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Epigenomics of Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is a large and growing public health problem. It is characterized by the accumulation of amyloid-β peptides and abnormally phosphorylated tau proteins that are associated with cognitive decline and dementia. Much has been learned about the genomics of AD from linkage analyses and more recently, genome-wide association studies. Several but not all aspects of the genomic landscape are involved in amyloid-metabolism. The moderate concordance of disease among twins suggests other factors, potentially epigenomic factors, are related to AD. We are at the earliest stages of examining the relation of the epigenome to the clinical and pathologic phenotypes that characterize AD. Our literature review suggests that there is some evidence of age-related changes in human brain methylation. Unfortunately, studies of AD have been relatively small with limited coverage of methylation sites and microRNA, let alone other epigenomic marks. We are in the midst of two large studies of human brains including coverage of more than 420,000 autosomal cytosine-guanine dinucleotides (CGs) with the Illumina Infinium HumanMethylation 450K BeadArray, and histone acetylation with chromatin immunoprecipitation-sequencing. We present descriptive data to help inform other researchers what to expect from these approaches in order to better design and power their studies. We then discuss future directions to inform on the epigenomic architecture of AD.

Keywords

Epigenomics; Alzheimer's disease; methylation; histone acetylation; chromatin states

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AGING AND DEMENTIA

Persons over the age of 65 are the most rapidly growing segment of the population throughout the developed world as a result of increasing life expectancy and declining fertility over the past century. This demographic trend will continue throughout the 21st century, and similar trends are forecast for developing countries in the latter half of this century. Thus, the world's population is aging rapidly making common chronic conditions of aging an urgent and growing public health problem. Looming large on this landscape is age-related cognitive decline, which can progress to mild cognitive impairment (MCI), i.e., obvious cognitive impairment in which most activities of daily living remain intact, followed by dementia, i.e., cognitive impairment contributing to impaired activities of daily living. The incidence and prevalence of dementia increase markedly with age. There are numerous causes of dementia. The most common cause is Alzheimer's disease (AD); however, cerebrovascular disease (CVD), Lewy body disease (LBD), and hippocampal sclerosis (HS) are also well-recognized causes. More recently, TAR DNA-binding protein 43 (TDP-43), originally thought to be a relatively rare pathology, has been shown to be quite common and also associated with dementia in aging populations. Thus, while Alzheimer's disease may play a large role in dementia, many other processes contribute to aging-related cognitive decline.

The projected impact of dementia in general and AD in particular on both health care costs and pension plan has recently captured the attention of economists, politicians and other policy makers.[1–5] This is of particular concern in Western European countries with publically funded health and pension systems. Thus, the USA and many European countries now have or are developing "National Plans."[6–12] France was the first country with a National Dementia Plan, launched in 2001. The Netherlands launched their National Dementia Plan in 2008, followed by the UK in 2009, Denmark in 2010, and others have followed suit. The US Congress unanimously passed the National Alzheimer's Project Act (NAPA; P.L. 111-375) in 2010 and the initial plan was unveiled in 2012. British Prime Minister David Cameron, G8 president in 2013, announced at a G8 Dementia Summit, that the G8 would lead a global plan to address AD.[13–14]

CLINICAL AND NEUROPATHOLOGIC FEATURES OF ALZHEIMER'S DISEASE

Clinical manifestations

AD is the most common cause of dementia in old age. However, progress over the past quarter century has led to new conceptualization of AD as a chronic disease that begins with a subclinical pathophysiologic process, followed by subtle cognitive decline, MCI due to AD, and finally to AD dementia which is now considered end stage AD.[15–17] The core feature of AD is memory loss. Memory is the recording, storage, and retrieval of information and accounts for all behavioral changes based on prior experience from single cell organisms to complex human behaviors. There are many forms of memory. The clinical hallmark of AD is progressive loss of episodic memory, the explicit recall of the times, places, and associated contextual characteristics that comprise the autobiography of one's

life. However, all cognitive abilities are eventually affected, and in many cases, multiple cognitive abilities are affected early in the disease process. The pace of cognitive decline varies widely and can range from a year to two decades. Although defined by its effect on cognition, AD affects a wide range of other abilities including gait, physical frailty and other motor abilities, smell, and sleep, and can be accompanied by depression, delusions and hallucinations.[18–22]

About 5 million people in the US are affected by AD dementia.[23–25] While the CDC reported fewer than 90,000 deaths based on death certificates in 2010, a recent study estimated that more than 500,000 excess deaths were associated with AD in 2010, putting it on a par with cancer and heart disease.[26] If MCI due to AD was included in these estimates based on the new consensus criteria both numbers would be higher.[27–28]

Pathologic features of AD

The neuropathologic hallmarks of AD are the extracellular deposition of a 40-42 amino acid amyloid-β protein in the form of plaques and the phosphorylation of intracellular microtubule associated tau proteins in the form of neurofibrillary tangles.[29] In line with the recently accepted chronic disease model of AD, the pathologic indicators can be present in people without cognitive impairment, in addition to those with MCI due to AD and AD dementia.[29–30] Further, there is good evidence that the deposition of amyloid-β precedes the accumulation of tangles and that both develop pathologies accumulate prior to the onset of overt cognitive impairment.[31–32] Further, using positron emission tomography, amyloid is seen in persons at genetic risk years prior to their estimated disease onset.[33] There are a variety of other ancillary changes in the brain such as degeneration of the cholinergic basal forebrain and other subcortical and brainstem nuclei, inflammation, and oxidative stress.[34–40]

Mixed disease and AD dementia and MCI

AD is among the most common chronic diseases of aging and its occurrence is strikingly related to age. Thus, it often is accompanied by co-morbid conditions, including other diseases that cause dementia. These conditions include CVD, LBD, HS, and more recently, TDP-43.[41–44] The vast majority of data suggest that these pathologies have an additive effect on the odds of dementia, although one study reported that AD and CVD had a multiplicative effect on the odds of dementia.[45–49] The result is that mixed diseases are the most common cause of dementia, including AD dementia.[50–51] Thus, it may be best to categorize the contribution of AD and other conditions to dementia. For example, the term vascular dementia is no longer preferred and has been replaced by vascular cognitive impairment.[52] Further, although the five conditions listed above appear to account for nearly all cases of dementia, it turns out that they only account for about half of the variability of cognitive decline.[53] These observations have profound implications for how we interpret studies of risk factors for AD. Specifically, it means that some risk factors for AD may be related to a co-morbid disease such as diabetes with cerebrovascular disease.[54–55] By contrast, other risk factors for AD may be unrelated to any extant pathology such as early and late cognitive activity.[56–57] These observations are equally important when considering genomic and epigenomic risk factors as will be discussed below.

HERITABILITY OF AD

Evidence of the heritability of AD comes from both family studies and twin studies.

