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REPORT

Genetic and epigenetic study of an Alzheimer's disease family with monozygotic triplets

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Age at onset of Alzheimer's disease is highly variable, and its modifiers (genetic or environmental) could act through epigenetic changes, such as DNA methylation at CpG sites. DNA methylation is also linked to ageing—the strongest Alzheimer's disease risk factor. DNA methylation age can be calculated using age-related CpGs and might reflect biological ageing. We conducted a clinical, genetic and epigenetic investigation of a unique Ashkenazi Jewish family with monozygotic triplets, two of whom developed Alzheimer's disease at ages 73 and 76, while the third at age 85 has no cognitive complaints or deficits in daily activities. One of their offspring developed Alzheimer's disease at age 50. Targeted sequencing of 80 genes associated with neurodegeneration revealed that the triplets and the affected offspring are heterozygous carriers of the risk *APOE* ε4 allele, as well as rare substitutions in *APP* (p.S198P), *NOTCH3* (p.H1235L) and *SORL1* (p.W1563C). In addition, we catalogued 52 possibly damaging rare variants detected by NeuroX array in affected individuals. Analysis of family members on a genome-wide DNA methylation chip revealed that the DNA methylation age of the triplets was 6–10 years younger than chronological age, while it was 9 years older in the offspring with early-onset Alzheimer's disease, suggesting accelerated ageing.

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Abbreviations: DNAm = DNA methylation; MAF = minor allele frequency; TorCA = Toronto Cognitive Assessment

Introduction

Alzheimer's disease is the most common neurodegenerative disease with a brain pathology characterized by neuronal loss, inflammation, amyloid plaques (consisting of amyloid-β peptides encoded by *APP*), and tau inclusions (encoded by *MAPT*). Rare mutations in *APP*, *PSEN1* and *PSEN2* could cause autosomal dominant early-onset Alzheimer's disease (<65 years); however, ~50% of early-onset sporadic Alzheimer's disease cases or families with a mix of early/late-onset cases are genetically unexplained. Common late-onset Alzheimer's disease is associated with 28 well-confirmed Alzheimer's disease loci, including the *APOE* ε4 allele with the largest risk (Ghani *et al.*, 2018).

Alzheimer's disease age at onset is highly variable (e.g. 39–85 years in *APP* carriers), suggesting the existence of genetic or environmental modifiers (Ghani *et al.*, 2018), both of which could act through epigenetic changes, such as DNA methylation (DNAm) at CpG sites. There is not a strict dichotomy between the action of genetic and epigenetic factors (Zhang *et al.*, 2016, 2018). For instance, the Exome Aggregation Consortium (ExAC) reported that CpGs are the most mutable sites in the genome (Lek *et al.*, 2016).

DNAm is also linked to ageing—the strongest Alzheimer's disease risk factor. The collective assessment of DNAm at 353 CpGs generates DNAm age, which is an accurate predictor of chronological age across multiple tissues, including blood and brain (Horvath, 2013). Deviation of DNAm age from chronological age (DNAm-age acceleration) may be linked to biological ageing via changes in gene expression, and was associated with several neurodegenerative diseases, including Parkinson's disease (Horvath and Ritz, 2015; Picillo *et al.*, 2018) and *C9orf72*-related disease (Zhang *et al.*, 2017). Accelerated DNAm age was also linked to the degree of amyloid pathology or cognitive decline (Levine *et al.*, 2015); and reported to be a significant predictor of dementia (Degerman *et al.*, 2017).

Alzheimer's disease concordance in monozygotic twins is 80%, suggesting high heritability (Martin *et al.*, 1997). Monozygotic siblings provide the best opportunity to investigate risk/protective factors in disease development. Here we conducted clinical, genetic and epigenetic analyses of a unique Alzheimer's disease family with monozygotic triplets.

