

Epigenetic modifications: mechanisms of disease and biomarkers of food allergy

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The rise in IgE-mediated food allergy in recent times is the likely result of gene–environment interactions mediated via epigenetic pathways. As epigenetic modifications, including DNA methylation, are at the interface between the environment and the genome, they may be ideal biomarkers of modifiable disease pathways. High-throughput methylation profiling of immune cell subtypes or whole blood from patients allows the identification of disease specific epigenetic variants. If faithfully tracking with disease parameters, these ‘signatures’ may have clinical applications as biomarkers of disease or therapeutic response. Development of such tools will depend on a number of factors, including determining the most appropriate experimental approach, analysis methodology, patient groups, and informative target cells/tissues. Here we discuss these potential applications and their implications for food allergy practise.

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Current Opinion in Immunology 2016, 42:9–15

This review comes from a themed issue on **Allergy and hypersensitivity**

Edited by **James McCluskey** and **Robyn E O’Hehir**

<http://dx.doi.org/10.1016/j.coi.2016.05.005>

0952-7915/Published by Elsevier Ltd.

Introduction

Epigenetic modifications to the genome are key mediators of gene–environment interactions and potential biomarkers of modifiable disease pathways. Owing to their position between the genome and the environment they represent the cumulative contribution of both genetic

predisposition and environmental exposures. Studies have now shown that epigenetic variants, primarily in the pattern of DNA methylation, are associated with food allergy and other allergic diseases. ‘Signatures’ of aberrant methylation have been identified as specific markers of disease-related processes, including immune cell phenotypes, biological aging and environmental exposures, each modifiable by underlying host genotype. Methylation signatures might therefore be developed with clinical utility for discriminating phenotypes of food (and other) allergy. We review here the role of epigenetic mechanisms in allergic diseases and food allergy, highlighting recent advances in the application of epigenetic biomarkers for predicting complex phenotypes.

Genes, environment and the aetiology of IgE-mediated food allergy

The prevalence of IgE-mediated food allergy has risen in many regions of the world over the last 15–20 years, now affecting 3–10% of children in developed countries [1]. The majority of this burden has fallen on children under 4 years of age, indicating the developing immune system is particularly vulnerable [2]. As with other complex diseases, both genetic and environmental risk factors have been implicated. Genetic studies indicate food allergies are polygenic disorders involving potentially hundreds of genes, with heritability estimates ranging from ~15% to 30% for food specific IgE [3] and ~80% for peanut allergy [4]. The largest genetic predisposition is linked to functional polymorphisms within the human leukocyte antigen (HLA)-*DQ* and *HLA-DR* gene regions [5]. Candidate gene studies have also revealed associations with genes that encode products associated with skin barrier integrity [6–8] and immune function [9–12]. Collectively, genetic associations confer a low to moderate level of predisposition, negating a major role for genetics in the recent rise in prevalence of food allergies; most likely attributable to rapid changes in environmental exposures [13].

The modern environment is firmly implicated in the rise of allergic diseases. Epidemiological studies have identified a number of risk factors, such as declining biodiversity [14], increasingly indoor lifestyle (low vitamin D levels) [15], less traditional diets [16], increased consumption of processed foods [17] and changes in infant feeding practises [18], amongst others. Many of these risk factors are also implicated in immune development more generally [19].

Other factors linked to atopic diseases, such as duration of breastfeeding [20], mode of delivery [21^{*}], early life viral infections [22] and antibiotic exposures [23] could also conceivably influence the risk for food allergy. Despite this, only a proportion of children subjected to variation in these exposures develop clinical food allergy, suggesting that some individuals are more genetically susceptible than others, but only in certain environments. Thus the complex interplay between genetic predisposition and environmental exposures during early immune development are central to disease aetiology. These gene–environment interactions provide an explanation for how individuals of differing genotypes respond to environmental variation in differing ways. For example, vitamin D deficiency in childhood is a risk factor for the development of food allergy, but individuals carrying the rs7041T allele are protected [24]. Similarly, carriers of the flaggrin loss-of-function mutation are more susceptible to food allergy following exposure to household peanut protein [25].

Traditional models of gene–environment interactions are now being expanded to include epigenetic mechanisms of gene regulation as additional components of disease. Epigenetic processes regulate all gene activity in all cells and are sensitive to both genetic and environmental variation. Thus, epigenetic mechanisms serve to integrate both genetic and environmental signals in a manner that modulates the functional output of the genome [26].

