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NAT gene polymorphisms and susceptibility to Alzheimer's disease: identification of a novel *NAT1* allelic variant

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Abstract

Background: Alzheimer's disease is multifactorial, having environmental, toxicological and genetic risk factors. Impaired folate and homocysteine metabolism has been hypothesised to increase risk. In addition to its xenobiotic-metabolising capacity, human arylamine *N*-acetyltransferase type-1 (NAT1) acetylates the folate catabolite *para*-aminobenzoylglutamate and is implicated in folate metabolism. The purpose of this study was to determine whether polymorphisms in the human *NAT* genes influence susceptibility to Alzheimer's disease.

Methods: Elderly individuals with and without Alzheimer's disease were genotyped at the polymorphic *NAT1* (147 cases; 111 controls) and *NAT2* (45 cases; 63 controls) loci by polymerase chain reaction-restriction fragment length polymorphism, and the genotype and allele frequencies were compared using the chi-squared test.

Results: Although a trend towards fast NAT2 acetylator-associated Alzheimer's disease susceptibility was indicated and the *NAT1*10/1*10* genotype was observed only in cases of Alzheimer's disease (6/147, 4.1%), no significant difference in the frequency of *NAT2* ($p = 0.835$) or *NAT1* ($p = 0.371$) genotypes was observed between cases and controls. In addition, a novel *NAT1* variant, *NAT1*11B*, was identified.

Conclusions: These results suggest that genetic polymorphisms in *NAT1* and *NAT2* do not influence susceptibility to Alzheimer's disease, although the increase in frequency of the *NAT1*10* allele in Alzheimer's disease is worthy of further investigation. Due to its similarity with the *NAT1*11A* allele, *NAT1*11B* is likely to encode an enzyme with reduced NAT1 activity.

Background

Alzheimer's disease (AD) is a common multifactorial disease of the elderly, which results in progressive neurodegeneration causing severe and permanent cognitive impairment [1]. As a result of family pedigree analyses and case-control studies, multiple genetic risk factors such as mutations in the β-amyloid precursor protein (*APP*) [2], presenilin type-1 (*PS1*) [3], presenilin type-2 (*PS2*) [4] and apolipoprotein E (*APOE*) [5] genes have been identified. Genetic epidemiology studies have however, estimated that only 30% to 50% of the population risk for AD can be attributed to genetic factors [6]. This along with the

low concordance rate of AD observed in monozygotic twins [7,8] suggests that environmental factors may also influence AD susceptibility. This may be the case particularly for later-onset 'sporadic' cases of the disease, which account for the majority (approximately 90%) of AD cases but show only modest familial clustering [6].

Many putative environmental risk factors for AD have been proposed. In particular, low blood levels of folate and elevated serum total homocysteine have been associated with increased risk of AD [9-11]. Differences in the level of serum homocysteine and folate were not associated with increasing duration of the symptoms of AD [10], which suggests that the observed level of these biochemical markers was not due to progression of the disease. Therefore, impaired folate and homocysteine (one-carbon) metabolism has been hypothesised as a risk factor in AD [10]. Such altered folate and homocysteine metabolism could arise as a result of genetic mutations in enzymes of folate metabolism.

The association between impaired folate and homocysteine metabolism and AD is particularly interesting in view of the putative endogenous role of the human phase II xenobiotic-metabolising enzyme arylamine *N*-acetyltransferase type-1 (NAT1)(and its murine homologue NAT2), in folate catabolism [12-15], and our previous demonstration of the expression of murine NAT2 in particular cell types, such as the cytoplasm and dendrites of the Purkinjie cells of the cerebellum in the adult mouse brain [16]. There has as yet been no description of the pattern of human NAT1 expression in the adult brain, although human NAT1 (the human equivalent of murine NAT2) activity has been identified in the brain early in embryonic development [17]. Human NAT1 is polymorphic [18] and inter-individual variation in NAT1 activity [19] may modulate individual folate levels. *NAT1* may therefore be a potential low penetrance gene which can modify individual risk of AD.

