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Epigenetic Changes During Food-Specific Immunotherapy

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Abstract
Purpose of Review The prevalence and severity of IgE-mediated food allergy has increased dramatically over the last 15 years and is becoming a global health problem. Multiple lines of evidence suggest that epigenetic modifications of the genome resulting from gene-environment interactions have a key role in the increased prevalence of atopic disease. In this review, we describe the recent evidence suggesting how epigenetic changes mediate susceptibility to food allergies, and discuss how immunotherapy (IT) may reverse these effects. We discuss the areas of the epigenome as yet unexplored in terms of food allergy and IT such as histone modification and chromatin accessibility, and new techniques that may be utilized in future studies.
Recent Findings Recent findings provide strong evidence that DNA methylation of certain promoter regions such as Forkhead box protein 3 is associated with clinical reactivity, and further, can be changed during IT treatment. Reports on other epigenetic changes are limited but also show evidence of significant change based on both disease status and treatment.
Summary In comparison to epigenetic studies focusing on asthma and allergic rhinitis, food allergy remains understudied. However, within the next decade, it is likely that epigenetic modifications may be used as biomarkers to aid in diagnosis and treatment of food-allergic patients. DNA methylation at specific loci has shown associations between food challenge outcomes, successful desensitization treatment, and overall phenotype compared to healthy controls.

Keywords Food allergy · Immunotherapy · Epigenetics · DNA methylation · Atopy

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Introduction

Food allergy (FA) prevalence has increased by a staggering rate of more than 18% between 1997 and 2007 in the USA [1]. Today, it is estimated that FA likely affects nearly 5% of all adults and 8% of children [2]. In this review, we discuss the role of epigenetics in FA. Epigenetics is the study of modifications that elicit changes in gene expression without altering the DNA sequence. Specifically, epigenetic changes such as DNA methylation (DNAm) have been shown to occur during food-specific immunotherapy (oral immunotherapy (OIT)), which currently has multiple ongoing phase II and III clinical trials. Additionally, we examine how epigenetic testing of DNA methylation could be used as a biomarker during OIT with the potential to predict outcomes of desensitization or sustained unresponsiveness. Finally, we explore other areas of epigenetics not yet explored in FA that could provide valuable information about risk and development.

DNA Methylation, Epigenetics, and Gene Regulation

The known epigenetic modification of DNA in mammals is the methylation or hydroxymethylation of cytosine at the fifth carbon in CpG dinucleotides [3]. CpG dinucleotides, which are cytosines followed by a guanine linked by a phosphate along the 5′→3′ direction, are different in function than GpC dinucleotides. Genomic regions with high G-C content rich in CpG motifs, termed CpG islands, are often found in regulatory regions [4, 5]. The proximity of CpG islands to gene promoters as well as their methylation status influences regulation of gene expression [6]. Increased methylation is associated with gene silencing along with genome stability, whereas hypomethylation leads to active transcription [7]. The relationship between DNAm and human disease, highlighting the nuances between methylation in cancers and autoimmune disease may be found in an in-depth review by Robertson [8].

Studies have shown that both genetics and environmental factors can alter the epigenetic profile [9, 10] and may provide the missing link to understanding environmental–genetic interactions and FA risk. Numerous studies associate many environmental factors with FA development including timing of food introduction and feeding pattern [11, 12, 13••, 14–19], diet and nutrition [20–22], exposure to environmental pollutants and tobacco smoke [23–26], prematurity and low birth weight [27, 28], microbial exposure [29–34], and race/ethnicity [35–37]. Hong and Wang have comprehensively reviewed these environmental factors and their associations with the development of FA [38]. These studies implicate many factors that could potentially modulate an epigenetic profile. Understanding the relationship between epigenetics and environmental exposure would provide profound insight to the rise and etiology of FA.

DNA Methylation and Allergy

Liu et al. published a twin study looking at concordance rates of food sensitization in twins, suggesting that both genetic and environmental factors play a role in inducing allergen sensitization [39]. Since then, results of genome-wide association studies (GWAS) and epigenome-wide association studies (EWAS) have supported correlations between genetic regions and CpG island methylation status near gene promoters and FA.

The first GWAS of a well-defined FA was performed in a U.S. cohort of children and their biological parents [40••]. The authors performed a three-stage GWAS looking for genetic associations with FA, particularly focused on milk, egg, and peanut allergies (PA). The study found that genetic variants in the HLA-DR and HLA-DQ gene region were significantly associated with PA in children of European ancestry. The GWAS revealed that this gene region accounted for approximately 20% of PA in the study population [40••]. Previous studies that associated mutations in HLA genes with PA and other autoimmune disorders lend further support to these findings [41–44]. Although a strong genetic base for PA has been identified via GWAS, it is also clear that genetics are neither sufficient nor required for development of the allergy as not all participants with the identified genetic risk factor developed PA.

