European Journal of Human Genetics (2002) 10, 292–296

www.nature.com/ejhg

ARTICLE

Genetic analysis of Paraoxonase (PON1) locus reveals an increased frequency of Arg192 allele in centenarians

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Human Paraoxonase (PON1) is a High-Density Lipoprotein (HDL)-associated esterase that hydrolyses lipo-peroxides. PON1 has recently attracted attention as a protective factor against oxidative modification of LDL and may therefore play an important role in the prevention of the atherosclerotic process. Two polymorphisms have been extensively studied: a Leucine (L allele) to Methionine (M allele) substitution at codon 55, and a Glutamine (A allele) to Arginine (B allele) substitution at codon 192. We have examined these two aminoacidic changes in 579 people aged 20 to 65 years old, and 308 centenarians. We found that the percentage of carriers of the B allele at codon 192 (B+ individuals) is higher in centenarians than in controls (0.539 *vs* 0.447), moreover we found that among the B+ individuals, the phenomenon was due to an increase of people carrying M alleles at codon 55 locus. In conclusion, we propose that genetic variability at PON1 locus affects survival at extreme advanced age.
European Journal of Human Genetics (2002) **10,** 292–296. doi:10.1038/sj.ejhg.5200806

Keywords: polymorphism; human longevity; apolipoproteins; aging

Introduction

A great deal of data indicate that risk factors for the major causes of mortality in the elderly, (cardiovascular diseases, diabetes) are associated with alterations in anti-oxidant, inflammatory and lipidic profile.[1–3] Particular importance has been attributed to the role of peroxidation of LDL in atherogenesis and to the capability of lipoprotein-associated proteins of modulating local inflammatory response.[4]

Serum Paraoxonase 1 (PON1) has been claimed to play a central role in the scenario. The enzyme is a High Density Lipoprotein (HDL)-bound Arylesterase which hydrolises lipo-peroxides and it is therefore responsible for the protective effects of HDL on peroxidation of Low Density Lipoproteins (LDL).[5] Indeed, oxydised LDL are capable of stimulating the production of foam cells, are cytotoxic towards arterial wall cells, and induce macrophagic cytokines, resulting in highly proinflammatory and proatherogenic.[6] The inter-individual variability of PON1 serum activity has been thought to modulate the risk to develop atherogenesis. In fact, the

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Received 12 December 2001; revised 8 March 2002; accepted 20 March 2002

variability of the PON1 gene at codons 192 (Gln/Arg=A/B alleles) and 55 (Leu/Meth=L/M alleles) has been associated with enzymatic activity and concentration.[7,8] Consistently, a large number of studies suggested an association between cardiovascular diseases and the Arg_{192} variant.[9] Even Leu_{55} has been associated with cardiovascular disease susceptibility.[9] Recently, the role of Arg_{192} allele on CHD (cardiovascular heart disease) risk in the Italian population has been questioned[10] and recent data indicate that PON1 genotype affects the vasculature responsiveness to triglycerids.[11] Interestingly, when the impact of A/B and L/M alleles of PON1 on mortality of individuals aged over 85 years of age was analysed, no significant effect has been found, leading to hypothesise that PON1 variants have not a major effect on the risk of fatal cardiovascular disease at the population level.[12]

In this study we wanted to test the hypothesis that PON1 genotypes affect long term survival, i.e. whether allele, genotype and combined genotypes frequency distributions of centenarians are different from those found in young individuals.

Materials and methods

Subjects

A sample of 579 Italian unrelated young individuals (347 males and 232 females, median age=40.5 years) ranging from 20 to 65 years of age and a sample of 308 Italian unrelated centenarians (241 females and 67 males) were analysed. The samples were recruited in both Northern Italy (368 young subjects and 216 centenarians) and Southern Italy (211 young subjects and 92 centenarians). Control subjects were apparently healthy at the time of blood collection judging from clinical examination and recent clinical history. All the subjects gave their informed consent.