Polymorphisms in the arylamine *N*-acetyltransferase type-2 (*NAT2*) gene, which encodes the phase II xenobiotic-metabolising isozyme NAT2, have been linked with increased susceptibility to multifactorial neurodegenerative disorders such as Parkinson's disease [20,21]. In this case the impaired ability of the individual to handle environmental xenobiotics or neurotoxins acting on the brain has been hypothesised to contribute to the development of the disease. In a similar manner human NAT2, and in addition human NAT1, in their more traditionally recognized role as phase II xenobiotic-metabolising enzymes [22], may also be modulators of AD risk as a result of chemical insult. An association study carried out by Rocha et al., [23] has previously indicated that human *NAT2* may be a potential low penetrance gene in AD pathogenesis. In view of these findings we undertook a study to genotype an elderly group of individuals with and without a history of AD for major alleles at the *NAT1* and *NAT2* loci, to investigate the role of *NAT1* and *NAT2* polymorphisms in AD susceptibility.

Methods

Study populations

Genomic DNA from 148 elderly Caucasian individuals with AD and 90 elderly Caucasian individuals without AD, from the Oxford Project to Investigate Memory and Ageing (OPTIMA) cohort, was available for investigation. OPTIMA is a longitudinal study of normal, non-institutionalised elderly volunteers with good cognitive function, and elderly patients with memory problems from the Oxfordshire community (UK), and has been described in detail elsewhere [10,24]. Briefly, the OPTIMA study follows the AD course of an individual, from initial diagnosis to *post mortem* confirmation. Every year each subject is examined by neurological and neuropsychological tests, brain scans and biochemical analysis of blood and cerebrospinal fluid. Cognitive evaluation is undertaken using the Cambridge Examination for Mental Disorders of the Elderly [25]. This includes the Cambridge Cognitive Examination (CAMCOG), a neuropsychological test which includes elements of the Mini-Mental State Examination and assesses a broad range of cognitive functions, such as memory, language, attention, perception, praxis and thinking. A cut-off value of <80/107 CAMCOG points discriminates between demented and normal subjects. Clinical diagnosis is made according to the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association criteria [1]. *Post mortem*, the brain is examined by histochemical and biochemical methods to confirm disease pathology using Consortium to Establish a Registry for Alzheimer's Disease criteria [26]. In this study the AD case individuals from the OPTIMA cohort included: 101 living patients with a clinical diagnosis of probable or possible AD, 30 patients with histologically confirmed pure AD with the absence of any additional disease pathology, 16 patients with histologically confirmed AD with the coexistence of vascular or Parkinson's disease pathology and 1 patient who demonstrated clinical symptoms of AD which were not confirmed *post mortem*. The non-demented control individuals from the OPTIMA cohort included: 83 living subjects with no clinically diagnosed symptoms of AD, 4 subjects with the absence of AD confirmed *post mortem* and 3 subjects who demonstrated no symptoms of AD which was not confirmed *post mortem*. The OPTIMA study had ethical approval from the central Oxford and Psychiatric Research Ethics Committees and informed consent was obtained in writing from all subjects.

Genomic DNA from 22 elderly volunteers from the Foresight Challenge cohort was also available for investigation. Foresight Challenge is a cohort of normal, non-institutionalised elderly volunteers with good cognitive function from the Oxfordshire community (UK)(non-black and Caucasian), recruited for a three-year longitudinal study aimed at further defining early markers or predictors of cognitive impairment and their relationship to the subsequent development of dementia [27]. Participants were excluded if they: (a) scored ≤80/107 points on the CAMCOG or ≤24/30 points on the Mini-Mental State Examinations [25] at the initial screening visit, (b) reported significant progressive subjective memory complaints, (c) lived in institutional care, or (d) were unable to complete the Cambridge Examination for Mental Disorders of the Elderly [25]. For the purpose of this study the individuals in the case and control groups were regarded as homogeneous groups. Subjects with other causes of dementia were excluded from the study.

Materials

All chemicals were purchased from Sigma-Aldrich Company Ltd., Merck Ltd. or BDH Laboratory Supplies, UK. Molecular biology reagents were purchased from Promega, Roche Molecular Biochemicals and New England Biolabs Inc., UK. Oligonucleotides were synthesized by Sigma-Genosys Ltd., UK.

DNA extraction

Genomic DNA from each AD case (n = 148) and control (n = 90) subject in the OPTIMA cohort, and from each control subject (n = 22) in the Foresight Challenge cohort was prepared from the buffy coat fraction of EDTA blood [28] using the Wizard® Genomic DNA Purification Kit (Promega, UK), and was stored at 4°C in TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM Na_2EDTA).

