Blood methylomic signatures of pre-symptomatic dementia in elderly subjects with Type 2 Diabetes Mellitus

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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 (T2D). Here we present a longitudinal study of genome-wide DNA methylation in whole blood from 18 elderly individuals with T2D who developed pre-symptomatic 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 eight CpG sites, being consistently differentially methylated above our nominal significance threshold prior to symptoms at baseline and at 18 month follow up, after a diagnosis of pre-symptomatic 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 to unaffected controls.

1. Introduction

Alzheimer's disease (AD) is a chronic, currently incurable, neurodegenerative disorder with more than 26 million cases worldwide, and accounting for ~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 characterised by the accumulation of extracellular amyloid plaques, and intracellular neurofibrillary tangles, leading to selective neuronal cell loss, behavioural 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 the majority of AD cases are sporadic, occur later in life (age>65 years) and have no known cause, the disease is associated with several vascular risk factors, especially type 2 diabetes mellitus (T2D) (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,Hye, et al., 2014,O'Bryant, et al., 2010,O'Bryant, et al., 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 AD, and even persons with mild cognitive impairment (MCI), from elderly controls with normal cognition. Recently we have started to examine whether epigenetic (DNA methylation) changes are seen in white blood cells in AD sufferers compared to elderly non-demented 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 current study was to identify DNA methylation differences in whole blood obtained from a longitudinal analysis of T2DM patients developing pre-symptomatic dementia symptoms over an 18 month period, compared to those remaining cognitively normal.

2. Methods

2.1 Subjects and Samples

This study builds on the longitudinal Israel Diabetes and Cognitive Decline (IDCD) study, which investigates the effects of long-term T2D-related characteristics on cognitive decline (Ravona-Springer, et al., 2013). The IDCD study design and subject selection is described in detail in the Supplementary Methods. The Clinical Dementia Rating (CDR) scale and neurological 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. pre-symptomatic dementia; converters) confirmed by a multidisciplinary diagnostic consensus conference. Eighteen control subjects (non-converters), i.e. 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 summarised in Supplementary Table 1.

2.2 Methylomic Profiling

Genomic DNA was isolated in individuals at baseline and follow up from whole blood stored in EDTA collection tubes using a standard phenol-chloroform extraction method and tested for degradation and purity prior to analysis. 500ng DNA 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, CA, USA). All samples were grouped by individual with their age and sex-matched pair processed alongside. All samples were processed in a single batch of six 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 (QC) 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 was pre-processed using *wateRmelon* (version 1.4.0) using the *dasen* function as previously described (Pidsley, et al., 2013), with two samples being excluded at baseline for failing QC (Supplementary Table 1). Non-CpG SNP probes, non-specific 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 non-converters and converters at both baseline and 18 month follow-up using linear models at each time point separately, whilst controlling for the effects of age, sex, Hbac1 and years of education. The Genomic Regions Enrichment of Annotations Tool (GREAT) (McLean, et al., 2010) was used to annotate probes with genes within 5000bp upstream, and 1000bp downstream. Similarities of DNA methylation differences across datasets was assessed using Pearson's correlation. Raw microarray data are available in the online Gene Expression Omnibus (GEO) (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 non-converters, 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 (Figure 1A; *0.856*, *P*=5.84E-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 (Figure 1B; *r*=0.872, *P*=1.49E-32). Furthermore, using a nominal p-value threshold ($p \leq 0.001$), we identified eight probes that were differentially methylated at both time points (Table 1; Supplementary Figure 1). This group of DMPs may represent early and consistent markers of cognitive change. Of these, four DMPs were hypermethylated in converters at both time points and four 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 post-mortem 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 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 neuropathological changes observed in the brain.

Having previously identified a number of CpG sites that are differentially methylated in whole blood in AD patients compared to elderly non-demented control subjects (Lunnon, et al., 2014), we were interested in investigating whether any of the DMPs identified in the current 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 Figure 2; *r*=0.165, *P*=0.101). Interestingly, however, there

was a significant correlation between DNA methylation differences at the top-ranked DMPs identified post-conversion with differences seen at the same CpG sites between control and clinically-defined AD subjects (Supplementary Figure 3; *r*=0.32, *P*=1.29E-3). This indicates that the differences seen in converters at follow-up, after they display symptoms of pre-symptomatic dementia, reflect differences identified in clinically-presenting AD patients. Differences between converters and non-converters 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 prior to the emergence of clinical symptoms, is of upmost importance, particularly in 'high risk' groups. This study identified a number of DMPs in blood samples from T2D patients after they had developed pre-symptomatic 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 pre-symptomatic dementia. These loci were altered prior to the emergence of clinical symptoms, and remained altered after conversion. Interestingly one of these CpG sites reside close to a gene 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 (EWAS) has yet to be established, but given the non-independence of DNA methylation across CpG sites and the non-variable nature of most probes on the 450K array (Mill and Heijmans, 2013), it is likely that a Bonferroni correction is overly stringent. The intra-individual 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 two independent time points and in an independent study comparing non-demented 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.