Martino et al. investigated whether variation in DNAm underscores the suboptimal neonatal $CD4^+$ T cell gene expression associated with the development of FA [45•], including impaired T cell expansion and reduced IFN-γ production [46–49]. Martino et al. previously found that the immature neonatal $CD4^+$ T cell response involves altered expression of T cell activation genes that signal through the NF-κB pathway [49]. In their follow-up study, the authors examined genome-wide DNAm profiles in $CD4^+$ T cells from 12 children with FA and from 12 nonallergic controls at birth and again at 12 months. They found that the dysregulation of DNAm at MAPK signaling-associated genes during early $CD4^+$ T cell development may contribute to suboptimal T lymphocyte responses in early childhood associated with the development of FA [45•].

Most recently, Hong et al. compared DNAm alterations in patients with active cow's milk allergy (CMA) with controls. They found that CMA patients have methylation changes affecting their TH1-TH2 balance, specifically at regions already seen in other atopic disease associated with IL1RL1, IL5RA, IL4, CCL18, and STAT4 [50••]. They also found three other regions related to NDFIP2, EVL, and TRAPPC9 that were associated with CMA and not reported previously. Hong et al. argue that these new regions are biologically plausible, as they are all related to TH1-TH2 balance. These studies supported the findings of Canani et al. in children with CMA [50••], discussed in detail below. This brings to light the complexity of methylation as it relates to allergic disease and suggests that multiple regions could be associated with atopy in general, with different regions potentially having association with food-specific allergy.

Epigenetics and Specific Immunotherapy

Although relatively few studies have examined epigenetic changes before and after specific immunotherapy, the findings have generally supported the same notion: that TH2-related gene promoter regions are more methylated post-treatment, while TH1 promoter regions become less methylated. These studies are discussed in detail below and summarized in Table 1. Looking at environmental allergen sensitization, Swamy et al. investigated sublingual immunotherapy (SLIT) to timothy grass and dust mite [51•]. Subjects undergoing immunotherapy showed significant decreases in their rhinoconjunctivitis scores, overall medication usage, and overall response to allergen based on skin prick test (SPT) or nasal disk challenge. Compared to control, dual SLIT promoted allergen-specific suppressive forkhead box protein 3

Table 1 Studies of epigenetic changes associated with FA

Study (year)	Study details	No. of patients	Age (mean)	Allergy	Cell type	IT status
Swamy et al. (2012)	Single-center, randomized, double-blind, controlled phase I study	Active (20); placebo control (10)	Active (30 years); placebo control (25 years)	DM, TG	PBMC memory T cells	SLIT
Martino et al. (2014)	Retrospective genome-wide DNA methylation profile study	Food allergy (30); nonallergic controls (30)	Blood samples collected at age birth and 1 year	IgE-mediated food allergy	CD4+ T cells	N/A
Syed et al. (2014)	Phase 1 single-site peanut immunotherapy study	Active (23); controls (20)	Active (10.4 years); control (12.0 years)	Peanut	PBMCs, basophils	OIT
Canani et al. (2015)	Study of DNA methylation of CpGs in the promoter regions of genes	CMA at diagnosis (10), Outgrown CMA (20); Healthy controls (10)	CMA at diagnosis (5.5 months); outgrown CMA (16.9 months); healthy (9.0 months)	CM	PBMC	N/A
Martino et al. (2015)	Genome-wide DNA methylation profiling study	Discovery cohort: food allergic (29); food sensitized ($n = 29$); nonallergic controls (13) Replication cohort: food allergic ($n = 12$); nonfood allergic (12)	Discovery cohort: 11–15 months Replication samples: birth and 12 months	EW, Peanut	PBMC	N/A
Hong et al. (2016)	Epigenome-wide association study	Discovery cohort: allergic (106); controls (77) Replication cohorts: Chicago replication cohort: allergic (5); controls (20) Boston replication cohort: allergic (8); controls (132)	Discovery cohort: Allergic (4.2 years); Control (5.5 years) Chicago replication cohort: Allergic (7.2 years); control (9.1 years) Boston replication cohort: allergic (3.8 years); controls (3.0 years)	CM	Whole blood	N/A

CM cow's milk, *DM* dust mite, *TG* timothy grass, *EW* egg white

(FOXP3$^+$) memory regulatory T cells through reduced DNA methylation of CpG sites within the FOXP3 promoter locus. This suggests a correlation between long-term tolerance and epigenetic modification of the FOXP3 region.