NAT1 genotyping

The human *NAT1*3*, *NAT1*4*, *NAT1*10*, *NAT1*11A* and *NAT1*14A* alleles (those unfamiliar with *NAT* allele nomenclature should see http://www.louisville.edu/medschool/pharmacology/NAT.html) were identified by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and allele-specific PCR (AS-PCR) step-by-step genotyping procedure [29]. To summarise, a fragment of 344 bp between nucleotides 769 and 1113 was amplified with primers N769 (5'-ACTCTGAGTGAGGTAGAAATA-3') and N1113 (5'-ACAGGCCATCTTTAGAA-3'). As a result of *Mbo* II sites at nucleotides 945 and 969, the alleles *NAT1*3*, *NAT1*10* and *NAT1*14A* produce fragments of 176 bp, 24 bp and 144 bp on digestion with *Mbo* II (for *NAT1*11A*, as a result of a 9 bp deletion, the fragments are 176 bp, 24 bp and 135 bp). The primer N1113 introduces an additional *Mbo* II site into *NAT1*4* such that the 144 bp fragment is cleaved to 125 bp and 19 bp. Subsequently, *NAT1*3* was distinguished from *NAT1*10* and *NAT1*14A* by an allele-specific PCR. *NAT1*10* and *NAT1*14A* were distinguished by a further PCR and digestion with *Bsa*O I [29]. Nucleotides which are changed from the *NAT1*4* sequence (EMBL Accession Number AJ307007) are underlined.

The *NAT1*14A* allele (G560A, T1088A, C1095A) was confirmed and distinguished from the similar *NAT1*14B* allele (G560A), by further analysis using AS-PCR and nested PCR-RFLP. This analysis first amplified only *NAT1*10* and *NAT1*14A* allele sequences, and then identified whether the G560A variation was present. AS-PCR amplification was performed in a 100 μl reaction using 1X PCR reaction buffer, 250 ng genomic DNA, 2.5 mM $MgCl_2$ 0.2 mM dNTPs, 2.5 U *Taq* DNA polymerase and 25 pmoles each of primers N539 (5'-TCCTAGAAGACAGCAACGACC-3') and N1110b (5'-GGCCATCTTTAAAATACATTTT-3')(with annealing temperature 54°C and a product extension of 1 minute at 72°C). Primer N1110b, which contains T1088 annealed to and amplified a 571 bp product from the *NAT1*10* and *NAT1*14A* allele sequences only. The PCR product from the first round of amplification was diluted 1 in 20, and 1 μl used as template in a PCR with primers N539 and N714 (5'-GTGAAGCCCACCAAACAG-3'), followed by *Bsa*O I digestion of the 175 bp amplified product [29]. *NAT1*14A* contains G560A; therefore no *Bsa*O I site was generated. *NAT1*10* contains G560, therefore a *Bsa*O I site was generated resulting in fragments of 155 bp and 20 bp.

NAT2 genotyping

The human *NAT2*4*, *NAT2*5A*, *NAT2*5B*, *NAT2*5C*, *NAT2*6A* and *NAT2*7B* alleles were identified by genotyping using the PCR-RFLP method of Hickman & Sim [30], and Hickman et al. [31]. This method is more than 95% accurate at predicting the NAT2 phenotype in a Caucasian population [31]. This method of genotyping is unable to distinguish between the *NAT2*4*/*NAT2*5B* and the *NAT2*5A*/*NAT2*5C* genotypes. However, since the expected frequency of the *NAT2*4* (19% to 27%) and *NAT2*5B* (38% to 45%) alleles in a Caucasian population is much greater than the expected frequency of the *NAT2*5A* (1.0% to 4.2%) and *NAT2*5C* (1.0% to 4.3%) alleles [32], this method is sufficient to confer an accurate genotype in greater than 99% of cases if the *NAT2*4*/*NAT2*5B* genotype is inferred. Genotyping of both *NAT1* and *NAT2* was carried out blind with respect to the disease status of the subjects.

