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Role of the PON polymorphisms on progression of chronic hepatitis and cirrhosis

[Kronik hepatit ve siroz gelişiminde PON polimorfizmlerinin rolü]

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ABSTRACT

Aim and Background: The probable role of PON1-192, PON1-55, PON2-148, PON2-311 polymorphisms of the PON enzyme family, which are play a role in antioxidant pathways in the progression of chronic hepatitis to cirrhosis was investigated.

Methods: The patient population included 64 chronic hepatitis patients without cirrhosis and 30 patients with cirrhosis which were diagnosed by biopsy compared to a control group (n=68) of normal healthy volunteers. All patients were recruited from the outpatient clinic of Gastroenterology, Celal Bayar University Faculty of Medicine. Genomic DNA of leukocytes was isolated by using a commercial isolation kit. PCR analysis was assessed for PON1-192, PON1-55, PON2-148, PON2-311 genotypes and the products digested with HinfI restriction enzyme to see the allelic polymorphisms. The PCR products were resolved on electrophoresis 2% agarose gel and visualized with a Syngene (USA) image analysis system. Odds ratios in 95% confidence interval were calculated for the two genotypes. For the statistical analyses SPSS version 10.0 was used.

Results: The prevalence of PON1-192, PON1-55, PON2-148 genotypes were very different between the groups but was not statistically significant. The frequency of the "SS" and "SC" genotypes of PON2-311 in patients with hepatitis and cirrhosis was higher than the control group and their Odds Ratios were statistically significant.

Conclusion: The prevalence of PON2 gene "SS" and "SC" genotypes of in patients was higher than that of healthy volunteers (ORs were 3,855 and 2,404 respectively). Similarly SS" and "SC" genotypes of in patients with cirrhosis was higher than that of patients with hepatitis (ORs were 3,436 and 2,223 respectively) These results suggested that the "SS" and "SC" genotypes of PON2 gene might cause a susceptibility for developing of hepatitis and progression of this condition to cirrhosis. It can be also speculated that "CC" genotype may be protective for the progression of disease.

Key words: paraoxonase, PON, polymorphism, chronic hepatitis, cirrhosis

ÖZET

Amaç ve kapsam: Antioksidan ve antiinflamatuvar yollarda işlev gösteren PON enzim ailesinden PON1-192, PON1-55, PON2-148 ve PON2-311 genlerine ait polimorfik genotiplerin kronik hepatitden siroz gelişiminde olası rolünün araştırılması amaçlanmıştır.

Materyal-metod: Celal Bayar Üniversitesi Tıp Fakültesi Gastroenteroloji Bilim Dalı tarafından takip edilen siroz olmayan kronik hepatitli 64 hasta ile biyopsi ile tanısı konulmuş siroz olan 30 hasta, ve kontrol grubu olarak hepatit markırları negatif olan (n=68) sağlıklı gönüllü birey çalışmamıza dahil edilmiştir. Periferik kan örneklerinden, lökosit genomik DNA'sı ticari izolasyon kiti ile ayrıldı. PCR ile amplifikasyonu yapılan örnekler HinfI restriksiyon enzimi ile kesilerek ürünler %2'lik agaroz jel elektroforezinde yürütüldü ve Syngene (ABD) marka image analiz sistemi ile görüntülendi. Veriler, Odds oranları %95 güven aralığı içerisinde SPSS 10.0 programı ile istatistiksel olarak değerlendirilmiştir.

Bulgular: Çalışmamıza dahil ettiğimiz gruplarımız arasında PON1-192, PON1-55 ve PON2-148 genlerine ait genotipik farklılıklar olmasına rağmen istatistiksel olarak anlamlı bir ilişki bulunamadı. PON2-311 geni için ise "SS" ve "SC" genotiplerinin frekansları hepatitli ve sirozlu hastalarda yüksek bulundu ve istatistiksel çalışmada da bu genotiplere ait frekansların Odds oranları anlamlı görüldü.

Sonuç: Siroz gelişmiş hepatitli hastalarda PON2-311 geninin "SS" ve "SC" genotiplerinin siroz gelişmemiş hepatitli hastalarla kontrol grubuna göre istatistiksel olarak anlamlı ölçüde yüksek görülmesi hepatit ve siroz gelişimine "SS" ve "SC" genotiplerinin katkısı olabileceği ve/veya "CC" genotipinin siroz gelişimini önleyici bir rolü olabileceği sonucuna varıldı. Daha geniş popülasyonlarda yapılacak çalışmalarda diğer genlerin polimorfik yapıları açısından da istatistiksel olarak anlamlı sonuçlar bulunabileceği kanısına varılmıştır.