Materials and methods

Participants

A Canadian Alzheimer's disease family of Ashkenazi Jewish origin (Fig. 1) was recruited from Baycrest Health Sciences (Toronto). The patients met the National Institute on Aging and Alzheimer's Association criteria for probable Alzheimer's disease (McKhann *et al.*, 2011). We also used 192 neurologically normal Canadian controls (59% female; average age 74 ± 8 years) (Li *et al.*, 2008), as well as whole-exome sequencing data from Washington Heights-Inwood Columbia Aging Project (WHICAP), including 1397 Alzheimer's disease cases and 2198 controls (>65 years old) residing in northern Manhattan, NY (Tosto *et al.*, 2019). Informed consent was obtained from each participant in accordance with the Research Ethics Boards.

Genetic analyses

Blood genomic DNA was extracted using a QIAGEN kit. Genotyping was conducted for family members (the triplets, one affected and one unaffected offspring), as well as 192 Canadian controls. First, we used a next-generation sequencing panel developed by the Ontario Neurodegenerative Disease Research Initiative (ONDRISeq) that targets 80 genes associated with neurodegeneration (Farhan *et al.*, 2016). ONDRISeq data were generated/processed as reported (Dilliott *et al.*, 2018), and interrogated for copy number variants by VarSeq® (Golden Helix, Bozeman, MT, USA), which uses normalized depth of coverage analysis to identify large-scale deletions/duplications (Iacocca *et al.*, 2017). ONDRISeq data of the 192 controls were provided to the algorithm, from which 49 with the lowest per cent difference in coverage data were selected as a reference. A coverage ratio ⩽0.7 and a z-score of ⩽−5.0 indicates heterozygous deletions, while a ratio ⩾1.30 and z-score ⩾5.0 suggests duplications. We also used the NeuroX array (Illumina), which includes the Exome BeadChip (~240 000 variants) and ~24 000 variants tailored to study neurodegenerative diseases (Ghani *et al.*, 2015). *APOE* genotypes were based on rs429358 and rs7412 by ONDRISeq. *MAPT* H1/H2 haplotypes and *C9orf72* repeat number were obtained as reported (Bruni *et al.*, 2007; Zhang *et al.*, 2017).

