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Paraoxonase Gene Mutations in Amyotrophic Lateral Sclerosis

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Abstract

Three clustered, homologous paraoxonase genes (*PON1*, *PON2* and *PON3*) have roles in preventing lipid oxidation and detoxifying organophosphates. Recent reports describe a genetic association between the *PON* genes and sporadic amyotrophic lateral sclerosis (ALS). We now report that in genomic DNA from individuals with familial and sporadic ALS we have identified at least seven *PON* gene mutations that are predicted to alter PON function.

Introduction

The paraoxonase gene cluster consists of three adjacent genes on chromosome 7q21.3-q22.1. Of these, *PON1* is most intensively studied. *PON1* and *PON3* are primarily expressed in the liver and secreted into the blood through their association with HDL; in contrast, *PON2* is ubiquitously expressed¹. The interaction of PON1 with HDL requires the amino terminal retained signal sequence of the PON1 protein². Almost all serum PON1 protein is bound to HDL³. PON1 has a broad spectrum of substrates including organophosphates (paraoxon, chlorpyrifos oxon and diazoxon), nerve toxins (soman and sarin), and aromatic

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esters (phenyl acetate)¹. Several organophosphate compounds are neurotoxins commonly found in insecticides, nerve agents, foods, and other household items. The ability to detoxify organophosphates is not shared by PON2 or PON3^{4, 5}. Because organophosphates are not produced in the body, it is postulated that the physiological function of PON1's is to protect LDL from oxidation⁶. All three PON proteins share the ability to hydrolyze lactones (cyclic esters)⁷. Because the PON proteins reduce oxidation and detoxify neurotoxins, the *PON* cluster has been intensively studied for a possible role in ALS. Six studies have shown a genetic association between single nucleotide polymorphisms in the *PON* genes and sporadic ALS,^{8–13} although a meta-analysis of the all published data¹⁴ failed to detect this association. To extend these studies, we have sequenced the *PON* genes in familial and sporadic ALS (FALS and SALS) to identify potentially causal mutations.

Subjects and Methods

All samples were collected with IRB approval. The FALS were previously screened for mutations in *SOD1*, *TARDBP* and *FUS* and consist of 255 Caucasians (97.3%), 3 Asians (1.2%), 1 African-American (0.38%) and 1 sample of mixed origin (0.38%). All SALS and control samples were Caucasian in origin. The coding regions of each *PON* gene were amplified using primers located in adjacent intronic or noncoding regions. Primer sequences are reported in Supplemental Table 2. PCR reactions were bi-directionally sequenced and aligned, and variations were identified with the Polyphred software. Genotyping was performed using custom Taqman SNP Genotyping Assays (Applied Biosystems). Primer and probe sequences are reported in Supplemental Table 3. All samples displaying the mutant genotype were confirmed by bi-directional DNA sequencing. A more extensive description of the methods is available in Supplemental Appendix 1.

Results

To identify DNA mutations that predispose to ALS, we sequenced the coding region of *PON1* in 260 familial ALS (FALS) and 188 sporadic ALS (SALS) cases. Our analysis revealed eight heterozygous rare variants in 15 case samples; seven variants were not present in dbSNP build 130, while one (c.269T>C) is reported as rs72552788. We presume that this SNP is rare, inasmuch as it was not detected in 591 control DNA samples included in the SNP500Cancer Database (<http://snp500cancer.nci.nih.gov>); moreover, no allele frequencies for this SNP are reported in dbSNP. Sequencing of *PON1* in 188 controls and 188 SALS did not reveal additional rare variants. To test further the possibility that these SNPs are benign polymorphisms, we genotyped each of the eight variants by TaqMan SNP assays in a panel of 1,159 control DNA samples and an additional set of 996 SALS DNAs. Five of the eight mutations were not present in control samples, suggesting that they contribute to ALS pathogenesis (Table 1). Three variants were detected in controls (N19D, M127R, and A201V) with a frequency ~50% lower than cases; these variants demonstrated a low to moderate level of evolutionary conservation (Supplemental Fig. 1).