Syed et al. investigated potential genetic and epigenetic biomarkers to distinguish between outcomes following immunotherapy treatment [52••]. Although the study did not find any genetic differences, they found that FOXP3 DNA methylation levels in regulatory T cells were significantly lower in patients who remained nonreactive to peanuts after 3 months of discontinued OIT than those who were intolerant after the 3-month grace period. Furthermore, peanut-allergic patients in the control group who had not received OIT exhibited much higher levels of DNAm than the OIT groups [52••]. This data suggests that antigen-induced Tregs play a key role in modulating immune response in OIT. Moreover, these results suggest that peanut tolerance via OIT is associated with decreased DNAm levels in the FOXP3 gene. Results indicate that epigenetic biomarkers may be able to predict immunotherapy success and assist in optimizing OIT protocols to increase rates of clinical tolerance. This is an exciting development given the blood test necessary to monitor DNA methylation is relatively inexpensive and uses common lab equipment.

Canani et al. found that the DNA methylation profiles of TH2 and TH1 cytokine genes clearly separated active CMA patients from healthy controls [53••]. They reasoned that using a profile of multiple promoters is a more realistic approach to a diagnostic tool considering the complexity of allergic response. They observed opposite patterns when comparing subjects with active IgE-mediated CMA with healthy controls and children who outgrew CMA. IL-4 and IL-5 DNA methylation were significantly lower, and IL-10 and IFN-γ DNA methylation was higher in active IgE-mediated CMA patients. Further, gene promoter DNA methylation rates of all cytokines and respective serum levels were strongly correlated. These profiles were able to show distinct differences between healthy controls and active CMA patients. The profile was less able to differentiate recently tolerant individuals and healthy controls as they had similar, but not identical profiles. Active allergy had increased methylation of TH1 regions, and decreased methylation of TH2 regions, which created a potential TH2 bias. Tolerance gained by using extra hydrolyzed casein formula with *Lactobacillus rhamnosus* GG (LGG), a probiotic currently being considered as a biologic for FA [54–57], has resulted in varied improvements to immunotherapy protocols including changes in overall methylation. If DNAm of TH1/TH2 loci could be more clearly associated with disease phenotype, DNAm could be used as a quantitative assessment of varying types of immunotherapy. Beyond the clinical relevance on the success of immunotherapy, it remains elusive which variant of food-specific immunotherapy is the best in terms of clinical outcome.

Martino et al. sought to find correlations between DNAm in a defined profile of specific allergy-related promoters and food challenge outcomes [58••]. The 96 CpG sites they isolated, which had a minimum of 5% difference in methylation between FA/food sensitization (FS) groups, outperformed sIgE and SPT in predicting oral food challenge (OFC) outcome. FA status was predicted in the replication cohort with accuracy of 79.2%. This study provides further evidence that continued research into methylation biomarkers could yield a diagnostic assay of a higher predictive power than currently available. Clinical reactive phenotype was associated with general hypermethylation of these sites. A sum of these methylation markers as a predictive measure to differentiate FA vs FS subjects had 96.55% specificity and 89.66% sensitivity, an improvement to both IgE and SPT. The authors suggest using a combination of specific IgE (sIgE), SPT, and a methylation profile to create a decision tree to help decrease the number of OFC. Using this as a diagnostic tool could provide a clear clinical benefit to current practices.

Future Directions

Limitations to Current Epigenetic Research

Current studies of epigenetics in FA share some limitations. First, these studies involve a low number of participants. While the cost of genomic testing has decreased drastically over the last decade, it is still a large factor contributing to study sizes consisting of small numbers of patients. Therefore, more data is needed to confirm previous findings. Moreover, analysis of next-generation sequencing (NGS) data presents difficulties in differentiating between significant change and epigenetic noise given many confounding factors such as diet and exposure [18–24]. To best control for noise, a validation cohort, i.e., replicate study, is essential for validating any potential conclusions. Twin studies, such as the one by Liu et al., can also provide a better-controlled cohort by minimizing many of the confounding factors [36].

Additionally, the cell types analyzed have not been consistent between the various studies. While whole blood is the most likely candidate for use as a diagnostic tool due to ease of access and cost, epigenetic regulation may be cell-type specific [47]. Further research into methylation profiles of specific cell types is needed to provide a more complete picture on the changes elicited from epigenetic gene regulation.

When utilizing NGS data, one must acknowledge the specific challenges that potentially limit their clinical utility. A next-generation sequencer provides massively parallel high-

throughput data. However, massively parallel data could leave artifacts or bias through enriching or depleting certain regions during creation of the DNA library, or from the hardware itself [59]. Experimental design should also control for the batch effect, which can skew data between runs of an NGS machine [60]. Differences between sequencer platforms, and even between sequencer models, require consideration in devising controls for metadata analysis [61].

