Short Conceptual Overview

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Epigenetic considerations of the *APOE* gene

Abstract: The apolipoprotein E (*APOE*) gene is robustly linked with numerous physiological conditions, including healthy aging, altered cardiovascular fitness, and cognitive function. These connections have been established primarily by phenotype-genotype association studies using *APOE*'s three common genetic variants ($\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$). These variants encode for the three apoE protein isoforms (E2, E3, and E4), which have slightly different structures and, consequently, distinct functions in lipid metabolism. However, the differential lipid binding and transferring properties of these isoforms cannot fully explain the association of *APOE* with such a wide range of physiological phenotypes. One potential explanation for *APOE*'s pleiotropic roles may lie in its unique epigenetic properties. In this article, we present a brief review of the *APOE* gene and protein, its disease associations, and epigenetic components, with a focus on DNA methylation. We close with a discussion of the prospective epigenetic implications of *APOE* in disease.

Keywords: *APOE*; DNA methylation; disease; epigenetics.

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Introduction

The human apolipoprotein E gene (*APOE*) gene is located on chromosome 19 (19q13.32) – it consists of four exons that transcribe and translate into the 317 amino acid apoE protein (Figure 1A). The apoE protein is a component of several lipoproteins and plays a key role in lipid metabolism, including the redistribution of lipoproteins and cholesterol. The liver produces the majority of circulating apoE, which binds lipids and interacts with cell-surface membrane receptors to initiate cellular uptake of lipoprotein particles by the liver and other tissues (1). ApoE is also abundantly present in the central nervous system (2), where it promotes the transport of lipids to and from damaged neurons and thereby conducts important functions in neuronal maintenance, repair, and homeostasis (3, 4).

Human apoE is a polymorphic protein, and the presence of either an arginine or a cysteine at amino acid positions 112 and 158 defines three common isoforms: E2, E3, and E4, which are encoded by the $\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$ genetic variants, respectively (5) (Figure 2A). These isoforms are metabolically distinct and differ in both their affinity for lipoprotein particles and their binding to low-density lipoprotein (LDL) receptors (6). The isoforms influence total serum and LDL cholesterol levels (7), and thus, *APOE* has been linked with a higher risk of cardiovascular diseases (CVD) (8–12). Additionally, *APOE*'s $\varepsilon 2/\varepsilon 3/\varepsilon 4$ allelic variants are determined by two single nucleotide polymorphisms (SNPs, rs429358 and rs7412) in the 3′-exon coding region of the gene (13) (Figures 1A and 2A) and have been associated with risk of Alzheimer's disease (AD). The $\varepsilon 3$ allele is the most common of the three alleles with a frequency range of 0.7–0.9 across ethnic populations. Within Caucasians, the $\varepsilon 4$ allele frequency in healthy controls is 0.14–0.16, however, this frequency is significantly elevated in AD patients (ranging from 0.36 to 0.42) (14–16). The inheritance of the $\varepsilon 4$ allele increases a person's risk of developing AD in a gene dose-dependent manner and predisposes them to an earlier age of onset (14, 17). Conversely, the *APOE* $\varepsilon 2$ allele appears to have a modest protective effect for AD (AlzGene, http://www.alzgene.org/). Besides AD and CVD, genetic studies have also connected *APOE* and its $\varepsilon 2/\varepsilon 3/\varepsilon 4$ alleles to multiple physiological conditions and disorders, including aging (18, 19), diabetes (20), dysbetalipoproteinemia (21), frontotemporal dementia (22), fragile X-associated ataxia (23), glomerulopathy (24), Lewy body dementia (25), metabolic syndrome (26), retinal-related disorders (27), Parkinson