Epigenetic modifications as additional mediators of gene–environment interactions

Epigenetic modifications represent the cumulative contribution of both genes and environment to underlying gene activity (expression). Modifications to DNA (5'-cytosine methylation, 5'-cytosine hydroxymethylation, non-cytosine methylation, etc.) or DNA associated proteins (histone acetylation, methylation, sumoylation, etc.) collectively mediate the phenotypic expression of the genome by regulating chromatin structure and gene expression during cell growth and differentiation. These modifications can occur at regulatory genomic domains such as enhancers, silencers, insulator elements, gene promoters and splice junctions. A simple analogy can be drawn with the elements of an electrical circuit board — the logic board itself represents the hard-wired genome, which is identical in every cell type, and contains the capacity to transmit information. The transistor components that act as switches mediating potential throughout the circuit are analogous to epigenetic modifications. These transistor switches can be 'on', 'off' or partially on, controlling the current that flows through the circuit. Similarly, epigenetic modifications can induce permissive or repressive effects on gene expression that collectively give rise to unique cell and tissue-specific transcriptional profiles and cellular functions.

A proportion of the epigenetic profile is unique within specific cell and tissue types, and these specific patterns are generally well conserved between individuals in a population. During haematopoiesis, permissive epigenetic modifications are established at lineage marker genes in corresponding differentiated cells of that lineage, whereas repressive modifications are established at the same locus in other cell types [27]. These patterns are sufficiently distinct in the case of DNA methylation to enable robust predictions about the proportion of different cell types within a sample of whole blood from the 'average' DNA pattern measured in that sample [28].

A proportion of the epigenome is also influenced by the underlying genetic sequence and exhibits inter-individual variability. Average heritability estimates for DNA methylation at specific sites within the genome, primarily at cytosine-guanine (CpG) dinucleotides, are approximately 20% [29]. The genetic contribution to epigenetic modifications is multifaceted beginning with variants within genes that code for proteins that establish and regulate the plethora of epigenetic marks. Genetic variation can also occur by nucleotide sequence changes that abolish cognate sequence motifs recognised by these enzymes and proteins. Single Nucleotide Polymorphism (SNP) variation has been shown to strongly influence proximal epigenetic loci for both DNA methylation and histone modifications [30]. These *cis*-genetic effects tend to strongly influence DNA methylation preferentially at loci that exhibit intermediate levels of methylation, as opposed to sites that are highly or lowly methylated, and tend to be shared across different tissues, although not always the case [31]. Genetic variants that specifically influence DNA methylation are referred to as methylation quantitative trait loci (meQTL or mQTL) and can influence not only methylation directly, but also modulate the effects of environmental exposures on the DNA methylation profile, particularly early in life [32].

Much attention has been focused on the proportion of the epigenome that is environmentally sensitive and mitotically heritable. Seminal experiments in mice and humans indicate external factors such as dietary micronutrients [33], xenobiotic chemicals [34], stress [35], assisted reproductive technology [36] and radiation exposure [37^{**}] can alter epigenetically determined patterns of gene expression in a lasting manner. There is evidence, mostly from animal models [38], that these effects can persist across generations depending upon the timing of exposures [39]. These effects have generated much excitement as potential explanations for thresholds of disease risk; for example, many children outgrow their food allergies despite genetic predisposition, suggesting their genes 'learn' or 'adapt' to the environment over time. Whether changes in epigenetic modifications are behind this is as yet unclear but nonetheless of particular interest.

Epigenetic pathways that contribute to food allergy

Many risk factors for the subsequent development of allergic diseases and asthma are associated with changes in epigenetic modifications, including tobacco smoke exposure [40], air pollution exposure [41], dietary exposures (omega-3, folic acid) [42] and variations in the microbiome [43]. Allergy-related phenotypes, atopic dermatitis [44], asthma [45] and IgE production [46] have now been linked to variations in DNA methylation. Our own genome-wide assessments of DNA methylation profiles from children with IgE-mediated food allergy have uncovered differentially methylated loci in total CD4+ T-lymphocytes [47]. In a study of 12 children who developed IgE-mediated food allergies to egg or peanut at 12-months, and 12 age-matched non-allergic controls we identified 136 CpG loci linked to food allergy, 44 of which were annotated to within 10 base pairs of common single nucleotide polymorphism variants (SNP) and exhibited genetic effects [47]. These loci were annotated to genes at *HLA-DQB1* and most likely influenced by underlying variants within the HLA. Support for this has come from a recent genome-wide study of peanut allergic children, which linked DNA methylation levels at the same, and other HLA genes, to nearby SNP variants that mediate the genetic risk for peanut allergy *via* DNA methylation [5].

