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MINI-SYMPOSIUM

Epigenome-wide association studies in Alzheimer's disease; achievements and challenges

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Abstract

Alzheimer's disease (AD) represents a devastating progressive neurodegenerative disease with a complex pathophysiology, affecting millions of people worldwide. Recent epigenome-wide association studies suggest a key role for epigenetic mechanisms in its development and course. Despite the fact that current evidence on the role of epigenetic dysregulation in aging and AD is convincing, the pioneering field of neuroepigenetics is still facing many challenges that need to be addressed to fundamentally increase our understanding about the underlying mechanisms of this neurodegenerative disorder. This perspective paper describes the current state of play for epigenetic research into AD and discusses how new methodological advances in the field of epigenetics and related data science disciplines could further spur the development of novel therapeutic agents and biomarker assays.

EPIGENOME-SCALE STUDIES IN ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a chronic neurodegenerative disorder characterized by the deposition of amyloid-beta (Aβ) plaques, neurofibrillary tangles (NFTs) of hyperphosphorylated tau protein, which ultimately lead to neuronal cell death. Genetic research exploring the etiology of AD has helped elucidate some of the pathogenic mechanisms underlying the disorder. Mutations in the *APP*, *PSEN1* and *PSEN2* genes are key players in developing early onset familial AD, whereas *APOE* was discovered as the most important genetic risk factor for late onset sporadic AD (1). Genome-wide association studies (GWAS) have helped identify several AD loci that are common in the general population, but show small risk effects (the most recent genetic studies of AD are summarized elsewhere in this mini-symposium series (2)). However, as only one third of AD cases can be explained by common variants or missing heritability, it is highly likely that nongenetic factors play a substantial role in the development and course of the disorder. As such, the multitude of nongenetic risk factors for developing sporadic AD suggests the involvement of epigenetic mechanisms (3).

Epigenetic mechanisms refer to processes that regulate gene expression without altering the DNA sequence. Changes in the epigenome are acquired throughout life and are subject to alterations based on the environment that the cell or organism is exposed to. There are various types of epigenetic modifications, which include DNA modifications, histone modifications, as well as noncoding RNAs (ncRNAs) (4). This perspective particularly focuses on the DNA modification of 5-methylcytosine (5mC) as it has been the best characterized in the context of AD, discussing recent scientific achievements and challenges in the context of AD.

Over the last decade, major technological advances have allowed the first epigenome-wide association studies (EWAS) of DNA methylation (herein termed methylome-wide association studies [MWAS]) in AD brain tissue (for a timely review, see (5); in addition, see (6)). The first MWAS on AD made use of Illumina Infinium Human Methylation 27K arrays to study more than 27 000 CpG sites in 14 475 genes within the frontal cortex (7) (see Table 1). The most significant AD-associated differentially methylated cytosine-phosphate-guanine (CpG) site was located within the *TMEM59* gene, and displayed DNA hypomethylation when comparing 12 AD patients with 12 age- and gender-matched controls. A similar 27K MWAS on 15 AD patients

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Table 1. Summary of findings from AD MWAS.

Author	Year	Sample size	Method	Tissue type	DNA treatment and modification identified	Top differentially methylated loci
Bakulski *et al* (6)	2012	24	Illumina Methylation 27K array	Frontal cortex	Bisulfite treatment (total DNA modifications [5mc+5hmc])	*TMEM59*
Sanchez-Mut *et al* (7)	2014	20	Illumina Methylation 27K array	Hippocampus	Bisulfite treatment (total DNA modifications [5mc+5hmc])	*DUSP22*
Lunnon *et al* (5)	2014	122	Illumina Methylation 450K array	Superior temporal gyrus Prefrontal cortex Cerebellum Blood	Bisulfite treatment (total DNA modifications [5mc+5hmc])	*ANK1*
De Jaeger *et al* (8)	2014	708	Illumina Methylation 450K array	Dorsolateral prefrontal cortex	Bisulfite treatment (total DNA modifications [5mc+5hmc])	*ABCA7 BIN1* *ANK1, CDH23* *DIP2A* *RHBDF2* *RPL13* *SERPINF1* *SERPINF2*
Watson *et al* (10)	2016	68	Illumina Methylation 450K array	Superior Temporal Gyrus	Bisulfite treatment (total DNA modifications [5mc+5hmc])	*MOV10L1* *B3GALT4* *DUSP6* *TBX15* *OXT*
Gasparoni *et al* (13)	2018	128	Illumina Methylation 450K array	Bulk Brain Tissue (Frontal + Temporal Cortex)	Bisulfite treatment (total DNA modifications [5mc+5hmc])	*CLU* *SYNJ2* *NCOR2* *RAI1* *CXXC5* *INPP5A* *MCF2L* *ANK1* *MAP2* *LRRC8B* *STK32C* *S100B*
Smith *et al* (33).	2018	147	Illumina Methylation 450K array	Prefrontal cortex Superior temporal gyrus	Bisulfite treatment (total DNA modifications [5mc+5hmc])	*HOXA3*
Semick *et al* (11)	2019	73	Illumina Methylation 450K array	Hippocampus Entorhinal cortex Dorsolateral prefrontal cortex Cerebellum	Bisulfite treatment (total DNA modifications [5mc+5hmc])	*ANK1* *ANKRD30B* *WDR81* *SERPINF2* *MYO1C*
Smith *et al* (12)	2019	96	Illumina Methylation 450K array	Entorhinal cortex	Oxidative-bisulfite treatment (5mc, 5hmc and UC independently	*WNT5B* *FBXL16* *ANK1* *ARID5B* *ALLC*
Lardenoije *et al* (23)	2019	80	Illumina Methylation 450K array	Middle temporal gyrus Blood	Oxidative-bisulfite treatment (5mc, 5hmc and UC independently)	*OXT* *CHRNB1* *RHBDF2* *C3*