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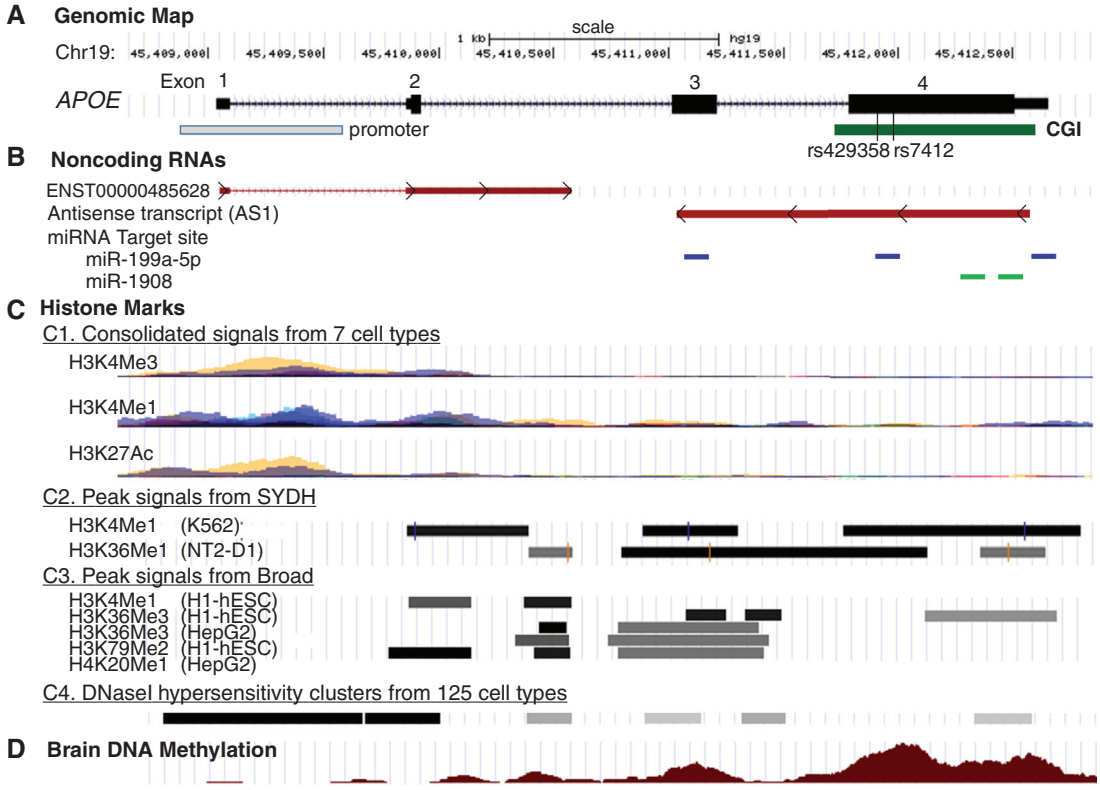

Figure 1: An overview of *APOE*'s epigenetic signatures.
(A) Genomic map of *APOE* exons, promoter, CGI, and two CpG SNPs. (B) Genomic position of noncoding RNAs. (C) Genomic position of histone marks and DNase I hypersensitivity sites from ENCODE project (47). (D) DNA methylation profiles of UCSF brain project (67).

A

Variant	rs429358	rs7412	CpG
ε2	T (Cys)	T (Cys)	–1
ε3	T (Cys)	C (Arg)	
ε4	C (Arg)	C (Arg)	+1

B rs429358

ε2/ε3	GACGTG**T**GCGGCCGCC
ε4	GACGTG**C**GCGGCCGCC

C rs7412

ε2	CGATGACCTGAGAAG**T**GCCTGGCAGTGTACCAGGCCG
ε3/ε4	CGATGACCTGAGAAG**C**GCCTGGCAGTGTACCAGGCCG

Figure 2: Sequence context of the two CpG SNPs that determine *APOE*'s ε2/ε3/ε4 alleles.
(A) Haplotype composition of the two CpG SNPs: rs429358 and rs7412. Their encoded amino acids are shown in the parentheses. (B) The C nucleotide of rs429358 introduces an extra CpG into a 12-bp CpG-enriched region. (C) The T nucleotide of rs7412 eliminates a CpG and open up a 33-bp CpG-depleted region of the CGI. CpG dinucleotides are underlined. SNPs are in bold type.

disease (28), posttraumatic stress disorder (29), primary progressive aphasia (30), schizophrenia (31), stroke (32), traumatic brain injury (33), and vascular dementia (34). Whether *APOE* plays a direct or indirect role in the pathophysiology of these divergent conditions is unclear.

