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# Epigenetic studies in Alzheimer's disease: current findings, caveats and considerations for future studies

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## Abstract

Alzheimer's disease (AD) is a sporadic, chronic neurodegenerative disease, usually occurring late in life. The last decade has witnessed tremendous advances in our understanding about the genetic basis of AD, but a large amount of the variance in disease risk remains to be explained. Epigenetic mechanisms, which developmentally regulate gene expression via modifications to DNA, histone proteins and chromatin, have been hypothesised to play a role in other complex neurobiological diseases, and studies to identify genome-wide epigenetic changes in AD are currently under way. However, the simple brute-force approach that has been successfully employed in genome-wide association studies is unlikely to be successful in epigenome-wide association studies of neurodegeneration. A more academic approach to understanding the role of epigenetic variation in AD is required, with careful consideration of study design, methodological approaches, tissue-specificity, and causal inference. In this article we review the empirical literature supporting a role for epigenetic processes in AD, and discuss important considerations and future directions for this new and emerging field of research.

### Keywords

Dementia; DNA methylation; brain; neurodegeneration; genetics

Alzheimer's disease (AD) is a chronic, currently incurable, neurodegenerative disorder that accounts for over 60% of dementia cases, with more than 26 million cases worldwide (Brookmeyer et al., 2007; Knapp et al., 2007). AD is a slowly progressive disorder characterized by increasingly severe behavioural changes, resulting in loss of independence, mounting intensive care requirements and ultimately, death.

AD pathogenesis appears to be initiated by the production, accumulation and oligomerization of amyloid-beta protein (Aβ), forming extracellular amyloid plaques that lead to the other neuropathological hallmarks of the disease including tangles of intracellular hyperphosphorylated tau, gliosis, synaptic dysfunction and eventually cell death (Hardy et al., 2002). The neurodegeneration associated with AD is believed to start many decades before clinical onset; during this preclinical phase the plaque and tangle load in the brain increases until a threshold level is reached and cognitive impairment becomes manifest (Blennow et al., 2006; Sperling et al., 2011). Different regions of the brain show differential vulnerability to AD, with some regions being particularly affected and others relatively resistant; both plaques and tangles occur first and most extensively in brain areas involved in learning, memory, and emotional behaviours. Regions such as the entorhinal cortex, the

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hippocampus and the basal nucleus of Meynert, for example, are characterized by considerable neuropathological damage (Wenk 2003). Other areas such as the cerebellum, however, are relatively resistant to neuronal damage with little or no tangle formation, tau pathology or neuronal loss, even in the context of extensive plaque formation.

While the neuropathological manifestation of AD has been well characterized in post-mortem brain tissue, little is known about either the underlying risk factors for the disorder or the precise mechanisms involved in disease progression. Given the high heritability estimates (60–80%) for AD derived from quantitative genetic analyses (Gatz et al., 2006), current approaches to understanding etiology have primarily focused on uncovering a genetic contribution to the disorder. Although autosomal dominant mutations in three genes (*APP*, *PSEN1*, and *PSEN2*) can explain early-onset (<65 years) familial AD, these account for only 5–10% of the total disease burden. Most cases of AD are late-onset (>65 years), non-Mendelian and highly sporadic, with susceptibility attributed to the action of highly prevalent genetic variants of low penetrance. Recent advances in our ability to interrogate genetic variation across the genome, in conjunction with the collection of large sample cohorts, has heralded the advent of genome-wide association studies (GWAS) aimed at identifying these genetic risk factors (Gandhi et al., 2010). Although common sequence variants in a number of genes (e.g. *ABCA7*, *CLU*, *CR1*, *CD33*, *PICALM*, *MS4A6A*, *MS4A4E*, *CD2AP* and *BIN1*) have been now robustly associated with AD via GWAS and subsequent meta-analyses (Harold et al., 2009; Hollingworth et al., 2011; Naj et al., 2011; Sleegers et al., 2010), they account for only a small proportion of attributable risk and the mechanism behind their action remains unknown. Moreover, recently discovered rare mutations in the *TREM2* gene have been shown to increase the risk of developing AD up to three fold (Guerreiro et al., 2012; Jonsson et al., 2012; Neumann et al., 2012), although the functional significance of these variants is yet to be understood. To date, the only common widely-replicated genetic risk for late-onset AD remains the ε4 allele of the *Apolipoprotein E* gene (*APOE*), accounting for about a fifth of the population-attributable risk for the disorder (Slooter et al., 1998). Although there have been numerous studies attempting to reveal the underlying mechanism for this association, precisely how *APOE ε4* influences AD onset and progression has yet to be elucidated. Despite considerable research effort, therefore, we are still a long way from realising the post-genomic promises of novel diagnostic and therapeutic strategies for AD. Recently, increased understanding about the functional complexity of the genome has led to growing recognition about the likely role of non-sequence-based “epigenetic” variation in health and disease (Bernstein et al., 2012). This article will briefly introduce epigenetic mechanisms, focussing primarily on DNA methylation and its relevance to AD, before discussing future directions for this emerging field of research.

