



Brief communication

Blood methylomic signatures of presymptomatic dementia in elderly subjects with type 2 diabetes mellitus



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ARTICLE INFO

Article history:

Accepted 15 October 2014

Available online 24 December 2014

Keywords:

DNA methylation

Biomarker

Epigenetics

Alzheimer's disease (AD)

Blood

Type 2 diabetes

Dementia

ABSTRACT

Due to an aging population, the incidence of dementia is steadily rising. The ability to identify early markers in blood, which appear before the onset of clinical symptoms is of considerable interest to allow early intervention, particularly in “high risk” groups such as those with type 2 diabetes. Here, we present a longitudinal study of genome-wide DNA methylation in whole blood from 18 elderly individuals with type 2 diabetes who developed presymptomatic dementia within an 18-month period following baseline assessment and 18 age-, sex-, and education-matched controls who maintained normal cognitive function. We identified a significant overlap in methylomic differences between groups at baseline and follow-up, with 8 CpG sites being consistently differentially methylated above our nominal significance threshold before symptoms at baseline and at 18 months follow up, after a diagnosis of presymptomatic dementia. Finally, we report a significant overlap between DNA methylation differences identified in converters, only after they develop symptoms of dementia, with differences at the same loci in blood samples from patients with clinically diagnosed Alzheimer's disease compared with unaffected control subjects.

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1. Introduction

Alzheimer's disease (AD) is a chronic, currently incurable, neurodegenerative disorder with more than 26 million cases worldwide and accounting for approximately 60% of dementia cases (Brookmeyer et al., 2007). With an increasingly aging population, new estimates for dementia incidence predict >115 million cases worldwide by 2050 (Prince et al., 2013). AD is characterized by the accumulation of extracellular amyloid plaques and intracellular neurofibrillary tangles, leading to selective neuronal cell

loss, behavioral and personality changes, and ultimately death after many years of suffering. Although much progress has been made in understanding the molecular pathology of AD, the treatments currently available only temporarily alleviate some symptoms and do not modify the underlying pathology. By the time an individual becomes symptomatic, there is already considerable neuronal cell loss, plaque deposition, and neurofibrillary tangle burden within the brain, which can appear years before a clinical diagnosis is made (Jack et al., 2010). Although most of the AD cases are sporadic, occur later in life (aged >65 years) and have no known cause, the disease is associated with several vascular risk factors, especially type 2 diabetes (T2D) mellitus (Arvanitakis et al., 2004; Stewart and Liolitsa, 1999), which more than doubles the risk of developing AD (Schnaider Beeri et al., 2004).

A number of studies have aimed to identify blood-based biomarkers for AD. These have identified changes in the abundance of plasma or serum proteins (Hye et al., 2006, 2014; O'Bryant et al., 2010, 2011; Ray et al., 2007; Thambisetty et al., 2008) and specific gene expression signatures (Booij et al., 2011; Fehlbaum-Beurdeley et al., 2012; Lunnon et al., 2013; Rye et al., 2011), some of which differentiate

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AD patients, and even persons with mild cognitive impairment, from elderly control subjects with normal cognition. Recently, we have started to examine whether epigenetic (DNA methylation) changes are seen in white blood cells in AD sufferers compared with elderly nondemented control subjects (Lunnon et al., 2014).

The ability to identify early peripheral molecular signatures associated with the onset of dementia in “high risk” groups is of particular importance for the development of preventive interventions. To this end, the aim of the present study was to identify DNA methylation differences in whole blood obtained from a longitudinal analysis of T2DM patients developing presymptomatic dementia symptoms over an 18-month period, compared with those remaining cognitively normal.

2. Methods

2.1. Subjects and samples

This study builds on the longitudinal Israel Diabetes and Cognitive Decline study, which investigates the effects of long-term T2D-related characteristics on cognitive decline (Ravona-Springer et al., 2013). The Israel Diabetes and Cognitive Decline study design and subject selection is described in detail in the [Supplementary Methods](#). The Clinical Dementia Rating (CDR) scale and neurologic and psychiatric assessments were used to define intact cognition (CDR = 0) at study entry. For the purposes of this study, we chose the first 18 subjects whose cognition at 18-months follow-up declined to CDR = 0.5 (i.e., presymptomatic dementia; converters) confirmed by a multidisciplinary diagnostic consensus conference. Eighteen control subjects (nonconverters), that is, individuals whose normal cognition was maintained at follow up based on a CDR = 0 and confirmed in consensus conference, were matched to the converters for age, sex, and number of years of education. Subject characteristics are summarized in [Supplementary Table 1](#).