Four of the mutations identified only in ALS cases are missense: C42R, L90P, M127I, and P315T. The M127I site displays evolutionary variation at this position. Since the M127R variant was identified in control samples, this change may likely reflect a benign polymorphism. In contrast, the wild-type residue for the remaining missense changes is highly conserved across multiple species including zebrafish (Fig. 1). Given that PON1 contains a single disulfide bridge between Cys42 and Cys353, and that this is required for PON1 activity,¹⁵ it is predicted that the C42R mutant will disrupt the bridge and impair PON1 function. The L90P and P315T mutations create or destroy a proline residue, which contains a ring structure with conformational rigidity that can destroy alpha helices. Based on these properties, we expect these mutations to change PON1 protein folding. The fourth

mutation (c.74+3A>C) disrupts the 5' human splice consensus sequence MAG|GTRAGT. The purine (R) base is present in 94% of splice sites and is highly evolutionarily conserved in PON1 (Fig. 1). The substitution with a pyrimidine will likely alter the primary sequence or expression of the PON1 protein. Cell lines or tissue was not available to directly assess the influence of this mutation. Additional DNA was available from an affected sibling of a FALS harboring the P315T mutation. Genotyping demonstrated that the affected sibling also carries the mutation. DNA was not available from any other affected family members (Supplemental Table 1 and Supplemental Fig. 2). All five mutations were observed in FALS, while only one mutation, L90P, was also observed in a single SALS case. These results suggest that mutations in *PON1* contribute more significantly to FALS than SALS. There was also no ethnic commonality between the mutated samples (Supplemental Table 1) suggesting that alteration to *PON1* are not unique to a single population.

We also sequenced the *PON2* and *PON3* genes in 166 FALS cases and identified five novel variants (two in *PON2* and three in *PON3* (Table 1 and Supplemental Table 1). As in the studies of *PON1*, we evaluated the ALS-specificity of these variants by genotyping each in a panel of 1,159 control and 1,184 SALS DNA samples, as well as an additional 94 FALS samples. One *PON2* mutation, C42Y, was not identified in control samples (Table 1). This mutation was homozygous in a proband whose parents were asymptomatic first cousins, strongly suggesting a recessive model of inheritance. The mutated residue corresponds to the C42R mutation identified in PON1, suggesting that this amino acid is of critical importance to PON function. Additionally, two of three novel PON3 mutations were not observed in control samples, but were identified in SALS, suggesting that they also contribute to ALS pathogenesis. In particular, a D230N mutation was observed in two distinct FALS, and a single SALS case. The identification of this mutation in two unrelated FALS cases further suggest that this variant is pathogenic. In all three cases, the PON2 and PON3 mutations were highly conserved, further highlighting the importance of the mutated amino acids (Fig. 1). We also detected a frameshift variant in *PON2* (c.286delA, R96GfxX5; Supplemental Table 1) that is predicted to generate a truncated PON2 protein of 101 amino acids. Because this was heterozygously present in FALS (1/260), SALS (6/1,184) and controls (4/1,159), it is likely to be a benign polymorphism.

In total, from nine FALS and three SALS cases we identified eight coding sequence mutations that were present in the PON genes but not in controls. The overall percentage of PON gene mutations in our FALS panel is approximately 3.5%. Because our panel excluded all cases with *SOD1*, *TARDBP* and *FUS* mutations, which represent ~30% of all FALS, we estimate that mutations in the PON gene cluster represent ~2.5% of all FALS cases.

Discussion

The hydrolytic activity of PON1 activity varies substantially (eight- to ten-fold) depending on the genotype of PON1 coding SNPs in the population and is influenced by exogenous factors including smoking, diet, and lipid-controlling medication¹. Given this wide variation in hydrolytic activity, we presume that if the PON1 mutations predispose to ALS by reducing hydrolysis, such activity reductions must be severe. This suggests that the PON1 mutants may act as dominant negative inhibitors by oligomerizing and inactivating wild-type PON1. This view is consistent with the observations that native PON1 may exist in multiple oligomeric states^{16, 17}, as predicted by its variable molecular mass (70–500 kDa). Oligomerization is promoted by the interaction of the PON1 amino terminal region with HDL particles¹⁶. That these PON1 mutations exert a dominant negative influence on PON1 activity is consistent with a report that serum from an individual with a heterozygous L90P PON1 mutation showed profoundly reduced hydrolysis of diazoxonase and paraoxonase¹⁸.