Sequencing of novel NAT1 variant

PCR amplification of a 1614 nucleotide region of *NAT1* for cloning and sequencing of a *NAT1* variant was performed in a 100 μl reaction using 1 μg genomic DNA, 2.5 U high-fidelity *Pfu* DNA Polymerase (Promega, UK), 1X

Pfu DNA Polymerase reaction buffer with 2 mM $MgSO_4$, 0.2 mM dNTPs, and 25 pmoles each of primers N-438 (5'-TTGTATAAGGCTCAGCTAAAAGGG-3') and N1176 (5'-GGAATTCAACAATAAACCAACAT-3')(with annealing temperature 56°C and a product extension of 2 minutes at 72°C). Following gel purification, the amplified PCR product was A-tailed with dATP, ligated into the pGEM®-T Easy Vector (Promega, UK), and transformed into high efficiency JM109 Competent *E. coli* cells (Promega, UK) by heat shock. Positive clones were identified by blue-white selection on LB agar plates containing 100 μg/ml ampicillin, 0.1 mM isopropyl-β-D-thiogalactoside (Melford Laboratories Ltd., UK) and 40 μg/ml X-Gal. The identity of the cloned *NAT1* sequence was confirmed by *NAT1* genotyping of plasmid DNA. The cloned *NAT1* inserts (two different clones for each allele) were sequenced using M13F (5'-AGGGTTTTCCCAGTCACGA-3') and M13R (5'-ACACAGGAAACAGCTATGAC-3') sequencing primers, and the *NAT1*-specific primer N714. Fluorescent DNA sequencing was performed by the DNA Sequencing Facility, Department of Biochemistry, University of Oxford, using an ABI 377XL Prism DNA Sequencer with ABI BigDye™ terminators (Warrington, UK). Sequencing chromatograms were analysed using Chromas version 1.45 (Conor McCarthy, Griffith University, Australia).

Statistical analysis

NAT allele and genotype frequencies for the AD case and control groups were calculated and the *NAT* genotype distribution for AD case and control groups was tested for Hardy-Weinberg equilibrium. Any deviations from this equilibrium were assessed using a chi-squared test. *NAT* allele and genotype distributions of the AD case and control groups were compared using the chi-squared test, and *p* values of less than 0.05 were considered to be significant. The strength of association between inheritance of a particular *NAT* allele or genotype and AD was assessed by calculation of crude odds ratios (OR) and 95% confidence intervals (CI).

Results

The *NAT1* genotype of each individual in the Alzheimer's case group (n = 147) and the control group (n = 111) was determined and the frequency of *NAT1* alleles and *NAT1* genotypes is illustrated in Table 1 and Table 2 respectively. The distribution of alleles demonstrates that *NAT1*4* is the most common allele in both groups (75–79%), whilst *NAT1*10* is the next most frequent (15–19%). The same pattern has been observed in all Caucasian populations that have been studied [32]. The genotypes observed were determined to be in Hardy-Weinberg equilibrium.

Table 1: *NAT1* allele frequency in AD cases and controls.

NAT1 ALLELE	AD CASES (*N* = 147)	CONTROLS (*N* = 111)	OR (95% CI)
*1*4*	220 (0.75)	176 (0.79)	1.00
*1*3*	9 (0.03)	7 (0.03)	1.03 (0.38, 2.82)
*1*10*	57 (0.19)	33 (0.15)	1.38 (0.86, 2.22)
*1*11A*	3 (0.01)	2 (0.01)	1.20 (0.20, 7.26)
*1*14A*	5 (0.02)	4 (0.02)	1.00 (0.27, 3.78)
TOTAL	294 (1.00)	222 (1.00)	-

The number and frequency (in brackets) of *NAT1* alleles in 147 AD cases and 111 controls is shown. The OR of AD to non-disease is given with 95% CI for each *NAT1* allele (with *NAT1*4* as the reference group).

Table 2: *NAT1* genotype frequency in AD cases and controls.