2.2. Methylation profiling

Genomic DNA was isolated in individuals at baseline and follow-up from whole blood stored in ethylenediaminetetraacetic acid collection tubes using a standard phenol-chloroform extraction method and tested for degradation and purity before analysis. DNA (500 ng) from each sample was treated with sodium bisulfite using the Zymo EZ96 DNA methylation kit (Zymo Research, CA, USA) according to the manufacturer’s standard protocol. Samples were assessed using the Illumina Infinium HumanMethylation450K BeadChip (Illumina Inc, CA, USA) using the Illumina HiScan System (Illumina). All samples were grouped by individual with their age- and sex-matched pair processed alongside. All samples were processed in a single batch of 6 BeadChips. Illumina Genome Studio software was used to extract the raw signal intensities of each probe (without background correction or normalization).

2.3. Data analysis

All computations and statistical analyses were performed within the R statistical environment (version 2.15.3) (R Development Core Team, 2012) and Bioconductor 2.14 (Gentleman et al., 2004). Signal intensities were imported into R using the methylumi package (Davis et al., 2012). Initial quality control checks were performed to assess concordance between reported and genotyped gender. Non-CpG SNP probes on the array were used to confirm that longitudinal samples were sourced from the same individual. Data were pre-processed using *wateRmelon* (version 1.4.0) using the *dasen* function as previously described (Pidsley et al., 2013), with samples being excluded for failing quality control checks detailed in

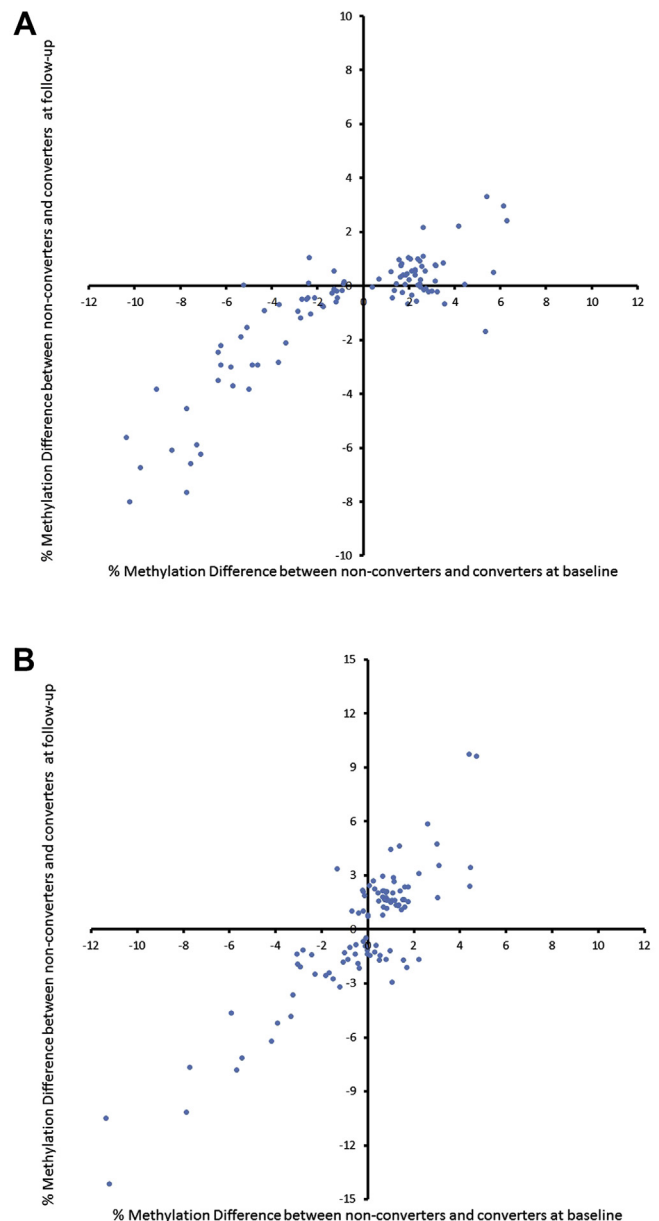


Fig. 1. Correlation of the percentage methylation differences for the 100 top-ranked DMPs across both time points. DNA methylation differences for the 100 most significant DMPs at baseline (shown in [Supplementary Table 2](#)) (x-axis) are significantly correlated ($r = 0.856$, $p = 5.84 \times 10^{-30}$) with DNA methylation differences in the same probes at 18 months follow up (y-axis) (A). Similarly, DNA methylation differences for the 100 most significant DMPs at 18 months follow-up (shown in [Supplementary Table 3](#)) (y-axis) are significantly correlated ($r = 0.872$, $p = 1.49 \times 10^{-32}$) with DNA methylation differences in the same probes at baseline (x-axis) (B). Abbreviation: DMP, differentially methylated position.