Because its activity, pattern of expression, localization, and incorporation into HDL mimics that of PON1, it is possible that the adverse consequences of PON3 mutants will be mechanistically similar to those of mutant forms of PON1. It is therefore of interest that the ALS-associated variants in both PON1 and PON3 are present on only one allele; heterozygous variants in either gene are potentially able to inhibit activity via a dominant negative mechanism. By contrast, PON2 is an intracellular enzyme that does not interact with HDL and that exists in a predominantly monomeric state. That an apparently benign, heterozygous frameshift mutation in PON2 is present within the general population argues that haploinsufficiency of PON2 is not obviously pathogenic. In turn, this argues that the PON2 mutants will only be pathogenic when they lead to more profound loss of function than is predicted by eliminating one functional PON2 allele. This prediction is consistent with our finding that the ALS-associated variant in PON2 is present as a homozygous defect.

As with all genetic based studies, the possibility exists that the identified changes represent rare variants as opposed to pathogenic mutations. This is especially true when the approach is based on candidate gene sequencing as opposed to linkage analysis. However, several lines of evidence suggest that the changes reported here are indeed pathogenic. With the exception of the PON1 M127I, all of the amino acid residues modified by the ALS-specific variants show a high level of evolutionary conservation. In particular, the PON1 C42R mutation (and presumably, by homology, the PON2 C42Y mutation) destroys a cysteine bond essential for activity. Furthermore, the L90P has been previously identified in a heterozygous individual demonstrating a very low diazoxonase/paraoxonase activity¹⁸. Additional evidence is derived from the presence of a homozygous PON2 C42Y mutation within FALS descendent from unaffected first cousins. The prediction that progeny of first cousins are homozygous for consanguineous alleles at only ~3.1% of the genome further suggests that this variant is pathogenic. Moreover, three variants were observed in unrelated ALS cases, consistent with the view that they disease-related: the PON3 D230N mutation was identified within two unrelated cases of FALS cases and one of SALS, while both PON1 L90P and PON3 D121N mutations were observed in one case each of FALS and SALS. Lastly, all these variants were not detected in over 1,100 controls.

While, in our view, these observations suggest that the identified FALS-associated *PON* variants are disease-causing mutations, with the evidence in hand we cannot formally exclude the possibility that they represent risk factors, especially because segregation could be proven only in a small pedigree. Moreover, if the penetrance of a disease-causing mutation is incomplete, as happens in many FALS pedigrees, it is difficult to distinguish between a causative mutation with low penetrance and a risk factor. One criterion suggesting that a rare variant influences inherited susceptibility is that it is over- (or under-) representation in disease vs control cohorts; this is underscored if a similar disease association exists for a group of variants affecting the same gene or set of genes with related functions¹⁹. We observed a statistically significant difference between FALS vs controls whether we consider variants in *PON1* only (13/260 vs. 8/1159, $p<0.0001$; two-tailed Fisher's exact test), *PON3* only (5/260 vs. 3/1159, $p=0.0069$), or all the rare variants identified in the *PON* cluster (20/260 vs. 15/1159, $p<0.0001$). We also observed a statistically significant difference between FALS vs. SALS whether we consider variants in *PON1* ($p=0.0007$), *PON3* ($p=0.0064$), or all rare variants ($p<0.0001$), again suggesting that mutations in the *PON* genes contribute more significantly to FALS than SALS.

There are several reports of an association between SALS and the *PON* cluster^{8–13}, yet genome-wide association studies (GWAS) and a recent meta-analysis of the all published data¹⁴ failed to detect an association between SNPs in the *PON* locus and susceptibility to SALS. We believe this discrepancy reflects at least two factors. First, there is almost

certainly heterogeneity among the different populations combined in the meta-analysis; if the ALS-associated variants differ in the different populations, the ALS associations will not be detected in an aggregate meta-analysis. Second, case-control genome wide SNP studies are intended to identify common variants, on the assumption that the risk of complex disorders is influenced by common, weakly associated alleles¹⁹. GWAS studies depend on the linkage disequilibrium of such common, deleterious variants to a common, linked polymorphic marker, which produces a differential allele frequency between cases and controls and will not detect rare deleterious variants or those common variants that lack linkage disequilibrium to a common marker. As an alternative, the identification rare deleterious variants requires the sequencing of a candidate gene(s) in a large disease population and then testing the frequency of each in a case/control population¹⁹. This approach has been successfully taken for variants influencing colorectal adenomas²⁰, 21 and HDL cholesterol levels²². Here, we have taken the same approach and identified the *PON* genes as a contributor to ALS.