## Beyond genetic variation: a role for epigenetics in AD?

Epigenetic processes mediate the reversible regulation of gene expression, occurring independently of DNA sequence, acting principally through chemical modifications to DNA and nucleosomal histone proteins. Epigenetic modifications regulate normal cellular development and differentiation and are necessary for the long-term regulation of gene function (Henikoff et al., 1997). DNA methylation is the best characterized and most stable epigenetic modification modulating the transcription of mammalian genomes, and because it can be robustly assessed using standardly-extracted genomic DNA resources is the focus of most human epidemiological epigenetic research to date. The methylation of CpG dinucleotides at the 5’ position on the pyrimidine ring, to form 5-methylcytosine (5-mC), can disrupt the cell’s transcriptional machinery by blocking the binding of transcription factors and attracting methyl-binding proteins that initiate chromatin compaction and bring about gene silencing (Klose et al., 2006). This is particularly true within CpG Islands (CGIs)

located within the 5' promoters of many constitutively expressed housekeeping control genes. Recent data suggests that the relationship between DNA methylation and transcription may be more complex, with gene body methylation often being associated with active gene expression (Aran et al., 2011; Ball et al., 2009; Hellman et al., 2007; Lister et al., 2009; Rauch et al., 2009) and alternative splicing (Flores et al., 2012; Lyko et al., 2010). Other modifications to DNA have been recently described, for example 5-hydroxymethylcytosine (5-hmC) (Penn et al., 1972; Tahiliani et al., 2009; Wyatt et al., 1953), 5-formylcytosine (5-fC) and 5-carboxylcytosine(5-caC) (Inoue et al., 2011; Ito et al., 2011); although their relative abundance in the genome is yet to be determined, there is some evidence for an enrichment of 5-hmC in specific regions of the brain (Globisch et al., 2010; Jin et al., 2011; Kriaucionis et al., 2009; Li et al., 2011; Munzel et al., 2010). Epigenetic regulation via the post-translational modification of histone proteins is another essential cellular mechanism regulating gene expression, with a spectrum of distinct histone modifications acting to dynamically alter chromatin structure and influence transcription (Jenuwein et al., 2001; Strahl et al., 2000)

Epigenetic mechanisms orchestrate a diverse range of important neurobiological and cognitive processes in the brain – e.g. neurogenesis and brain development (Ma et al., 2010), neuronal activity (Guo et al., 2011), learning and memory (Lubin et al., 2008) and circadian rhythm (Nakahata et al., 2007) – and disruption to these processes is likely to play a profound role in health and disease. Aberrant patterns of DNA methylation, for example, have been hypothesized to be involved in an increasing number of human neurobiological disease phenotypes including autism (Wong et al., 2013), psychosis (Mill et al., 2008), major depressive disorder (Mill et al., 2007) and recently AD (Balazs et al., 2011; Chouliaras et al., 2010; Mastroeni et al., 2011; Mill 2011).

