proportion of this IL-13-mediated epigenetic signature was mirrored in freshly isolated airway epithelial cells of asthmatic compared to non-asthmatic subjects. The authors hypothesize that these epigenetic changes highlight the most long lasting effects of IL-13 exposure. Importantly, this epigenetic signature clustered in pro-fibrotic and pro-inflammatory pathways, thereby potentially implicating epigenetic modifications in the development and persistence of key features of asthma in the airways.³⁴

What have we learnt from asthma epigenetics?

At a first glance, the scenario emerging from DNA methylation studies in human asthma is not overly encouraging.^{35,36} With few exceptions, the regions where disease-associated differential methylation was detected are spread throughout the genome, with no obvious functional links to asthma and/or allergy-related pathways and no or minimal replication across studies. Moreover, even when statistically significant, disease-associated differences in DNA methylation were typically very modest, of the order of a few percent, raising questions about their biological significance.

There are, however, some extenuating circumstances. Studies are difficult to compare because of their heterogeneity in design and phenotypic characterization. These in turn reflect the fact that oftentimes these studies were a byproduct rather than a primary goal of previously existing data collections, made possible by the relatively simple technical requirements of genome-wide DNA methylation analyses. As a result, the questions these studies asked (and the answers they got) often appear contrived. The numbers of cases and controls in each study also vary greatly, reflecting the lack of firm criteria to define population sizes adequate to generate robust results. The tissues/cells on which these studies were performed also deserve a comment. Because of availability and ease of access, most studies relied on DNA isolated from unfractionated peripheral blood leukocytes or peripheral blood mononuclear cells. Such an approach may be problematic if the cells bearing a given mark are present in different proportions among cases and controls, but on the other hand available methods to use the DNA methylation data to infer cell proportions³⁷ remain controversial.

On balance, we feel that asthma and allergy epigenetics is still in its infancy and several technological and design hurdles will have to be overcome for the field to bloom. Thus, collection of more robust information will require the development of array platforms with higher coverage and ultimately the coming of age of next-

generation sequencing and mapping methods that can efficiently handle data from bisulfite-converted DNA. For study design, it is desirable that the emphasis shift more and more towards the search for marks predictive of, as opposed to associated with, the disease phenotype of interest. This move appears especially appropriate in allergic diseases, which are typically triggered in response to environmental signals and interact with the child's developmental programs. In this respect, strong expectations are associated with studies in well-phenotyped mother-child cohorts that collect samples from birth throughout the first decade of life and carefully assess environmental exposures to chemicals, diet and microbes for both the mothers and their children. While the ability to obtain samples other than cord and peripheral blood from such populations may remain limited, such cohorts will allow for the analysis of *epigenetic trajectories over time*, a theme that is exquisitely relevant to the developmental processes that epigenetic mechanisms primarily regulate. Such birth cohorts will also allow investigating the relationships between the fetal methylome and the methylome and exposures of the mother (for instance, to smoking or to specific environmental microbial profiles), and simultaneously, early (and later) life allergic disease outcomes. Indeed, the link between environmental exposures, epigenetic marks and immune phenotypes appears to be robust.³⁸ Several such birth cohorts [e.g., the Tucson Infant Immune Study (IIS),³⁹ the Wisconsin Childhood Origins of Asthma (COAST) study,⁴⁰ the Danish Copenhagen Prospective Study of Asthma in Childhood (COPSAC)⁴¹ and COPSAC2000,⁴¹ the UK Manchester Asthma and Allergy Study (MAAS),⁴² the Growing Up in Singapore Towards healthy Outcomes (GUSTO) cohort⁴³] already exist, and research along these lines is already beginning. Because the unique time- and environment-dependent nature of epigenetic marks appears better suited for the analysis of prenatal and early post-natal trajectories to disease than for conventional, static assessments of disease risk, we expect this second generation of epigenetic studies will avoid most of the early pitfalls, and will return exciting results that will begin to highlight the role of epigenetics in asthma and allergic disease pathogenesis.

Conflict of interest

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