NAT1 GENOTYPE	AD CASES (*N* = 147)	CONTROLS (*N* = 111)	EXPECTED	OR (95% CI)
*1*4/1*4*	81 (0.55)	65 (0.59)	0.63	1.00
*1*4/1*10*	43 (0.29)	33 (0.30)	0.24	1.05 (0.60, 1.83)
*1*4/1*3*	7 (0.05)	7 (0.06)	0.05	0.80 (0.27, 2.40)
*1*4/1*14A*	5 (0.03)	4 (0.04)	0.03	1.00 (0.26, 3.89)
*1*4/1*11A*	3 (0.02)	2 (0.02)	0.01	1.20 (0.20, 7.42)
*1*10/1*10*	6 (0.04)	0 (0)	0.02	-
*1*3/1*10*	2 (0.01)	0 (0)	0.01	-
Others	0 (0)	0 (0)	0.01	-
TOTAL	147 (1.00)	111 (1.00)	1.00	-

The number and frequency (in brackets) of *NAT1* genotypes in 147 AD cases and 111 controls is shown. Expected genotype frequency was calculated based on the allele frequency in the control group (Table 1). 'Others' includes additional *NAT1* genotypes containing the alleles *1*4*, *1*3*, *1*10*, *1*11A* and *1*14A* expected at low frequency which were not observed in these AD cases and controls. The OR of AD to non-disease is given with 95% CI for each *NAT1* genotype (with *NAT1*4/NAT1*4* as the reference group).

Table 3: *NAT2* allele frequency in AD cases and controls.

NAT2 ALLELE	AD CASES (*N* = 45)	CONTROLS (*N* = 63)	OR (95% CI)
*2*4*	20 (0.22)	26 (0.21)	1.00
*2*5A*	1 (0.01)	4 (0.03)	0.33 (0.03, 3.14)
*2*5B*	35 (0.39)	53 (0.42)	0.86 (0.42, 1.77)
*2*5C*	1 (0.01)	2 (0.02)	0.65 (0.06, 7.69)
*2*6A*	32 (0.36)	39 (0.31)	1.07 (0.51, 2.25)
*2*7B*	1 (0.01)	2 (0.02)	0.65 (0.06, 7.69)
TOTAL	90 (1.00)	126 (1.00)	-

The number and frequency (in brackets) of *NAT2* alleles in 45 AD cases and 63 controls is shown. The OR of AD to non-disease is given with 95% CI for each *NAT2* allele (with *NAT2*4* as the reference group).

Although a chi-squared comparison of the two groups being investigated demonstrates that the allele and genotype frequency of the two groups does not differ significantly (χ^2 = 1.84, p = 0.765 and χ^2 = 6.48, p = 0.371 respectively) the number of *NAT1*10* alleles is slightly elevated in the Alzheimer's patient population. Comparing the *NAT1*10* homozygote genotype frequency, there are 6 out of 147 Alzheimer's cases with this genotype, whilst there are no control individuals with this genotype out of 111 investigated. Although the numbers are small, it is a finding worthy of further investigation, especially since the *NAT1*10* allele may have an effect on the level of NAT1 expression [33].

NAT2 genotyping was carried out on a random subgroup of the AD cases (n = 45) and of the controls (n = 63). The frequency of *NAT2* alleles and *NAT2* genotypes (which were in Hardy-Weinberg equilibrium) is illustrated in Table 3 and Table 4 respectively. *NAT2*5B* is the most common allele in both groups (39–42%), whilst *NAT2*6A* is the next most frequent allele (31–36%), as seen in other Caucasian populations that have been studied [32]. A direct chi-squared comparison of the *NAT2* allele and genotype frequencies in the Alzheimer's case and control groups indicates that they are not significantly different (χ^2 = 1.95, p = 0.857 and χ^2 = 5.76, p = 0.835 respectively). In a Caucasian population, an individual's NAT2 phenotype can be predicted quite accurately (in more than 95% of cases) by their *NAT2* genotype [31]. Therefore the Alzheimer's cases and control individuals were classified as fast or slow acetylators and compared (Table 5). Although a small increase in the frequency of the NAT2 fast acetylator phenotype was observed in the AD cases (38%) compared with the controls (35%), which is in agreement with a similar study carried out in a Portuguese sporadic AD population by Rocha et al. [23], once again no significant difference between the Alzheimer's case and control group was observed (χ^2 = 0.09, p = 0.761). Other studies have also demonstrated a lack of association between the NAT2 acetylator phenotype and risk of AD including those by Steventon et al. [34] and Ladero et al. [35]. However, in these cases it has been suggested that administration of therapeutic agents such as tacrine, an acetylcholinesterase inhibitor used in the treatment of AD, which is thought to be metabolised by CYP1A2 [36,37] may have affected the acetylation of sulphamethazine which was used to phenotype NAT2.