[Supplementary Table 1](#). Non-CpG SNP probes, nonspecific probes, and probes that have been reported to contain common (MAF >5%) SNPs in the CpG position or single base extension position were flagged and removed from analyses, leaving 388,850 probes (Chen et al., 2013).

We identified differentially methylated positions (DMPs) by comparing nonconverters and converters at both baseline and 18-month follow-up using linear models at each time point separately, while controlling for the effects of age, sex, Hba1c, and years of education. The Genomic Regions Enrichment of Annotations Tool (GREAT) (McLean et al., 2010) was used to annotate probes with

Table 1
CpG sites consistently differentially methylated at baseline and 18-month follow-up representing robust markers of conversion

Probe	Position	CpG density	Illumina annotation	Genes upstream		Genes downstream		Baseline				18-mo follow-up			
				p	%	p	%	p	%	p	%	p	%	p	%
cg11609366	8:146018277	Island	RPL8	RPL8 (-473)	HIF1AN (+121, 724)	2.07 × 10 ⁻⁴	8.05	9.10	1.04	5.8 × 10 ⁻⁴	8.08	9.11	1.03		
cg03613178	10:102417364	N_Shore		PAX2 (-88, 103)	TIME117 (+628, 108)	8.46 × 10 ⁻⁵	66.91	59.16	-7.75	9.65 × 10 ⁻⁵	66.69	59.04	-7.65		
cg11964314	12:44858069			NELL2 (+412, 563)	CBFA2T3 (+9211)	9.34 × 10 ⁻⁴	70.06	65.87	-4.19	8.08 × 10 ⁻⁵	71.04	64.87	-6.17		
cg08317738	16:89034292	Island	CBFA2T3	PABPN1L (-101, 225)	SPG7 (+22, 485)	8.11 × 10 ⁻⁴	44.93	33.70	-11.24	1.54 × 10 ⁻⁴	46.24	32.13	-14.11		
cg03586649	16:89597289	S_Shelf	SPG7	RPL13 (-29, 775)	MAT2K6 (+492, 952)	6.11 × 10 ⁻⁴	89.35	90.69	1.340	6.97 × 10 ⁻⁵	89.36	90.74	1.39		
cg26999431	17:67903789			KCNJ16 (-167, 636)	PQLC1 (+73, 206)	7.09 × 10 ⁻⁴	83.37	84.94	1.57	2.12 × 10 ⁻⁴	83.35	85.01	1.67		
cg16488953	18:77638446	Island	KCNJ2	KCNJ2 (+14, 779)	BHLHE23 (-22, 424)	4.37 × 10 ⁻⁴	95.13	95.73	0.61	8.61 × 10 ⁻⁴	95.24	95.67	0.43		
cg085664027	20:61660810	Island		BIRC7 (-206, 465)		9.62 × 10 ⁻⁴	76.02	86.88	10.86	2.84 × 10 ⁻⁴	75.41	87.84	12.43		

Probes that had a difference in methylation between converters and nonconverters at both time points were selected ($p < 0.001$). Shown for each DMP is chromosomal location (Hg19), Illumina gene annotation, up and/or downstream genes (from GREAT annotation), p -value from linear model at baseline and at follow up and normalized mean group methylation values and difference between groups after controlling for the effect of age, sex, Hba1 and years of education (Δ). Data are organized in genomic by location.

Key: Δ , difference between groups; % C, percentage methylation in converters; DMP, differentially methylated position; GREAT, Genomic Regions Enrichment of Annotations Tool; % NC, percentage methylation in nonconverters.

upstream and downstream genes. Similarities of DNA methylation differences across data sets were assessed using the Pearson correlation. Raw microarray data are available in the online Gene Expression Omnibus (accession number: GSE62003).