Family studies

Familial aggregation in AD has been recognized for many years with persons with a first degree relative with AD having a two- to four-fold risk of AD.[58–61] Interestingly, because AD is so common in older persons and genomic risk factors tend to have variable penetrance, AD risk among first degree relatives varies by age such that the risk is highest among relatives of probands with earlier ages of onset compared to relatives of probands with later ages of onset.[62] Further, AD endophenotypes such as impairment of episodic memory and plasma amyloid-β levels have also been shown to be heritable.[63–64] Finally, in rare and primarily early onset cases, the disease has been found to run in families in an autosomal dominant fashion.[65–67]

Twin Studies

Numerous twin studies also demonstrate the heritability of AD.[68–71] However, the concordance rate varies widely and is substantially less than 100%, suggesting that factors other than genomic sequence influence disease occurrence.

GENETICS OF AD

Genetic linkage studies employed in the 1990's successfully yielded the identification of mutations in three genes that contribute to autosomal dominant early onset AD, and two common polymorphisms associated with late-onset AD. Following the sequencing of the human genome and the development of genome-wide association studies (GWAS), many additional polymorphisms were identified in the past five years.

Mutations and cytogenetic alterations

It has long been known that adults with trisomy 21 (Down's syndrome) develop the clinical and pathologic features of AD in mid life.[72–78] This led to an intensive search for a mutation on chromosome 21.[79] A mutation in the gene that encodes the amyloid precursor protein (*APP*) was eventually identified.[80–82] This was followed by linkage to chromosome 14, and the eventual discovery of a mutation in *PSEN1* that codes for the protein presenilin-1.[83–87] The third gene, *PSEN2* which codes for the protein presenilin-2, was identified after linkage to chromosome 1.[88–90]

Polymorphisms

Linkage to chromosome 19 resulted in the identification of two polymorphisms in the apolipoprotein E gene associated with AD: the *APOE* ε4 haplotype is associated with an increased risk of AD, and the *APOE* ε2 haplotype is associated with decreased risk.[91–94] Over the past five years, the GWAS era has yielded a number of additional polymorphisms. There are now nine loci containing susceptibility alleles that are considered confirmed including (*ABCA7*, *BIN1*, *CD33*, *CLU*, *CR1*, *CD2AP*, *EPHA1*, *MS4A6A-MS4A4E* and *PICALM that have* been confirmed by a number of different studies.[95–100] The most recent

GWAS with nearly 75,000 subjects identified these, as well as *SORL1* (which had previously been suggested to be a susceptibility locus), and 11 novel loci including *HLA-DRB5/HLA-DRB1*, *PTK2B*, *SLC24A4-RIN3*, *DSG2*, *MEF2C*, *INPP5D*, *NME8*, *ZCWPW1*, *CELF1*, *FERMT2*, and *CASS4*.[101]

Biologic mechanisms linking genomic risk factors to AD

Mutations in *APP*, *PSEN1* and *PSEN2* played a crucial role in advancing our understanding of AD. APP is a transmembrane protein that, when cleaved, yields the amyloid-β 1-42 peptide that represents an essential pathologic hallmark of AD. APP cleavage is accomplished by a set of three secretases, α, β, and γ.[102–106] β-secretase is the protein BACE-1.[107–108] Cleavage with this secretase is a prerequisite for generating the amyloid-β peptide. α-secretase cleaves within the amyloid-β 1-42 peptide sequence preventing the generation of the amyloid-β protein. By contrast, γ-secretase cleaves elsewhere and leaves the amyloid-β fragment intact allowing the generation of the amyloid-β peptide. Presenilin-1 and -2 are both components of γ-secretase and lead to the development of AD. Mutations in *BACE* are not known to cause AD.

There is extensive evidence that *APOE* is somehow involved in amyloid deposition most likely via clearance of amyloid-β.[109–113] It is likely, however, that two or more mechanisms are involved. Despite the fact that *APOE* ε4 is sufficiently common to make it a practical genotype-specific target for drug development and more than two decades of research, *APOE* ε4 remains a research tool and has very limited clinical utility.[114] Identifying and validating therapeutic targets in the pathway(s) linking these genomic variants to AD is among the most urgent issues in the field.

The relation of the other genetic risk factors to classic AD pathology has only recently been the subject of investigation. Approaches include associations with neuropathologic traits at autopsy or with imaging or biofluid biomarkers. The data to date suggest that some but not all of the newly discovered genomic variants are related to classic AD pathology such as amyloid and that the effect sizes tend to be small, as one might suspect in view of the strength of associations in the GWAS. The results are variable but include *CR1*, *ABCA7*, *CD2AP*, *CD33*, *PICALM* and SORL1.[115–121]

EPIGENETICS OF AD

The highly variable and relatively low concordance rates for AD among identical twins clearly indicate that factors in addition to sequence variation must play an important role in AD susceptibility, raising the possibility of a role for epigenetics. In the only study of a pair of monozygotic twins discordant for AD, a lower global 5-methylcytosine fluorescence intensity was seen in the temporal neocortex of the affected twin providing further support for a role of epigenetics (Table 2, reference 146). Further, there is evidence supporting age-related differences in human blood and brain methylation in advanced age (see below).

Nearly all epigenomic studies of AD to date are relatively small, targeted, and limited to one epigenomic mark. There are a few custom microarrays and data from first generation DNA methylation chip technology, e.g., Illumina Infinium HumanMethylation27 BeadArray that

interrogates a very small and non-random fraction of the methylome. Thus, the landscape is either biased or sparse and resembles the early days of genomics prior to the large GWAS studies. Most candidate SNP studies were simply not replicated, and it took large unbiased studies to identify the common genetic variants other than *APOE ε4*. However, the epigenome is much more complicated with a relatively large number of epigenomic marks. We also have limited knowledge regarding their correlation structure, their cell type specificity, and the potential for reverse causality (where the trait of interest such as AD influences the epigenome). So, the complexities of interrogating the epigenome relative to GWAS are compounded many fold. These issues make it impossible to draw any definitive conclusions regarding the relation of the epigenome to AD at this time. Below, we briefly discuss studies published to date and then share some descriptive work relating to different approaches of measuring epigenomic profiles in brain and to assessing data from a large number of human brains profiled with the Illumina Infinium HumanMethylation450K BeadArray, the one platform capable of high-throughput work. There is as yet no published data of such large-scale studies. Thus, while this field holds great promise, it is nascent, and the first generation of large studies are beginning to define the key parameters needed to perform epigenomic epidemiology studies.

DNA methylation

Cytosine-guanine (CG) dinucleotides are prominent sites for DNA methylation and are variably distributed across the genome. There are an estimated 23 million CG sites in the human genome, which can be interrogated by a variety of approaches. Interrogating the whole methylome remains labor intensive and expensive and is not yet amenable to high throughput.

We found 20 studies from 1999 to date on the relation of DNA methylation to age (Table 1).[122–141] Of these, six were of brain and the rest of peripheral biospecimens. Of the brain studies, only three had sample sizes greater than 100. One of these studies was a targeted study of 50 CG sites using PCR.[124] The other two studies used the Illumina Infinium HumanMethylation27 BeadArray.[130,133] All three studies showed relatively robust positive correlations between methylation and age.