Discussion

It is hypothesized that variation in NAT1 activity may alter the risk of AD (which has been associated with low blood folate levels [9-11]), via the postulated role of NAT1 in folate metabolism. Together, the results of the present study suggest that genetic polymorphisms in *NAT1* and *NAT2* do not influence susceptibility to AD. It should be noted that the study presented here is preliminary and its statistical power is limited due to the relatively small number of samples analysed, particularly the study of *NAT2* genotype and risk of AD. Therefore much larger case-control investigations, using more highly automated detection methods such as the LightCycler real-time PCR methods of Blömeke et al. [38] and Wikman et al. [39] will help to validate the results shown here. In order to detect a 1.5 fold increased risk of AD associated with the *NAT1*10* allele, approximately 650 cases and 650 controls would need to be studied to give 80% power of achieving significance at the 5% level. Similarly, in order to detect a 1.5 fold increased risk of AD associated with the *NAT2*4* allele, approximately 520 cases and 520 controls would need to be studied.

It is hypothesised that expression of a high activity NAT2 enzyme might increase the metabolic activation of environmental compounds (including neurotoxins), contributing to the neuronal tissue degeneration characteristic of AD. However, as no environmental compounds or neurotoxins currently suspected of contributing to the development of AD are known to be metabolised by the NAT enzymes, the identification of endogenous NAT substrates

Table 4: *NAT2* genotype frequency in AD cases and controls.

NAT2 GENOTYPE	AD CASES (*N* = 45)	CONTROLS (*N* = 63)	EXPECTED	OR (95% CI)
*2*4/2*4*	3 (0.07)	4 (0.06)	0.04	1.00
*2*4/2*5A*	1 (0.02)	4 (0.06)	0.01	0.33 (0.02, 4.74)
*2*4/2*5B*	6 (0.13)	10 (0.16)	0.17	0.80 (0.13, 4.87)
*2*4/2*6A*	6 (0.13)	4 (0.06)	0.13	2.00 (0.28, 14.20)
*2*4/2*7B*	1 (0.02)	0 (0)	0.01	-
*2*5B/2*5B*	6 (0.13)	8 (0.13)	0.18	1.00 (0.16, 6.26)
*2*5B/2*5C*	1 (0.02)	1 (0.02)	0.01	1.33 (0.06, 31.12)
*2*5B/2*6A*	16 (0.36)	25 (0.40)	0.26	0.85 (0.17, 4.33)
*2*6A/2*6A*	5 (0.11)	5 (0.08)	0.10	1.33 (0.19, 9.31)
*2*5B/2*7B*	0 (0)	1 (0.02)	0.01	-
*2*5C/2*7B*	0 (0)	1 (0.02)	0.00	-
Others	0 (0)	0 (0)	0.08	-
TOTAL	45 (1.00)	63 (1.00)	1.00	-

The number and frequency (in brackets) of *NAT2* genotypes in 45 AD cases and 63 controls is shown. Expected genotype frequency was calculated based on the allele frequency in the control group (Table 3). 'Others' includes additional *NAT2* genotypes containing the alleles *2*4*, *2*5A*, *2*5B*, *2*5C*, *2*6A* and *2*7B* expected at low frequency which were not observed in these AD cases and controls. The OR of AD to non-disease is given with 95% CI for each *NAT2* genotype (with *NAT2*4/NAT2*4* as the reference group).

Table 5: NAT2 phenotype distribution in AD cases and controls.

NAT2 PHENOTYPE	AD CASES (*N* = 45)	CONTROLS (*N* = 63)	OR (95% CI)
fast acetylator	17 (0.38)	22 (0.35)	1.00
slow acetylator	28 (0.62)	41 (0.65)	0.88 (0.40, 1.96)
TOTAL	45 (1.00)	63 (1.00)	-

The number and frequency (in brackets) of fast and slow NAT2 acetylators in 45 AD cases and 63 controls is shown. Based on their genotype, AD cases and controls were classified as either 'fast' or 'slow' NAT2 acetylators. Individuals homozygous or heterozygous for the *NAT2*4* allele were assigned fast NAT2 acetylators, individuals who carried two 'slow' *NAT2* alleles (*NAT2*5A*, *NAT2*5B*, *NAT2*5C*, *NAT2*6A* and *NAT2*7B*) in any combination were assigned slow NAT2 acetylators [31]. The OR of AD to non-disease is given with 95% CI for each NAT2 phenotype (with NAT2 fast acetylator as the reference group).

or NAT substrates which may be precursors of neurotoxic derivatives, will be important to strengthen the hypothesis that NAT1 and NAT2 may be risk factors for AD as a result of chemical insult.