Given the multiplicity of PON substrates, it is difficult to ascertain which altered functions of the mutant PON genes are significant in ALS pathogenesis. Because there are ALS-related mutations in all three enzymes, the pathogenic mechanism is likely to reflect some property shared by mutant PON1, PON2 and PON3 proteins. While this might be the loss of some shared function (e.g. anti-oxidative capacity), we cannot exclude the possibility that the mutants have acquired a novel, toxic function. Because in ALS, motor neurons are exposed to abnormal oxidative stresses such as lipoperoxidation²³ and signs of ER stress are increased in the spinal cord of SALS patients²⁴, it is plausible that loss of anti-oxidative capacity of the paraoxonases could be neurotoxic. Alternatively, the PON mutations might contribute to the development of ALS by impairing metabolism of OP or other unknown exogenous toxins. This premise is supported by the recent discovery that mutations in the neuropathy target esterase (NTE), a gene coding for a protein targeted by OP in Organophosphorus-Induced Delayed Neuropathy (OPIDN), can cause motor neuron disease²⁵. Understanding how these mutant forms of PON contribute to neurodegeneration will aid in the development of novel therapeutic strategies to attenuate motor neuron cell death in ALS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Evolutionary Conservation of PON Mutations in Familial and Sporadic ALS

This illustrates the evolutionary conservation of the amino acids implicated by the 8 identified mutations. For each, the mutated amino acid (or nucleotide for the splicing mutation) is shown in red. Chromatograms displaying the mutation are shown below. The position indicates the base pair position within the cDNA. All mutations were heterozygous except for the homozygous C42Y *PON2* mutation. Each mutation was confirmed by bi-directional sequencing and 5' nuclease assay genotyping.

Table 1

Paraoxonase Gene Variants in Familial and Sporadic ALS.

Gene	Exon	bp change	Variant	Variant Dose	Direct Sequencing			Total		
					FALS	SALS	CTRL	FALS	SALS	CTRL
			<i>ALS only</i>							
PON1	1	c.74+3A>C	Intron 1 (+3)	Heterozygous	1/260	0/188	0/188	1/260	0/1184	0/1159
PON1	2	c.124T>C	C42R	Heterozygous	1/260	0/188	0/188	1/260	0/1184	0/1159
PON1	4	c.269T>C	L90P	Heterozygous	1/260	0/188	0/188	1/260	1/1184	0/1159
PON1	5	c.438G>T	M127I	Heterozygous	1/260	0/188	0/188	1/260	0/1184	0/1159
PON1	9	c.943C>A	P315T	Heterozygous	1/260	0/188	0/188	1/260	0/1184	0/1159
PON2	2	c.95G>A	C42Y	Homozygous	1/166	-	-	1/260	0/1184	0/1159
PON3	4	c.361G>A	D121N	Heterozygous	1/166	-	-	1/260	1/1184	0/1159
PON3	6	c.688G>A	D230N	Heterozygous	1/166	-	-	2/260	1/1184	0/1159
Subtotal								9/260	3/1184	0/1159
			<i>ALS and controls</i>							
PON1	1	c.55A>G	N19D	Heterozygous	2/260	0/188	1/188	2/260	6/1184	3/1159
PON1	5	c.437T>G	M127R	Heterozygous	2/260	1/188	0/188	2/260	6/1184	2/1159
PON1	6	c.602C>T	A201V	Heterozygous	4/260	0/188	1/188	4/260	3/1184	3/1159
PON2	4	c.286delA	R96GfsX5	Heterozygous	1/166	-	-	1/260	6/1184	4/1159
PON3	9	c.971G>A	G324D	Heterozygous	2/166	-	-	2/260	1/1184	3/1159
Subtotal								11/260	22/1184	15/1159
Total								20/260	25/1184	15/1159