Abbreviations: 5hmc = hydroxymethylation; 5mc = methylation; UC = unmodified cytosine.

and 5 non-demented controls identified Braak-associated DNA methylation alterations in four loci, including two residing in *DUSP22*, displaying DNA hypermethylation, concomitant with decreased *DUSP22* RNA expression, with increasing Braak stage (8). Subsequent approaches made use of the Illumina Infinium Human Methylation 450K array, which interrogates more than 485 000 CpG sites. Several larger sample-size 450K studies (N = 68–740 depending on study), primarily focusing on cortical brain tissue, provided converging evidence for numerous genes displaying differential methylation in AD, that is, *ANK1*, *C10orf105*, *CDH23*, *DIP2A*, *LOC100507547*, *PPT2*,

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PPT2-EGFL8, *PRDM16*, *PRRT1*, *RHBDF2*, *RNF39*, *RPL13*, *SERPINF1* and *SERPINF2* (9–12).

Interestingly, of the genes identified, *ANK1, CHD23, DIP2A, RHBDF2, RPL13, SERPINF1* and *SERPINF2* were also found to display significant AD-associated gene expression changes, supporting a potential functional role for DNA methylation in AD (10). Differential methylation of *ANK1* has been established in five studies (6,9,12–14). Furthermore, two genes of the dual specificity phosphatase (DUSP) family of proteins were shown to be differentially methylated in two independent studies as well (8,11). An overview of key findings and study designs for all MWAS published to date can be found in Table 1. Notably, a recent cross-cortical meta-analysis performed on nine previously published MWAS data sets consisting of 1408 donors, identified 220 CpGs significantly associated with AD neuropathology. These probes were annotated to 121 unique genes, of which 96 had not been previously reported in AD MWAS (15).

Although these MWAS findings have been highly valuable for improving our understanding of the molecular etiology of AD, there are a number of limitations to these approaches. Such challenges would need to be addressed properly in future endeavors to allow for appropriate reliable interpretation of generated data in order to significantly increase our understanding about the disease processes underlying AD. In this mini-symposium article, we showcase DNA methylation EWAS in AD, addressing several achievements and challenges in this respect in more detail. While this particular perspective article focuses on DNA methylation studies, there is also a substantial body of literature on other levels of epigenetic regulation, including ncRNAs such as microRNAs (miRNAs). For a timely review, in the context of AD, see (16). Notably, many of the considerations referred to below do apply to other epigenetic processes as well.

GENOMIC COVERAGE

Current state-of-the-art EWAS platforms used for studying DNA methylation alterations in AD have mostly been microarray based. This approach has been largely successful owing to their sensitivity and relative ease of execution in terms of analysis and standardization of protocols, allowing for a reliable comparison between studies. Ultimately, the common platform used across the MWAS to date would also lend itself well to meta-analyses, the first of which has just been undertaken in the case of AD (15). However, despite a significant increase in CpG coverage on the Illumina microarrays over recent years, current EWAS platforms still interrogate only a small proportion of CpG sites and predominantly assess CpG-rich promoter regulatory regions, hence, missing a large proportion of the human DNA methylome. As an example, even though the latest Illumina Infinium Methylation EPIC BeadChip array, which allows for the interrogation of over 850 000 CpG sites across the genome, shows improved coverage of regulatory elements, such as enhancers, when compared to its predecessors, even now, only a limited proportion of distal and proximal regulatory elements are represented (17). For a carefully annotated description of CpG coverage on Illumina arrays, see (18) and (19). These EWAS microarray platforms further lack the possibility to thoroughly assess DNA methylation at non-CpG (cytosine and adenine) sites. The use of (more expensive) next-generation sequencing-based approaches allowing for methylation assessment across the entire genome shows great promise in this respect, although these come with other major challenges as well, including those related to power (requiring larger sample sizes in view of multiple testing; see below), sequencing depth and associated costs.

