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The neuropeptide genes *TAC1*, *TAC3*, *TAC4*, *VIP* and *PACAP* (*ADCYAP1*), and susceptibility to multiple sclerosis

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Abstract

The related immunomodulatory neuropeptides vasoactive intestinal peptide (VIP) and pituitary adenylyl cyclase activating peptide (PACAP; gene symbol *ADCYAP1*) have recently been proposed as novel therapeutics for the treatment of multiple sclerosis (MS). These neuropeptides, as well as those belonging to the tachykinin family exert pleiotropic effects, many of which are of relevance to central nervous system inflammation. In the present study, we have analysed 14 single nucleotide polymorphisms (SNPs) and 4 microsatellite markers in the *VIP*, *ADCYAP1*, *TAC3* and *TAC4* genes for susceptibility to MS in a case-control collection from Northern Ireland. Following correction for multiple comparisons, we did not find any significant associations between single polymorphic markers or multiple-marker haplotypes and susceptibility to MS. Furthermore, we analysed 2 SNPs in the *TAC1* gene in a set of Sardinian trio MS families, based on our previous observation of association of these SNPs with MS in the Northern Irish (Genes Immun. 2005, 6, 265–270). Analysis of these SNPs in the Sardinians was not significant though a similar trend to that originally observed in the Northern Irish was present. Meta-analysis of the Sardinian and Northern Irish *TAC1* SNP genotype data revealed a Mantel–Haenszel Common OR Estimate for the *TAC1* intron 1 SNP *rs2072100* of 0.76 (95% CI 0.63–0.92; $P=0.005$; A allele) and for the *TAC1* promoter SNP *rs7793277* of 0.76 (95% CI 0.615–0.95; $P=0.014$; C allele). Our data advocate a need for further exploration of the *TAC1* gene region in MS.

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1. Introduction

A number of neuropeptides with immunomodulatory effects relevant to inflammation in the central nervous system have been identified. Of these, the related neuropeptides vasoactive intestinal peptide (VIP) and pituitary adenylyl cyclase activating peptide (PACAP) have recently been proposed as novel therapeutics for the treatment of multiple

sclerosis (Abad et al., 2006). VIP was reported to reduce incidence and severity of experimental allergic encephalomyelitis (EAE), to suppress EAE neuropathology and to prevent recurrence of the disease (Gonzalez-Rey et al., 2006). Similarly, PACAP was reported to exert beneficial effects on clinical and pathological manifestations of EAE, probably through suppression of antigen presenting cell functions (Kato et al., 2004). VIP and PACAP exert a number of effects in the central nervous system (CNS) that could generally be regarded as being anti-inflammatory and neuroprotective (Gressens et al., 1997; Gozes et al., 1997; Gomariz et al., 2001). Upon axotomy, expression of PACAP is rapidly

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induced (Mulder et al., 1999). VIP and PACAP, released by injured neurons act as “neuron survival factors” by down-regulating synthesis of pro-inflammatory factors such as iNOS, IL-1 β , TNF- α and chemokines by activated microglia (Delgado et al., 2002a,b, 2003). Activated microglia have been implicated in early stages of myelin destruction in MS (Trapp et al., 1999a). Axon trans-section in MS occurs at sites of inflammation, and correlates with neurological disability (Trapp et al., 1999b; Anthony et al., 2000). Thus, dysregulation of the VIP/PACAP neuroimmune circuitry might be of relevance to processes leading to inflammatory demyelination and axon loss in MS. Notwithstanding this rather convincing biological data, a putative role for the VIP and PACAP (*ADCYAP1*) genes in genetic susceptibility to MS has not yet been explored.

We have recently reported association between a two-marker haplotype spanning the promoter-intron-1 region of the neuropeptide tachykinin-1 (*TAC1*) gene on chromosome 7q21-22 and multiple sclerosis in Northern Ireland (Vandenbroeck et al., 2002; Cunningham et al., 2005). *TAC1* generates the neuropeptide substance P, known to display a range of immunomodulatory activities some of which are of relevance to CNS inflammation (McCluskey and Lampson, 2001; Annunziata et al., 2002). Two further tachykinins with overlapping and distinct functions have been recognized. The *TAC3* precursor gives rise to a mature peptide of 10 amino acids that is identical to neurokinin B of other mammalian species. *TAC4*, originally called hemokinin-1, was discovered in 2000 as a survival factor for B cell precursors (Zhang et al., 2000).

