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Brief communication

Increased DNA methylation near TREM2 is consistently seen in the superior temporal gyrus in Alzheimer's disease brain

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ABSTRACT

Although mutations within the *TREM2* gene have been robustly associated with Alzheimer's disease, it is not known whether alterations in the regulation of this gene are also involved in pathogenesis. Here, we present data demonstrating increased DNA methylation in the superior temporal gyrus in Alzheimer's disease brain at a CpG site located 289 bp upstream of the transcription start site of the *TREM2* gene in 3 independent study cohorts using 2 different technologies (Illumina Infinium 450K methylation beadchip and pyrosequencing). A meta-analysis across all 3 cohorts reveals consistent AD-associated hypermethylation (p = 3.47E-08). This study highlights that extending genetic studies of *TREM2* in AD to investigate epigenetic changes may nominate additional mechanisms by which disruption to this gene increases risk.

1. Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disease characterized by the accumulation of amyloid plaques and neurofibrillary tangles within the brain, ultimately leading to neuronal cell loss. This is accompanied by changes in behavior and personality followed by progressive cognitive decline. Although the neuropathology that characterizes the disease has been well described, little is known about the underlying mechanisms that drive disease onset and progression. As quantitative genetic analyses demonstrated high heritability estimates (58%–79%) for AD (Gatz et al., 2006), initial approaches to understand etiology focused on uncovering a genetic contribution to the disorder. In recent years, large cohort collections and the relatively inexpensive cost of assessing genetic variation through genome-wide association studies have allowed the identification of numerous common variants that are associated with increased risk of developing AD. Although common sequence variants in a number of genes have been robustly associated with AD via genome-wide association studies and subsequent meta-analyses (Escott-Price et al., 2015; Harold et al., 2009; Hollingworth et al., 2011; Lambert et al., 2013; Naj et al., 2011; Sleegers et al., 2010), collectively common single-nucleotide polymorphisms (SNPs) account for only 33% of attributable risks (Ridge et al., 2013) and the mechanism behind their action remains largely unknown.

With the advent of whole genome and exome sequencing, recent efforts have focused on identifying rare variants for AD with a larger effect size. The most robust locus identified through these studies is the rs75932628 SNP within the *TREM2* gene, which leads to a R47H substitution and has been nominated as a risk loci in numerous studies of AD (Forabosco et al., 2013; Guerreiro et al., 2013; Jonsson et al., 2013; Neumann and Daly, 2013). The minor T allele at this locus significantly increases the risk of AD (odds ratio 2.92) (Jonsson et al., 2013). Although the genetic epidemiology of *TREM2* in AD has now been extensively explored, only 1 study to date has explored whether alterations in the regulation of this gene, independent of genotype, may be associated with AD (Celarain et al., 2016). Epigenetic processes occur independently of DNA sequence variation, dynamically regulating gene expression, and are mediated principally through chemical modifications to DNA and nucleosomal histone proteins. DNA methylation is the best characterized modification modulating the transcription of

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mammalian genomes, and the first genome-scale studies to assess DNA methylation (epigenome-wide association studies [EWAS]) in AD brain have recently been published (De Jager et al., 2014; Lunnon et al., 2014). One of these studies highlighted hypermethylation at cg25748868, which is annotated to the *TREM2* gene, in the superior temporal gyrus (STG) associated with increased Braak stage (Lunnon et al., 2014). The aim of the present study was to measure DNA methylation in the STG at this locus in 2 further independent AD sample cohorts and to perform a meta-analysis on the 3 cohorts.

