SHORT REPORT

Paraoxonase promoter and intronic variants modify risk of sporadic amyotrophic lateral sclerosis

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Background: The paraoxonases, PON1–3, play a major protective role both against environmental toxins and as part of the antioxidant defence system. Recently, non-synonymous coding single nucleotide polymorphisms (SNPs), known to lower serum PON activity, have been associated with sporadic ALS (SALS) in a Polish population. A separate trio based study described a detrimental allele at the PON3 intronic variant INS2+3651 (rs10487132). Association between PON gene cluster variants and SALS requires external validation in an independent dataset.

Aims: To examine the association of the promoter SNPs $PON1_{-162G>A}$ and $PON1_{-108T>C}$; the non-synonymous functional SNPs $PON1_{Q192R \text{ and } L55M}$ and $PON2_{C311S \text{ and } A148G}$; and the intronic marker $PON3_{INS2+3651A>G}$, with SALS in a genetically homogenous population.

Methods: 221 Irish patients with SALS and 202 unrelated control subjects were genotyped using KASPar chemistries. Statistical analyses and haplotype estimations were conducted using Haploview and Unphased software. Multiple permutation testing, as implemented in Unphased, was applied to haplotype p values to correct for multiple hypotheses.

Results: Two of the seven SNPs were associated with SALS in the Irish population: $PON1_{55M}$ (OR 1.52, p = 0.006) and $PON3_{INS2+3651\ G}$ (OR 1.36, p = 0.03). Two locus haplotype analysis showed association only when both of these risk alleles were present (OR 1.7, p = 0.005), suggesting a potential effect modification. Low functioning promoter variants were observed to influence this effect when compared with wild-type.

Conclusions: These data provide additional evidence that genetic variation across the paroxanase loci may be common susceptibility factors for SALS.

Epidemiological evidence suggests that environmental factors may contribute to the risk of sporadic amyotrophic lateral sclerosis (SALS). Such factors include insecticides, pesticides, arylesterases[1 2] and oxidants in cigarette smoke.[3] The incidence of SALS is increased in Gulf war veterans who may have been exposed to exogenous neurotoxins.[4] These observations have led to the proposition that genetic determinants known to increase susceptibility to exogenous compounds may also increase the risk of SALS.[5]

The human paraoxonase (PON) gene cluster is located on chromosome 7q21.3. The cluster comprises three genes ordered PON1, PON3 and PON2, with PON1 being the most centromeric.[6] The PON1 enzyme acts as the major protective mechanism by which toxic exogenous compounds are hydrolysed in serum.[7 8] PON1 has four common functional polymorphisms, two non-synonymous coding single nucleotide polymorphisms (SNPs) ($PON1_{Q192R}$ and $PON1_{L55M}$) which alter enzyme activity,[9 10] and two promoter SNPs ($PON1_{-108T>C}$ and $PON1_{-162G>A}$) which affect expression levels.[11] PON2 and PON3 do not appear to have detoxifying activity, but along with PON1 have important roles in protecting against lipid peroxidation.[13 14] Non-synonymous coding polymorphisms associated with PON2 activity include $PON2_{C311S}$ and $PON2_{A148G}$.[15 16] The risk alleles, associated with lower PON activity, are $PON1_{192R,\ 55M,\ -108T \text{ and } -162G}$ and $PON2_{311C \text{ and } 148G}$.[9–16]

Two recent studies have reported associations between PON gene cluster polymorphisms and increased risk for SALS. The first reported an association of $PON1_{192R}$ and $PON2_{311C}$ with SALS in a Polish population.[17] A separate haplotype study of trio pedigrees in the US observed an important intronic marker in PON3 ($PON3_{INS2+3651A>G}$ or rs10487132).[18]

Reproduction of association in independent populations is of substantial importance to support the hypothesis that PON cluster polymorphisms modify the risk for SALS. Here we test for the association of PON cluster variants with SALS in an Irish population. We selected variants with previous association or an established biological role in PON kinetics, and explored the influence of PON promoter polymorphisms and the PON3 intronic variant.

METHODS

Subjects

The study population comprised 221 Irish patients with sporadic ALS (52.9% male; mean age 58.8 (SD 12.8) years) and 202 spousal/population control subjects (50.9% male; mean age 54.9 (14.3) years). All patients fulfilled the El Escorial criteria for clinically definite or probable ALS. Cases with familial ALS, based on a positive family history, were excluded. Control subjects had no history of neurological disease and no family history of ALS. All cases and controls were of Irish Caucasian ethnicity. Informed consent was obtained and the study approved by the Beaumont Hospital Ethics and Medical Research Committee (protocol 49/05).