Naturally, due to the fact that *APOE*'s disease associations are dependent upon the ε2/ε3/ε4 alleles, one would speculate that these associations are most likely attributed to the apoE protein isoforms. However, the major biological effect of apoE – transportation of lipid particles – cannot readily explain a large portion of these disparate phenotypes. Take AD for example, mainstream research has focused on apoE isoform-specific differences in protein structure and function for the past two decades. Numerous hypotheses have been proposed for how apoE4 might increase the risk of AD, including roles in Aβ aggregation and clearance (35, 36), apoE domain-induced neurotoxicity (37), apoE expression (38) and apoE lipidation states (39), neuroprotection (3, 40), neuroinflammation (41), and tau hyper-phosphorylation (42). Yet, the precise molecular mechanism(s) by which the ε4 allele exerts its detrimental effect in AD remains elusive, and no unified consensus has been reached within the field. Nevertheless, the ε4 allele has been identified as the strongest genetic risk factor for AD in multiple studies and across many ethnic groups. Thus, there is strong reason to

deduce that the $\varepsilon 4$ allele may contribute additional biological consequences to AD (and other disorders as well), beyond simply coding for the apoE4 isoform. Because a genetic association signal with disease also reflects the target site's sequence architecture such as epigenetic code, *APOE* and its disease association may very well be partially attributed to epigenetic alterations.

Epigenetic signature of *APOE*

Epigenetics is defined as the regulation of genomic functions that do not impinge on the nucleotide sequence. Epigenetic signals and marks enable temporal integration of regulatory events through dynamic mechanisms in response to environmental stimuli. These mechanisms include DNA methylation, noncoding RNAs, and histone modification/chromatin conformation. Because DNA methylation is the best-studied epigenetic signature of *APOE*, we will discuss this discipline last and in the most detail.

Noncoding RNA

Noncoding RNAs control gene expression by several processes, ranging from natural antisense RNA- and microRNA-induced degradation of mRNAs to long noncoding RNA-mediated modification of chromatin. These RNA transcripts can be targets of or contributors to epigenetic regulation that modulates cellular and tissue development. Dysregulation of noncoding RNA can lead to a wide range of diseases (43).

There are several lines of evidence supporting the involvement of noncoding RNAs with *APOE*. First, based on the Ensembl data (http://uswest.ensembl.org), *APOE* has five alternatively spliced transcripts. Among them, one transcript (ENST00000485628) has no sizeable open reading frame and contains only exon 1 and exon 2 followed by a partial 659 bp intron 2 (Figure 1B). Second, a natural antisense RNA transcript, encoded by exon 4 of *APOE*, has been reported in the study of Seitz et al. (44) (Figure 1B). This antisense transcript was identified from human liver and was speculated to be involved in the regulation of *APOE*. Third, a miRNA network including miR199A and miR1908, with binding sites spread across *APOE*'s exon 3 to the 3′UTR (Figure 1B), has been identified through lab-based reporter assays (45). This miRNA network has a combined effect of silencing apoE signaling, and can lead to enhanced endothelial cell recruitment as well as metastatic invasion. Additionally, presence of noncoding RNAs at the 3′ region of *APOE* (the intragene region between *APOE* and *APOC1*) is evident in UCSC genome browser's UniGene and SIB Alt-Splicing tracks (http://genome.ucsc.edu). However, due to the heavy repetitive sequence nature of the same region, such transcripts could have been misaligned. Most of these *APOE*-related noncoding RNAs have neither been extensively studied nor independently validated, leaving their biological consequences and tissue specificity largely unknown.

Chromatin remodeling and histone marks

Another common epigenetic mechanism is chromatin remodeling, featured by covalent histone modifications. This process facilitates gene transcription by loosening the histone-DNA complex and consequently allowing other proteins such as transcription factors access to their binding sites on DNA. Alternatively, the modified histone can assume a closed confirmation, blocking protein access to the DNA, resulting in down-regulation of gene expression. Such remodeling is typically initiated by either post-translational modification of the amino acids that compose the histone proteins or through methylation of neighboring DNA (46).

Experimental data on this aspect of *APOE* is scarce. To the best of our knowledge, no published literature has addressed this discipline, with the exception of the ENCODE project (47). Using chromatin immunoprecipitation and next-generation DNA sequencing (Chip-seq), three common histone marks, H3K4Me3 (near promoters), H3K4Me1 (near regulatory elements), and H3K27Ac (near active regulatory elements) have been identified in *APOE* across seven cell types. These data have been displayed in ENCODE's integrated regulation track under the UCSC genome browser. The genomic positions of these three histone marks are mainly concentrated in a region spanning from the 5′ end of *APOE* through the second intron (Figure 1, C1). This region coincides with the *APOE* promoter, indicating the presence of chromatin remodeling hotspots with a main function in the transcriptional regulation of *APOE*. Additionally, based on the ENCODE's histone modification track, there is evidence of histone marks outside of the promoter. These marks reside in a region extending from intron 2 to the 3′-end of *APOE*. Chip-seq peaks of these histone marks are shown in Figure 1, C2 (Stanford/Yale/Davis/Harvard data), and C3 (Broad Institute data). The overall results generated from different institutes are not

robustly consistent, probably due to usage of different antibodies, cell types, and analysis algorithms. Despite inconsistencies, these data provide valuable candidate sites for future studies into chromatin remodeling at the *APOE* locus.