Several epidemiological and clinical features of AD suggest an epigenetic contribution to etiology. These include monozygotic (MZ) twin discordance in both AD diagnosis (Gatz et al., 2006; Plomin et al., 1994) and age of onset (Cook et al., 1981; Nee et al., 1999), the seemingly sporadic onset of symptoms late in life (Jost et al., 1995), sexual dimorphism in disease progression (Lapane et al., 2001) and evidence of parent-of-origin effects in both disease transmission (Edland et al., 1996) and genetic association studies (Bassett et al., 2006). There are striking age-associated epigenetic changes in the human brain (Hernandez et al., 2011; Horvath et al., 2012), including within the *APP* and *MAPT* genes (Tohgi et al., 1999a; Tohgi et al., 1999b; West et al., 1995), and the first candidate-based gene studies of DNA methylation in AD report significant age-specific epigenetic drift at several loci previously implicated in the disorder (Siegmund et al., 2007; Wang et al., 2008). Finally, recent studies have described altered epigenetic regulation in other chronic neurodegenerative diseases related to AD (Urdinguio et al., 2009); for example histone hypoacetylation and DNA hypomethylation across the TNF-α gene promoter, resulting in TNF-α overexpression (Pieper et al., 2008), have been associated with Parkinson's disease (PD), and histone trimethylation and hypoacetylation, resulting in altered expression of the dopamine D2 receptor (Ryu et al., 2006; Sadri-Vakili et al., 2007), has been identified in Huntington's disease (HD).

## Epigenetic Studies of AD: The Current State of Play

Despite considerable speculation about the role of epigenetic dysfunction in AD, this is a relatively nascent area of investigation; compared to other complex disorders such as cancer, where an epigenetic contribution to disease is well established, little empirical research has been undertaken. Several recent studies have investigated DNA methylation in AD using a variety of molecular approaches, as reviewed in Table I. Using immunohistochemistry, for example, Mastroeni and colleagues report that global levels of 5mC and 5hmC are

significantly lower in neurons in the entorhinal cortex in AD patients compared to non-demented elderly controls (Chouliaras et al., 2013; Mastroeni et al., 2010). The same group examined a single pair of monozygotic twins discordant for AD, demonstrating a global reduction in 5mC levels in cortical neurons in the affected twin (Mastroeni et al., 2009), and a decrease in both 5hmC and 5mC in hippocampal neurons and glia (Chouliaras et al., 2013).

It is hard to draw any conclusions about specific AD-associated epigenetic changes from the limited existing literature. Most analyses have assessed only small numbers of samples, and different studies have used a range of different cell- and tissue-types. These studies have primarily focused on only one epigenetic modification (i.e. DNA methylation) and profiled very specific genomic regions (i.e. promoter CpG islands associated with *a priori* candidate genes). Despite these limitations, the available data provides some preliminary insights about the molecular mechanisms involved in AD. For example, a recent study demonstrated that a number of neuroinflammatory genes are hypomethylated and show increased expression in AD, whilst some neuron-specific genes are hypermethylated and are transcriptionally repressed (Rao et al., 2012). Recently the first study to take a more systematic genome-wide approach, assessing AD-associated changes at >27,000 CpG sites in the prefrontal cortex, identified 948 CpG sites in the vicinity of 918 genes, demonstrating small but nominally-significant AD-associated DNA methylation differences (Bakulski et al., 2012).