In our study we also observed 92 additional loci did not exhibit obvious SNP effects but varied significantly between cases and controls. These loci were annotated to 49 unique genes involved in T-cell activation, including the MAP kinase canonical pathway, a major pathway induced by T-cell receptor stimulation and involved in T-cell responses including T-helper type 2 (Th2) differentiation [48]. Importantly, methylation difference between food allergy cases and controls were associated with variations in gene expression. These effects were also detectable in cord blood CD4+ cells from the same children suggesting potential markers of 'pre-existing disease risk'. The origin and nature of these DNA methylation differences is unclear; they could represent environmentally induced effects from in utero environmental exposures, or they could represent *cis* genetic effects. The dynamic nature of epigenetic patterns makes it difficult to ascertain whether associations with phenotypic traits are a cause or consequence of disease process. Further studies are now needed to characterize epigenetic pathways of food allergy, and are likely to reveal new mechanistic insights into disease aetiology. Moreover, aberrations in epigenetic modifications may have important clinical implications, as they may be useful for stratifying patients, or represent novel targets for therapeutic or lifestyle intervention. Therefore, in addition to promising new mechanistic insights, epigenetic studies may reveal new markers with clinical utility.

Epigenetic biomarkers and clinical utility

Disease-associated epigenetic loci have emerged as useful clinical tools for diagnosis and prognosis, mostly in the

oncology field [49]. However, it is important to realise that a causal relationship between an epigenetic biomarker and phenotypic trait is not a prerequisite for clinical utility. Thus, when undertaking an epigenetic analysis in any complex disease (such as food allergy) the scope of the investigation should be tailored to the ultimate goal. Factors to consider include; (i) the timing and (ii) cell/tissue of origin for investigation, (iii) sample size and (iv), the methodology to be employed. Each will vary according to whether the goal is to explore aetiology, develop a clinical biomarker, or both. For example, whereas specific epigenetic changes in multiple cell sub-populations of blood (e.g., Naïve, or regulatory CD4+ T cells) may play a key role in the development of food allergy and therefore warrant investigation to understand disease aetiology, a diagnostic test that requires sorting of these low abundance cell populations would be of far less utility than one applicable to a small amount of unsorted whole blood, such as that obtainable by finger prick.

In the case of clinically relevant biomarkers, it is also important to distinguish the type of marker to be developed. A diagnostic biomarker aims to identify DNA methylation variants associated with a clinical condition, often in the absence of overt clinical symptoms (e.g. allergic response). On the other hand, prognostic biomarkers predict the progression of disease and outcome (e.g. acquisition of tolerance in allergy). Predictive biomarkers may provide valuable information on an individual's responsiveness to a specific treatment.

There are a multitude of DNA methylation biomarkers that have been described (reviewed in [49]), but few have been validated/replicated and most are considered experimental. The reasons for this are multifaceted but generally reflect the need for such markers to meet criteria of both sensitivity (limited false negatives) and specificity (limited false positives) at multiple stages of investigation. Epigenetic marks linked to disease in a single study have *potential as a biomarker*. Independent replication will then yield a *validated biomarker* and further replication in independent cohorts will produce a *replicated biomarker* or *candidate clinical biomarker*. Finally, if successful in clinical trials, the biomarker will become a *proven clinical biomarker* [49]. DNA methylation biomarkers that have proven utility are generally only found in the field of cancer with several approved for use in diagnosis [50]. The successful development of methylation-based diagnostics in cancer is a promising advance in the field, although methylation differences between cancer patients and controls are generally much larger (more than 50%) than that seen in common non-communicable diseases like allergy (typically less than 5% when assessed in whole blood). Approaches that target multiple informative sites from patient DNA in combination may be more suited to the allergy setting.

Fortunately, the advent of genome-wide technologies such as DNA methylation microarrays and Next-Generation Sequencing technologies offer useful platforms to discover aberrant patterns of DNA methylation in patient groups of interest. High-throughput screening of patient samples allows the identification of DNA methylation sites that correlate with clinical outcomes of interest. These loci can be combined using a variety of algorithmic approaches to derive a prediction model that can be harnessed to resolve phenotypes of interest. We recently explored the utility of this approach for addressing the clinical challenge of resolving symptomatic food allergy from asymptomatic sensitisation.