The chromosomal locations of the *VIP* gene (6q26-27), *ADCYAP1* (18p11), *TAC3* (12q13-21) and *TAC4* (17q21.33) have all been highlighted as potential regions of interest in some individual genome-wide linkage and/or association studies (Kuokkanen et al., 1997; Akesson et al., 2002; Ban et al., 2002). In a meta-analysis of raw genotype data from three full genome scans, of these, only the *VIP* and *TAC4* chromosome regions emerged more strongly with non-parametric linkage (NPL) scores of 2.01 (6qtel) and 2.30 (17q22), respectively (The Transatlantic Multiple Sclerosis Genetics Cooperative, 2001). In the recent high-density screen for linkage in MS, of these regions, only 17q23 showed suggestive evidence for linkage (International Multiple Sclerosis Genetics Consortium, 2005).

The present study was designed in two parts. First, in order to shed light on a potential role of the neuropeptide genes *VIP*, *TAC3*, *TAC4* and *ADCYAP1* in susceptibility to MS, we have genotyped a selection of 4 microsatellite and 14 single nucleotide polymorphisms (SNPs) located in these genes in a collection of multiple sclerosis patients and healthy control subjects from Northern Ireland. We primarily analysed SNPs in *VIP*, *TAC3*, *TAC4* and *PACAP* for association with susceptibility to MS as single markers, and used haplotype analysis only as subsidiary to the single-marker analysis. Second, starting from our previous study in which we demonstrated significant association of the *TAC1* SNPs *rs7793277* and

rs2072100 with MS in the Northern Irish collection (Cunningham et al., 2005), we have scrutinized these SNPs in an independent trio family-based collection from Sardinia.

2. Materials, subjects and methods

2.1. Patients and controls

Northern Irish collection: A total of 451 MS patients and 206 healthy controls all of Northern Irish origin were employed in this study. The control group is composed of a combination of both (i) non-affected friend clinical attendee and (ii) blood bank donors. Both of these groups are assumed to neither have MS or any other neurological condition, and are residents of Northern Ireland. The ratio of males to females in the cases and controls were 1:2.08 and 1:1.13, respectively. **Sardinian family collection:** A total of 199 trio families (maternal, paternal and affected MS patient) from a Sardinian population were implemented for the *TAC1* part of the study. All patients were diagnosed as having MS according to the Poser criteria (Poser et al., 1983). Clinical and demographic data have been published before (Cunningham et al., 2005; Goris et al., 2002).

2.2. Genotyping

Polymorphic sites within the four candidate genes *TAC3*, *TAC4*, *VIP* and *ADCYAP1* were selected from the NCBI database. An initial sequencing strategy was employed for the *TAC3* gene to screen for polymorphisms in the exon sequences and immediately surrounding regions in 24 individuals (12 MS/12 Controls). Post-PCR cycle sequencing reactions were carried out using Big Dye v3.1 (Applied Biosystems, Foster City, USA) performed on an ABI Prism 3100 (Applied Biosystems, USA) and the data generated were examined by means of Sequencer v4.05 (Gene Codes Corp; MI, USA).

SNP genotyping was performed using either (i) Taqman SNP Genotyping Assays (Applied Biosystems, USA), in conjunction with a DNA Opticon 2 (MJ Research; Waltham) or (ii) a Fluorescence Polarisation Single Base Extension (FP-SBE) method (Kwok, 2002), adopted for a number of SNPs using AcycloPrime assays (PerkinElmer, USA) and performed on an Analyst AD (Molecular Devices; Sunnyvale, CA). Taqman assays were performed using the Quantitect Probe PCR master mix (Qiagen, USA) in 10 μ l reaction volumes. Amplification of templates for FP-SBE genotyping was performed using standard amplification conditions using HotStarTaq polymerase (Qiagen, USA) prior to enzymatic cleanup. FP-SBE analysis was performed as per manufacturer's protocol. The genotyping of *rs8192597* and *rs2856966* was performed by a direct sequencing approach as these SNPs are in close proximity. A list of all genotyping assays, primers and probes are given in Table 1. Primer and probes used for *TAC1* are those previously described (Cunningham et al., 2005).