## Examining the Epigenome in AD: Study Design Issues

Recent advances in microarray and genomic sequencing technologies mean that genome-scale studies of the epigenome across much larger sample collections are now feasible, particularly for DNA methylation, and a number of epigenome-wide association studies (EWAS) for AD are currently underway. It is important to recognize, however, that the simple brute-force ‘science by numbers’ approach that has been successfully employed in genetic studies of AD is unlikely to be directly translatable to epigenetic epidemiology (Heijmans et al., 2012; Mill et al., 2013). In reality, studies aiming to identify epigenetic changes in complex diseases such as AD needs to consider a number of important issues. These, together with potential solutions, are discussed below and presented in Table II.

### 1. Technological Caveats

To date, the primary focus of epigenetic studies in AD has been on cytosine methylation at a small proportion of the CpG sites present in the human genome. The majority of probes on the recently released Illumina 450K Methylation Beadchip array, the current workhorse for EWAS analyses, for example, are located in CpG-rich promoters and may not be optimal for identifying the most phenotypically-relevant epigenetic variation. Recent studies highlight the importance of epigenetic modifications occurring outside of promoter CpG islands; in fact functionally-relevant epigenomic variation may primarily occur at non-promoter CpG islands, low CG-content promoters, and the gene body (Davies et al., 2012), in addition to intermediate CG density ‘shores’ flanking CpG islands (Hansen et al., 2011). Non-CpG DNA methylation may also be important to assess; for example, a recent study highlighted how ~25% of DNA methylation in embryonic stem cells (ESCs) occurs at non-CpG sites (Lister et al., 2009).

### 2. Alternative Epigenetic Marks

A number of additional DNA modifications (5-hmC, 5-fC, and 5-caC) have recently received considerable attention. 5-hmC, for example, is believed to result from the active demethylation of methylated cytosine, and is particularly abundant in neurons within the

healthy brain (Globisch et al., 2010; Jin et al., 2011; Kriaucionis et al., 2009; Li et al., 2011; Munzel et al., 2010) and enriched in genes with synapse-related functions (Khare et al., 2012). Initial data suggest that some hydroxymethylated-CpG sites may be stable during aging, whilst other loci are more dynamically altered (Szulwach et al., 2011). Although a handful of recent reviews have alluded to a role for 5hmC in AD (Irier et al., 2012; van den Hove et al., 2012), and a recent study demonstrated a global decrease in 5hmC in AD hippocampus, there is at present a lack of empirical research, particularly at specific CpG loci, and further investigation of 5hmC in the context of neurobiological phenotypes such as AD is warranted. Importantly, many of the existing methods used to interrogate the methylome (i.e. those based on sodium bisulfite conversion or methylation-sensitive restriction enzyme cleavage) are unable to specifically discriminate between the different cytosine modifications (Ito et al., 2011). Post-translational histone modifications are another major source of epigenetic regulation that have been largely neglected in epidemiologically-informative study designs of AD, in part because of the difficulties associated with assessing these in available sample resources. Research using murine models of AD suggest a tangible role for histone alterations in AD with reduced histone H4 acetylation (Ricobaraza et al., 2009) and elevated histone deacetylase 2 (HDAC2) levels (Graff et al., 2012) being linked to AD-related phenotypes. HDAC2, for example, was found to be associated with the promoter regions of genes involved in memory, increasing H4K12 acetylation and ultimately increasing gene transcription (Graff et al., 2012). Furthermore, levels of HDAC2 were found to be significantly upregulated in neurons in the CA1 field of the hippocampus in human AD brain post mortem (Graff et al., 2012). Another histone modifier, HDAC6, was recently found to be upregulated in the temporal cortex of patients with frontotemporal lobar degeneration with TDP-43 inclusions (FTLD-TDP) but not in patients with AD or Dementia with Lewy Bodies (DLB) (Odagiri et al., 2013), indicating some disease specificity in epigenetic changes.