3. Results and discussion

The top-ranked DMPs at baseline and 18-month follow-up are shown in [Supplementary Tables 2 and 3](#). A particular interest was the identification of stable DNA methylation differences between converters and nonconverters, detectable at both time points. In this regard, it is notable that DNA methylation differences for the 100 top-ranked DMPs at baseline are strongly correlated with DNA methylation differences at the same probes at 18-month follow-up ([Fig. 1A](#); $r = 0.856$, $p = 5.84 \times 10^{-30}$). Similarly, DNA methylation changes for the 100 top-ranked DMPs at follow-up are strongly correlated with DNA methylation differences at the same probes at baseline ([Fig. 1B](#); $r = 0.872$, $p = 1.49 \times 10^{-32}$). Furthermore, using a nominal p -value threshold ($p \leq 0.001$), we identified 8 probes that were differentially methylated at both time points ([Table 1](#); [Supplementary Fig. 1](#)). This group of DMPs may represent early and consistent markers of cognitive change. Of these, 4 DMPs were hypermethylated in converters at both time points and 4 were hypomethylated at both time points. One of these probes is located in close proximity to *RPL13*. Interestingly, DNA methylation in the vicinity of *RPL13* has been previously associated with AD pathology in postmortem brain ([De Jager et al., 2014](#); [Lunnon et al., 2014](#)). Although the other identified loci have not been robustly associated with dementia, they could still represent novel biomarkers. We have previously shown that the most significant DMPs in blood between AD patients and non-demented control individuals are, as expected, very distinct from those seen in the brain ([Lunnon et al., 2014](#)), and it is plausible that novel DMPs identified in this study represent a peripheral response by leukocytes to early disease changes in the brain, rather than a direct reflection of neuropathologic changes observed in the brain.

Having previously identified a number of CpG sites that are differentially methylated in whole blood in AD patients compared with elderly non-demented control subjects ([Lunnon et al., 2014](#)), we were interested in investigating whether any of the DMPs identified in the present study overlapped with loci differentially methylated in clinically diagnosed AD patients. We found no significant correlation between DNA methylation differences at the 100 top-ranked converter-associated DMPs at baseline with our previously reported AD-associated DMPs ([Supplementary Fig. 2](#); $r = -0.165$, $p = 0.101$). Interestingly, however, there was a significant correlation between DNA methylation differences at the top-ranked DMPs identified postconversion with differences seen at the same CpG sites between control and clinically defined AD subjects ([Supplementary Fig. 3](#); $r = 0.32$, $p = 1.29 \times 10^{-3}$). This indicates that the differences seen in converters at follow-up, after they display symptoms of presymptomatic dementia, reflect differences identified in clinically presenting AD patients. Differences between converters and nonconverters at baseline, however, are not seen in clinically recognized AD patients. This reinforces the hypothesis that epigenetic differences identified in the blood from AD patients most likely reflect peripheral responses to the disorder, rather than causally related variation. Such changes are, however, potentially useful as biomarkers of underlying neuropathology.

4. Conclusions

Given the predicted increase in dementia incidence, the identification of early and robust markers of disease, which are detectable before the emergence of clinical symptoms, is of utmost importance,

particularly in “high risk” groups. This study identified a number of DMPs in blood samples from T2D patients after they had developed presymptomatic dementia, which are also altered in white blood cells from AD patients and could thus represent early markers of dementia. This study also demonstrated robust alterations at several CpG sites in blood samples from T2D patients at baseline who developed presymptomatic dementia. These loci were altered before the emergence of clinical symptoms and remained altered after conversion. Interestingly, one of these CpG sites resides close to a gene at which DNA methylation has been previously associated with dementia (*RPL13*) (De Jager et al., 2014; Lunnon et al., 2014). Although the other identified CpG sites have not been robustly associated before with dementia, they still could represent part of an early peripheral response to dementia and serve as potential biomarkers for early cognitive changes. Although the changes reported in this study do not reach genome-wide significance, this is not surprising given the relatively small number of samples. An optimum level of significance for epigenome-wide association studies has yet to be established, but given the nonindependence of DNA methylation across CpG sites and the nonvariable nature of most probes on the 450 K array (Mill and Heijmans, 2013), it is likely that a Bonferroni correction is overly stringent. The intraindividual, longitudinal, repeated-measure design used in this study is relatively robust and controls for many potential confounders in epigenetic epidemiology. Although, we were able to validate DMPs at 2 independent time points and in an independent study comparing nondemented control and clinically diagnosed AD patients, future research is needed to validate the findings from this pilot study in larger independent sample cohorts and to determine the exact specificity and timing of these changes.

Disclosure statement

The authors declare that they have no conflicts of interest in regard to this work.

Acknowledgements

This work was funded by NIH grant R01AG036039 to Jonathan Mill, NIH grant R01AG034087 to Michal Schnaider Beeri, and the Helen Bader Foundation, the Leroy Schecter Foundation, and the BrightFocus Foundation. The authors thank the Israel Diabetes and Cognitive Decline study subjects who made this research possible.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2014.12.023.

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