We also found 28 studies from 1995 to date on the relation of DNA methylation to AD (Table 2).[124,142–168] Of these,20 were of brain and the remainder of peripheral tissues. Only one of the studies of brain had a sample size exceeding 100.[161] Only one study of brain used the Illumina HumanMethylation27 BeadArray, and the remainder interrogated far fewer CG sites.[155] The study of 12 cases and 12 matched controls using the Illumina platform reported that the AD brains were slightly hypomethylated. The remaining studies were quite variable with some suggesting hypermethylation, others hypomethylation, and some without obvious differences.

Histone acetylation

We found only a single 2012 study of histone marks, H3K18/K23 acetylation in persons with and without AD (Table 3).[169] The study employed selective reaction monitoring liquid chromatography-mass spectrometry (SRM LC-MS) to quantify histone acetylation in 11 AD

and 4 control brains and found down-regulation of these marks in AD brains. One study found that protein level of histone deacetylase 2 (HDAC2) was elevated in AD brains.[170]

microRNA

We identified 18 AD studies of microRNA (miR) from 2008 to present (Table 4).[171–188] Of these all but two were in human brain.[183,186] None of the studies had a sample size in excess of 100. Further, only one of the studies of human brain interrogated more than one or a few targeted miR's.[186] Overall, the results of studies of brain tissue were highly variable with some miR's up-regulated and others down-regulated.

Overall, it is clear that the study of epigenomics in AD is a nascent field, without robust results at this time. The studies of aging-related changes in the brain's epigenome do appear to have returned reasonable evidence supporting that many CG sites throughout the methylome are more highly methylated with advancing age. However, it is not yet clear how this relates to brain function or addresses the relevance of these changes to the local chromatin architecture at specific sites.

EXPERIENCE WITH HIGH TRHOUGHPUT APPROACHES TO THE EPIGENOME IN HUMAN BRAIN

The review of the literature to date suggests that there is very little published epigenomic data from the currently available high throughput approaches to the epigenome from large numbers of human brains. Over the past few years, we have generated DNA methylation data from several hundred human brains using the Illumina Infinium HumanMethylation450 BeadArray and genome wide histone acetylation data using chromatin immunoprecipitation-sequencing (ChIP-Seq). Here we describe our experience and share some basic descriptive information with the reader. The data are intended to inform future researchers regarding what they can expect from these approaches in order to better design and power studies of the brain methylome.

Brain DNA methylation using Infinium Humanmethylation450k Beadchip

We are unaware of any published studies of human brain using the Illumina Infinium HumanMethylation450 BeadArray, which is the current platform of choice for high throughput methylation studies. Here, we describe the basic characteristics of DNA methylation profiles from 740 postmortem human brains from participants in two ongoing longitudinal clinical pathologic studies of aging and dementia, the Religious Orders Study and the Rush Memory and Aging Project.[189–190] All participants signed an informed consent and an anatomical gift act for organ donation. Both studies were approved by the Institutional Review Board of Rush University Medical Center and conform to ethical guidelines for human research. While very little of these data have been published in peer-reviewed journals, additional data presented at national and international meetings are also in the public domain.[191–197]

Their average age was 88 (SD=6.7, range 66–108), 36% (N=269) were males, and 330 had AD or other dementia, 175 mild cognitive impairment, and 235 were without cognitive

impairment proximate to death. DNA was extracted from frozen dorsolateral prefrontal cortex (DLPFC) tissue. Methylation data was generated using the Illumina Infinium HumanMethylation450K BeadArray. For each brain sample, our dataset, after quality control, consists of methylation measures of a total of 420,132 autosomal CGs which are distributed across the entire genome.

First, we observe that DNA methylation at these 420,132 CG sites features a distinct bimodal distribution such that approximately a third of CGs are extremely hyper-methylated (i.e., mean methylation > 0.8) and another third are extremely hypo-methylated (i.e., mean methylation 0.2) (Figure 1). This bimodal pattern is preserved in all 22 autosomes (Figure 2). Since the coverage offered by the Illumina chip is targeting methylation sites primarily at CG islands and genic regions, we examine the distribution of CGs and methylation pattern by CG islands as well as surrounding shelves and shores. A total of 135,303 (32.2%) CGs are located in CG islands, 98,653 (23.5%) in CG shores, 39,141 (9.3%) in CG shelves and the remaining 147,035 (35.0%) in regions not related to CG islands. Interestingly, the distribution of mean methylation varies across the regions, as shown in figure 3. For example, in the case of CG island related features, only the CGs sites in the north and south shores (regions flanking a CG island) show a bimodal pattern of methylation levels, while the distributions in the CG island proper and the more distal north and south shelves are more polarized. Specifically, the majority of CGs (82.9%) in shelves are hypermethylated (i.e. mean methylation 0.5). By contrast, 83.8% of CGs in CG islands are hypo-methylated, among which 75.7% are extremely hypo-methylated. Notably, the mean methylation level in CG islands is much lower when compared with non-CG island regions (0.20 ± 0.29 vs. 0.62 ± 0.30; $p<0.0001$).

DNA methylation is an attractive epigenomic mark to study because it is relatively simple to extract from tissue and because the Illumina platform offers an option for automated high-throughput data generation, which minimizes technical variation in the data. However, the platform only samples a small fraction of the epigenome and does not differentiate between cytosine methylation and hydroxymethylation. These marks are distinct, and their relative role is just beginning to be explored. Thus, while DNA methylation provides a reasonable platform for the first generation of epigenomic studies, it is not sufficient, and alternative strategies are needed.

Histone acetylation data and other chromatin MARKS with ChIP-Seq

In contrast to methylation, profiled on DNA which is easily extracted from human tissues, profiling histone marks requires the capture of chromatin fragments that bear the mark of interest using immunoprecipitation. This process is time-consuming and not currently automated for high throughput. Its success is very dependent on the antibody clone and batch selected for the experiment, and use of the appropriate tissue type. Below, we illustrate our evaluation of different human brain-derived starting material, i.e., fresh frozen vs. paraformaldehyde fixed human brain. We also describe the experimental pipeline for chromatin immunoprecipitation followed by sequencing (ChIP-Seq) that yields genome-wide data on the location of a target chromatin mark, in this case H3K9Ac. Again, the data presented here are intended to inform future researchers regarding what they can expect

from these methods in order to better design and power studies of human brain histone marks. While very little of these data have been published in peer-reviewed journals, additional data presented at national and international meetings are also in the public domain.[198–199]

To generate ChiP-Seq data from the brain samples that were used in the DNA methylation experiment described above, we first identified the Millipore anti-H3K9ac mAb (catalog # 06-942, lot: 31636) as a robust monoclonal antibody for ChIP experiments out of four different antibodies tested and used a single batch of this antibody for all experiments that explore the pattern of H3K9Ac in the human dorsolateral prefrontal cortex. This particular mark was selected given evidence suggesting that (1) histone deacetylases may have an effect on AD pathology,[200] and (2) this mark accumulates in sharply defined, discrete areas at the transcription start site of actively transcribed genes as well as enhancers of such genes. For our pilot study, we explored the utility of using fresh vs. fixed brain tissue. Approximately 500mg of fixed tissue and 50mg of frozen tissue were needed to generate similar DNA yields of about 50 ug per sample (Supplementary Table 1). The larger fixed tissue requirement suggests that longer periods of fixation reduce the ability to completely reverse cross-linking within a sample fixed at autopsy.