## 3 Tissue Specificity Issues

A major caveat when studying epigenetic variation associated with AD, a disease that is primarily manifest in specific regions of the brain, is the tissue- (and cellular-) specificity of the epigenome. Distinct differentially methylated regions (DMRs) are observed when comparing multiple brain regions in the normal brain (Davies et al., 2012; Ladd-Acosta et al., 2007). Although germline epimutations or changes occurring very early in development may be manifest across tissues (Martin et al., 2011), AD is by definition characterized by progressive changes in the abundance and function of specific brain cells, particularly in the hippocampus with where there is selective neuronal cell loss (West et al., 1994; Zarow et al., 2005), the activation of glia (Meda et al., 2001), and increased density of microglia surrounding amyloid plaques (Arends et al., 2000; Rodriguez et al., 2010). Although identifying disease-related changes in the hippocampus post-mortem is important, the absence of certain neuronal populations due to apoptosis and the presence of “activated” microglia in such regions will make the biological interpretation of methylomic data generated on whole tissue difficult. When heterogeneous tissues, such as the brain, are used for genome-wide quantitative trait analyses, alterations in one cell type may oppose or dilute those in another, potentially obscuring important cell-specific changes (Blalock et al., 2011). Although gene expression analyses have highlighted clear transcriptomic differences between individual cell types in the human brain (Johnson et al., 2009; Khaitovich et al., 2004; Roth et al., 2006), detailed studies of cell-specific DNA methylation have yet to be conducted. To date no study has examined methylomic variation in pure populations of neurons, astrocytes, and microglia across multiple unaffected individuals; such a resource would be invaluable for interpreting epigenetic changes at a genome-wide level when comparing diseased and control brain tissue (Mill 2011). A recent study has made progress in this regard by developing an algorithm to determine the relative proportions of neurons to

total glia in methylomic data from brain tissue (Guintivano et al., 2013). Furthermore, a number of methods for isolating specific cell-types from brain tissue have been developed, including laser capture microdissection (LCM) (Blalock et al., 2011; Ginsberg et al., 2010; Pietersen et al., 2009; Suarez-Quian et al., 1999), fluorescence-activated cell sorting (FACS) (Matevossian et al., 2008; Nunes et al., 2003; Uchida et al., 2000), magnetic affinity cell sorting (MACS) (Yu et al., 2004) and density gradients (Barksdale et al., 2010; Olah et al., 2012; Whittemore et al., 1993). Such methods have been previously criticised in gene expression studies, however, due to the possibility of cell transcriptional changes occurring during isolation, and their applicability to epigenetic studies needs confirmation.

## 4. Additional Considerations

Another issue is the limited availability of high quality post-mortem tissue samples from AD patients and, in particular, suitably matched control subjects. In the transcriptomics field, a number of peri-mortem and post-mortem factors are known to affect RNA integrity and subsequent downstream analyses (Barton et al., 1993; Stan et al., 2006), yet the degree to which these factors may influence epigenomic analyses of the brain has not yet been systematically addressed. Although studies of histone modifications and/or chromatin structure are likely to be confounded by similar peri- and post-mortem factors, DNA methylation is a relatively stable chemical modifications to genomic DNA and may be more robustly examined for AD-associated changes (Pidsley et al., 2011).

The issue of determining causality is a major issue in epigenetic epidemiology (Martin et al., 2011; Mill et al., 2013), but is difficult to address in research using human post-mortem samples for obvious reasons. For example it is likely that the disease process itself or treatments may cause epigenetic changes, and the associations identified in EWAS analyses could represent a secondary effect of pathogenesis (Relton et al., 2012) or the medication (Boks et al., 2012) used to treat it. Our ability to detect true AD-associated DMRs is limited by the fact that, to some degree, AD pathology is also evident in non-demented “pre-clinical” control samples, and a greater availability of donor brains from persons with mild cognitive impairment would allow the assessment of DMRs in early disease. Alternatively a comparison of DMRs in late-onset AD brain to DMRs in early-onset familial AD brain could help address causality, as would a comparison of DMRs in other dementias with overlapping pathology. Repeated longitudinal profiling of the epigenome using accessible tissues such as peripheral blood is one potential approach for assessing causality. Given the tissue-specific nature of epigenetic marks, discussed above, recent data suggesting that some inter-individual variation in DNA methylation may be conserved across brain and blood has important implications for epigenetic studies of complex neurobiological phenotypes (Davies et al., 2012). At the transcriptomic level it has been shown that differentially expressed loci identified in blood reflect differences observed in AD brain (Lunnon et al., 2012), further suggesting that molecular biomarkers of disease may have some utility in epidemiological studies.