Table 1
Polymorphic markers in neuropeptide genes and genotyping information

Gene	Marker	Position in gene (genomic location)	Taqman assay	PCR primers	Genotyping probes (5'→3') used in FP-SBE	Typing info
<i>VIP</i>	<i>D6S290</i> ^c	3' from gene (153176367–153176701)		5'[FAM]GTT TGC TGG ATG AGT GG 3' 5' GAT TTG GTG AAT GCT CTG 3'		
	<i>rs1407267</i> ^c	5' from gene (153162565)		5' TTT TGT ATT GGG AAA AGC TAC TA 3' 5' CAA ACT AGA GGT TGA AAC AGT TC 3'	5' TTT CTT CTT ATC ATT CAG CAG TCA TTT 3' 5' AGC AAC TTA ATT GAG GGT CAC AGA 3'	
	<i>rs2791580</i> ^c	Intron 6 (153169617))		5' AAT GGA AAG AGG AGG TAA AGA 3' 5' CAC CGA AGA CAC CGA TT 3'	5' TCT CAC TTA TCT ACC TAT ACT AC 3' (G→C) 5' AGG TTG AAA ATC ACT GCT TCA 3'	
	<i>rs688136</i> ^b	Exon 9, UTR (153172175)	C_3250639	5' CTA GAA GCT GCT CTC TTG GTA T 3' 5' GGA TAT TGA AGT TGT TTT CTT GA 3'		
	<i>rs1321968</i> ^c	Exon 9, UTR (153172351)		5' ATA TAT TTT ATG CCT AAA GCA ACA 3' 5' GCA GGC TTT TTA TGA GTT AAA G 3'	5' TGT ATT CTA GCT AAT GTA ATA ACT GTG A 3' 5' ACT CTC AAA TAC TAT ATA CAA TGT AAA 3' (T→A)	
<i>TAC3</i>	<i>D12S1906</i> ^c	3' UTR (55690085–55690334)		5'[FAM]AGT TTC CAC ACC AGG GTC A 3' 5' TGC ATT AGG AAG ACC TCT TTC C 3'		NP
	<i>rs733629</i> ^a	Exon 7, NS: E → K (55692711)	C_620360			
	<i>rs17119327</i> ^c	Intron 3 (55695007)		5' TTT CCA CAG TTC CTT TAC GAA 3' 5' ACT CTG CCC ACC TCC TAT AAT 3'	5' AAG GTT TTG AAA TGA CAG CAA TTA 3' 5' CAT CTT AAA AGC ATT TGT ACC GAT 3'	
	<i>rs2291855</i> ^c	Exon 1, UTR (55696458)		5' CTC CAC TCG GTT TCT CTC TT 3' 5' CAT CTG CTT TAT TCC CTC CT 3'	5' CAG ACC ACC CAG CCC C 3' 5' CTA CAG GTG CTT TGT GCT CAC 3'	
	<i>D12S1691</i> ^c	5' from gene (55792049–55792368)		5'[TET]GGT AAA CAC TGA GAC ACG CC 3' 5' TGA TGA CNC AGA AGT TGA GC 3'		
<i>TAC4</i>	<i>D17S1795</i> ^c	Intron 1 (45279978–45280344)		5'[HEX]AGT GCC AGA GAT ATA CCG TG3' 5' GTC TGC AAG GCA AGT TGT C 3'		
	<i>rs4794068</i> ^b	Intron 1 (45277340)	C_27945803	5' GAC ACT TCC CAT GAT AGA GCA 3' 5' TTT AAT CTT CAC AAC CAC CAT AAC 3'		
	<i>rs883010</i> ^b	3' from gene (45271796)	C_7476514	5' CTC TAA TCG TTG ATC GTT 3' 5' CTC ACT TGA GCT GTG ACC CT 3'		
<i>ADCYAP1</i>	<i>rs8192597</i> ^d	Exon 3, S: A → A (897675)		5' TGT CGT TGC CTC CTC CTT ACC T 3' 5' CAC TGC CCC TCC CGT CA 3'		Upper Primer
<i>(PACAP)</i>	<i>rs2856966</i> ^d	Exon 3, NS: G → D (897710)		5' TGT CGT TGC CTC CTC CTT ACC T 3' 5' CAC TGC CCC TCC CGT CA 3'		Upper Primer
	<i>rs2231187</i> ^a	Exon 5, S: K→K (899561)	C_25474227			
	<i>rs1610037</i> ^a	Exon 6, UTR (900635)	C_7494714			
	<i>rs948234</i> ^a	3' from gene (904304)	C_7494712			
<i>TAC1</i>	<i>D7S554</i> ^c	5' from gene (96969506–96969842)		5'[HEX]GTC TAA TTA CCC ACA TTT CCC T 3' 5'ATG TTC CAT ATT TAA AAG ACT CAG TGA 3'		
	<i>rs7793277</i> ^c	Proximal promoter region (97004236)		5' GCC CTC TTC CAG GTT ACA GAC TGT 3' 5' GCG GTA CAC TCT CCT GAC CTG TC 3'	5' ATA TTG TCT CCT TGT TAT ATC CTC ACA T 3' 5' GCT CTC TCA TTC TTT CCT GC 3'	
	<i>rs2072100</i> ^a	Intron 1 (97006435)	C_2560484			