DNA methylation and a cytosine-phosphate-guanine island

Methylation is a normally occurring modification to DNA in eukaryotic organisms. In mammalian cells, it is characterized by the biochemical addition of a methyl group (CH_3) to the cytosine's 5th carbon in cytosine-phosphate-guanine (CpG) dinucleotide via a methyltransferase enzyme (48). This process is an evolutionarily conserved feature that provides an additional layer of information for biological processes, including embryogenesis, development, genomic imprinting, silencing of transposable elements, and regulation of gene transcription (49–54). Although CpG dinucleotides occur rather infrequently in mammalian genomes (approximately one-fourth of the expected frequency), some segments of the genome are highly enriched with CpG dinucleotides. These segments, defined as CpG islands (CGIs), are typically 500–2000 bp long and commonly correspond to transcriptional start sites (55–57). The CGIs represent one of the most critical regulatory elements in the human genome with major functional roles in gene expression and regulation (57–59). Based on the UCSC genome browser, which applies a quantitative annotation algorithm (60) to define candidate CGI regions, there are 30477 CGIs present in the most updated version (GRCh38/hg38) of the human genome. Approximately 67% of these CGIs overlap with UCSC genes, and they can be separated into three subgroups according to their relative position to genes, i.e., the 5′- (first exon/promoter overlapping), the intragenic, and the 3′- (last exon overlapping) CGIs. Most gene body CGIs belong to the 5′-CGI subgroup, which are present in virtually all housekeeping genes (61, 62).

The genomic sequence of *APOE*, including its promoter (63), is approximately 4 kb in size (chr19:45408714-45412650, hg19). This region contains 172 CpG dinucleotides. Based on combined data from the published literature (64–66), ENCODE's DNA methylation track, and UCSF's brain DNA methylation data (67) from the UCSC genome browser (Figure 1D), the methylation profiles of *APOE* can be roughly divided into three general groups. The promoter region is hypo-methylated (<10% methylation), the intronic and exonic (except exon 4) regions are intermediately methylated, and the exon 4 is hyper-methylated (>50% methylation).

Interestingly, *APOE* has a single well-defined CGI that does not reside in the promoter region; instead, it overlaps with the 3′-exon (or exon 4) of *APOE* (see Figure 1A). Such a 3′-CGI is very rare in the human genome, representing <1% of total CGIs (62, 67). This *APOE* CGI is also conserved in other mammals including chimps, mice, rats, cows, and dogs (UCSC Genome Browser), which suggests that it holds a critical functional role. The human *APOE* CGI consists of 90 CpG sites that are hyper-methylated in almost all tissues (ENCODE's DNA methylation tracks in UCSC Genome Browser) except for testis (64). In our own study, we have determined the DNA methylation profiles from 75 CpG sites of the *APOE* CGI in whole blood lymphocytes and postmortem brain tissues using bisulfite pyrosequencing (66). All of the CpG sites and samples analyzed were highly methylated (>75% average methylation). The methylation profiles showed consistent up/down patterns between samples and tissues, suggesting inherent epigenetic regulation at the level of the individual CpG site. While we found the patterns of the methylation to be similar across postmortem brain tissues, we detected significant overall differences in the mean methylation levels between brain regions. Methylation levels were lower in brain regions (frontal lobe, temporal lobe, and hippocampus) that are affected the most by AD. Conversely, the highest methylation levels were observed in the cerebellum, a region lacking profound pathological changes in the same disease. These results suggest that a correlation may exist between the methylation levels of the *APOE* CGI and the vulnerability of brain regions to disease.