Finally the generation of new cellular and animal models, which reflect the genetic diversity observed in the general population are likely to become important for understanding the role of epigenetic mechanisms in AD. Rodent models in particular enable researchers to exclude potential confounding variables (e.g. age, sex, medication, and the environment) in epigenomic analyses, and specific brain regions can be easily isolated. There are, however, some caveats: although *in vivo* transgenic animal studies provide considerable insight into the molecular changes that occur as a result of particular neuropathological situations that arise in AD, they are generally not true models of late-onset AD because they do not display overt neurodegeneration (Holcomb et al., 1998; Irizarry et al., 1997a; Irizarry et al., 1997b; Stein et al., 2002). Another pitfall of many transgenic models is that they are, in reality,

models of familial AD, with pathology driven by mutations within the APP, PSEN1 or PSEN2 genes.

## Considerations for future studies: The added value of an integrated "omics" approach

The integration of epigenomic data with genetic and other "omic" data modalities will be vital in understanding the causes and downstream consequences of disease-associated epigenetic changes on AD pathology (Meaburn et al., 2010; Mill 2011). Of particular relevance to the etiology of complex disease phenotypes like AD is increasing evidence for the widespread occurrence of allele-specific DNA methylation (ASM) occurring outside of classically imprinted autosomal regions (and the X-chromosome in females) (Meaburn et al., 2010; Schalkwyk et al., 2010). A key observation is that the majority of observed ASM is associated with genetic variation in *cis* and has a significant influence on gene transcription, although a noticeable proportion is also non-*cis* in nature and mediated by parental origin, stochastic, developmental, or environmentally-induced factors. We propose that the interpretation of GWAS data can be improved by incorporating such 'epiallelic' information into analyses (Meaburn et al., 2010); whilst genotype-mediated DNA methylation (controlled by so-called methylation QTLs) can provide a functional mechanism for apparently non-coding genetic variation, other epigenetic patterns may complicate the direct identification of disease-associated loci, contributing towards the 'missing heritability' of complex disease by masking direct associations between genotype and phenotype. Of note, a recent study reported an enrichment of *cis*-regulatory mQTLs among susceptibility variants identified in a GWAS of bipolar disorder (Gamazon et al., 2012), and the utility of an integrated genetic-epigenetic approach is exemplified by the mapping of haplotype-specific methylation at the GWAS-nominated *FTO* risk locus in the context of type 2 diabetes and obesity (Bell et al., 2010). Because epigenetic processes may be influenced by a spectrum of external environmental factors including diet, toxins, drugs, and stress (Dolinoy et al., 2008), the observation that polymorphisms can also exert an effect on gene function via epigenetic processes occurring in *cis* suggests a common pathway behind both genetic and environmental effects and a potential mechanism for gene-environment interaction.