STATISTICAL POWER

Existing MWAS findings should be interpreted with caution, as most published studies seem (severely) underpowered. Power calculations for EPIC-based DNA methylation studies demonstrate that existing studies with data on ~1000 samples are adequately powered to detect small differences at the majority of sites (20). Furthermore, for future endeavors that may use next generation sequencing approaches to increase coverage, increased samples sizes will be required. Evidently, when taking into account the intrinsic methylation susceptibility of specific CpG sites (ie, likelihood of an individual CpG to be methylated, and the nucleotide distance between neighboring CpG sites (21)), or when assessing differentially methylated regions (DMRs) consisting of adjacent differentially methylated positions (DMPs), smaller sample sizes are sufficient to reach genome-wide significance. In this respect, Bonferroni correction for the number of probes on the array is often presumed to be too conservative as DNA methylation values at neighboring probes are generally known to be correlated. As such, in addition to false-discovery rate (FDR) correction, recent endeavors attempt to estimate the number of independent tests performed in an EWAS and adjust the significance level accordingly (20,22). Altogether, although it is important to consider sample size when interpreting EWAS results, it is worth noting that different CpG sites show different variance and they will, therefore, be differently powered at the same sample size.

SPECIFICITY OF THE EPIGENETIC MARKS ASSESSED

Accumulating evidence suggests an important role for other types of cytosine modifications such as 5-hydroxymethylcytosine (5hmC) in the human brain (23). However, the great majority of MWAS on AD published to date have made use of sodium bisulfite-treated DNA, which is unable to discriminate between 5mC and its first oxidation product, 5hmC. Recently, the first EWAS simultaneously assessing DNA methylation and hydroxymethylation in isolation in AD by making use of oxidative-bisulfite DNA treatment was published (13). This study highlighted different levels

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of 5mC, 5hmC or unmodified cytosine (UC) in a number of different genes, including *WNT5B*, *ANK1*, *ARID5B*, *FBXL16*, *ALLC* and *JAG2*. More specifically, in view of the earlier MWAS findings on *ANK1*, this study demonstrated that *ANK1* DNA modification alterations in AD are as a result of DNA hypermethylation and hypohydroxymethylation, suggestive of a loss of active DNA demethylation of *ANK1* in AD. Recently, a similar approach was used in AD and control individuals using cortical tissue from the middle temporal gyrus and peripheral whole blood samples and led to the identification of a common DMR associated with AD, close to the transcription start site of *OXT* (24). The study suggested *OXT* 5mC and 5hmC levels change in opposite directions within the middle temporal gyrus in AD. Interestingly, the detection of a differentially methylated region near *OXT* is in line with a recent report of a nearly identical AD-associated *OXT* DMR in the superior temporal gyrus, which is located directly above the middle temporal gyrus (11).

While earlier studies on DNA modification changes have primarily focused on 5mC and 5hmC, other marks such as 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which both represent oxidized derivatives of 5hmC, are heavily understudied. Little is known about the frequency or functionality of these modifications in the healthy or diseased human brain. Further studies are therefore required to develop a better understanding on their potential role in the development and course of AD. Alternative, more specific technologies such as DNA-immunoprecipitation (IP) sequencing approaches with DNA being captured by specific antibodies to distinct DNA modifications hold great promise for assessing these epigenetic marks. In addition, direct DNA sequencing using novel third generation sequencing platforms, such as the Oxford Nanopore Technologies (ONT) minion/promethion or the Pacific Biosciences (Pacbio) Sequel, allows calling of both single nucleotide polymorphisms (SNPs) and a range of different DNA modifications by sequencing native DNA without prior amplification via PCR or labeling the sample chemically, which represents another promising approach (25). Evidently, these more advanced sequencing approaches do still require distinct, more challenging data science investments for calling of different DNA modifications.

CELL-TYPE SPECIFICITY AND COMPOSITION

A major issue that challenges the field of neuroepigenetics is that of tissue and cell-type specificity of epigenetic modifications. AD is characterized by neuronal loss, and activation of glia cells, concomitant with alterations in the cell-type composition of brain samples, which challenges the correct interpretation of results when making use of heterogeneous bulk tissue samples. As such, cell-type specific modifications in one cell-type could, for example, mask changes in another (26). Aside from cell-type specific changes in activity or changes in cellular proportions that can occur during the development and course of AD, differences in cell-type compositions between samples derived from different individuals that arise as a result of tissue sampling is also a consideration. A workable, though often expensive and tedious, solution to this issue could be specifically profiling cell-type specific samples, to be acquired via, for example, laser capture microdissection (LCM), fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS). This could even exploit single-cell sequencing technologies to investigate cell-specific epigenomes (27). Alternatively, one can use advanced bioinformatic approaches to correct for cell-type composition in MWAS data generated in bulk (unsorted) tissue. As an example, one can estimate neuron-glia proportions based on DNA methylation values in EWAS data and this approach has been adopted by many in the AD epigenetics field (28-30). Akin to the issues associated with studying epigenetic changes in the brain, cell type specificity issues also apply to studies making use of whole blood samples and these can be addressed in similar ways, for example, by adjusting data for cell-type specific DNA methylation markers (20,21).