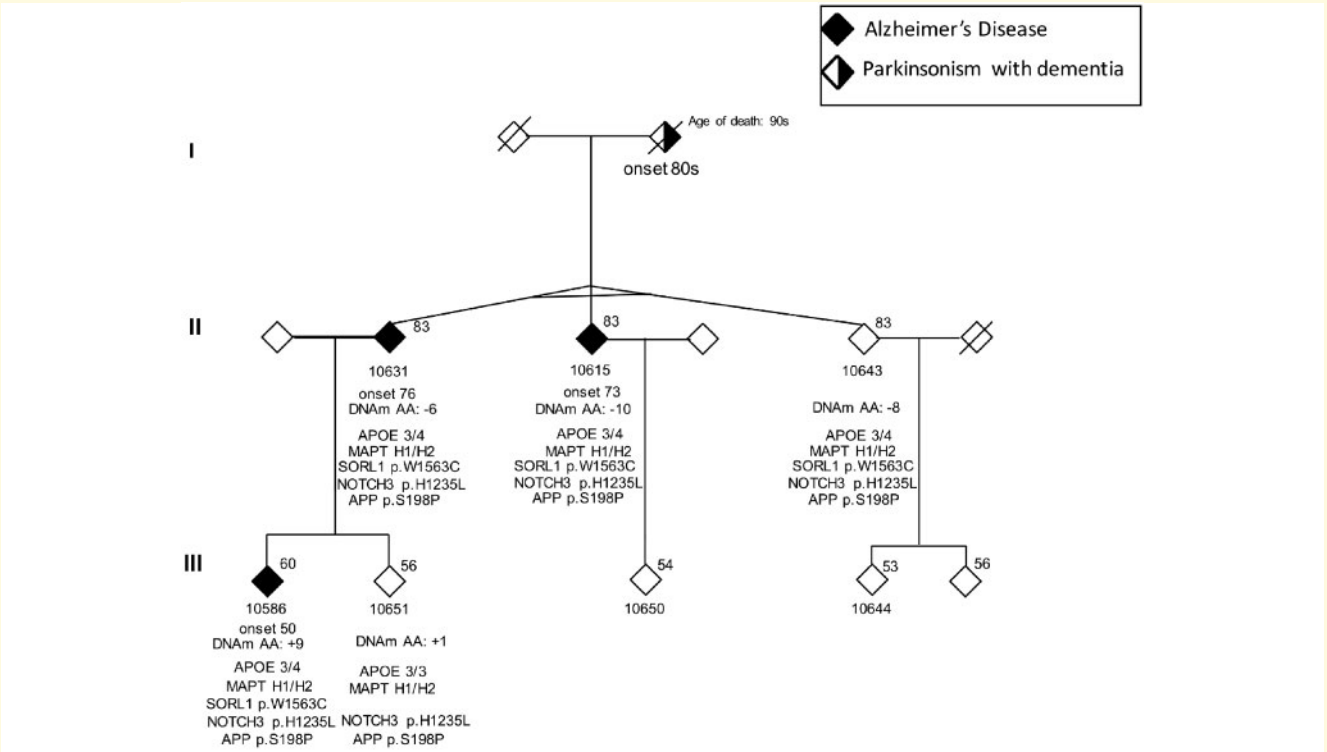

Figure 1 Pedigree of the Ashkenazi family with monozygotic triplets. Gender is masked. Age at time of sample collection is shown above the symbol. Age of onset, DNAm-age acceleration (AA) and genetic findings by ONDRISeq are indicated below the symbol.

Minor allele frequencies (MAF) were acquired from gnomAD, including the Ashkenazi Jewish cohort (http://gnomad.broadinstitute.org/), ExAC (http://exac.broadinstitute.org/), and the Healthy Exome (HEX) database (https://www.alzforum.org/exomes/hex) containing ~500 neuropathologically normal autopsy cases (age >60). The variant filtering process included two steps. First, both the ONDRISeq (Table 2) and NeuroX results were filtered for rare variants with MAF <0.005 (ExAC-ALL). Second, NeuroX variants that were predicted to be damaging by both SIFT and PolyPhen-2 were selected for Supplementary Table 3, which includes allele frequencies for the above mentioned datasets.

Epigenetic analyses

DNAm analysis was conducted as reported (Zhang *et al.*, 2017). Briefly, DNA was bisulfite converted using the Imprint DNA modification kit (Sigma), and assessed on genome-wide Infinium MethylationEPIC chip (Illumina) at the Centre for Applied Genomics (Toronto). Raw DNAm data was processed using GenomeStudio (Illumina). DNAm-age calculator tool (https://dnamage.genetics.ucla.edu) analysed 334 age-related CpGs, which includes 90% of the CpGs from the discontinued 450K BeadChip and has a similar capability to estimate DNAm-age (Logue *et al.*, 2017).

Data availability

The data that support the findings of this study are available on request from the corresponding authors (E.R., M.F.). The data are not publicly available because of information that could compromise the privacy of the research participants.

Results

Clinical findings

The family structure is presented in Fig. 1. The triplet's mother (deceased at age 97) had a history of parkinsonism with dementia. Current age of the triplets is 85. Two of them (Patients 10615 and 10631) were diagnosed with late-onset Alzheimer's disease in their mid-seventies, while triplet Subject 10643 still has no cognitive complaints or deficits in daily living activities, and functions normally based on self/family-reporting. The offspring (Patient 10586) of triplet Subject 10631 developed early-onset Alzheimer's disease at age 50. Imaging results are presented in Supplementary Figs 1–3. Detailed clinical findings are available in Supplementary Table 1, and performance profiles on the Toronto Cognitive Assessment (TorCA) (Freedman *et al.*, 2018) are presented in Supplementary Fig. 4.

Briefly, the progressive memory problems of Patient 10615 started at age 73. CT brain scan was normal at age 75, but a single photon emission computed tomography (SPECT) scan showed mild symmetrical decreased perfusion in the posterior parietal lobes compatible with Alzheimer's disease. At that time, Mini-Mental State

Examination (MMSE) score was 23/30 and score on the Behavioural Neurology Assessment – Short Form (Darvesh *et al.*, 2005) was 70/114 (cut-off for dementia is 82). At age 85, the MMSE score was 26/30 and TorCA total score was 202 (impaired <257). The progressive memory problems of Patient 10631 (monozygotic sibling of Patient 10615) started at age 76. SPECT scan at age 80 showed decreased bilateral parietotemporal lobe perfusion. MMSE score was 23/30 and TorCA score was 219. At age 85, MMSE was 21/30 and TorCA total score dropped to 165. For both siblings, insight, ability to show empathy and sympathy were reduced. Their medical history is also remarkable for hypertension and long-standing obsessive-compulsive behaviour.