Starting from a well-described ChIP-Seq protocol,[201] we first optimized DNA fragmentation, a key step in constructing libraries following enrichment by ChIP of target sequences. We did not observe the expected increase in the targeted 150–700 bp DNA fragments with increasing sonication in fixed tissue specimens but did document this trend in the frozen tissue specimens (Supplementary Figure 1). Neither the post mortem interval nor the fixation interval strongly influenced the performance of the fixed material in this respect; Supplementary Table 2 reports these parameters for all tested samples. The optimum fragment size range is the length of 1–3 nucleosomes, or 150–700bp. As illustrated in Supplementary Figure 1B, a sonication time of 4–6 minutes appears to be optimal to enhance the proportion of DNA fragments in the target range while avoiding an increase in the <150 bp fragment that enhance the background. Further, we tested two methods of tissue homogenization and find a 10% increase in the target range of fragment size, with a corresponding 10% decrease in the >700 bp fragment range and no change in the low size range when using the Tissue Lyser II approach compared to the standard Dounce homogenizer. There is no decrease in total DNA yields using the Tissue Lyser II approach (Supplementary Figure 1 B). Libraries produced from fixed and frozen tissue were sequenced.

Sequencing data from fresh *vs.* fixed tissue

Sequencing data were produced using the Illumina Genome Analyzer II platform. Libraries were not multiplexed. Supplementary Table 3 reports the summary statistics of sequencing output in terms of total reads produced, reads which passed Illumina quality filter, its percentage and number of filtered reads with percentage, which were successfully aligned to the human reference genome and number of duplicate reads from each sample. Given rapid improvements in sequencing technology, the number of output reads per lane increased from our pilot 1 study to our pilot 2 study for which data were generated 3 months apart.

As shown in Supplementary Table 3, sequence data were of high quality, with, on average, more than ~80% of reads from the sequencing passing the Illumina quality filter. 97% of these filtered reads were uniquely mapped to the human reference genome. Using the Picard pipeline, we also assessed the quality of sequencing by looking at the quality score distribution and sequencing coverage in different regions of genome based on GC-content. The normalized coverage of mapped sequence reads to genomic regions with varying GC-content is reported in Supplementary Figure 2 (A, B and C). The control sample without immunoprecipitation provides an estimate of the un-enriched genome-wide baseline distribution of sequences; it has the expected slight bias against GC-content that reflects the genome-wide skew in GC content found in the sequenced human genome (Supplementary Figure 2A). Given that H3K9Ac, our target epigenomic mark, is enriched near the transcription start site of genes that are, on average, GC-rich relative to the genome-wide average, we expect an enrichment for GC content in sequences selected by ChIP. While the library generated from a fixed tissue sample (Supplementary Figure 2B) shows a slight enrichment in GC content, we see the expected very substantial skew towards greater GC content in the frozen sample (Supplementary Figure 2C) that suggests successful enrichment by ChIP. Supplementary Figure 2D illustrates the much better enrichment in target sequence that is achieved when using frozen rather than fixed tissue sample as a target for ChIP.

To visualize the ChIP enrichment and further assess the quality of our data production pipeline, we generated a read tag density distribution plot using the Integrated Genomic Viewer (IGV; http://www.broadinstitute.org/igv/) (Supplementary Figure 3). Here, we see that samples from fixed brain tissue have a similar sequence read distribution as the control sample without ChIP, consistent with the lack of significant enrichment in GC content documented in Figure 2B. On the other hand, frozen samples show very clear and distinct peaks of H3K9Ac that are similar across samples from different individuals.

Overall, this pilot study illustrates the finding that fresh-frozen autopsy tissue is the preferred substrate for ChIP-seq data generation: fixed material failed to generate data in our pilot study. While the protocol based on fixed material can doubtlessly be improved and optimized, it is clearly not as easy to use as frozen material; nonetheless, optimization is indicated in circumstances where tissue availability is limited to fixed material.

Reference Chromatin maps

Through a collaboration with the NIH's Epigenomic Roadmap, we next generated ChiP-Seq data for six chromatin marks in two subjects who were cognitively non-impaired at the time of death and displayed minimal burden of age-related neuropathologies at the time of death. Six different chromatin marks were targeted, including three marks associated with condensed, transcriptionally repressed chromatin (H3K36me3, H3K9me3, and H3K27me3) and three marks associated with transcriptionally active regions (H3K4me3, H3K9ac, and H3K4me1). For each mark, we therefore have genome-wide data regarding the location where each of these chromatin modifications was found. This information can be collapsed by assessing each 200 bp segment of the genome and annotating the extent to which each of these marks is found in each segment and using the resulting pattern to estimate the state of the chromatin found in that particular segment. Each state reflects the extent to which a

segment of DNA is accessible for transcription and what function it may exert. For example, the ChromHMM software can be used to derive such a chromatin map of profiled tissues, and we can bin the segments into different states. We employed an 11-state profile. However, the number is increasing and is now a 15-state model and is likely to increase soon. Seven maps were generated in the Roadmap effort: the targeted regions include the angular gyrus, anterior caudate, cingulate gyrus, dorsolateral prefrontal cortex, inferior temporal cortex, mid-hippocampus and substantia nigra. Thus, we have a sampling of the state of chromatin in the gray matter of different cortical and subcortical regions. Further, Figure 4 illustrates that the proportions of CG sites with different mean methylation varies by chromatin state. A majority (more than 60%) of CG sites in weak and transcription regions are extremely hyper-methylated, while more than 90% of CG sites in strong promoters are extremely hypo-methylated. Thus, information can be integrated across different types of epigenomic data to interpret the association of given regions of the genome with SNPs, RNA expression and ultimately a trait of interest such as clinical or pathologic AD. Overall, these chromatin states and methylation patterns, and the extent to which they vary across individuals, will rapidly enrich our understanding of the basic organization of the epigenome.