Genotyping

DNA was extracted from blood lymphocytes by the salting-out method.[13] Polymerase chain reactions were performed using primer sequences derived from published data and specific for the amplification of regions surrounding codon 192 and codon 55 sites.[14] The amplification reaction for codon 192 locus was performed on a Perkin-Elmer Cetus 9700 thermal cycler with initial denaturation at 94°C for 4 min, followed by 40 cycles, each one comprised of denaturation at 93°C for 1 min, annealing at 64°C for 40 s and extension at 72°C for 1 min, with a final extension time of 10 min at 72°C. The PCR products were digested with AlwI (New England Biolabs) for 4 h at 37°C, and the samples were electrophoresed in 2% agarose gels. Amplification of the codon 55 polymorphism was performed on a Perkin-Elmer Cetus 9700 thermal cycler with initial denaturation at 96°C for 3 min, followed by 35 cycles, each one comprised of denaturation at 95°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min, with a final extension time of 5 min at 72°C. The PCR products were digested with *Nla*III (New England Biolabs) for 4 h at 37°C, and the samples were electrophoresed in 2% agarose gels. Some genotypes at both loci were confirmed by direct sequencing of amplified DNA fragments with the automatic sequencer (ABI PRISM 310, Perkin Elmer Biosystem).

Statistical analysis

Allelic, genotypic and combined genotypes frequency distributions were compared by Monte Carlo χ^2. Sex-specific age-related differences were assessed by Mantel-Haenszel test, implemented with SPSS package (SPSS, Chicago, IL, USA). HWE (Hardy-Weinberg Equilibrium) was checked by Monte Carlo Markov Chain.[15] Estimation of maximum-likelihood multi-locus haplotype frequencies was calculated using an EM (Expectation Maximization) algorithm, implemented with Arlequin package.[16] Standard Deviations were calculated by bootstrap (1000 replications). Difference in haplotype frequency distribution was assessed by Fisher exact test.

Results

PON1 192 alleles and genotypes in young people and centenarians

No significant difference was present between Northern and Southern Italians either in centenarians or in controls (data in http://biologia.unical.it/labs/genetica.html). Table 1 reports genotypes and alleles of the PON1 polymorphism at codon 192 in the whole Italian sample. In both the age-classes the observed genotypes were in agreement with those expected at Hardy-Weinberg Equilibrium ($P>0.05$). Significant differences between young people and centenarians were evident when allele and genotype frequency distributions were compared (χ^2=5.429, df=1, P=0.020 for allele frequency, and χ^2=6.798, df=2, P=0.034 for genotype frequency). In particular, the proportion of B+ subjects (AB+BB individuals) was increased in centenarians in comparison to young people (χ^2=6.765, df=1, P=0.011). The role of gender as a confounding variable was tested by MH test, and no significant result was found (data not shown).

PON1 55 alleles and genotypes in young people and centenarians

Again no geographic difference was observed either in centenarians or in young control subjects for PON1 codon 55 variants (data in http://biologia.unical.it/labs/genetica.html). Table 2 shows genotypes and alleles in the whole Italian sample. In both centenarians and young people the observed genotypes were in agreement with those expected at Hardy-Weinberg Equilibrium ($P>0.05$). Unlike what has been observed at codon 192, allele and genotype frequency distributions were not statistically different between young people and centenarians. Moreover, the M+ subjects (LM plus MM individuals) were present at the same proportion in the two age-classes (0.598 *vs* 0.607, respectively).

Table 1 Genotype and allele frequency distributions at PON1 192 locus

	Young subjects (n=*579*) n *(%±s.e.)*	*Centenarians* (n=*308*) n *(%±s.e.)*
Genotypes[a]	Observed Genotypes[a]	Observed Genotypes[a]
AA subjects	320 (0.553±0.021)	142 (0.461±0.028)
AB subjects	212 (0.366±0.020)	137 (0.445±0.028)
BB subjects	47 (0.081±0.011)	29 (0.094±0.017)
B+ subjects[b]	259 (0.447±0.021)	166 (0.539±0.028)
Alleles[c]		
A allele	852 (0.735±0.013)	421 (0.683±0.019)
B allele	306 (0.265±0.013)	195 (0.317±0.019)

[a]χ^2=6.798, df=2, *P*=0.034; [b]χ^2=6.765, df=1, *P*=0.011; [c]χ^2=5.429, df=1, *P*=0.020.