2. Materials and methods

2.1. Sample information

STG brain tissue was obtained from 3 independent sample cohorts from 3 different brain banks. Cohort 1 consisted of 95 samples acquired from the MRC London Neurodegenerative Disease Brain Bank (http://www.kcl.ac.uk/iop/depts/cn/research/MRC-London-Neurodegenerative-Diseases-Brain-Bank/MRC-London-Neurodegenerative-Diseases-Brain-Bank.aspx). Cohort 2 consisted of 103 samples acquired from the Mount Sinai Alzheimer's Disease and Schizophrenia Brain Bank (http://icahn.mssm.edu/research/labs/neuropathology-and-brain-banking) (Haroutunian et al., 1998). Cohort 3 consisted of 192 samples acquired from the Thomas Willis Oxford Brain Collection (http://www.medsci.ox.ac.uk/optima/information-for-patients-and-the-public/the-thomas-willis-oxford-brain-collection) (Esiri, 1993). For all cohorts, samples were classified as either controls (Braak 0–II) or AD cases (Braak V–VI). All samples were dissected by trained specialists, snap frozen, and stored at −80 °C. Further information about the samples is provided in Table 1.

2.2. DNA isolation and sodium bisulfite treatment

Genomic DNA was isolated from ~100 mg of each dissected brain region using a standard phenol–chloroform extraction method and tested for degradation and purity before the analysis. Five hundred–nanogram DNA from each sample was sodium bisulfite treated using the Zymo EZ-96 DNA methylation kit (Zymo Research) according to the manufacturer's standard protocol.

2.3. Genome-wide DNA methylation analysis

Samples from cohorts 1 and 2 were assessed using the Illumina Infinium 450K methylation beadchip (Illumina) using an Illumina HiScan System (Illumina). All samples were assigned a unique code for the purpose of the experiment and randomized with respect to sex and disease status to avoid batch effects and processed in batches of 4 BeadChips. Illumina Genome Studio software was used to extract the raw signal intensities of each probe (without background correction or normalization). All computations and statistical analyses were performed using R 3.2.1 (R Development Core Team, 2015) and bioconductor 3.1 (Gentleman et al., 2004). Data were loaded into R using the methylumi package (Davis et al., 2014) as a methylumi object. Initial quality control checks were performed using functions in the methylumi package to assess concordance between reported and genotyped gender. Data were preprocessed in the R package wateRmelon using the dasen function as previously described (Pidsley et al., 2013). EWAS data for cohorts 1 and 2 are available on Gene Expression Omnibus (GEO) under accession numbers GSE59685 and GSE80970, respectively.

2.4. Bisulfite-pyrosequencing analysis

Bisulfite pyrosequencing was used to quantify DNA methylation at cg25748868 (chr6:41,131,213) (GRCh37 Hg18) upstream of the *TREM2* transcription start site (TSS) in cohort 3. A single amplicon (134 bp) was amplified using primers designed using the PyroMark Assay Design software 2.0 (Qiagen) and tested for specificity in our laboratories (forward primer = GAGGGTTTTGGTTTTTA AAGGTATAG; reverse primer = TACAAAACCTAACCCAAAAATCAC, sequencing primer = ATTTTTGTAAGGTTGAAATTAGA). DNA methylation was quantified using the Pyromark Q24 system (Qiagen) following the manufacturer's standard instructions and the Pyro Q24 CpG 2.0.6 software.

2.5. Genotyping

Genotyping for rs75932628 within exon 2 of *TREM2* was carried out by LGC Genomics, Herts, UK.

2.6. Statistical analyses

All 3 cohorts were analyzed independently. Data were adjusted for the effects of age and gender, and linear regression models were used to compare control brain samples (Braak scores 0–II) with AD brain samples (Braak scores V–VI) using the linear model function in R. A Fisher's meta-analysis of p values was performed in the MetaDE package within R (Wang et al., 2012). We later estimated neuron/glia proportions in data generated on the Illumina Infinium 450K methylation beadchip using the Cell Epigenotype Specific Model algorithm (Guintivano et al., 2013) and reanalyzed the data in cohorts 1 and 2 using this estimate as an additional covariate.

