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Original scientific paper

DISTRIBUTION OF PARAOXONASE 1 CODING REGION POLYMORPHISMS IN SERBIAN POPULATION

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Serum paraoxonase 1 (PON1) in humans is a protein component of high-density lipoprotein (HDL) particles that protects against oxidative damage, detoxifies toxic metabolites of organophosphorus pesticides and nerve agents and activates or inactivates specific drugs. It has been reported that *PON1* gene coding region polymorphisms, L55M and Q192R, could influence both expression level and catalytic activity of PON1, and their

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link with a broad spectrum of diseases has been described. The aim of this study was to determine the frequencies of *PON1* coding region polymorphisms Q192R and L55M in Serbian population. The most frequent alleles were Q (0.77) for Q192R and L (0.68) for L55M. Genotypes QQ (0.60) and LL (0.47) and combined genotype QQ/LL (0.26) were the most frequent in examined population.

Key words: coding region polymorphisms, paraoxonase 1, Serbian population

INTRODUCTION

Paraoxonase1 (PON1) is a calcium-dependent esterase composed of 354 amino acids (45 kDa) belonging to a family of proteins that includes PON2 and PON3 (PRIMO-PARMO *et al.,* 1996). The genes coding for the PON family are located on human chromosome 7 (q21.22). PON1 is synthesized in the liver and secreted into the blood where is associated with high-density lipoprotein (HDL) particles (MACKNESS *et al.,* 1998). It is apparently associated with less than 10% of the total HDL.

In vivo, a wide interindividual variation in serum PON1 concentration and activity is observed. This variation is largely explained by common genetic polymorphisms in the *PON1* gene. Two polymorphisms are present in the *PON1* coding sequence: a Gln(Q)/Arg(R) substitution at position 192 (Q192R), and a Leu(L)/Met(M) substitution at position 55 (L55M) (COSTA *et al.,* 2005). The *PON1* Q/R polymorphism significantly affects the catalytic efficiency of PON1. Initial studies demonstrated that the R192 allozyme hydrolyzes paraoxon more readily than Q192 (COSTA *et al.,* 2005). Further studies showed that this polymorphism was substrate dependent, as the Q192 alloform was found to hydrolyze diazoxon, sarin and soman more rapidly than R192 *in vitro* (DAVIES *et al.,* 1996). Some studies have shown that under physiological conditions, both PON1 alloforms hydrolyze diazoxon with nearly equivalent catalytic efficiencies (LI *et al*., 2000). The *PON1* L/M polymorphism has been associated with plasma PON1 protein levels, with M55 being associated with low plasma PON1 (BLATTER *et al*., 1997; MACKNESS *et al*., 1998).

Nearly 200 polymorphisms have been described in the *PON1* gene, some in the coding regions and others in introns and regulatory regions of the gene (JARVIK *et al*., 2003).

Although its natural substrate is still unclear, PON1 is capable of hydrolyzing toxic metabolites of organophosphorus (OP) compounds, including paraoxon (a catabolite of the insecticide parathion), diazoxon and chlorpyrifos oxon, detoxifies various neurotoxic agents like sarin and soman and hydrolyses the aliphatic lactones such as dihydrocoumarin, γ-butyrolactone and homocysteine thiolactone. (BILLECKE *et al*., 2000). Several experimental studies have shown, however, that the effect of PON1 on the toxicity of OP compounds varies with the particular compound (COSTA *et al*., 2005).

PON1 has been widely investigated, especially for its involvement in atherosclerosis and age-related diseases. The Q192R polymorphism has been associated with coronary artery disease, stroke, familial hypercholesterolemia, Parkinson's disease, and the onset of hypertension (MARCHEGIANI *et al*., 2008). Also, the L55 variant has been associated not only with variations in plasma levels of total and LDL cholesterol and in levels of PON1 message, protein, and activity toward paraoxon, but also with a variety of pathological conditions such as stroke, coronary artery disease (CAD), and Parkinson's disease (MARCHEGIANI *et al.,* 2008)

PON1 is assumed to be involved in the lipid metabolism. It has been reported to hydrolyze lipid peroxides in the arterial wall, preventing oxidation of low density lipoproteins, inactivates LDL-derived oxidized phospholipids once they are formed and prevents oxidation of HDL phospholipids (COSTA *et al*., 2003) and, thus, protecting against development of atherosclerosis (DURRINGTON *et al*., 2001). The analysis of literature on *PON1* suggests that this gene is likely a leading actor in determining the rate and the quality of the aging process, probably due to the capability to counteract oxidative stress (MARCHEGIANI *et al*., 2008).