Table 2 Genotype and allele frequency distributions at PON1 55 locus

	Young subjects (n=*579*) n *(%±s.e.)*	*Centenarians* (n=*308*) n *(%±s.e.)*
Genotypes[a]	Observed Genotypes[a]	Observed Genotypes[a]
LL subjects	233 (0.402±0.020)	121 (0.393±0.028)
LM subjects	262 (0.453±0.021)	155 (0.503±0.028)
MM subjects	84 (0.145±0.015)	32 (0.104±0.017)
M+ subjects[b]	346 (0.598±0.020)	187 (0.607±0.028)
Alleles[c]		
L allele	728 (0.629±0.014)	397 (0.644±0.019)
M allele	430 (0.371±0.014)	219 (0.356±0.019)

[a]χ^2=3.754, df=2, *P*=0.155; [b]χ^2=0.077, df=1, *P*=0.829; [c]χ^2=0.433, df=1, *P*=0.535.

Combined analysis of PON1 192/55 loci in young people and centenarians

Since the haplotype estimation from population data is found on the assumption of homogeneity of the population, and is therefore very sensitive to possible stratification, haplotype analysis in young people and centenarians was carried out first in Northern and Southern Italians separately (data in http://biologia.unical.it/labs/genetica.html). As no significant geographic difference was observed either in young people or in centenarians, the following analyses were carried out in the whole Italian sample. The results are shown in Table 3. A significant difference in estimated haplotype frequency distributions was found between young people and centenarians (Fisher Exact Test, *P*=0.012). The difference was predominately due to an increase of BM haplotype in centenarians (1.4%) in comparison to that found in young people (0.3%).

We therefore thought it worthwhile to investigate the combination of genotypes at codons 192 and 55 (Table 4), and we found that their distributions were significantly different between young subjects and centenarians (χ^2=11.525, d.f.=3, *P*=0.010). In particular, we found that B+ subjects (Arg_{192}+) could be sub-grouped on the basis of the genotype at codon 55 as M+ and M-, and that only B+M+ individuals (AB.LM, AB.MM, BB.LM) were increased in centenarians (0.173 in young people *vs* 0.263 in centenarians), whilst the proportion of B+M- individuals

Table 3 PON1 codon192/codon 55 haplotypes frequency distribution

	Haplotype frequency±s.d.					
	Young subjects (1158 chromosomes)			*Centenarians (616 chromosomes)*		
Haplotype[a]	*Mle*	*Exp*	*D*[b]	*Mle*	*Exp*	*D*[c]
AL	0.367±0.014	0.462±0.015	−0.0955	0.341±0.018	0.440±0.020	−0.0996
AM	0.368±0.016	0.273±0.013	+0.0955	0.342±0.019	0.243±0.017	+0.0996
BL	0.262±0.014	0.167±0.011	+0.0955	0.303±0.017	0.204±0.016	+0.0996
BM	0.003±0.002	0.098±0.009	−0.0955	0.014±0.006	0.113±0.013	−0.0996

[a]χ^2=10.5, df=3, *P*=0.012; [b]D′=97.4%, χ^2=232.855, df=1, *P*<0.001; [c]D′=88.5%, χ^2=123.1642, df=1, *P*<0.001; Mle: Maximum likelihood estimation; Exp: expected.

Table 4 Genotype combinations at PON1 codon 192 and codon 55

	Young subjects (n=*579*) n *(%±s.e.)*	*Centenarians* (n=*308*) n *(%±s.e.)*
Genotype Combinations[a]	Observed genotype combinations	Observed genotype combinations
B+M+subjects (AB.LM+AB.MM+BB.LM)	100 (0.173±0.016)	81 (0.263±0.025)
B+M−subjects (AB.LL+BB.LL)	159 (0.275±0.018)	85 (0.276±0.025)
B−M+subjects (AA.LM+AA.MM)	246 (0.424±0.021)	106 (0.344±0.027)
B−M−subjects (AA.LL)	74 (0.128±0.014)	36 (0.117±0.018)

[a]χ^2=11.525, df=3, *P*=0.010.