Other Epigenetic Areas to Explore

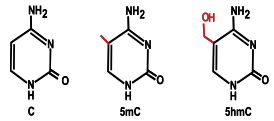
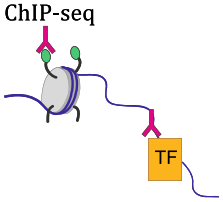
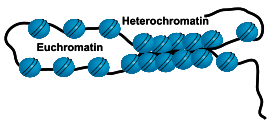
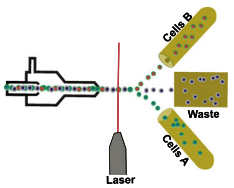
Beyond DNA methylation, explained earlier in the review and the most well studied epigenetic change, microRNA, histone modification, and chromatin accessibility are other types of epigenetic regulation. However, there has not yet been a peer reviewed study examining these other types of epigenetic regulation in food-specific immunotherapy. A large problem currently faced by researchers when exploring this avenue of epigenetics is that methylation of DNA, histone modification, and chromatin accessibility are interrelated. An in-depth explanation on each epigenetic mechanism is beyond the scope of this review. Different reviews on each of these topics have been provided in each paragraph below to aid in the understanding behind the mechanism of each epigenetic mark, and the protocol created to explore it. As approaches to generate epigenetic data are complex and involved, a summary of each approach with their area of interest and limitations is shown in Table 2.

DNA methylation has been shown to recruit enzymes for histone modification, and vice versa [62–64]. Additionally, chromatin accessibility is related to histone modifications, as chromatin is made up of a histone octamer, which helps organize the DNA into the structural nucleosome [65] While the exact mechanism and relation between these pieces of epigenetics is not fully understood, there is evidence of independent associations between histone modification or chromatin accessibility and gene expression [66, 67]. This evidence brings validity to exploring each mechanism in combination with food-specific immunotherapy.

Core histones have long N-terminal tails protruding from the nucleosome, which can undergo posttranslational modifications that alter their interaction with DNA and nuclear proteins. Research has shown a strong relation between covalent histone modifications and gene expression [65]. Histone modification has also been shown to predict RNA expression that is not reflected in chromatin accessibility or DNA methylation [67]. As a general rule, histone acetylation and phosphorylation are associated with an active state. Histone methylation, on the other hand, has diverse function in the control of gene activity, depending on the amino acid and the number of methyl groups added [68]. Highly expressed genes are associated with greater permissive histone modifications, and less frequently transcribed genes are associated with repressive changes and more tightly packaged chromatin, although the relationship between gene expression status and histone modification is not absolute [68]. In addition to influencing chromatin structure, recruitment of chromatin remodeling complexes by covalently modified amino acid on histone tails may also help target gene loci for preinitiation of transcription genes [69, 70]. The addition or removal of the various chemical elements on histones is catalyzed by histone-modifying complexes such as histone acetyl transferase (HAT) and histone deacetylase (HDAC), which add and remove acetyl groups on histone residues, respectively. More information on histone modification can be found in a review by Tessarz et al. [71]. ChIP sequencing is the protocol of choice for looking at histone modifications [72]. ChIP-seq utilizes antibodies to target specific loci of proteins or transcription factors. With this technique, Wei et al. found evidence that specific histone modifications, H3K4me4 and H3k27me3, were correlated with the determination of T cells, which could lead to a TH1/TH2 imbalance [73•]. This provides evidence that histone modifications can independently skew the immune response in an atopic individual toward TH2.

Chromatin, the complex of DNA and nucleic proteins in the nucleus, is another central target of epigenetic modifications. Transcriptionally inactive heterochromatin is packed densely, and is “closed,” whereas active euchromatin is less condensed and “open.” Further details on the mechanisms involved in chromatin structure and the inheritance of epigenetic information are provided in a review by Margueron et al. [74]. The core component of chromatin is the histone octamer which organizes DNA in structural units called nucleosomes [65]. The histone octamer consists of two dimers of core histones H2A and H2B and two dimers of core histones H3 and H4. Chromatin accessibility and change is a dynamic process, regulated by histones and ATP-dependent chromatin remodeling complexes that move, eject, or restructure nucleosomes. The “open” or “closed” state of the chromatin near a particular gene can be revealed through ATAC-sequencing [75]. ATAC-seq takes advantage of a specialized transposase, Tn5, which binds to the end of a transposon and allows a sequence to be “cut and pasted” somewhere else in the genome [76]. Further details of mechanisms and areas of interest in NGS sequencing of chromatin accessibility can be found in a review by Tsompana and Bucks [77]. ATAC-seq targets open chromatin by cutting and ligating with adapters allowing the open chromatin to then be expanded and sequenced. An exploratory study by Qu et al. analyzing multiple samples from 12 individuals indicated that ATAC-seq could provide a reference for comparing disease-associated regulomes in T cells isolated from standard blood draws [66]. There are currently no peer-reviewed articles with a focus on ATAC-seq and atopic disease.