3. Results

We recently published an EWAS of AD demonstrating increased DNA methylation in the STG associated with the Braak stage in cohort 1 at cg25748868 (nominal p = 8.81E-05) (Lunnon et al., 2014), which was observed to a lesser extent in the entorhinal cortex (p = 0.0323) but not in the prefrontal cortex or cerebellum ($p > 0.05$). Cg25748868 is located 289 bp upstream of the TSS of the *TREM2* gene. Given the interest in this gene in AD due to the

Table 1
Sample demographics for the study

	Cohort 1 (London)		Cohort 2 (Mount Sinai)		Cohort 3 (Oxford)	
	Controls (Braak 0–II)	AD cases (Braak V–VI)	Controls (Braak 0–II)	AD cases (Braak V–VI)	Controls (Braak 0–II)	AD cases (Braak V–VI)
Number of individuals	29	66	59	44	75	117
Gender (male and/or female)	13/16	26/40	31/28	12/32	44/31	50/67
Age at death (±SD)	77.6 (12.80)	85.4 (8.13)	82.1 (7.56)	88.0 (7.53)	84.1 (7.72)	78.4 (9.27)
Method used to assess DNA methylation	Illumina 450K		Illumina 450K		Pyrosequencing	

Key: AD, Alzheimer's disease; SD, standard deviation.

the recently described novel risk variant, we were keen to examine whether we could replicate this differentially methylated position (DMP) in independent AD brain samples. As our previous study in cohort 1 used linear regression models to assess DNA methylation associated with Braak stage in 113 individuals with an even representation of samples across the entire Braak spectrum and as validation cohort 3 only had samples available with a Braak stage ≤II or ≥V, we initially reanalyzed our data in cohort 1 using a case (Braak ≥V) or control (Braak ≤II) analysis (N = 95 individuals in these groups) to allow a comparable analysis across all 3 cohorts. In our case–control analysis in cohort 1, we observed a similar association of increased DNA methylation at cg25748868 in the STG (Fig. 1A; DNA methylation difference = 1.4%, $p = 1.06E-04$) as reported in our published quantitative Braak stage model data. One potential confounder of epigenetic data generated in a heterogeneous tissue such as the brain is that methylomic differences may simply reflect a difference in cellular abundance between cases and controls. As such, we utilized a published bioinformatic algorithm that can predict neuron/glia proportions in data generated from the Illumina Infinium 450K methylation beadchip (Guintivano et al., 2013). When we included these estimates as covariates in the model, we still observed a significant increase in DNA methylation at this loci in cases ($p = 6.31E-04$). We next assessed this specific probe in a second independent cohort of 103 STG samples (cohort 2), again identifying increased DNA methylation associated with AD at this locus (Fig. 1A; DNA methylation difference = 0.73%, $p = 0.020$), which still remained significant when we controlled for neuron/glia proportions ($p = 0.026$).

To independently validate our findings using another technology, we quantified DNA methylation at the same site using bisulfite pyrosequencing in a third cohort of 192 STG samples (cohort 3), again finding significantly higher DNA methylation associated with AD (Fig. 1A; DNA methylation difference = 0.72%, $p = 4.01E-06$). A meta-analysis of this DMP across the 3 cohorts showed significant hypermethylation associated with AD (Fig. 1B; $p = 3.47E-08$). As the rare SNP at rs75932628 has previously been shown to increase AD risk (Guerreiro et al., 2013; Jonsson et al., 2013), we were interested to investigate whether this SNP is also associated with DNA methylation at cg25748868. When we compared DNA methylation levels at cg25758868 between carriers and noncarriers of the SNP across all cohorts, we saw no significant difference between the groups ($p = 0.488$). However, as the presence of the T allele is rare within the population [minor allele frequency = 0.002 (Genomes Project Consortium et al., 2012)], we only observed heterozygosity in 11 individuals across the study (2 individuals in cohort 1, 6 individuals in cohort 2, and individuals in cohort 3).