At present, the suggestion for an endogenous role for human NAT1 has focused on the ability of human NAT1 to acetylate the folate catabolite *para*-aminobenzoylglutamate [12,13]. However, it is possible that NAT1 may play a role in the metabolism of other, as yet unidentified arylamines. Experiments in which the murine gene equivalent to human *NAT1* (murine *NAT2*) has been knocked out indicate that superficially the mice are well [40]. However, in view of the distribution of murine NAT2 in the nervous system [16], it is important that subtle tests involving behaviour are carried out and the histology of the nervous system is studied in order to determine the role of murine NAT2 in the nervous system and identify whether there might be compensating factors at play in the genetically modified mice. In addition, mice over-expressing the human *NAT1* gene show developmental abnormalities [41].

Whilst identifying the *NAT1* alleles, a pattern was observed in one individual that was not consistent with any known *NAT1* genotype. Only individuals with the *NAT1*4* allele generate restriction fragments of 176 bp and 125 bp following PCR amplification of the region corresponding to nucleotides 769 to 1113, and digestion with the restriction enzyme *Mbo* II. This allele (*NAT1*4*) is present in the individual indicated by the open arrow in Figure 1(a). However, most other known *NAT1* alleles (in which the primer N1113 does not generate an additional *Mbo* II restriction site) generate fragments of 176 bp and 144 bp, apart from *NAT1*11*, in which the corresponding fragment is 135 bp due to a 9 base pair deletion. In the case of the individual identified by the open arrow, an additional band of 116 bp was identified which was not