Many food-sensitised children that produce an IgE-mediated response to dietary antigens are not allergic, and this discrepancy between food sensitisation and food allergy creates diagnostic difficulties [51]. In a pilot study of 58 food-sensitised children, half of whom were clinically allergic by oral food challenge, we performed genome-wide DNA methylation profiling from DNA derived from peripheral blood mononuclear cells [52]. Using applied statistical learning we identified 96 CpG sites annotated to 73 protein-coding genes that exhibited predictive utility for discriminating symptomatic allergy from asymptomatic allergic sensitisation. In an area under the curve analysis we compared the predictive utility of the methylation signature to serum IgE and skin prick test wheal size for predicting the known clinical phenotype (symptomatic clinical food allergy versus asymptomatic antigen sensitivity). Patient methylation signatures predicted food challenge outcomes with 89.7% sensitivity and 96.6% specificity, and the signature was found to be reproducible in a second cohort.

The attractiveness of this approach is the potential to capture both genetic and environmental variability. A recent study of 572 children aged 3–5 years examined epigenetic signatures of prenatal tobacco smoke, consisting of 26 CpG sites that have reproducibly been associated with smoking exposure in a number of cohorts. The investigators examined the potential for 26 aberrantly methylated CpG to act as biomarkers of prenatal smoking exposure [53^{*}]. They were able to combine the 26 CpG into a DNA methylation signature that accurately predicted prenatal smoking exposure status in early childhood, suggesting a blood derived DNA methylation signature of in utero exposure to tobacco smoked. In a landmark study by Horvath and colleagues, a signature of 353 CpG sites was discovered using 8000 samples from 51 tissues and cell types that accurately predicted chronological age with a median error of 3.6 years across all cell types [54^{**}]. The relevance of this to human health is becoming increasingly clear, with epigenetic age predicting all-cause mortality risk independent of a combination of risk factors such as smoking status, CVD and diabetes in adults [55]. When measured in blood this has also

shown utility in tracking the molecular aging associated with obesity [56] and may be useful as a marker to track the trajectory of health from early age to later life [57]. The striking feature of this age-associated methylation signature is its reproducibility across many tissues, such that it can accurately be determined in whole blood or saliva. Given the demonstrated stability of DNA methylation, this will likely include samples stored for days (or longer) at room temperature.

These studies have broad-reaching implications. For example, DNA methylation signatures that reflect environmental exposures, programmed *in utero* or otherwise, could be useful biomarkers of modifiable pathways around which to design intervention strategies. These signatures could also reflect changes in cellular composition associated with disease progression. Beyond medical science the application of DNA methylation signatures for forensics is already being explored for the potential to exploit methylation biomarkers on DNA recovered at the scene of a crime to predict the age, tissue type and smoking status of a perpetrator [58].

Challenges for developing methylation signatures with clinical utility

Successful development of methylation signatures requires consideration of a number of parameters. The goals of the experiment and the clinical utility should determine the appropriate choices around these parameters. Experimental design should address a clear clinical need, and the choice of appropriate patient groups should align closely to these goals. Investigators should consider the utility of the signature — will it be used as a screening tool, or a second line test? The choice of cell or tissue type is often a trade-off between relevance to disease, practicality and accessibility, though for a truly useful clinical test the latter predominate. Mixed cell populations like blood are easily accessible but owing to the tissue specificity of DNA methylation, reflect a mixture of the unique methylation signature for each of its constituent cell types. Effect sizes from whole blood methylation profiles are often smaller due to the inherent noise, which can also mask the ability to detect associations. Purified cell types may be preferable but add to cost and handling time, which may not align with the end goal. Methods have been developed to de-convolute mixed cell DNA methylation signatures providing surrogate covariates of estimated cell proportions [59,60].

The statistical approach should be appropriate for the goals of the experiment. Differential methylation analysis is typically suited for investigations into disease aetiology by identifying novel associations at the genome-wide level. There may be other loci that exhibit less of an association and do not meet the criteria for genome-wide significance, but exhibit some correlation with phenotypes of interest. Statistical approaches that

simultaneously model all methylation sites might therefore be more suited for problems of class classification or class prediction [61]. Spurious associations are a constant worry and replication is the only way to assess robustness — key to the development of any truly relevant test.

Conclusions

Epigenetic modifications are now recognised as additional components of disease under an expanded model of gene–environment interactions. Characterizing the epigenetic pathways associated with allergic diseases has the potential to both reveal novel aetiological insights, and new markers with clinical potential for patient stratification, or therapeutic targets. The integration of epigenomic information alongside conventional clinical information to facilitate disease prediction is becoming a realistic possibility, although examples of such approaches are only just emerging. Large well-phenotyped cohorts with longitudinal data will be required to enable characterization of epigenetic pathways with clinical utility. Although epigenetic biomarkers may be associated with a variety of clinical outcomes of interest, they will need to meet already well-established rigorous criteria to support routine use for risk or outcome prediction in clinical practise.

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