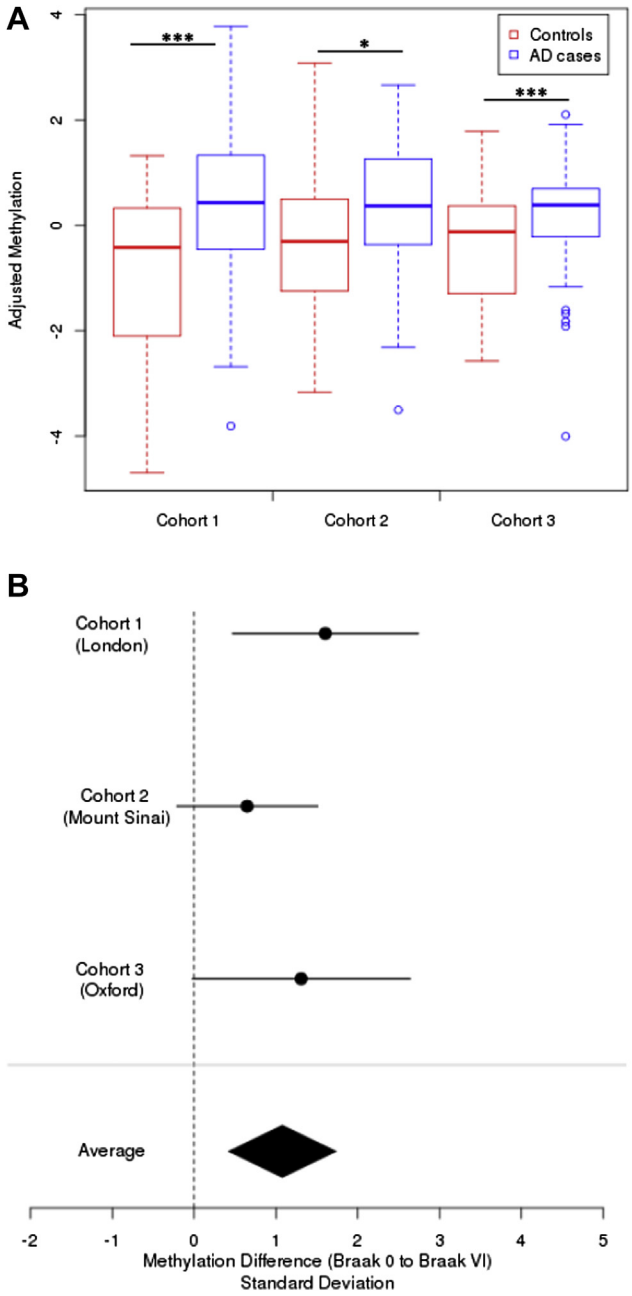

Fig. 1. DNA methylation is consistently increased in AD samples compared to controls in the STG at chr6:41,131,213. (A) Adjusted methylation levels of cg25748868 in the STG in controls (Braak stage 0–II) and AD (Braak stage V–VI) samples from 3 cohorts. In all 3 cohorts, DNA methylation at this loci is increased in AD cases relative to controls. (B) Forest plot of meta-analysis of effect size of cg25748868 across the 3 cohorts. Key: $^{*}p < 0.05$ and $^{***}p < 0.005$. Abbreviations: AD, Alzheimer's disease; STG, superior temporal gyrus.

4. Discussion

Mutations in *TREM2* have been robustly associated with AD risk. In addition to the well-described mutation at rs75932628, other rare mutations have also been described; for example, D87N (rs142232675) also increases susceptibility to AD (Guerreiro et al., 2013). More recently, common variants across the TREM gene cluster have been associated with AD pathology or susceptibility, including rs6910730 in *TREM1*, rs9381040 and rs6916710 in *TREML2*, and rs7759295 and rs6922617 which are intergenic within the TREM gene cluster (Benitez et al., 2014; Chan et al., 2015; Cruchaga et al., 2013; Lambert et al., 2013; Replogle et al., 2015). In the present study, we observed a small but consistent increase in DNA methylation at a CpG site 289 bp upstream of the TSS in the *TREM2* gene in the STG in AD brain across all cohorts. The *TREM2* gene resides on chromosome 6 which is 4680 bp in length (chr6:41,126,244–41,130,924) and contains 5 exons, and it is known to be expressed as 3 transcript variants. The Illumina Infinium 450K methylation beadchip has limited coverage of the *TREM2* gene, with only 7 probes spanning the gene. When we looked for AD-associated differential methylation at the remaining 6 probes within the gene, we saw no significant difference in either cohort 1 or 2. *TREM2* is expressed by macrophages and microglia, and its expression, particularly within the cytoplasm, is dramatically increased in activated microglia (Sessa et al., 2004). *TREM2* is predominantly expressed intracellularly, in the Golgi complex and in cytoplasmic vesicles, but *TREM2* must be