## Looking beyond biology: Implications for Diagnostics and Therapeutics

Aside from identifying novel mechanistic pathways involved in the etiology of AD, epigenomic analyses ultimately promise the development of novel translational clinical tools for AD. At present a number of transcriptomic biomarkers for AD have already been developed, with specific clinical utility for the early diagnosis of the disease (Booij et al., 2011; Fehlbaum-Beurdeley et al., 2010; Lunnon et al., 2013; Rye et al., 2011), and monitoring drug response in clinical trials (Fehlbaum-Beurdeley et al., 2012). Given the evidence for similar gene expression changes in AD brain and blood, the relative stability of DNA methylation compared to RNA, and recent reports that some inter-individual variation in DNA methylation may be consistent across different brain regions and blood (Davies et al., 2012), DNA methylation biomarkers could prove to be a robust and reliable alternative biomarker for early diagnosis of AD. In the cancer field, hypermethylation of methylguanine-DNA methyltransferase (MGMT) in glioma and glutathione S-transferase pi 1(GSTP1) in prostate cancer have been proposed as potential candidate biomarkers for diagnosis, with other loci proposed to predict both survival times and sensitivity and response to new medications (Heyn et al., 2012). At present, new pharmacological strategies are desperately required for AD, with current medications merely treating the symptoms of disease, often ineffectively. Because epigenetic changes are potentially reversible the identification of AD-associated epigenomic marks could yield potentially new therapeutic targets for treating the disease. Agents that actively influence the epigenome are already

licensed for clinical use in oncology, with more in development (Nebbioso et al., 2012). Into the future, a better understanding about the role of epigenetic processes in neurodegeneration will hopefully enable similar drugs to be developed for the treatment of AD, directly targeting the molecular switches involved in the etiology of the disorder.

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**Table I**

Summary of empirical epigenetic studies in Alzheimer's disease.

| Reference | Methodology | Approach | Samples | N | Primary Findings |
|---|---|---|---|---|---|
| **(Rao et al., 2012)** | Real-Time PCR (MSRE-digested DNA) | Nine candidate genes previously identified as differentially expressed in AD | Human frontal cortex (BA9) | 10 AD<br>10 CTL | Hypomethylation of inflammatory genes NF-ʁβ and COX-2 and hypermethylation of neuronal genes BDNF and synaptophysin in AD |
| **(Mastroeni et al., 2009)** | Immunofluorescence | Global methylation (in monozygotic twin-pair discordant for AD) | Human temporal neocortex and cerebellum | 1 AD<br>1 CTL | Global hypomethylation in neuronal nuclei in neocortex in AD |
| **(Mastroeni et al., 2010)** | Immunofluorescence | Global methylation in AD and elderly control samples | Human temporal cortex and cerebellum | 20 AD<br>20 CTL | Global hypomethylation in neuronal nuclei in entorhinal cortex in AD |
| **(Chouliaras et al., 2013)** | Immunofluorescence | Global and cell-specific methylation and hydroxymethyation in AD and elderly control samples as well as monzygotic twin-pair discordant for AD | Human hippocampus (CA1, CA3 and DG) | 10 AD<br>10 CTL<br>1 AD<br>1 CTL | Global decrease in 5mC and 5hmC in both glia and neurons in AD compared to control. |
| **(Bakulski et al., 2012)** | Illumina Infinium Human Methylation 27K BeadArrays | Genome-wide analysis of >27,000 CpG sites | Human prefrontal cortex | 12 AD<br>12 CTL | 918 differentially methylated genes. The highest ranking gene (TMEM59) was confirmed by RT-PCR in an additional 13 AD and 13 CTL samples |
| **(Wang et al., 2008)** | Sequenom Epityper (MALDI-TOF mass spectrometry) | Twelve Candidate genes associated with AD | Human prefrontal cortex | 24 AD<br>10 CTL | Greater age-specific epigenetic drift from "normal" in AD |
| **(Siegmund et al., 2007)** | Real-Time PCR (Bisulfite-treated DNA) | Fifty candidate genes related to CNS growth and development | Human temporal neocortex | 18 AD<br>63 CTL | Hypomethylation of S100A2 and hypermethylation of SORBS3 in AD |
| **(West et al., 1995)** | Southern Blot | One candidate gene previously associated with AD | Human frontal cortex (BA38) | 1 AD<br>1 PD<br>1 CTL | Hypomethylation of APP gene in AD |
| **(Furuya et al., 2012)** | Sequenom Epityper (MALDI-TOF mass spectrometry) | Correlation of mRNA and promoter methylation of synaptic protein SNAP25 | Human entorhinal cortex, auditory cortex and hippocampus | 10 AD<br>10 CTL | No DNA methylation changes in SNAP25 promoter |
| **(Zhang et al., 2012)** | Targeted proteomics, LC-MS/MS-TMT quantitative proteomics and Western Blotting | Comparison of histone acetylation levels using three methods | Human temporal lobe | 11 AD<br>4 CTL | Decreased acetylation of Histone H3 in AD |
| **(Ogawa et al., 2003)** | Immunohistochemistry | Comparison of Histone H3 phosphorylation in AD | Human hippocampus | 17 AD<br>9 CTL | Increased phosphorylation of Histone H3 in neurons (cytoplasmic) in AD |
| **(Graff et al., 2012)** | Immunohistochemistry | Comparison of HDAC1, 2 and 3 levels in CA1 field | Human hippocampus | 19 AD<br>7 CTL | Increased levels of HDAC2 in CA1 neurons in AD |