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Disclosure Statement

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

References

Arvanitakis, Z., Wilson, R.S., Bienias, J.L., Evans, D.A., Bennett, D.A. 2004. Diabetes mellitus and risk of Alzheimer disease and decline in cognitive function. Archives of neurology 61(5), 661-6.

Booij, B.B., Lindahl, T., Wetterberg, P., Skaane, N.V., Saebo, S., Feten, G., Rye, P.D., Kristiansen, L.I., Hagen, N., Jensen, M., Bardsen, K., Winblad, B., Sharma, P., Lonneborg, A. 2011. A gene expression pattern in blood for the early detection of Alzheimer's disease. Journal of Alzheimer's disease : JAD 23(1), 109-19.

Brookmeyer, R., Johnson, E., Ziegler-Graham, K., Arrighi, H.M. 2007. Forecasting the global burden of Alzheimer's disease. Alzheimer's & dementia : the journal of the Alzheimer's Association 3(3), 186-91.

Chen, Y.A., Lemire, M., Choufani, S., Butcher, D.T., Grafodatskaya, D., Zanke, B.W., Gallinger, S., Hudson, T.J., Weksberg, R. 2013. Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation450 microarray. Epigenetics : official journal of the DNA Methylation Society 8(2), 203-9.

Davis, S., Du, P., Bilke, S., Triche, J., =., Bootwalla, M. 2012. Methylumi: Handle Illumina Methylation Data 2012..

. R package version 220.

De Jager, P.L., Srivastava, G., Lunnon, K., Schalkwyk, L.C., Yu, L., Eaton, M.L., Keenan, B.T., Ernst, J., McCabe, C., Tang, A., Raj, T., Replogle, J., Brodeur, W., Gabriel, S., Chai, H., Younkin, C., Younkin, S.G., Zou, F., Szyf, M., Epstein, C.B., Schneider, J.A., Myers, A.J., Ertekin-Taner, N., Bernstein, B.E., Meissner, A., Chibnik, L.B., Kellis, M., Mill, J., Bennett, D.A. 2014. Alzheimer's disease pathology is associated with early alterations in brain DNA methylation at ANK1, BIN1 and other loci. Nature medicine ([Submitted]).

Fehlbaum-Beurdeley, P., Sol, O., Desire, L., Touchon, J., Dantoine, T., Vercelletto, M., Gabelle, A., Jarrige, A.C., Haddad, R., Lemarie, J.C., Zhou, W., Hampel, H., Einstein, R., Vellas, B. 2012. Validation of AclarusDx, a Blood-Based Transcriptomic Signature for the Diagnosis of Alzheimer's Disease. Journal of Alzheimer's disease : JAD 32(1), 169-81.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y., Zhang, J. 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome biology 5(10), R80.

Hye, A., Lynham, S., Thambisetty, M., Causevic, M., Campbell, J., Byers, H.L., Hooper, C., Rijsdijk, F., Tabrizi, S.J., Banner, S., Shaw, C.E., Foy, C., Poppe, M., Archer, N., Hamilton, G., Powell, J., Brown, R.G., Sham, P., Ward, M., Lovestone, S. 2006. Proteome-based plasma biomarkers for Alzheimer's disease. Brain : a journal of neurology 129(Pt 11), 3042-50.

Hye, A., Riddoch-Contreras, J., Baird, A., Ashton, N., Bazenet, C., Leung, R., Westman, E., Simmons, A., Dobson, R., Sattlecker, M., Lupton, M.K., Lunnon, K., Keohane, A., Ward, M., Pike, I., Zucht, H., Pepin, D., Zheng, W., Tunnicliffe, A., Richardson, J., Gauthie, S., Soininen, H., Kłoszewska, I., Mecocci, P., Tsolaki, M., Vellas, B., Lovestone, S. 2014. Plasma proteins predict conversion to dementia from prodromal disease. . Alzheimer's & dementia : the journal of the Alzheimer's Association Nov; 10(6), 799-80.

Jack, C.R., Jr., Knopman, D.S., Jagust, W.J., Shaw, L.M., Aisen, P.S., Weiner, M.W., Petersen, R.C., Trojanowski, J.Q. 2010. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. Lancet neurology 9(1), 119-28.

Lunnon, K., Sattlecker, M., Furney, S., Coppola, G., Simmons, A., Proitsi, P., Lupton, M.K., Lourdusamy, A., Johnston, C., Soininen, H., Kloszewska, I., Mecocci, P., Tsolaki, M., Vellas, B., Geschwind, D., Lovestone, S., Dobson, R., Hodges, A. 2013. A blood gene expression marker of early Alzheimer's disease. Journal of Alzheimer's disease : JAD 33(3), 737-53.