Genetic variations (e.g., a CpG-altering SNP) can alter DNA methylation states, and these variants represent an important class of regulatory elements that connect genetic changes with epigenetic variability. Remarkably, the $\varepsilon 2/\varepsilon 3/\varepsilon 4$ alleles of *APOE* are determined by two CpG-altering SNPs (rs429358 and rs7412) that reside within the core region of the *APOE* CGI (Figure 1A). When compared to $\varepsilon 2$ and $\varepsilon 3$, the $\varepsilon 4$ allele introduces one more CpG and further saturates a small 12 bp region with 4 CpG sites (Figure 2B); in contrast, the $\varepsilon 2$ allele removes 1 CpG and opens up a 33-bp CpG-free region (Figure 2C). Therefore, these two SNPs not only change the regional CpG load but are likely to affect the overall DNA methylation landscape of the CGI. Such changes in CpG load are expected to alter the binding profiles of methyl CpG-binding domain proteins which bind specifically to methylated DNA through their unique amino acid motif (68). Alternatively,

the methylation status of exons can fine-tune exonic protein binding, which in turn affects other biological processes such as pre-mRNA processing (69). Additionally, there is an evidence of histone marks and a DNase I hypersensitivity cluster (an indirect indicator of protein binding) within the *APOE* CGI (Figure 1C), suggesting that the *APOE* CGI (and exon 4) is a site for protein binding and chromatin remodeling. Lastly, *APOE* CGI methylation differences between individuals with and without AD increase with age. This could be a consequence of environmental stimuli influencing DNA methylation gradually with aging (70). Taken together, inheritance of different $\varepsilon 2/\varepsilon 3/\varepsilon 4$ alleles in the *APOE* CGI might present different methylation landscapes, which could be further modified by environmental stimuli and the aging process. Such changes can potentially alter protein binding, leading to diverse biological consequences, and possibly even influence the pathophysiological processes of multiple diseases.

Environmental stimuli that influence *APOE*

A number of environmental and lifestyle factors have been found to interact with the effects of *APOE* genotype on disease. For example, exercise (71), education (72), and vitamin D status (73) have all been shown to impact the effects of *APOE* genotype on cognitive functions. Also, lifestyle factors such as alcohol consumption and physical activity were found to significantly interact with *APOE* genotype in determining plasma lipid concentrations in a gender-specific manner (74). Given that epigenetic mechanisms seem to bridge the gap between genetics and environment as they pertain to disease, it is plausible that epigenetic mechanisms underlie the above observations. However no studies, to our knowledge, have fully explored this avenue.

Expert opinion

APOE has an unusual epigenetic makeup, represented by a CGI located at 3′-end of the gene. The critical genetic variants ($\varepsilon 2/\varepsilon 3/\varepsilon 4$) of *APOE*, besides simply coding for the apoE isoforms, also contribute to the epigenetic alterations of the *APOE* CGI. Thus, interplay between genetic and epigenetic variations is conceivably one of the molecular mechanisms behind *APOE*'s association with multiple physiology conditions and diseases. DNA methylation of the *APOE* CGI and its $\varepsilon 2/\varepsilon 3/\varepsilon 4$ variants adds another layer of embedded biological instruction, possibly involving protein binding, chromatin remodeling, and specific RNA regulation in an age-dependent course. However, unlike genetic variation, epigenetic changes are cell-type specific, reversible, and susceptible to both inherited and environmental influences. Thus, some key questions need to be addressed to definitively establish *APOE*'s role in epigenetics. For example, why is there a CGI located at the 3′-end of *APOE*, and why is this CGI hyper-methylated in almost all cell types? Can such DNA methylation be modulated by exposure to environmental cues, and/or be reversible in a cell-type-specific spatial and temporal manner? What are the biological effects and consequence of this CGI in *APOE*-associated diseases? In order to establish the role of epigenetics in *APOE*'s function, several approaches could be implemented. One direction is to identify disease-specific changes in *APOE*'s epigenetic marks. For example, if altered DNA methylation profiles of the *APOE* CGI from disease-relevant tissues (e.g., hippocampus of postmortem brain) could be identified in that disease (e.g., AD), when compared to age- and gender-matched controls, it would signify an epigenetic role of *APOE* in that disease. Once these disease-specific epigenetic marks/regions are identified, functional experiments (e.g., assays for protein binding or gene expression) could then be applied to study the biological consequences resulting from the altered epigenetic marks. Such results may provide a clue as to whether or not epigenetic changes in *APOE* are potential upstream effectors or merely down-stream consequences in disease pathophysiology. Another conceivable direction would be to determine *APOE*'s susceptibility to epigenetic changes. For example, one could use cellular models to identify key factors capable of changing the epigenetic marks of *APOE*. Such data would highlight physiological or environmental cues with the potential to modulate *APOE*'s epigenetic mechanisms, which could contribute to altered disease phenotypes. Further studies to decipher epigenetic regulation and modification of the *APOE*'s epigenetic marks are important; they may shed light on potential epigenetic preventions and interventions for AD, CVD, and other *APOE*-associated disorders.

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