Marker selection

Comprehensive tagSNP analysis of the PON gene cluster suggests that the majority of the *cis* effects on serum detoxifying activity are attributable to known non-synonymous and promoter sequence polymorphisms, which thus capture the functionally important genetic variation across the region.[12] Aiming to explore the influence of these functionally relevant PON cluster variants, along with those previously associated with SALS, we selected seven markers for study: the four non-synonymous polymorphisms known to alter enzyme kinetics ($PON1_{Q192R \text{ and } L55M}$ and $PON2_{C311S \text{ and } A148G}$), the two promoter polymorphisms known to influence expression levels ($PON1_{-162G>A \text{ and } -108T>C}$) and the PON3 intronic marker ($PON3_{INS2+3651A>G}$) noted from the study in the US.

Abbreviations: ALS, amyotrophic lateral sclerosis; HWE, Hardy–Weinberg equilibrium; LD, linkage diseqilibrium; PON, paraoxonase; SALS, sporadic amyotrophic lateral sclerosis; SNP, single nucleotide polymorphism

Table 1 Association statistics of the genotyped polymorphisms with risk for sporadic amyotrophic lateral sclerosis

SNP No	Variant	NCBI ref	Alleles	HWE	Risk allele (functional variant)	RAF (%) (cases; controls)	χ^2	p Value	OR (95% CI)
1	$PON1_{Q192R}$	rs662	A>G	0.78	G (192R)	28.3; 32.7	1.95	0.16	0.81 (0.6–1.09)
2	$PON1_{L55M}$	rs854560	T>A	0.64	A (55M)	36.6; 27.6	7.59	0.006*	1.52 (1.13–2.04)
3	$PON1_{-108T>C}$	rs705379	T>C	0.42	T	54.3; 54.7	0.01	0.93	0.99 (0.74–1.31)
4	$PON1_{-162G>A}$	rs705381	G>A	0.96	G	74.9; 73.7	0.15	0.7	1.06 (0.78–1.45)
5	$PON3_{IVS2+3651A>G}$	rs10487132	A>G	0.86	G	41.6; 34.3	4.46	0.03*	1.36 (1.02–1.81)
6	$PON2_{C311S}$	rs6954345	C>G	0.68	G (311C)	23.0; 25.4	0.62	0.43	0.88 (0.64–1.21)
7	$PON2_{A148G}$	rs12026	C>G	0.24	G (148G)	23.9; 24.0	0.001	0.97	0.99 (0.71–1.38)

HWE, Hardy–Weinberg equilibrium p value; NCBI, National Center for Biotechnology Information; RAF, risk allele frequency; SNP, single nucleotide polymorphism. *Significant p values.

Genotyping

DNA was extracted from peripheral blood. Genotyping was performed by KBiosciences (Herts, UK) using a KASPar PCR system. Quality control criteria were that genotypes formed three distinct clusters, water controls were negative and minor allele frequency was greater than 5%. The number of genotypes callable was 97.6%. All studied polymorphisms were in Hardy–Weinberg equilibrium (HWE) for the case, control and entire study populations.

Statistical analysis

Individual polymorphisms were analysed for association with SALS by the Pearson χ^2 test of independence. Estimations of departures from HWE were also calculated by the χ^2 test. For single point associations, $p<0.05$ was considered significant, and the loci found to be associated were entered into two locus haplotypes to investigate potential interactions. Pairwise linkage diseqilibrium (LD) was examined with the program Haploview (version 3.32).[19] Haplotype frequencies were estimated using the accelerated expectation–maximisation algorithm as implemented in Haploview (version 3.32) and Unphased (version 3.0.3).[20] Haplotypes incorporating one or more risk alleles are compared with the corresponding haplotype with no risk alleles present. The p values for association of haplotypes with SALS were corrected for multiple comparisons by use of a permutation procedure, as implemented by the program Unphased.[20] Multiple permutations were performed (up to 1000) until an even distribution curve of the permuted data was obtained. The permutation procedure gives a corrected p value for association so that values <0.05 can be considered significant.

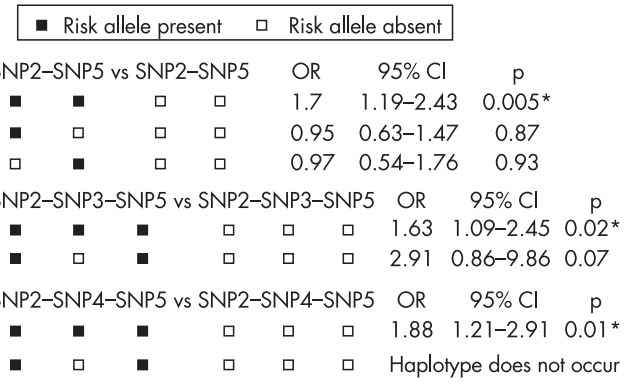

Figure 1 Tests of association with sporadic amyotrophic lateral sclerosis (SALS) for (A) two locus haplotypes comprising single nucleotide polymorphisms (SNPs) 2 and 5. (B) Three locus haplotypes with risk alleles present at SNPs 2 and 5, and low functioning versus wild-type promoter alleles at SNP3 (upper) and at SNP4 (lower). *Significant p value after correction for multiple testing.