Lunnon, K., Smith, R., Hannon, E.J., De Jager, P.L., Srivastava, G., Volta, M., Troakes, C., Al-Sarraj, S., Burrage, J., Macdonald, R., Condliffe, D., Katsel, P., Haroutunian, V., Kaminsky, Z., Joachim, C., Powell, J., Lovestone, S., Bennett, D.A., Schalkwyk, L.C., Mill, J. 2014. Cross-tissue methylomic profiling in Alzheimer's disease implicates a role for cortex-specific deregulation of ANK1 in neuropathology. Nature neuroscience Sept; 17(9), 1156-63.

McLean, C.Y., Bristor, D., Hiller, M., Clarke, S.L., Schaar, B.T., Lowe, C.B., Wenger, A.M., Bejerano, G. 2010. GREAT improves functional interpretation of cis-regulatory regions. Nature biotechnology 28(5), 495-501.

Mill, J., Heijmans, B.T. 2013. From promises to practical strategies in epigenetic epidemiology. Nature reviews Genetics Aug; 14(8), 585-94.

O'Bryant, S.E., Xiao, G., Barber, R., Reisch, J., Doody, R., Fairchild, T., Adams, P., Waring, S., Diaz-Arrastia, R. 2010. A serum protein-based algorithm for the detection of Alzheimer disease. Archives of neurology 67(9), 1077-81.

O'Bryant, S.E., Xiao, G., Barber, R., Reisch, J., Hall, J., Cullum, C.M., Doody, R., Fairchild, T., Adams, P., Wilhelmsen, K., Diaz-Arrastia, R. 2011. A blood-based algorithm for the detection of Alzheimer's disease. Dementia and geriatric cognitive disorders 32(1), 55-62.

Pidsley, R., Wong, C.C.Y., Volta, M., Lunnon, K., Mill, J., Schalkwyk, L.C. 2013. A data-driven approach to preprocessing Illumina 450K methylation array data. BMC genomics 14(May 1), 293.

Prince, M., Bryce, R., Albanese, E., Wimo, A., Ribeiro, W., Ferri, C.P. 2013. The global prevalence of dementia: a systematic review and metaanalysis. Alzheimer's & dementia : the journal of the Alzheimer's Association 9(1), 63-75 e2.

R Development Core Team. 2012. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria 2012.

Ravona-Springer, R., Heymann, A., Schmeidler, J., Guerrero-Berroa, E., Sano, M., Preiss, R., Koifman, K., Hoffman, H., Levy, A., Silverman, J.M., Schnaider-Beeri, M. 2013. Haptoglobin 1-1 genotype is associated with poorer cognitive functioning in the elderly with type 2 diabetes. Diabetes care 36(10), 3139-45.

Ray, S., Britschgi, M., Herbert, C., Takeda-Uchimura, Y., Boxer, A., Blennow, K., Friedman, L.F., Galasko, D.R., Jutel, M., Karydas, A., Kaye, J.A., Leszek, J., Miller, B.L., Minthon, L., Quinn, J.F., Rabinovici, G.D., Robinson, W.H., Sabbagh, M.N., So, Y.T., Sparks, D.L., Tabaton, M., Tinklenberg, J., Yesavage, J.A., Tibshirani, R., Wyss-Coray, T. 2007. Classification and prediction of clinical Alzheimer's diagnosis based on plasma signaling proteins. Nature medicine 13(11), 1359-62.

Rye, P.D., Booij, B.B., Grave, G., Lindahl, T., Kristiansen, L., Andersen, H.M., Horndalsveen, P.O., Nygaard, H.A., Naik, M., Hoprekstad, D., Wetterberg, P., Nilsson, C., Aarsland, D., Sharma, P., Lonneborg, A. 2011. A novel blood test for the early detection of Alzheimer's disease. Journal of Alzheimer's disease : JAD 23(1), 121-9.

Schnaider Beeri, M., Goldbourt, U., Silverman, J.M., Noy, S., Schmeidler, J., Ravona-Springer, R., Sverdlick, A., Davidson, M. 2004. Diabetes mellitus in midlife and the risk of dementia three decades later. Neurology 63(10), 1902-7.

Stewart, R., Liolitsa, D. 1999. Type 2 diabetes mellitus, cognitive impairment and dementia. Diabetic medicine : a journal of the British Diabetic Association 16(2), 93-112.

Thambisetty, M., Hye, A., Foy, C., Daly, E., Glover, A., Cooper, A., Simmons, A., Murphy, D., Lovestone, S. 2008. Proteome-based identification of plasma proteins associated with hippocampal metabolism in early Alzheimer's disease. Journal of neurology 255(11), 1712-20.

Figure 1: Correlation of the % 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*=5.84E-30) with DNA methylation differences in the same probes at 18 month follow up (Y-axis) (A). Similarly DNA methylation differences for the 100 most significant DMPs at 18 month follow-up (shown in Supplementary Table 3) (Y-axis) are significantly correlated (*r*=0.872, *P*=1.49E-32) with DNA methylation differences in the same probes at baseline (X-axis) (B).