PON1 activity in infants is low compared to adults, rendering them with lower metabolic and antioxidant capacities. This difference was larger in children with genotypes associated with low PON1 activities (−108TT, 192QQ, −909CC). In mothers, PON1 activities were elevated at delivery and during pregnancy compared to 7 years later when they were not pregnant .Genetic control of PON1 enzymatic activity varies in children compared to adults and is also affected by pregnancy status (HUEN *et al.,* 2010).

Rojas-Garcia and coworkers (ROJAC-GARCIA *et al.,* 2009) examined role of paraoxonase polymorphisms in the induction of micronucleus (MN) in paraoxon-treated human lymphocytes. They found that paraoxon had no effect on cell viability, but caused a significant dose-dependent increase in MN frequency. A significant difference was observed in the MN frequency only in lymphocytes from individuals with the QQ genotype treated with 5 μM paraoxon. In another study, paraoxon showed no significant effects in a dose-dependent increase in the sister chromatide exchanges (SCE) and in delay in the cell cycle. Also, no significant clastogenic effects and no difference in response were observed among individuals with different phenotypes of paraoxonase (SINGH *et a*l., 1984). Serhatlioglu (SERHATLIOGLU *et al.,* 2003) reported a decreased level of paraoxonase and arylesterase activities in radiology workers exposed for more than five years to ionizing radiation

In this study, we have evaluated the distribution of the *PON1* Q192R and L55M polymorphisms in healthy Serbian population, considering the important role that these polymorphisms may play in the genetic susceptibility to toxicity with OP pesticides and in initiation and/or progression of different diseases.

Table 1. Allele frequencies of coding region polymorphisms in PON1 (Q192R i L55M) worldwide

Population	Q192R		L55M		References
	Q	R	L	M	
Europe					
Finnish	0.69	0.31	0.67	0.33	CLARIMON *et al*., 2004
Dutch	0.68	0.32	0.63	0.37	LEUS *et al.,* 2001
Spanish	0.7	0.3	0.63	0.37	PARRA *et al*., 2006
Italians	0.65	0.35	0.66	0.34	SARDO *et al*., 2005
English	0.78	0.22	0.7	0.3	O'LEARY *et al*., 2005
Turkish	0.69	0.31	0.7	0.3	AYNACIOGLU *et al*., 1999
Croatian	0.77	0.23	0.66	0.34	GRDIC *et al*., 2008
Germans	0.72	0.28	-	-	GARDEMANN *et al*., 2000
French	0.76	0.24	-	-	RUIZ *et al*., 1995
Northern Irish	0.712	0.288	-	-	HERRMANN *et al*., 1996
Serbian	0.77	0.23	0.68	0.32	PRESENT STUDY
Asia					
Japanese	0.4	0.6	0.96	0.04	MOHAMED and CHIA, 2008
Koreans	0.38	0.620	0.94	0.06	HONG *et al.,* 2001
Chinese	0.42	0.58	0.95	0.05	MOHAMED and CHIA, 2008
Indians	0.67	0.33	-	-	SANGHERA *et al*., 1997
Iranian	0.69	0.31	0.59	0.41	SEPAHVAND *et al*., 2007
Saudi Arabian	0.73	0.27	-	-	NOGUEIRA *et al*., 1993
Israeli	0.67	0.33	0.61	0.39	BRYK *et al*., 2005
America					
Caucasian-Americans	0.73	0.27	0.64	0.36	BROPHY *et al.,* 2001
Canadians	0.73	0.27	0.64	0.36	MCKEOWN-EYSSEN *et al*., 2004
African-Americans	0.68	0.32	0.80	0.2	ERLICH *et al*., 2006
African-Americans	0.37	0.63	-	-	BROPHY *et al*., 2001
Amazonian	0.27	0.730	0.967	0.033	SANTOS *et al*., 2005
Ameridian tribes					
European-Brazilians	0.693	0.307	0.609	0.391	ALLEBRANDT *et al*., 2002
African-Brazilians	0.471	0.529	0.714	0.286	ALLEBRANDT *et al*., 2002
Caribean-Hispanics	0.540	0.460	0.71	0.29	CHEN *et al*., 2003
Mexicans	0.510	0.490	-	-	ROJAS-GARCIA *et al.,* 2005
Chileans					ACUNÃ *et al.,* 2004
NS	0.569	0.431	-	-	
ES	0.663	0.337	-	-	
Peruvians	0.539	0.461	-	-	CATAÑO *et al*., 2006
Africa					
Beninese	0.388	0.612	-	-	SCACCHI *et al*., 2003
Ethiopians	0.592	0.408	-	-	SCACCHI *et al.,* 2003
Egyptians			-	-	EL-FASAKHANY e*t al*., 2007