FUTURE DIRECTIONS

Technological improvements will soon allow better interrogation of the epigenome by increasing the proportion of the genome that can be sampled effectively in a high-throughput manner. This advance will enable the execution of studies on the large scale that is needed for disease-related studies. Studies to date suggest that many loci may be associated with AD, and the better coverage will therefore provide both (1) better resolution of the association within each locus and (2) a comprehensive assessment of the genome. On the other hand, improvements in our understanding of the correlation structure between different epigenomic features will enable us to more efficiently interrogate the methylome and structure of chromatin and to understand how these features relate to another. This information will also be leveraged to inform the design of the molecular networks that drive AD pathology: mapping the epigenomic features that relate to AD identifies regions of the genome in which transcriptional potential is altered in disease. A key challenge remains whether these epigenomic changes are a cause or an effect of the pathologic process, and therefore, it is essential to study subjects at risk of developing dementia as well as subjects with dementia. We have already demonstrated that subjects who are not impaired at the time of death but have AD pathology exhibit epigenomic changes seen in demented subjects, suggesting that these changes are not a feature of terminal dementia.[202] To assess whether these changes predate early evidence of Alzheimer pathology (such as amyloid pathology), we need to evaluate their relation to other risk factors such as genetic and experiential factors. However, early analyses suggest that genetic factors appear to have largely independent effects in relation to epigenomic factors. An evaluation of younger subjects without amyloid pathology but with life experiences such as depression that contribute to dementia risk will also be necessary to investigate the role of non-genetic factors. These critical data derived from human samples will need to be supplemented by careful dissection

of the causal chain events using *in vitro* models such as neuronal cells differentiated from induced pluripotent stem cells and murine and other models of AD.

The methylation data and reference chromatin map derived from different cortical and subcortical tissues are an excellent initial evaluation of epigenetic changes in different regions of the aging brain. However, the profiles are based on cortical tissue, and we cannot resolve cell-autonomous effects at this point. Initial efforts to estimate the neuronal burden within a brain sample have been published,[203] but we need a better set of reference epigenomic profiles for different cell types. Such resources will be essential to begin to identify the cells which are affected by a given epigenomic feature discovered to be associated with AD in profiles of CNS tissues that are performed today. Such data will begin to address important questions relating to the observation that the differences in methylation relating to AD may be modest (1% difference in methylation levels, on average): is this difference is due to a small proportion of cells of a common cell type that are changing? Is a rare cell population is changing en bloc? Or, do these small changes represent the presence of infiltrating inflammatory cells?

In coming years, larger studies using better technologies will enhance the data that we can analyze to understand how chromatin structure influences the role of susceptibility variants and whether it may mediate some of the experiential risk factors associated with AD susceptibility. DNA methylation is likely to remain the mark of choice, but the study of histone protein modifications will be necessary to understand the fine architecture of loci implicated in AD. This will be supplemented by ChIP-Seq studies targeting pertinent transcription factors that regulate gene expression within susceptibility loci. All of these results will inform the identification and refinement of the molecular networks that lead to AD and are influenced by AD pathology. Despite its many challenges, epigenomic studies of AD will play an important role in the research community's efforts to delineate the sequence of events leading from health to dementia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
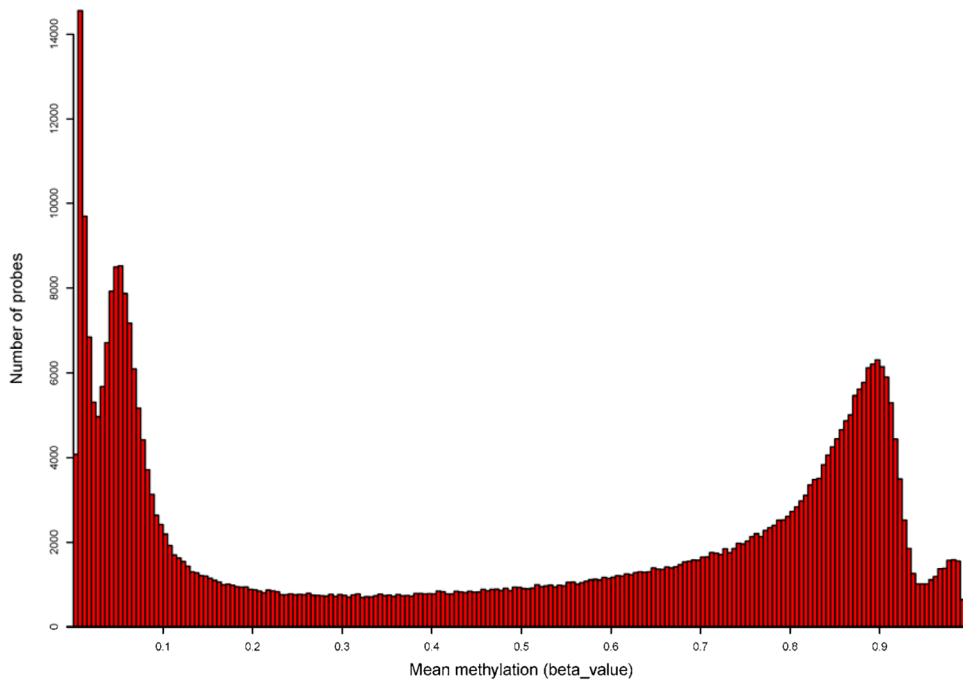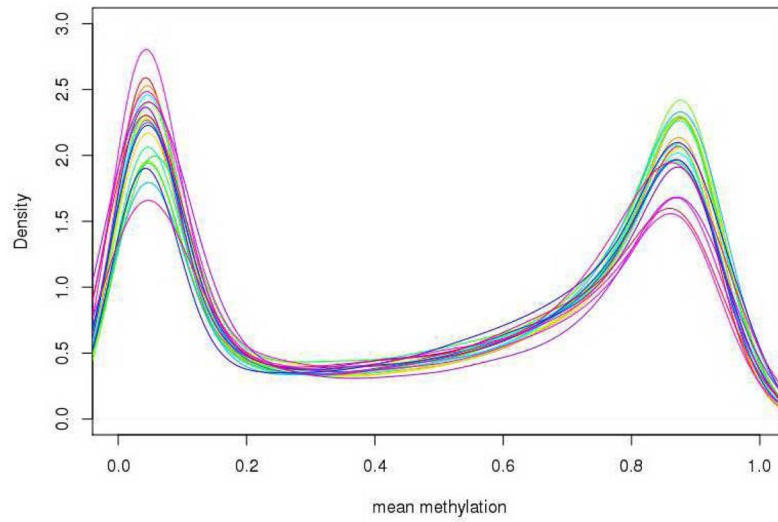Distribution of mean methylation of CGs in HumanMethylation450K.

Figure 2.
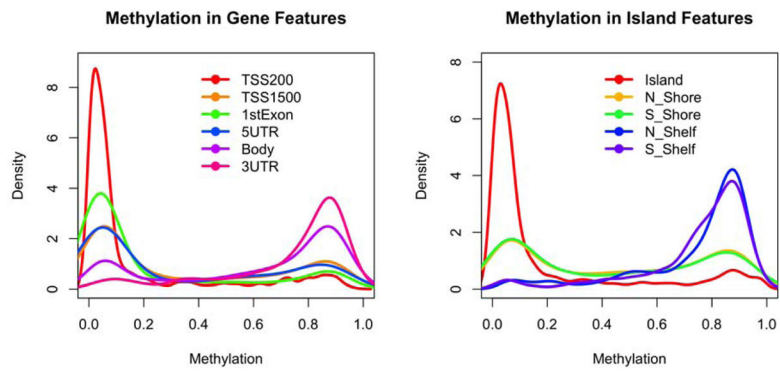Distribution of mean methylation of CGs in HumanMethylation450K by Autosomes.