RESULTS

Table 1 shows the allele frequencies and allele tests of association for each of the genotyped polymorphisms. $PON1_{55M}$ (SNP2, $p = 0.006$) was over-represented in SALS vs controls. A weaker association signal was observed at $PON3_{INS2+3651G}$ (SNP5, $p = 0.03$). The two positive markers were not in strong LD ($r^2 = 0.32$) (see supplementary fig; the supplementary fig can be viewed on the *J Neurol Neurosurg Psychiatry* website at http://www.jnnp.com/supplemental). The other five markers were negative in our population.

To further explore the influence of the two associated polymorphisms, we next investigated potential interaction between them (fig 1A). The two locus haplotype, comprised of risk alleles at both SNP2 and SNP5, showed association with SALS ($p = 0.005$) whereas the presence of the risk allele at SNP2 with the wild-type allele at SNP5 ($p = 0.87$) and the presence of the risk allele at SNP5 with the wild-type allele at SNP2 ($p = 0.93$) did not.

Finally, we investigated potential modification by low functioning promoter variants (SNPs 3 and 4) on the associated haplotype. We tested this by constructing three locus haplotypes consisting of low functioning versus wild-type promoter alleles at one locus, in addition to the risk alleles at SNPs 2 and 5 (fig 1B). The haplotype incorporating the low functioning SNP3 variant showed association ($p = 0.02$) whereas the haplotype incorporating wild-type SNP3 did not ($p = 0.07$). The haplotype incorporating the low functioning SNP4 variant was associated ($p = 0.01$) while that incorporating wild-type SNP4 was estimated not to occur in our population.

DISCUSSION

We found a strong association of $PON1_{L55M}$, and modest association of $PON3_{INS2+3651A>G}$, with SALS in the Irish population. The presence of two positive markers prompted us to examine their interaction, leading to identification of a two locus haplotype associated with 70% increased risk. These data add support to the previous reports of association between PON gene cluster variants and SALS.

It is unclear why $PON1_{L55M}$, rather than $PON1_{Q192R}$ reported by Slowik *et al*,[17] is associated in our population. The associated allele from both the Irish and Polish studies leads to an amino acid substitution resulting in the same effect of reduced serum PON1 activity. As the influence of these polymorphisms is substrate dependent,[8] it is possible that our population is exposed to a different deleterious agent, for which the $PON1_{L55M}$ variant, rather than $PON1_{Q192R}$, is critical. An alternative explanation may be that the positive markers from

each population are in LD with another, as yet unidentified, key variant which modifies risk.

Association of $PON3_{INS2+3651G}$ with SALS was described by Saeed *et al*[18] in a large American family based trio model, but not replicated in their smaller case control study group. This latter group exhibited different haplostructure with breakdown of strong LD across PON2–3, raising the possibility of a more genetically heterogeneous population. In contrast, we observed strong LD across PON2–3 (supplementary fig; the supplementary fig can be viewed on the *J Neurol Neurosurg Psychiatry* website at http://www.jnnp.com/supplemental) similar to that among the American trios.

We also observed a potential effect modification on the associated haplotype conferred by the presence of a low functioning PON1 promoter polymorphism. It is difficult to draw firm conclusions as the rarity of these haplotypes limited power for analysis, and one of the relevant haplotypes for comparison was not present. This rarity within the population may have arisen by selection given the protective role of PON1 from both environmental toxins and premature cardiovascular disease.[7 8 21]

Taken together, our findings support the suggestion that genetic variation across the paroxanase loci may be common susceptibility factors for SALS. Two biological explanations for this have been proposed.[17 18] Exposure to exogenous environmental toxins, in a genetically predisposed host, may precipitate motoneuron degeneration. However, in vitro studies have consistently reported that only PON1 exhibits such detoxifying properties.[13 14] Low paraoxonase activity may alternatively predispose to SALS by rendering the antioxidant defence system insufficient to protect against oxidative stress. All three paraoxonase enzymes exhibit antioxidant properties.[13 14] We observed associated polymorphisms in both PON1 and PON3, but haplotype comparisons suggested association only when both risk alleles were present. This supports the notion that the PON3 intronic variant could modify PON1 activity through *cis* effects, but it remains unclear whether the detoxifying or antioxidant activity of PON1 is impaired.

Association of common variations in PONs with SALS and other neurodegenerative diseases[22 23] may have important implications. If environmental substrates, such as insecticides and arylesterases, do indeed increase the risk of neurodegeneration, it follows that occupational health strategies to minimise exposure should be prioritised. Additionally, paraoxonases may have therapeutic potential. Further study will be required to assess the full interaction of environmental exposures, enzyme kinetics and genetic predisposition with risk for SALS.

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The supplementary figure can be viewed on the *J Neurol Neurosurg Psychiatry* website at http://www.jnnp.com/supplemental.

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