MATERIAL AND METHODS

The study included 122 healthy unrelated blood donors (78 male and 44 female), aged 22–50 years, livingin in different Serbian regions.

The study was approved by University Medical Hospital Zvezdara Ethics Committee.

Venous blood samples were collected in EDTA-coated tubes and were used for determination of *PON1* genotypes. Genomic DNA was extracted from EDTA-anticoagulated blood using the DNeasy Blood & Tissue Kit (Qiagen). Isolated DNA was stored at +4 °C until further analysis.

PON1 L55M and Q192R genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP). The primers used for the amplification of the both polymorphism were described by Rantala and coworkers (RANTALA *et al.,* 2002). PCR mixture (total volume 20 µl) contained PyroStart™ Fast PCR Master Mix (2X-concentrated solution containing hot start *Taq* DNA polymerase, reaction buffer, $MgCl_2$, and dNTP, Fermentas Life Sciences), 0.5µM of each primer (Metabion) and 0.2µg of genomic DNA. The PCR reaction was performed for each polymorphism. PCR thermocycling was suited for PyroStart™ Fast PCR Master Mix Protocol (provided by the manufacturer).

Digestion of PCR products was performed as previously described (RANTALA *et al*., 2002) with slight modifications. The L55M (169-bp) PCR product was digested with 2,5 U of Hin1II (Fermentas Life Sciences) at 37°C for 3h. Digestion resulted in 127- and 42-bp fragments for the M allele and a nondigested 169-bp fragment for the L allele. The Q192R (99-bp) PCR product was digested with 2,5 U of *Bsp*PI (Fermentas Life Sciences) at 37°C for 3h. Digestion with BspPI resulted in 63- and 36-bp fragments for the R allele and a nondigested 99-bp fragment for the Q allele. The digested fragments were separated on 3 % agarose gel, stained with ethidium bromide (0.5 µg/ml) and visualized under UV light.

Genotype and allele frequencies were determinated by counting-method, and *Hardy-Weinberg's equilibrium* was tested by the *Chi-square test* (χ^2 test).

RESULTS

This study represents genotype and allele frequencies for coding region polymorphisms of the *PON1* gene, Q192R and L55M, in Serbian population.

Results are summarized in Table 2 and Table 3. The observed frequencies for Q, R, L and M alleles were 77%, 23%, 68% and 32%, respectively (Table 1).

The most common genotype in Serbian population was QQ (60%) for Q192R polymorphism and LL (47%) for L55M polymorphism, and the least common was RR (6%) for both polymorphisms (Table 1).

Frequencies of combined genotypes are shown in Table 2. The most frequent genotype combinations were QQ/LL (26%) and QQ/LM (25%) respectively. The least frequent combined genotypes were both RR/LM and RR/MM (1%).

According to results of χ^2 test, Serbian population is in *Hardy-Weinberg equilibrium* for examined loci.