Genotyping assay applied: ^aTaqman assay pre-validated, ^bTaqman assay validated in house by direct sequencing of PCR amplified products, ^cFP-SBE, ^dDirect sequencing (both SNP's located within the one amplified region) and ^eCapillary Fragment Analysis. Where Taqman assays have been used their product reference is provided (i.e. C_number). In genotyping probes, base substitution is shown in (). Abbreviations: NP, appeared not polymorphic within the population studied; S, synonymous; NS, non-synonymous; UTR, untranslated.

Microsatellite repeat polymorphisms were examined by PCR amplification and electrophoresis through POP6 filled capillaries on an ABI Prism 3100 Genetic Analyser (Applied Biosystems, Foster City, USA) using GeneScan software (Applied Biosystems, Foster City, CA). Forward primers were labelled with fluorophores, and GeneScan 500 HD (Applied Biosystems) was used as an internal size standard. In addition, marker amplicons from an identical DNA pool comprising 16 individual DNA samples were included in the analysis as internal controls. Details on primers and amplification procedures can be found online at The Genome Database (www.gdb.org).

2.3. Analysis of data

Case-control: For each bi-allelic marker individual P -values were calculated using standard 3×2 and 2×2 χ^2 contingency tables comparing genotype and allele counts in cases against controls. All markers were tested for Hardy Weinberg Equilibrium (HWE). Examination of haplotype association was performed using the CHAPLIN program (Epstein and Satten, 2003) which relaxes the HWE assumption that is required if the expectation–maximisation (EM) algorithm is used for haplotype estimation. For multi-allelic markers the CLUMP program (T1 and T2) was used to test associations before and after combining rare alleles (Sham and Curtis, 1995). Bonferroni correction for multiple testing of 17 loci was performed (accounting for 14 SNPs and 3 microsatellite markers; the fourth microsatellite *D12S1906* appeared monomorphic in our collection and is therefore not included in the comparison). **Family data:** Analysis of the *TAC1* Sardinian family data was performed using the TDT approach (Spielman et al., 1993). The haplotype-based haplotype relative risk (HHRR) test was performed using the computer program TDTPhase (Dudbridge, 2003). The *TAC1* study in the Sardinians is not subject to multiple comparison concerns since this is a single-hypothesis replication attempt of an earlier study (Cunningham et al., 2005).

3. Results

A total of 14 SNPs enriched for location in coding and potential regulatory sequences of the *VIP*, *TAC3*, *TAC4* and *ADCYAP1* genes (Table 1) were selected for genotyping. 8 SNPs were located in exon sequences, and two of these were non-synonymous (*rs733629* E→K in *TAC3* and *rs2856966* G→D in *ADCYAP1*). All other SNPs were located in 5' and 3' terminal gene regions, or in introns. In order to boost the information content of the screen, we also included a number of multi-allelic microsatellite markers, and we selected those that were located as closely as possible to the genes under study (*D6S290* located <4 Kb 3' from *VIP*; *D12S1906* within the 3' UTR of *TAC3*; *D12S1691* located ~90 Kb 5' from *TAC3*; and *D17S1795* in the unique intron of *TAC4*). All markers were genotyped in the Northern Irish

collection. Table 2 summarizes allele counts and frequencies for all 14 SNPs scrutinized within the case-control collection. No statistically significant associations were found between any of the 14 SNPs and susceptibility to MS. Multi-marker haplotype association for each of the four genes was examined using the CHAPLIN program and was negative (results not shown).