Triplet Subject 10643 has remained free from dementia, despite some deficits on cognitive testing at age 85 (MMSE = 22/30). TorCA scores were obtained on two occasions (10.5 weeks apart) because of poor sleep before the first assessment. Both times TorCA total scores were impaired (179 and 198). Other medical history includes hypertension and sarcoma at age 60. There were no features of obsessive-compulsive disorder.

The progressive memory problems of Patient 10586 (offspring of monozygotic triplet Patient 10631) started at age 50. Brain MRI at age 52 showed a few small foci of non-specific subcortical white matter of uncertain significance (mainly in the left frontal lobe); and a SPECT scan showed moderate decreased perfusion within the left parietotemporal region consistent with Alzheimer's disease. At age 53, global hypoperfusion was slightly more prominent in the left frontal, temporal and parietal lobes. The score on the Behavioural Neurology Assessment–Short Form was 64/114; and MMSE score was 24/30, declining to 9/30 by age 55 and 4/30 by age 57. Behavioural symptoms (e.g. verbal aggression and resistance to care) started at age 59. Other medical history included Crohn's disease (inactive for years).

Genetic findings

The monozygosity of the triplets was confirmed by the identical ONDRISeq and NeuroX genotypes. The triplets and Alzheimer's disease-affected offspring are carriers of normal *C9orf72* alleles (⩽8 repeats), but heterozygous for the risk *APOE* ε4 allele and *MAPT* H1 haplotype (Table 1 and Fig. 1). None of the 80 genes included on ONDRISeq had deletions/duplications, but we detected three heterozygous substitutions in *NOTCH3* (p.H1235L), *APP* (p.S198P) and *SORL1* (p.W1563C) with MAF < 0.005 in ExAC-ALL (Table 2). The investigation of these variants in the WHICAP dataset did not reveal association with Alzheimer's disease (Supplementary Table 2). Although the WHICAP dataset could be under power to study very rare variants, considerations below also argue against the pathogenic nature of the substitutions in *NOTCH3* and *APP*, whereas the significance of *SORL1* p.W1563C in Alzheimer's disease could not be excluded.

The p.H1235L substitution in exon 22 of *NOTCH3* is deleterious by SIFT, but benign by PolyPhen-2; and is not rare in the Ashkenazi Jewish population (gnomAD MAF = 0.02; 10 368 chromosomes) (Table 2). It is mapped outside the mutation hotspot for *NOTCH3*-related dementia caused by the loss/gain of cysteine residues encoded by exons 2–5 or 7–11 (Joutel *et al.*, 2004). Moreover, *NOTCH3* mutations are associated with diffuse white matter abnormalities (absent on the neuroimages of the family members). Similarly, the p.S198P in exon 5 of *APP* is mapped outside the Alzheimer's disease mutation hot-spot (exons 16–17 encoding amyloid-β domain) (Ghani *et al.*, 2018). Although p.S198P is predicted to be damaging by SIFT and PolyPhen-2, it is not very rare in the Ashkenazi Jewish population (gnomAD MAF = 0.01) (Table 2). One of the WHICAP controls is homozygous for p.S198P, arguing against its pathogenic nature (Supplementary Table 2).

In contrast, the p.W1563C substitution in the Fibronectin type-III domain of *SORL1* is very rare in the Ashkenazi Jewish population (gnomAD MAF = 0.0005) and elderly controls (one Canadian, one WHICAP, and none in the HEX database). It is predicted to be damaging by SIFT, PolyPhen-2 (Table 2), and strongly damaging by the Combined Annotation Dependent Depletion score (>30) (Kircher *et al.*, 2014).