(AB.LL+BB.LL) did not change appreciably (0.275 in controls *vs* 0.276 in centenarians).

Discussion

Taking into account the physiological role of the enzyme Paraoxonase 1, which may play a central role in rate and quality of ageing,[17] we investigated the possible association of PON1 genetic variability with longevity, i.e. the capacity of reaching the extreme limits of human life span escaping major age-related diseases.

When the two variants A/B_{192} and L/M_{55} were analysed separately (Tables 1 and 2), we found that PON1 variability at 192 codon was able to discriminate between young people and centenarians. In particular, the frequency of B allele, and consequently of B+ individuals, increased from young people to centenarians, thus indicating that this allele decreases mortality in carriers. When the two variants A/B_{192} and L/M_{55} were analysed together (Table 4) only B+M+ individuals increased from young people to centenarians, thus indicating that this genotype combination decreases mortality in carriers.

It should be noted that, given the large sample size, the observed gene/longevity association is not highly significant. This finding implies that the effect of PON1 variability on the overall population mortality is rather slight, and this finding is in line with the results obtained in several gene/longevity association studies.[18] Moreover, the AB_{192} heterozygous genotype appears to contribute rather largely to the observed association (Table 1). A recently developed mathematical model explains the variation of heterozygosity in an ageing cohort in terms of differential survival as affected by the biological role of the gene.[19] According to the model, centenarians are expected to show increasing heterozygosity with respect to young subjects for stress-responder genes driving the individual adaptive capacity. Data in Table 1 suggest that PON1 is one of the genes affecting such adaptive capability, and therefore one of the genes affecting rate and quality of ageing.

The finding that the B_{192} allele is more frequent in centenarians than in young people seems to be contradictory with literature data reporting the role of this allele as risk factor in artery and coronary diseases, carotid stenosis, cardiovascular diseases, vascular dementia.[10,20–22] However, the data are still conflicting. What is more, these discrepancies may be related to different criteria of recruitment, different average age of the sample groups, different experimental design (for example the fact that in some studies A/B_{192} variants have been examined without taking into account other variants of PON1 gene, such as L/M_{55} variants). Probably, the population genetic history as well as the presence of other risk factor for CVD play a key role in determining association between A/B_{192} alleles and CVD. It is of interest that studies carried out in the Mediterranean area do not support the existence of a significant association between PON1 A/B_{192} genetic polymorphism and CVD.[10,21–23] On the other hand, carriers of B allele (B+ subjects) seem to be protected when other known risk factors for CVD are present, such as smoking,[24] familiar hypercolesterolemia,[25] low HDL-cholesterol levels.[26] Anyway, even if the role of the B allele as risk factor were ascertained, the presence of a genetic risk factor in the gene pool of centenarians is not unusual. The above cited mathematical model[19] is able to explain such 'paradoxes in centenarians' taking into account the biological and physiological role of risk alleles in survival, and the heterogeneity of the today population of centenarians. In any case, an increasing number of data indicate that long life expectancy is compatible with genetic risk factors,[27–30] when the individual genetic background is able to cope with stress and consequent age-related accumulation of somatic damage.[31]

PON1 activity is involved in the protection of LDL against oxidative/proinflammatory modifications which play a central role in the pathogenesis of arteriosclerosis and cardiovascular diseases.[6] As the increase of B+M+ individuals among centenarians suggests a genetically determined Paraoxonase activity advantageous for longevity, it could be hypothesised that such individuals are provided with an elicited capacity to counteract the deleterious effects of the accumulation of pro-inflammatory molecules and the increase of the proinflammatory status which accompany human aging.[31] Functional studies of correlation between PON1 genotype and PON1 activity in cells from centenarians may clarify this point.

Acknowledgements

This work was financed by Ministry of Health 'Progetto finalizzato 1997: la prevenzione della morbilità per malattie croniche nell'età avanzata. Il modello dei centenari' and 'Progetto finalizzato 1998: interazioni tra fattori di rischio cardiovascolari e fattori genetici dell'aterosclerosi e della trombosi: un approccio integrato genetico-funzionale in casistiche selezionate' and by COFIN 2000–2002 Genetics Determinants of Human Longevity.

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