Analysis of the *TAC3* gene was based on three SNPs and two microsatellite markers, *D12S1906* and *D12S1691* (Table 1). The *D12S1906* marker was found not to be polymorphic within our collection. *D12S1691* was found to be composed of 19 alleles (size range 189 to 227 bp), 10 of which had a frequency >5%. This marker was significantly associated with MS when analysed using the CLUMP program after pooling of minor alleles (<T2> $P=0.0114$). Bonferroni correction for multiple comparisons renders this P value insignificant.

We have recently demonstrated association of the *TAC1* intron 1 SNP *rs2072100* with MS in Northern Ireland ($P=0.009$ for comparison of AA homozygotes versus G carriers). Two-marker haplotypes of *rs2072100* and

Table 2
Neuropeptide SNP allele counts and frequencies within the Northern Irish sample collection

Gene	dbSNP	Genotyping success rate (%)	Alleles	Healthy control count (%) ^d	Unstratified MS count (%) ^d
<i>VIP</i>	<i>rs1407267</i>	97	G	381 (0.95)	809 (0.92)
			T	21 (0.05)	67 (0.08)
	<i>rs2791580</i>	96.5	C	360 (0.90)	777 (0.89)
			T	42 (0.10)	99 (0.11)
	<i>rs688136^a</i>	98	C	144 (0.36)	318 (0.36)
			T	258 (0.64)	558 (0.64)
<i>TAC3</i>	<i>rs1321968</i>	98	C	375 (0.93)	809 (0.92)
			A	27 (0.07)	67 (0.08)
	<i>rs733629</i>	100	C	27 (0.07)	68 (0.08)
			T	371 (0.93)	810 (0.92)
	<i>rs17119327</i>	98	G	358 (0.90)	776 (0.88)
			A	40 (0.10)	102 (0.12)
<i>TAC4</i>	<i>rs2291855</i>	97.5	C	272 (0.68)	647 (0.74)
			T	126 (0.32)	231 (0.26)
	<i>rs4794068^b</i>	99	G	247 (0.63)	547 (0.62)
			A	145 (0.37)	337 (0.38)
	<i>rs883010</i>	98	C	196 (0.50)	450 (0.51)
			A	196 (0.50)	434 (0.49)
<i>ADCYAP1</i>	<i>rs8192597^c</i>	98	G	114 (0.28)	235 (0.26)
			A	296 (0.72)	661 (0.74)
	<i>rs2856966</i>	98	G	89 (0.22)	196 (0.22)
			A	321 (0.78)	700 (0.78)
	<i>rs2231187</i>	99	G	126 (0.31)	312 (0.35)
			A	284 (0.69)	584 (0.65)
	<i>rs1610037</i>	100	G	121 (0.30)	273 (0.30)
			A	289 (0.70)	623 (0.70)
	<i>rs948234</i>	100	G	231 (0.56)	485 (0.54)
			A	179 (0.44)	411 (0.46)

^aDeviation from HWE in controls ($P<0.001$) and MS cases ($P=0.02$).

^bDeviation from HWE in controls ($P=0.017$) and MS cases ($P=0.022$).

^cDeviation from HWE in MS cases ($P=0.042$).

^dOnly those allele counts are represented for which full genotyping information is available on a per gene basis.

Table 3

Meta-analysis of the Odds Ratios for association of individual *TAC1* SNPs with Northern Irish and Sardinian MS^a

	<i>rs7793277*C</i>					<i>rs2072100*A</i>				
	Control or NT (%)	Case or T (%)	OR	95% CI	<i>P</i>	Control or NT (%)	Case or T (%)	OR	95% CI	<i>P</i>
Northern Irish ^b	101 (30.6%)	207 (25.4%)	0.77	0.58–1.03	0.07	191 (57.9%)	398 (48.9%)	0.70	0.54–0.90	0.006
Sardinian	101 (26.2%)	81 (21%)	0.75	0.54–1.05	0.09	177 (47.3%)	162 (44.3%)	0.85	0.63–1.13	0.27
Breslow–Day Homogeneity Test	–	–	–	–	0.89	–	–	–	–	0.32
Mantel–Haenszel Common OR	–	–	0.76	0.615–0.95	0.014	–	–	0.76	0.63–0.92	0.005

^aControl/case refers to the N Irish study; T (transmitted)/NT (not transmitted) to the Sardinian family study. Allele counts are presented together with % of the total allele count in each cohort.