Figure 3. Distribution of mean methylation level (across 740 samples) of CpG sites located at different genomic features

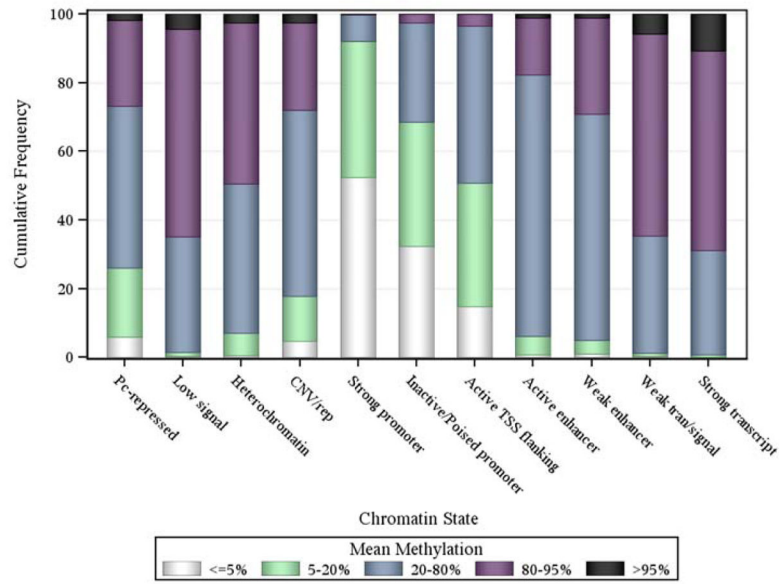(Left) Genic features; (Right) Island features.

Figure 4.
Proportions of CG sites with different mean methylation by chromatin

Table 1

Summary of studies on DNA Methylation and Age using Human Tissue

Reference	Methodology	Main Approach	Samples/Tissue	N	Primary Finding
122	PCR	Target gene analysis (APP)	Human parietal cortex (superior parietal lobule or supramarginal gyrus)	10 subjects (six males and four females, aged 35–90 years)	Methylcytosines decrease with age in the APP promoter region
123	PCR	Target gene analysis (tau)	Human parietal cortex	16 subjects (14 males and two females, aged 35–90 years)	Methylcytosines decrease with age in the promoter region of the tau gene
124	PCR	Target gene analysis (50 loci, covering primarily 5′ CG islands of genes related to CNS growth and development)	Human temporal cortex	17 fetal, 15 child and 93 adults.	Increased DNA methylation in 8 loci (GABRA2, GAD1, HOXA1, NEUROD1, NEUROD2, PGR, STK11, SYK) across the age span
125	Illumina GoldenGate Methylation assay	Selected CG sites in the array (280 high quality probes in 250 genes)	Whole blood	23 MZ twin-pairs; 23 DZ twin-pairs (N = 92) 96 healthy controls	22% of interrogated sites are related to age, including six loci of ACVR1, IL6, CARD15, PDGFRA, NFKB1, ELK3; 23% are highly heritable including AXL, GSTM1, TIAM1 and TEK
126	Illumina GoldenGate methylation bead arrays; Pyrosequencing	1,413 autosomal CGs in 773 genes	Nonpathologic human tissues	12 brain; 53 lung; 85 blood; 18 pleural; 11 head/neck anatomic site; 5 bladder; 6 kidney; 5 small intestine; 19 placenta	Methylation increases with age CG islands; decrease with age in non-CG islands site
127	Illumina HumanMethylatio n27 BeadChips	genome-wide DNA methylation analysis	Human whole blood; CD14+ monocytes and CD4+ T-cells; buccal cells	31 twin pairs and 31 singletons (blood); 25 singletons (monocytes and T-cells); 10 (buccal cells)	Aging-associated differentially methylated regions (aDMRs) are found in multiple tissues. Aging-associated hypermethylation occurs predominantly at bivalent chromatin domain promoters
128	Illumina HumanMethylatio n27 microarrays	genome-wide DNA methylation analysis	Mesenchymal stromal cells (MSC)	4 young (21–50 years) and 4 elderly donors (53–85 years)	295 CGs are hypermethylated, and 349 CGs are hypomethylated upon aging
129	Illumina HumanMethylatio n27 microarrays	genome-wide DNA methylation analysis	Saliva	34 male identical twin pairs between 21 and 55 years of age	88 CG sites near 80 genes are significantly correlated with age
130	Illumina HumanMethylatio n27 BeadChips	genome-wide DNA methylation analysis	Human frontal cortex, temporal cortex, pons and cerebellum	387 subjects between the ages of 1 and 102 years	Majority of associated CGs are within CG islands and methylation are positively correlated with age.
131	Illumina HumanMethylatio n27 BeadChips	genome-wide DNA methylation analysis	Human dermal fibroblasts	9 young (<23 years) and 6 elderly donors (>60 years)	75 CG sites differ their methylation level by more than 15% between the age groups
132	Bisulfite sequencing	Target gene analysis (MCHR1)	Blood	49	DNA methylation at MCHR1 is allele-specific, age-dependent,

Reference	Methodology	Main Approach	Samples/Tissue	N	Primary Finding
					BMI-associated and affects transcription BMI-associated and affects transcription
133	Illumina HumanMethylatio n27 BeadChips	genome-wide DNA methylation analysis	Human prefrontal cortex	108 subjects	DNA methylation shows unique temporal patterns across life.
134	Illumina Infinium HumanMethylatio n450 BeadChip	genome-wide DNA methylation analysis	Human whole blood	64 subjects	3 regions of ELOVL2, FHL2, and PENK genes strongly correlated with age
135	PCR	Longitudinal target genes analysis	Human blood	784 elderly men	Aging was associated with decreased methylation of GCR, iNOS and TLR2 and with increased methylation of IFNγ, F3, CRAT and OGG
136	MALDI-TOF mass spectrometry	4 target regions in the imprinted IGF2/H19 locus	Whole blood	376 age/sex matched individuals that differ in ancestral geographical origin and place of living (cohort 1); 31 MZ and 16 DZ twin couples of different ages, but homogeneous for geographic origin	No differences in methylation by ancestry/geography; In MZ twins intra-couple variations increased after the age of 60 years two IGF2/H19 regions
137	Illumina HumanMethylatio n27 BeadChips	genome-wide DNA methylation analysis	Whole blood	172 female twins aged 32 to 80; 44 younger adult MZ twins aged 20 to 61	Majority of age-related changes in methylation are not associated with phenotypic measures of healthy ageing in later life.
138	Whole-genome bisulfite sequencing (WGBS); and Illumina Infinium HumanMethylatio n450 BeadChip	genome-wide DNA methylation analysis	Blood	1 newborn (male Caucasian) and 1 centenarian [WGBS]; 19 newborns (Caucasians) and 19 nonagenarians (Caucasians)	More hypomethylated DNA sequences in the advanced age group
139	Illumina HumanMethylatio n27 BeadChips	genome-wide DNA methylation analysis	Blood	398 boys, aged 3–17 yr	significant age association with DNA methylation at 2078 loci
140	high-throughput ELISA-based method	Global DNA methylation	Peripheral blood leukocytes	126 older individuals	Methylation is lower in older age
141	Illumina Infinium HumanMethylatio n450 BeadChip	genome-wide DNA methylation analysis	Whole blood	656 human individuals, aged 19 to 101	Differences in aging rates explains epigenetic drift