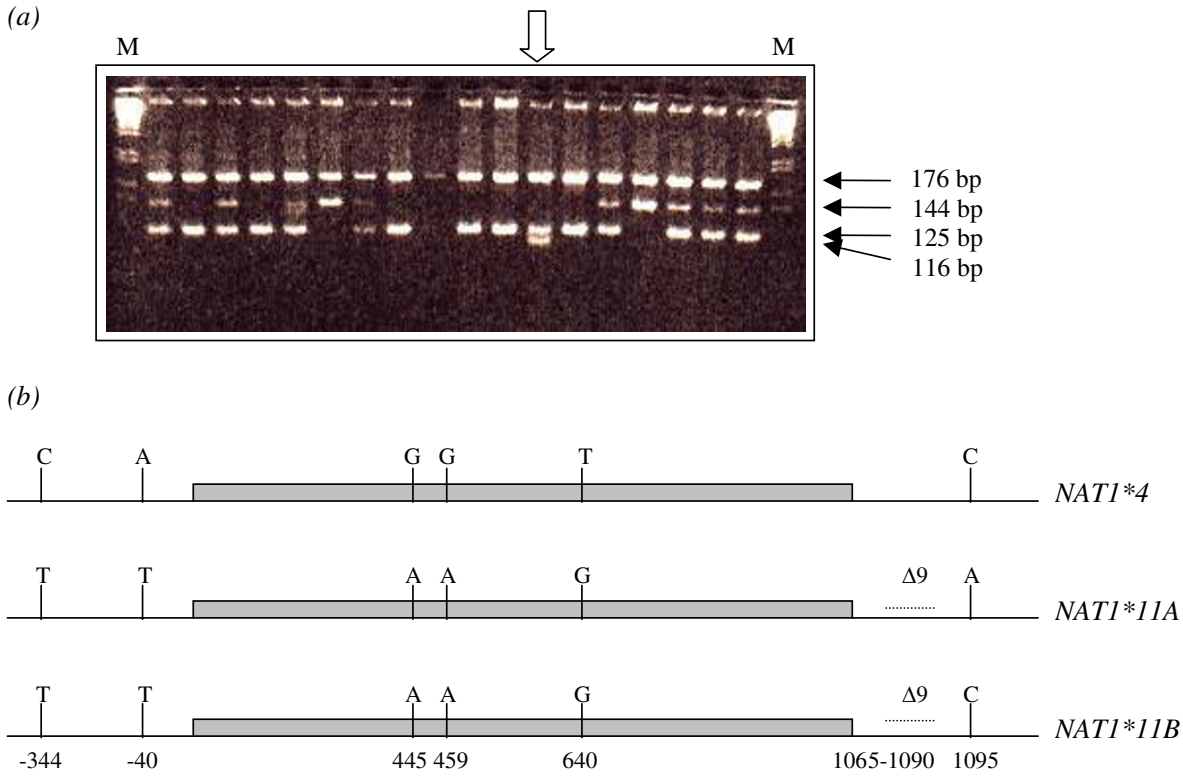

Figure 1
Identification of a novel *NAT1* allele. *(a)* A 344 bp PCR product of *NAT1* (amplified from genomic DNA using primers N769 and N1113, the latter introducing an additional *Mbo* II restriction site in *NAT1*4*) was digested with *Mbo* II and separated on a 4% (w/v) MetaPhor® agarose (FMC BioProducts, UK)/0.5X TBE gel, against a 1 kb DNA Ladder (Gibco BRL, UK)(Lane M). Detectable *NAT1*4* fragments are 176 bp and 125 bp. Fragments of other known alleles are 176 bp and 144 bp (*NAT1*3*, *NAT1*10, NAT1*14A*) or 176 bp and 135 bp (*NAT1*11A*). The individual marked with an open arrow had a novel restriction fragment (116 bp) which did not correspond to any known allelic combination. *(b)* Both *NAT1* alleles of the individual marked with an open arrow were sequenced across the coding and 5' and 3' flanking regions and the sequence of the novel allele (*NAT1*11B*) is illustrated in comparison with the most common Caucasian *NAT1* allele, *NAT1*4* (EMBL Accession Number AJ307007) and the most similar *NAT1* allele, *NAT1*11A*. *NAT1*11B* contains sequence variations identical to those of *NAT1*11A* (C-344T, A-40T, G445A, G459A, T640G and a 9 base deletion (Δ) in the nucleotide range 1065–1090, compared with the *NAT1*4* sequence), but contains a cytosine at nucleotide 1095. The 870 bp coding region of *NAT1* is shaded grey, with 438 nucleotides upstream and 306 nucleotides downstream also shown.

consistent with any of the then-known alleles. To confirm whether this restriction pattern was due to the presence of a novel allele, the *NAT1* alleles (a 1.6 Kb region) from the individual were cloned and sequenced. As a result, a novel allele was identified and defined as *NAT1*11B* (with C at 1095) due to its high sequence similarity with *NAT1*11A* (with A at 1095) (Figure 1(b)). *NAT1*11B* contains six mutations with respect to the *NAT1*4* sequence: C-344T, A-40T, G445A (V149I), G459A, T640G (S214A), 9 bp deletion in the nucleotide region 1065–1090. The functional consequences of this allele are likely to be similar to that of *NAT1*11A* where an association with low enzymic activity has been proposed as a result of the amino acid changes V149I and S214A [42,43]. Other changes in the novel allele compared with *NAT1*4* are outside the coding region (Figure 1(b)) and their effects have not yet been

established. Although this allele was identified in an AD patient, it is unlikely to play a significant role in the pathogenesis of AD since it was found in only one individual.

Conclusions

In conclusion, the study presented here, which to our knowledge includes the first known investigation of the association between *NAT1* genotype and susceptibility to AD, suggests that genetic polymorphisms in *NAT1* and *NAT2* do not influence susceptibility to AD. However, the inclusion of SNP analysis at positions 1088 and 1095 in a multi-variant analysis would be warranted based on the present studies since there is an indication that *NAT 1*10* is elevated in AD.

Competing interests

None declared.

Authors' contributions

NJ carried out the *NAT1* and *NAT2* genotyping and *NAT1* sequence analysis, performed the statistical analysis and drafted the manuscript. PB and VJ participated in the *NAT1* genotyping. MB participated in the design of the study and provided study samples. ES conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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