^bThe data of the Northern Irish sample collection have been published before but are reproduced for clarity (Cunningham et al., 2005).

rs7793277 — which is located in the promoter of *TAC1*, were also strongly associated with Northern Irish MS ($P=0.0003$; Cunningham et al., 2005). In the present study, individual association of these SNPs was analysed in 199 trio MS families from Sardinia using the TDT (Spielman et al., 1993). In the single-locus family tests, a trend toward under transmission of *rs7793277*C* ($T=81$, $NT=101$; NS) and *rs2072100*A* ($T=162$, $NT=177$; NS) was observed, which, whilst not attaining significance, is in keeping with the single locus analysis of the Northern Irish case-control (Cunningham et al., 2005). The P value of 0.09 for *rs7793277*C*, although not significant, can be considered as “suggestive” since the only hypothesis tested in this replication experiment is that of under-transmission of the C allele to patients with MS, which is not subject to correction for multiple comparisons. In view of the similar trends in both populations, we performed a meta-analysis of the Odds Ratios for both *TAC1* SNPs in both populations (Table 3), with the transmitted and untransmitted parental alleles in the Sardinian trios providing the case and control groups (as in the HHRR test). The Breslow–Day test showed homogeneity of OR’s for both SNPs, allowing calculation of a Maentel–Haenszel common OR of 0.76 for *rs7793277*C* ($P=0.014$) and of 0.76 for *rs2072100*A* ($P=0.005$).

4. Discussion

Analysis of 14 SNPs and 4 microsatellite markers located in the gene regions of the neuropeptides *TAC3*, *TAC4*, *VIP* and *ADCYAP1* did not demonstrate any significant associations in a Northern Irish collection of 451 MS patients and 206 controls. The power of the present study was limited due to the low minor allele frequencies at some of the gene loci included in this study, especially at *VIP* and *TAC3*. The International HapMap Project (<http://www.hapmap.org/index.html.en>), for instance, lists 17 genotyped SNPs in a 20 Kb region encompassing the *TAC3* gene (chr12:55683322..55703321). At least 10 of these appear to be monomorphic in the CEU collection, and the highest minor allele frequency (MAF) seen for any of the remaining SNPs is only 0.1 in CEU (e.g. for *rs17119327*A*, this value is in agreement with the frequency found in the Northern

Irish collection, Table 2). The ramification of these low minor allele frequencies is that the study design (451 MS, 206 controls) had a high chance of detecting a genetic effect with OR (genotypic relative risk) ranging from 1.8 to 2.2 (power 90%; 2-sided $P=0.05$ — not corrected for multiple comparisons and assuming that the genotyped locus is the actual disease susceptibility locus and not merely one in linkage disequilibrium with it) for the 5 SNPs in the *TAC3* and *VIP* genes with MAF ranging from 0.05 to 0.1 (Purcell et al., 2003). For the 9 remaining SNPs (with MAF ranging from 0.22 to 0.50), a more moderate genetic effect was theoretically detectable with OR ranging from 1.6 to 1.5. These latter values are more in line with the experimentally verified risks attributed to common polymorphic variants associated with multifactorial diseases, including *DLG5* in Inflammatory Bowel Disease (RR of 1.74), *IBD5* in Crohn’s Disease (RR of 1.3), and *CTLA4 CT60* in Graves’ Disease (RR of 1.5) (for review see Oksenberg and Rioux, 2006). However, if minor effects with RR of around 1.2 are to be considered, our study did not have enough power to rule these out.

The confirmatory study of *TAC1* in the Sardinian family set yielded ambivalent results, with as main result a not-significant trend toward under-transmission of *rs7793277*C* to children with MS. This is similar to the significant under-representation of this allele seen before in the Northern Irish MS collection (Cunningham et al., 2005). All in all, the results from the *TAC1* replication attempt in the present study, though as yet inconclusive, urge for a further analysis of *TAC1* in additional patient collections.

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