Table 2 Overview of approaches to study epigenetic regulation

Test	Summary and Benefits	Barriers and Limitations
CpG Methylation Pyrosequencing 	*Bisulphite treatment replaces non-methylated cytosine to uracil, leaving 5-methylcytosine.* -Can show methylation of large parts of genome, or specific region of interest -Most studied epigenetic change	-Difficulty differentiating between other DNA modifications like 5-hydroxymethylcytosine -False positives exist from incomplete conversion of cytosines -Treatment destroys DNA, reducing complexity
miRNA-seq 	*A variant of RNA-seq, miRNA-seq uses gel electrophoresis to control for small RNA fragments before sequencing, which can identify miRNA* -May show tissue specific expression patterns -Potential diagnostic for disease phenotype in allergic disease [81••] -High coverage of miRNA sequences	-Difficult to find the miRNA target mRNA -biased towards largely expressed miRNA -Long, involved protocol
ChIP-seq 	*Analyzes protein interactions (ex: transcription factors) with DNA by utilizing antibodies of the protein of interest. Able to look at histone modification in relation to gene regulation* -Allows for high precision -Determines binding sites and chromatin modifications	-Sequencing depth directly correlated to cost -Resolution dependent on fragment size -Antibody (and protein of interest) has to be known before testing
ATAC-seq 	*Utilizing the specialized Tn5 transposase, exposed DNA, which is considered chromatin-open, is cut and ligated with adapters* -Low cell count needed -Short protocol -Large coverage -Direct relation between specific read and transposon event	-Tn5 transposase has potential bias -New test largely unexplored -Need purified cell subsets for best results
Single Cell 	*Single cell analysis provides additional benefits to NGS.* -Can show differences between cell types and other subpopulations to allow for a better mechanistic understanding of the relationship between epigenetics and allergy.	-Hardware intensive -Very expensive -Precursor to other testing greatly extends protocol

MicroRNA (miRNA) are small RNA molecules that mediate posttranscriptional gene silencing and are highly conserved throughout evolution [78, 79]. They have the ability to mediate posttranscriptional gene silencing of target genes [80]. In an informative review, Lu et al. detailed the relation between regulatory mechanisms of allergic inflammation and specific miRNA [81••] miR-21 and miR-146 have been related to the skewing of the adaptive immune system to a TH2 response and to T cell activation [82–84]. MiR-223 levels are upregulated in patients with eosinophilic esophagitis (EoE). MiR-223 has been associated with development of eosinophils, a cell type associated with disease severity [85, 86••]. MiR-375 has been tied to modulation of IL-13-driven epithelial response and has been shown to be largely downregulated in EoE patients [86••]. A report by Shaoqing et al. compared the miRNA expression profile of the nasal mucosa from patients with allergic rhinitis and nonallergic control subjects who underwent surgery for nasal obstruction [87]. They found nine miRNAs with more than a twofold change between the allergic rhinitis group and control group, providing evidence to justify further exploration of miRNA in FA.

Single-cell microfluidics allow for individual cell analysis as well as sorting of specific cell types, which could be useful for many sequencing protocols [88]. A large limitation with epigenetic studies is the potential for significant epigenetic changes in cell subsets present in small numbers to not be

reflected in epigenetic changes observed in overall whole blood or PBMCs. With the advancement of microfluidics, we have an ability to explore the mechanism of epigenetic change in such specific underrepresented cell types.

Conclusion

Epigenetics play an important role in understanding mechanisms involved in allergy and immunotherapy and could be used to identify new safe and effective therapies or could be used as a diagnostic or biomarker. The canonical allergic pathway is exquisitely regulated by epigenetic mechanisms. However, DNA methylation, histone modification, and chromatin accessibility are related to each other, and their specific relations are not yet fully understood. Currently, these types of epigenetic marks are presented in the literature independently, but in practice, they occur together and have a close relation with each other. The progression of single cell sorting technology will allow researchers to tease apart the individual mechanisms involved. Despite this potential limitation, independent associations can still be made between disease phenotype and epigenetic status.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no conflicts of interest relevant to this manuscript.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of great importance

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