expressed at the cell surface in order to be functional. In response to cell stimulation, *TREM2* is exocytosed to the cell surface, where it is able to act as a functional receptor (Prada et al., 2006). Cell surface expressed *TREM2* is associated with its signaling counterpart *DAP12* (also called tyrosine kinase binding protein [TYROBP]) forming a molecular complex that promotes phagocytosis. In vitro, *TREM2* knockdown microglia have defective clearance of apoptotic neurons and increased synthesis of tumour necrosis factor (TNF)-α and iNOS, while overexpression of microglial *TREM2* increases phagocytic activity and reduces the production of the proinflammatory mediators TNF-α, interleukin (IL)-1β, and inducible nitric oxide synthase (iNOS) (Takahashi et al., 2005), indicating that *TREM2* may promote a phagocytic anti-inflammatory phenotype in microglia. Interestingly, one recent study highlighted an association of the inflammatory *CD33* AD risk allele with increased *TREM2* cell-surface expression, as well as higher cortical *TREM2* RNA expression with increasing amyloid pathology, supporting a potentially pathogenic role for increased *TREM2* expression in AD (Chan et al., 2015). Another recent study has demonstrated an increased expression of *TREM2* in the hippocampus in AD (Celarain et al., 2016). Interestingly, this study used bisulfite clonal sequencing and 5-hydroxymethylated DNA immunoprecipitation combined with quantitative real time-PCR (qRT-PCR) (5hMeDIP-qRT-PCR) to additionally assess DNA methylation and DNA hydroxymethylation in *TREM2* in the same samples. In line with our findings they reported increased DNA methylation in the *TREM2* TSS in AD. Further they reported a significant correlation of *TREM2* messenger RNA expression and 5-hydroxymethylation levels in exon 2 of *TREM2*. Although traditionally DNA methylation in CpG islands is associated with gene silencing, recent data suggest that the relationship between DNA methylation and transcription may be more complex, with gene body methylation often being associated with active gene expression and alternative splicing (Smith et al., 2016). Interestingly, the CpG site we investigated resides within a transcription binding site for the transcriptional repressor protein YY1.

5. Conclusions

This study further explored a DMP in *TREM2* identified in our recent EWAS in AD brain and demonstrated consistent hypermethylation in AD across 3 independent cohorts. This opens up the possibility that *TREM2* gene regulation may also be important in the etiology of AD, and further studies to examine other epigenetic mechanisms along the entire length of the *TREM2* gene should be undertaken. As noted earlier, the Illumina Infinium 450K methylation beadchip only contains 7 probes spanning the *TREM2* gene, and thus, it would be of interest to bisulfite sequence the entire gene to nominate additional differentially methylated loci that could not be identified in the current study. It will also be of considerable interest to correlate DNA methylation changes along the entire gene with levels of gene expression, given that the recent study by Celarain et al. (2016) identified increased *TREM2* gene expression and DNA methylation in AD hippocampus (Celarain et al., 2016). Although we investigated whether the DMP we identified may be influenced by genotype at rs759232628, which has been strongly associated with increased AD risk, the rarity of this SNP in the population meant that we were not sufficiently powered to make any firm conclusions and future work should be undertaken to examine DNA methylation in individuals harboring the risk allele in larger sample cohorts. Finally, although we have shown consistent hypermethylation at this DMP in AD brain, our experiments were performed in tissue homogenates, and as such, it is possible that this may represent cell proportion differences in people with AD, given the widespread neuronal loss at late stage disease. Although we were able to control for neuron/glia proportions in cohorts 1 and 2, in the future, further studies could be undertaken to measure DNA methylation in pure microglia isolated from AD brain, once these methods are available.

Disclosure statement

The authors have no actual or potential conflicts of interest.

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