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Table 2

Summary of studies on DNA Methylation and AD using Human Tissue

Reference	Methodology	Main Approach	Samples/Tissue	N	Primary Finding
142	Southern blot	Target gene analysis (APP)	Human temporal lobe (Brodmann area 38)	1 female AD, 1 female non-AD dementia, and 1 female control	A site in APP is not methylated in AD
143	Western Blot	Target analysis on PP2A(C)	Human middle frontal gyrus (Brodmann areas 9 or 46), superior temporal gyrus (Brodmann area 22), and the cerebellum	48 AD and 24 matched controls	Decreased PP2A(C) methylation levels in AD
124	PCR	Target gene analysis (50 loci, covering primarily 5′ CG islands of genes related to CNS growth and development)	Human temporal cortex	17 fetal, 15 child and 93 adults, among adults 18 AD.	increased DNA methylation for SORBS3 and S100A2 in AD
144	MALDI-TOF mass spectrometry	Target gene analysis (12 AD susceptibility loci)	Human prefrontal cortex	24 AD and 10 matched controls	significant epigenetic difference between AD and controls; age-specific epigenetic drift in AD; genes participating in amyloid-β processing (PSEN1, APOE) and methylation homeostasis (MTHFR, DNMT1) show a significant interindividual epigenetic variability
145	PCR	Target genes analysis (RAGE, ADORA2A, UCHL1, MAPT, PSEN1, and APP)	Human frontal cortex and hippocampus	AD including 17 stage I-II; 15 stage III-IV; and 12 V-VI, and 26 controls	No differences in CG methylation between cases and controls
146	Immunohistochemistry	Global methylation(5-methylcytosine [5mC])	Human temporal neocortex	A pair of monozygotic twins discordant for AD	Reduce level of DNA methylation in AD twin.
147	Immunohistochemistry	Global methylation (two DNA methylation markers of 5-methylcytosine and 5-methylcytidine, and eight methylation maintenance factors)	Human entorhinal cortex	20 AD and 20 controls	Reduce level of DNA methylation in AD.
148	Bisulfite sequencing	Target analysis on APP	Human cortex and cerebellum	6 familial AD cases	no methylation in APP associated CG-island
149	qPCR pyrosequencing	Target analysis on repetitive elements of Alu, LINE-1 and SAT-α	Human whole blood	43 AD and 38 matched controls	LINE-1 methylation was increased in AD
150	Illumina Infinium HumanMethylation27 BeadArray; and PCR	Genome wide DNA methylation association study (27,578 CGs); and target genes analysis	Human glioblastoma H4 cells and APP695-Swedish mutant	-	Reduced global DNA methylation in H4-sw cells. Target gene analysis shows CTIF, NXT2 are hypomethylated and DDR2

Reference	Methodology	Main Approach	Samples/Tissue	N	Primary Finding
					hypermethylated in H4-sw cells hypermethylated in H4-sw cells
151	real-time PCR	Target gene analysis (FAAH)	Human peripheral blood mononuclear cells	33 LOAD; 33 age/sex-matched controls	Methylation at the gene promoter is significantly less in LOAD.
152	real-time PCR	Target genes analysis	Human frontal cortex (Brodmann area 9)	10 AD and 10 age matched controls	CG islands in promoter regions for COX-2 and NF-κB are hypomethylated in AD, and those for BDNF and CREB are hypermethylated.
153	Mass spectrometry using the Sequenom EpiTYPER platform	Target gene analysis (SNAP25)	Human entorhinal, auditory cortices and hippocampus; and peripheral blood leukocytes	10 healthy elderly and 10 AD (brain); 22 young and 23 elderly controls and 24 AD (blood)	No difference in SNAP25 promoter DNA methylation
154	real-time PCR	Target gene analysis (PIN1)	Human peripheral blood mononuclear cells	32 LOAD; 28 age-matched controls	Methylation at the gene promoter is significantly less in LOAD.
155	Illumina Infinium HumanMethylation27 BeadArray	Genome wide DNA methylation association atudy (27,578 CGs)	Human frontal cortex	12 LOAD; 12 age/sex matched controls	948 CG sites from 918 genes show association with LOAD. The top hit is in promoter of TMEM59.
156	Real Time PCR and MALDI-TOF mass spectrometry	Target gene analysis (SORL1 and SIRL1)	Human entorhinal and auditory cortices and hippocampus; peripheral blood leukocytes	12 AD; 10 controls (brain); 36 AD; 25 young and 23 elderly controls(blood)	Neither genes show different in promoter DNA methylation in blood or brain
157	Custom microarray (Illumina's VeraCode GoldenGate DNA methylation assay); Pyrosequencing and Immunoblot analysis	The array includes 384 mouse genes, enriched in genes related to sensory perception, cognition, neuroplasticity, brain physiology and mental diseases. The average interrogated CGs is 2.7.	Human frontal cortex (Brodmann area 9]	20 AD [Braak stage V-VI] and 20 age/sex matched controls (human)	Four genes [TBXA2R, F2RL2, SORBS3 and SPTBN4] were hypermethylated in the frontal cortex of transgenic AD mice. Three of them were hypermethylated in humans.
158	dot blot immunochemistry	Global methylation (5-methylcytosine [5mC]; 5-hydroxymethylcytosine [5hmC], 5-formylcytosine [5fC] and 5-carboxylcytosine [5caC])	Human hippocampus/ parahippoc ampal gyrus (HPG) and cerebellum	5 age-matched controls, 5 preclinical AD (PAD) and 7 late-stage AD (LAD)	Increased levels of 5mC and 5hmC, and decreased levels of 5fC and 5caC in the HPG of AD subjects.
159	Immunohistoche mistry and image analysis	Global methylation (5-methylcytidine [5-mC] and 5-hydroxymethylcytidine [5-hmC]	Human hippocampus	10 AD, and 10 age matched controls; and a pair of monozygotic twins discordant for AD	Decrease in the hippocampal levels of 5-mC and 5-hmC in AD hippocampus.