Table 2. Allele and genotype frequencies of coding region polymorphisms of PON1 gene in Serbian population

Polymorphism	Allele	Frequency		Genotype	Frequency	
	N=244	n	%	N=122	n	%
	Q	187	77	QQ	73	60
PON1 Q192R	R	57	23	QR	41	34
				RR	8	6
	L	165	68	LL	54	47
PON1 L55M	M	79	32	LM	51	42
				MM	14	11

N- number of examinated alleles and genotypes; n - number of individuals having a certain allele and genotype

Table 3. Combined genotypes frequencies of coding region polymorphisms of PON1 gene in Serbian population

Combined genotype	Frequency	
	n	%
QQ/LL	32	26
QQ/LM	31	25
QQ/MM	10	8
QR/LL	19	16
QR/LM	19	16
QR/MM	3	2
RR/LL	6	5
RR/LM	1	1
RR/MM	1	1

n - number of individuals having a certain genotype

DISCUSSION

To our knowledge, this is the first study reporting the frequency of *PON1* polymorphisms Q192R and L55M in Serbian population.

Our findings are in agreement with previously reported data for European population, which showed predominance of Q192 and L55 alleles over R192 and M55 alleles. (Table 1). In Serbs, for the most frequent allelles, Q192 and L55, frequencies were 0.77 and 0.68 respectively, and for R192 and M55 alleles were 0.23 and 0.32 (Table 2).

Allele Q192 is more frequent in Indian, Iranian, Saudi Arabian and Israeli populations in Asia, in Canadian, European-Brazilians and in much more American and in Egyptian populations. It's slightly higher in Caribean-Hispanics, Mexicans, Chileans, Peruvians and Ethiopians (Table 1).

Allele R192 is predominant in Japanese, Korean, Chinese and Malaysian populations in Asia, in Afro–Americans where we have contradictory data, and in Amazonian-Ameridiand tribes in America, and also in Beninese in Africa (Table 1). From the *PON1* sequences of chimpanzees and an orangutan, Koda and coworkers (KODA *et al*., 2004) found that the ancestral type of codon 192 was R.

The variable distribution of allele frequencies appeared to be dependent on geographic locations (SCACCHI *et al.,* 2003) perhaps due to genetic drift, as well as ethnic groups (SANGHERA *et al*., 1998).

The Q192R polymorphism significantly affects the catalytic efficiency of PON1 associated with HDL in blood. Studies showed that this polymorphism was substrate dependent, as the Q192 allozyme was found to hydrolyze diazoxon, sarin and soman more rapidly than R192 *in vitro* (DAVIES *et al*., 1996), and R192 allozyme hydrolyzes paraoxon more readily than Q192 (COSTA *et al*., 2005). Also, Q192 hydrolyses phospholipid and cholesteryl ester hydroperoxides more efficiently than the R192 allozyme. (AVIRAM *et al.,* 1999).

It has been shown that HDL isolated from RR homozygotes is less effective in protecting LDL against lipid peroxidation than HDL from either QQ or QR genotypes (MACKNESS *et al.,* 1998). Genotype QQ may have protective effects against oxidative stress. Paraoxonase is lipid-dependent enzyme and its conformation within the hydrophobic environment of HDL is crucial for its activity (SORENSON *et al.,* 1999). Phospholipids stabilize PON1 enzyme and are required for its binding to HDLs lipoprotein surface. So, lipid peroxidation of that surface can affect PON1s binding and decrease enzyme activity.

LDL oxidation is a key process in the pathophysiology of atherosclerosis and the onset of cardiovascular diseases (STEINBERG *et al.,* 1989), and various studies of Caucasians have associated *PON1* Q192R polymorphism with coronary heart disease risk, whereas others have not (RUIZ *et al*., 1995; SERRATO *et al*., 1995).

For codon 55 polymorphism, populations worldwide show predominance of L55 over M55 allele, and Japanese, Korean, Chinese, Malaysian and Amazonian-Ameridian tribes show a very low frequencies of it (Table 1). The generally low frequency of the M allele was such that in some studies the MM genotype was not observed at all (SANTOS *et al*., 2005). In Serbian population, frequencies of LL, LM

and MM genotypes were 0.47, 0.42 and 0.11 respectively. The L55M mutation may considerably affect PON1's stability and thereby account for the lower enzymatic activity (MACKNESS *et al.*, 1998). This is due to the key role of L55 in packing the propeller's central tunnel, and of its neighboring residues (Glu53 and Asp54), which ligate both Ca1 and Ca2 – 'catalytic' and 'structural' calcium iones, respectively. (HAREL *et al.,* 2004). M55 isoform is unstable form unresistant to proteolysis, so we assume that allele M is not favored in human populations, but it is kept in populations in heterozygous individuals.