CAUSAL INFERENCE

At present, one of the major limitations of current endeavors investigating the role of epigenetic dysregulation in aging and AD, is the issue of causality. Alterations in DNA modifications could be either causal in the disease process or could themselves arise as a direct or indirect result of pathological or secondary behavioral and psychological changes associated with disease. Similarly, age, comorbidity or the use of therapeutic agents may have a profound impact on the epigenome, both in the brain and blood. In addition, epigenetic signatures identified in advanced AD cases, particularly in brain regions affected relatively late in the disease process, may provide limited information on causality. An interesting notion in this regard is the study of epigenomic changes with respect to the spatiotemporal spread of pathology in AD. For example, utilizing postmortem AD brain tissue derived from donors at different disease stages and comparing the epigenome of brain regions implicated in the early stages of AD (ie, brainstem) to those regions affected in more advanced phases (ie, cortical regions) in the same individuals, could allow the identification of the relative, spatiotemporal contribution of potentially causal, region-specific epigenetic alterations in the development and course of AD. Similarly, when assessing the blood epigenome, longitudinal studies capturing early stages of cognitive decline, and preferably also exposure to environmental factors throughout life (which may evidently also impact on the blood epigenome), are needed (31). Alternatively, rodent models of AD could also be used to explore causal epigenetic mechanism in AD, by studying alterations prior to the onset of pathology. However, one limitation in this regard is that these murine systems are models of familial AD, bearing autosomal dominant mutations and are not a true model of sporadic disease. Another approach to elucidate causal epigenetic mechanisms in AD would be through the use of epigenetic editing. The use of novel editing

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constructs based on the catalytically deactivated clustered regularly interspaced short palindromic repeats (CRISPR) associated protein 9 (ie, CRISPR-dCas9) fused to chromatin modifying enzymes such as DNA methyltransferase 3a (DNMT3A) or TET1, also known as EpiEffectors (32), allows adding, or removing, of methyl groups to specific DNA loci, respectively (33). The use of this system *in vitro* in, for example induced pluripotent stem cells could ultimately allow the field to determine the functional implications of epigenetic signatures identified in MWAS and to establish whether these represent a cause, a consequence, or both, of the disease process. Further information on this concept can be found elsewhere in this mini-symposium series [35]. As epigenetic changes are often reversible, the identification of causative epigenetic signatures may provide promising targets for future therapeutic interventions.

MULTI-OMICS APPROACHES

Importantly, the ability to identify specific and early signatures of AD is hindered by the substantial clinical heterogeneity among patients, for instance there is considerable variability in disease duration and cognitive decline rate between patients, which likely reflects inter-individual variation in genetics, exposure to environmental factors and underlying neuropathology. In recent years, several "big data" approaches have allowed the identification of robust genomic, epigenomic and transcriptomic changes in AD. However, these studies have largely been restricted to correlating a single layer of molecular information with respect to a single measure of disease severity, in a single tissue, and it is not yet possible to identify the exact mechanisms involved in the pathophysiology of AD or to predict a patient's disease trajectory with current peripheral biomarkers. The recent development of powerful computational frameworks now offers multilayer inter-regulatory approaches to understand the development and course of AD, while accounting for inter-individual differences in genotype and exposure to environmental risk factors. As such, multi-omics approaches—that include an integrative analysis of various layers of epigenetic regulation—and associated data science tools show great promise in the development of novel diagnostic tools and treatment strategies for AD, with further details on this approach provided elsewhere in this mini-symposium [36].

CONCLUDING REMARKS

There is increasing interest in the role of epigenetic dysregulation in aging and AD, with the primary focus of most EWAS studies being on DNA methylation. Platforms such as the Illumina Infinium Methylation 450K and EPIC BeadChip arrays have enabled cost-efficient, high-throughput profiling of methylomic variation across large numbers of samples. EWAS results should be interpreted with caution though, particularly when dealing with low sample-size studies. Future endeavors should aim for specific, adequately powered approaches, with large sample sizes and well-characterized environmental, medication and ante- and/or postmortem data, while considering tissue and cellular heterogeneity. Moreover, candidate signatures should be functionally validated to determine whether they could represent novel causal mechanisms.

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