Reference	Methodology	Main Approach	Samples/Tissue	N	Primary Finding
160	Immunohistochemistry	Global methylation (5-methylcytosine [5mC]; 5-hydroxymethylcytosine [5hmC])	Human middle frontal gyrus (MFG) and middle temporal gyrus (MTG)	13 AD (MFG) and 13 age matched controls; 29 AD (MTG) and 29 age matched controls	Hypermethylation and increased level of 5hmC in both AD MFG and MTG.
161	pyrosequencing	Target analysis on ACE, APOE, APP, BACE1, GSK3B, MAPT and PSEN1	Human inferior temporal lobe, the superior parietal lobe and the cerebellum	62 AD 106 Controls	15 CGs among APP, GSK3B, MAPT were differentially methylated in AD
162	methylation-sensitive high-resolution melting (MS-HRM) quantitative assay	Target analysis on repetitive elements of LINE-1	Human blood	28 Colombian LOAD and 30 controls	No differences in LINE-1 methylation levels
163	real-time PCR	Target analysis on 5-lipoxygenase (5-LOX)	Human blood	27 LOAD and 28 Age matched controls	Reduced methylation at 5-LOX gene promoter in AD
164	Mass spectrometry using the Sequenom EpiTYPER platform	Target analysis on CNP and DPYSL2	Human entorhinal and auditory cortices and hippocampus	10-11 AD; and 8-9 Controls	No differences in CNP and DPYSL2 promoter DNA methylation
165	385K DNA Methylation microarrays; Site-specific qPCR	Global and site specific methylation	Human neuroblastoma cells with and without Aβ treatment	-	Aβ changes DNA methylation of cell fate genes
166	mass spectrometry	Target analysis of two chaperones, HSPA8 and HSPA9	Human entorhinal and auditory cortices and hippocampus; peripheral blood leukocytes	12 AD; 10 controls (brain); 34 AD; 23 elderly controls(blood)	No DNA methylation differences are observed between AD and controls
167	Illumina Infinium HumanMethylati on450 BeadChip	Genome-wide DNA methylation analysis	Human buccal epithelial cells	10 adults with Down Syndrome and 10 controls	5 CpGs are correlated with cognitive function including two in the TSC2 gene.
168	Immunohistochemistry	Four cytosine modifications, including 5-methylcytosine (5-mC), 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC)	Human entorhinal cortex and cerebellum	13 AD and 8 controls	decrease in global 5-hmC in AD; and a significant difference in 5-fC between brain regions in AD

Table 3

Summary of studies on Histone Marker and AD using Human Tissue

Reference	Methodology	Main Approach	Samples/Tissue	N	Primary Finding
169	SRM-based targeted proteomics	H3 K18/K23 acetylation	Human temporal lobe tissue	5 AD with minor CAA; 6 AD with severe CAA, and 4 age matched controls	histone H3 K18/K23 acetylation is down-regulated in AD

Table 4

Summary of studies on miRNA and AD using Human Tissue

Reference	Methodology	Main Approach	Samples/Tissue	N	Primary Finding
170	Microarray	miRNA profiling	Human temporal cortex	34 dementia and 21 non-dementia (5 cases and 5 controls used for miRNA profiling)	miR-29a/b-1 level decreased in AD and is associated with abnormally high BACE1 protein
171	miRNA array and Northern blot	Target miRNA analysis	Human temporal cortex	6 AD, and 6 controls	Upregulated miRNA-9, miRNA-125b and miRNA-146a in AD
172	probe-specific Taqman miRNA assays	Target miRNA analysis	Human temporal cortex or cerebellum	19 dementia; 11 non-dementia	Decreased miR-106b expression in AD
173	microarrays	Genome-wide analysis	Human parietal lobe cortex	4 AD and 4 age matched controls	miRNA and target mRNA associations are changed in AD.
174	Real time qPCR	Target miRNA analysis (miR-29a, miR-29b and miR-338-3p)	Human frontal cortex	7 AD patients and 14 non-AD	miR-29a is down-regulated in AD
175	Real time qPCR	Target miRNA analysis (miR-485-5p)	Human parietal lobe and cerebellum (Set 1); Human superior frontal gyrus, entorhinal cortex, hippocampus and cerebellum (Set 2)	5 AD and 5 matched controls for each region (Set 1); 35 AD and 35 matched controls (Set 2)	miR-485-5p is down-regulated in AD
176	Fluorescent miRNA array and northern dot-blot	Target miRNA analysis (miR-146a)	Human superior temporal cortex (Brodmann area A22), hippocampus, brain stem, thalamus, or cerebellum	36 AD and 30 controls	miR-146a is up-regulated in AD, which differentially regulates IRAK-1 and IRAK-2 expression.
177	Real time qPCR	Target miRNA analysis (miR-107)	Human temporal cortex	19 elderly aged 73 and above	Lower level of miR-107 expression is associated with increased neuritic plaque counts and neurofibrillary tangles
178	Microarrays and northern blot	Target miRNA analysis	Human superior and middle temporal cortex (Brodmann Areas 21/22)	10 elderly females	Differential white matter and gray matter changes in miRNAs by AD pathologies
179	Real time qPCR	Target miRNA analysis (miR-137, -181c, -9, 29a, 29b-1, -15, -124)	Human neocortical brain samples	7 AD and 7 controls	Loss of miR-137, -181c, -9, and 29a/b-1 increases SPT and in turn Aβ levels
180	Real time qPCR	Target miRNA analysis (miR-124)	Human anterior temporal cortex	11 dementia and 11 non-demented controls	miR-124 is down-regulated in AD
181	Real time qPCR	Target miRNA analysis	Human frontal and temporal neocortex (Brodmann areas 6 and 22)	12 AD and 6 controls	Lower level of miR-128a/b in AD

Reference	Methodology	Main Approach	Samples/Tissue	N	Primary Finding
182	Real time qPCR	Target miRNA analysis (miR-137, -181c, -9, 29a, 29b)	Human blood serum	7 AD, 7 MCI, and 7 controls	miRNA levels were down-regulated in the sera of probable AD and amnestic MCI/probable early AD
183	Western blot	Target miRNA analysis	Human frontal cortex	20 AD and 5 age matched controls	Reduced miR-339-5p levels in AD brain
184	nCounter Human miRNA assay Kit	Medium-throughput miRNAs profiling	Human prefrontal cortex and hippocampus	42 AD (7 for each Braak Stage) and 7 controls; (prefrontal cortex) 41 LOAD; 23 controls (hippocampus)	deregulation of a subset of microRNAs in the hippocampus and prefrontal cortex in AD
185	IlluminaHiSeq 2000 sequencing; and Real time qPCR	next-generation sequencing; and target analysis of 12-miRNA signaturess	Human blood	48 AD and 5 age matched controls (sequencing); 202 (target analysis)	140 unique miRNAs show different expression levels in AD
186	Illumina Truseq Small RNA sequencing platform; qRT-PCR	Deep sequencing of miRNA sequences; Target analysis of miR-132	Human temporal cortex (Brodmann areas 21/22)	5 AD and 2 controls (sequencing) 8 AD and 8 controls (Validation)	No apparent difference in miRNA.
187	Real time qPCR	Target miRNA analysis (40 selected brain-enriched miRNAs)	Human temporal cortex	6 mild AD (Braak III), 6 severe AD (Braak VI) and age matched controls	miR-132 and miR-212 are downregulated in AD brains