Abbreviations: Methylation-sensitive restriction enzymes (MRSE), Brodmann area (BA), Alzheimer's disease (AD), Control (CTL), Pick's disease (PD), Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-ʁβ), Cyclooxygenase-2 (COX-2), Transmembrane protein 59 (TMEM59), Real-time polymerase chain reaction (RT-PCR), S100 calcium binding protein A2 (S100A2), Sorbin and SH3 domain containing 3 (SORBS3), Amyloid precursor protein (APP), Synaptosomal-associated protein 25 (SNAP25), Liquid chromatography-mass spectrometry (LC-MS), Mass spectrometry-tandem mass tags (MS-TMT), Histone deacetylase (HDAC)

## Table II

Study design issues for genome-wide epigenetic analyses in AD

| Issue | Problem(s) | Suggested solutions |
|---|---|---|
| **1. Technological Caveats** | Current EWAS microarray platforms interrogate only a small proportion of CpG sites and are primarily focused on CpG-rich promoter regulatory regions | Use next-generation sequencing-based approaches (e.g. WGBS or MeDIP-seq) to interrogate entire methylomes |
| | Inability to assess DNA methylation at non-CpG sites using standard EWAS microarray platforms | Use targeted sequencing-based approaches to identify both CpG and non-CpG DNA methylation across specific regions |
| | Inability to distinguish between different cytosine modifications using standard bisulfite-based approaches | Oxidative-bisulfite sequencing (to distinguish 5hmC from 5mC)<br>DNA-IP sequencing with DNA captured with specific antibody to each modification (only possible for 5hmC and 5mC at present) |
| **2. Alternative Epigenetic Marks** | Inability to distinguish between different cytosine modifications using standard bisulfite-based approaches | Oxidative-bisulfite sequencing (to distinguish 5hmC from 5mC) |
| | | DNA-IP sequencing with DNA captured with specific antibody to each modification (only possible for 5hmC and 5mC at present) |
| **3. Tissue Specificity Issues** | Differential patterns of DNA methylation across different regions of the brain potentially involved in disease | Cross-tissue study to identify DMRs |
| | Brain is a heterogeneous tissue and cell numbers change in disease | Validate findings in specific cell types isolated via LCM or FACS |
| **4. Additional Considerations** | Pre-, Peri- and Post-mortem factors could influence epigenetic profile in post-mortem brain tissue | Use large sample sizes with similar group characteristics and well characterized environmental, medication, and post-mortem data, regressing out effects in analyses |
| | Determining causality in disease is difficult in cross-sectional studies using post-mortem tissue | If peripheral methylomic biomarkers of AD are identified, longitudinal sampling of blood could address when DMRs first appear in relation to cognitive changes |
| | | Better animal/cellular models representing the genetic diversity observed in general population for investigating the functional consequences of specific epigenetic changes |