NeuroX confirmed the substitutions in *SORL1*, *NOTCH3* and *APP*. In addition, we catalogued 52 possibly damaging rare variants (five truncating variants) detected by NeuroX (24 in the triplets and 28 in the offspring with early-onset Alzheimer's disease) to be followed-up in large Alzheimer's disease datasets (Supplementary Table 3).

Table 1 Genotyping results of *C9orf72*, *MAPT* and *APOE* in the family members

DNA #	Status	Age of onset, years	*C9orf72* genotype	*MAPT* haplotype	*APOE* genotype
10631	Alzheimer's disease	76	2/8	H1/H2	4/3
10615	Alzheimer's disease	73	2/8	H1/H2	4/3
10643	Unaffected	NA	2/8	H1/H2	4/3
10586	Alzheimer's disease	50	2/2	H1/H2	4/3
10651	Unaffected	NA	2/8	H1/H2	3/3

NA = not applicable.

Table 2 Genetic analysis of the family using the ONDRISeq panel identified three heterozygous missense variants that are rare in ExAC-ALL database (MAF <0.005)

Gene	Variation (transcript)	Predicted effects	SNP	ExAC MAF			gnomAD MAF	HEX MAF	SIFT prediction (score)	PolyPhen-2 prediction (score)	Phenotype associated with the gene
				ALL	American	NFE	Ashkenazi Jewish				
SORL1	c.G4689C (NM_003105)	p.W1563C	rs138580875	0.0005	0.0005	0.0007	0.0005	0	D (0.003)	D (1)	AD
NOTCH3	c.A3704T (NM_000435)	p.H1235L	rs55882518	0.004	0.003	0.007	0.02	0.01	D (0.03)	B (0)	CADASIL
APP	c.T592C (NM_000484)	p.S198P	rs145081708	0.0005	0.0007	0.0007	0.01	0.001	D (0.009)	D (0.999)	AD

B = benign; CADASIL = cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; D = probably damaging or deleterious; ExAC = Exome Aggregation Consortium; HEX = Healthy Exome database; nFE = non-Finnish European; WHICAP = Washington Heights-Inwood Columbia Aging Project.

Table 3 The five age-related CpG sites

CpG ID	Gene symbol	Beta 10631	Beta 10615	Beta 10643	Beta 10586	Delta beta
cg00945507	*SEC61G*	0.27	0.28	0.33	0.69	0.39
cg12768605	*LYPD5*	0.32	0.32	0.32	0.52	0.20
cg13854874	*CHAF1B*	0.24	0.23	0.20	0.49	0.27
cg22901840	*DIRAS3*	0.70	0.74	0.68	0.48	-0.23
cg07770222	*C8orf31*	0.60	0.55	0.59	0.81	0.23

Presented are the age-related CpG sites that showed a >20% difference in DNAm levels between the patient with early-onset Alzheimer's disease (Patient 10586) and the triplets (Patients 10631, 10615 and 10643).

Epigenetic findings

In agreement with our prior study of monozygotic twins, demonstrating that many DNAm changes are genetically controlled (Zhang *et al.*, 2016), the genome-wide blood DNAm profiles of the monozygotic triplets are much more similar than between first-degree relatives, who share 50% of their genetic material (Supplementary Fig. 5). Four CpGs in *KCNS2*, *CADM1*, *RAB3IL1* and *MATN2* had DNAm difference >30% between the triplets affected by Alzheimer's disease in their seventies and the triplet without dementia at age 85 (Supplementary Table 4), warranting further investigation of these CpGs in Alzheimer's disease.

The discordance in age at onset between the triplets could not be attributed to DNAm-age acceleration (their DNAm age was 6–10 years younger than chronological age) (Fig. 1). In contrast, the DNAm age in the offspring with early-onset Alzheimer's disease (Patient 10586) was 9 years older than chronological age, suggesting accelerated ageing, which might be driven by five age-related CpGs that showed a >20% difference in DNAm levels between Patient 10586 and the triplets with late-onset Alzheimer's disease (Table 3). Notably, the DNAm age of the unaffected sibling Subject 10651 of Alzheimer's disease Patient 10586, was only 1 year older than the chronological age.