Unlike some examed populations (GRDIĆ *et al*., 2008; POH and MUNIANDY, 2007), all nine possible genotype combinations of two polymorphisms were seen in this population. (Table 3). The most frequent combinations were QQ/LL (0.26) and QQ/LM (0.25), and the leas frequent were RR/LM (0.01) and RR/MM (0.01).

As a result of ionizing radiation, Serhatlioglu (SERHATLIOGLU *et al*., 2003) reported a decreased level of paraoxonase and arylesterase activities in irradiated workers, and earlier cytogenetic analyses showed persistence of unstable structural chromosome aberrations, both dicentric (dic) and centric ring chromosomes (cR), long time after irradiation (PENDIC *et al.,* 1980). The results from Schröder et al, (SCHRÖDER *et al*., 2003) obtained from blood lymphocyte analyses from Gulf War and Balkan wars veterans exposed to depletet uranium (DU), showed statistically significant increase in the frequency of dic and cR. This information raised concern with respect to potential biological hazard from DU exposure on Balkan-countries populations, especially because of decreased antioxidant/detoxifying capacity of PON1 enzyme and chromosome unstabilities. In same study (SCHRÖDER *et al.,* 2003), frequency of SCEs was decreased compared with control. SCE assay is accepted to measure genotoxic exposure only to chemical toxicants, and ionizing radiation is inefficient in SCE production (BARJAKTAROVIĆ, 1984). MILLER *et al* (2002) have demonstrated in *in vitro* experimented that DU is able to catalyse reactions of hydrogen peroxide and ascorbate, generating oxidative DNA damage that can induce carcinogenic lesions. Probably, same reactions can lead to lipid peroxidation of HDL surface and decreased enzyme activity.

The results obtained in study of Rojas-Garcia and co-workers (ROJAC-GARCIA *et al.*, 2009) suggest that PON1 genotype might have an important role in the identification of individuals at risk for cancer development due to occupational exposure to pesticides, because they observed a significant difference in the MN frequency only in lymphocytes from individuals with the QQ genotype treated with 5 μM paraoxon. Enhanced sensitivity to the chromosome-damaging effects of ionizing radiation is a feature of many cancer-predisposing conditions, but to our knowledge, there is no data linking PON1 genotypes with higher sensitivity to ionizing radiation.

Paraoxonase 1 is a multi-purpose enzyme that has been shown to perform a variety of jobs in the body, including ridding the arteries of plaque-forming clumps of LDL that lead to artheriosclerosis, and degrading toxic chemicals such as pesticides and nerve gases. Results from this population-based study provide a basis for future examining the possible association of *PON1* gene polymorphisms with the development and/or progression of various diseases in Serbian population, and to

examine link between PON1 genotype and ionisation irradiation inside Serbia borders.

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DISTRIBUCIJA POLIMORFIZAMA U KODIRAJUĆEM REGIONU GENA ZA PARAOKSONAZU 1 U SRPSKOJ POPULACIJI

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I z v o d

Serumska paraoksonaza 1 (PON1) kod ljudi je proteinska komponenta lipoproteina visoke gustine (HDL) koja štiti od oksidativnog oštećenja, detoksifikuje toksične metabolite organofosfatnih pesticida i bojnih otrova i aktivira/inaktivira pojedine vrste lekova. Uočeno je da polimorfizmi u kodirajućem regionu *PON1* gena, L55M i Q192R, mogu uticati i na nivo ekspresije i na katalitičku aktivnost PON1, a i opisane su njihove povezanosit sa pojavom širokog spektra oboljenja. Cilj ove studije je bio da se odrede učestalosti polimorfizama u kodirajućem regionu *PON1* gena, L55M i Q192R, u populaciji ljudi koji žive na teritoriji Republike Srbije. Prema našim rezultatima, najzastupljeniji aleli su Q (0.77) za Q192R i L (0.68) za L55M. Genotipovi QQ (0.60) i LL (0.47) i kombinovani genotip QQ/LL (0.26) su najzastupljeniji u srpskoj populaciji.

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