Discussion

We report a family with monozygotic triplets, two of whom were diagnosed in their seventies with late-onset slow progressing Alzheimer's disease (mild dementia 9 and 12 years post-onset). In contrast, an offspring of one of the affected triplets has early-onset Alzheimer's disease, which progressed to severe dementia within 5 years. The unaffected triplet was impaired on cognitive testing, but is independent in daily activities at age 85 and thus did not meet criteria for dementia or mild cognitive impairment.

The *APOE* ε4 allele may explain the late-, but not the early-onset Alzheimer's disease in the family. Also, the pathogenic role of *SORL1* p.W1563C cannot be excluded. Notably, APOE and SORL1 interact in the amyloid-β pathway, and rare *SORL1* variants (p.N674S) could increase penetrance of Alzheimer's disease in *APOE* ε4 carriers (Louwersheimer *et al.*, 2017). Multiple evidence support *SORL1* as an Alzheimer's disease gene, and truncating *SORL1* variants were even suggested for consideration in clinical practice, similar to *PSEN1*, *PSEN2* and *APP* mutations (Rogaeva *et al.*, 2007; Holstege *et al.*, 2017). However, it is challenging to determine penetrance of rare substitutions, and their pathogenic impact remain unclear without functional investigations. A recent study sequenced *SORL1* in 1895 cases and 3206 controls, and proposed that the pathogenicity of the 181 detected variants could

be classified based on their predicted damaging effect and MAF in databases, which placed p.W1563C among the variants with uncertain significance (Holstege *et al.*, 2017).

Our study has limitations. A single, although unique, family has limited statistical power in the context of genome-wide data. Furthermore, we did not search for ultra-rare mutations that may have occurred after the blastocyst split (Weber-Lehmann *et al.*, 2014); and *de novo* mutations have been reported to contribute to the discordance between monozygotic twins for some neurological disorders, such as schizophrenia (Tang *et al.*, 2017). Both limitations will be resolved by analysing this family together with others in our ongoing whole-genome sequencing Alzheimer's disease project. We also could not exclude Alzheimer's disease-associated genetic variability in brain, although a recent study of brain samples reported that somatic variants in Alzheimer's disease genes is not a common Alzheimer's disease cause (Nicolas *et al.*, 2018). Finally, it would be important to estimate the polygenic risk score for individuals affected by late- versus early-onset Alzheimer's disease. However, association studies related to polygenic risk/hazard scores are currently done only in large European or North American Alzheimer's disease datasets (Leonenko *et al.*, 2019) and their utility is not yet clear for individual application in specific ethnic groups with a unique genetic makeup (e.g. Ashkenazi Jewish). Notably, of the 54 rare possibly damaging variants detected by NeuroX in our family (ExAC-ALL MAF <0.005), only 14 are also rare in the Ashkenazi Jewish population (Supplementary Table 3).

Environmental/ageing factors triggering epigenetic changes may also affect disease manifestation. It was reported that a cluster of genes with DNAm changes may influence biological networks, but DNAm at specific CpG sites may not be sufficient for modifying phenotypes in monozygotic twins discordant for major depression (Malki *et al.*, 2016) or amyotrophic lateral sclerosis (Zhang *et al.*, 2016). In the current study, DNAm age was similar between the monozygotic triplets (6–10 years younger than chronological age regardless of Alzheimer's disease onset/status). In contrast to the parental generation with late-onset Alzheimer's disease, DNAm-age was accelerated by 9 years in the offspring with early-onset Alzheimer's disease. This is in line with previous findings that DNAm age of the prefrontal cortex is associated with amyloid load and cognitive function (Levine *et al.*, 2015). A longitudinal study revealed that individuals at age 70–80 were epigenetically younger (by 2–3 years) than their chronological age compared to baseline at 55–65 age (Degerman *et al.*, 2017). However, this systematic deviation did not affect the conclusion that accelerated epigenetic age at the age of 55–80 years may increase risk of dementia. In the current study, correcting for the possible error would bring the DNAm-age of the monozygotic triplets to 3–7 years younger than chronological age and would still be noticeably different from the early-onset patient whose DNAm age was 69 at age 60. Future case-control Alzheimer's disease studies are required to assess if DNAm-age acceleration is associated with age at onset.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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