Anahtar kelimeler: Paraoksonaz, PON, polimorfizm, kronik hepatit, siroz

Introduction

Paraoxonase (PON) is a polymorphic enzyme of many tissues, as well as serum, where it is associated with HDL. Human serum paraoxonase enzyme is an ester hydrolase in 43 – 45 kDa molecular weights, associated with HDL, addicted to Ca and also called aryldialkylphosphatase which is synthesized in the liver [8, 10-12]. Ca is essential for the enzyme activity and stability and also plays a role in catalytic mechanisms. The N – terminal signal peptide in the structure of the paraoxonase is needed for the interaction with HDL. By the intervention of N – terminal signal peptide, paraoxonase enzyme adheres to phospholipids and lipoproteins [14, 15]. The paraoxonase enzyme is a glycoprotein which has 354 amino acid residues. The paraoxonase gene is located in the q 21-22 area of the chromosome 7. There are three members of the paraoxonase gene and protein family which are called; PON1, PON2 and PON3. There is no lysine residue on the 105 position of the PON2 and PON3 amino acid structure, and so some researchers suggest that they can not hydrolyze the paraoxon. Also PON2 and PON3 are not presented in plasma [10, 16, 17].

The paraoxonase (PON) gene cluster is located on human chromosome 7q21-22. PON 1 gene has several polymorphisms in the promoter and coding regions that identified and are known to influence gene expression levels. Human serum paraoxonase enzyme (PON1) has two genetic polymorphisms. These polymorphisms appears with the amino acid exchange on the 55 and 192 positions. Glutamine (Q allele) and arginine (R allele) are exchanged in the 192 position and methionine (M allele) and leucine (L allele) are exchanged in the 55 position. Q and also R isoenzymes of the PON1 protects LDL from oxidation. Indeed the R isoenzyme has a high affinity for paraoxon, whereas the Q isoenzyme has a lower affinity [5, 21-23]. PON2 has also two polymorphisms that appears with the exchange of the amino acids on 148 and 311 positions. Alanine (A allele) and glysine (G allele) are exchanged in 148 position and Serine (S allele) and cystein (C allele) are exchanged in 311 position.

Paraoxonase has three cystein amino acids. Two of them joins the formation of the disulfide bonds in the molecule, while the other cystein molecule in 284. position exists freely in the structure. It has been thought that the free cystein is located closely to the active side of the enzyme and it is necessary for the enzyme to connect to the substrate. Indeed, this free cystein molecule has a very important role in protecting LDL from oxidation, but there is no affect on the hydrolyzing of the organophosphates. In recent years it is shown that smoking inhibits PON1 enzyme activity by modifying the free thiol groups of the enzyme [7, 8, 10, 22]. The existence of the three cystein residues supports that PON1 is a serine esterase which uses nucleophilic cystein amino acids instead of the serine amino acids on the catalytic centre against the other serine esterases [17].

Paraoxonase hydrolyses the paraoxon which appears with oxidative desulfuration of the parathion, p-nitrophenole, and diethyl phosphate. The formation of the paraoxon is catalyzed by the cytochrome p-450 enzyme systems in the liver and other tissues [10, 30].

Initially, the enzyme was characterized as organophosphate hydrolase, thus given its name derived from its commonly used substrate paraoxon. PON has been shown to play an important role in lipid metabolism as an antioxidant-antiatherosclerotic molecule through (a) hydrolysation of active oxidized phospholipids (phospholipase A2-like activity), (b) destruction of platelet activating factor, lipid hydroperoxides and H_2O_2 (peroxidase-like activity), (c) reduction of monocyte chemo taxis and adhesion to endothelial cells, and (d) preservation of HDL integrity and function [1-4]. PON1 also protects LDL–cholesterol from the oxidation which is induced by Cu and free radicals [5-7]. PON1 hydrolyses the 25 % of the hydrogen peroxide (H_2O_2) had been formed under the oxidative stress in the development of the atherosclerosis. This property shows the peroxidase activity of the PON1 [8, 9].

Paraoxonase enzyme can be shown in various tissues like liver, kidney, or intestine and serum [10,18]. Non-genetic factors like diet, acute phase reactants, pregnancy, hormones, smoking and the use of simvastatin modulates PON1 levels in serum [19,20]. The decrease of the paraoxonase enzyme activity in the myocardial infarction, familial hypercholesterolemia, diabetes mellitus and chronic renal impairments was shown by many studies [30, 38, 39, 40, 41].

Reactive oxygen metabolites and nitrogen derivatives play an important role in the beginning and the development of liver disease regardless from the etiology. Reactive oxygen metabolites also play an important role in the transcription and activation of many cytokines and growth factors. The main sources of free radicals are the result of the activity of the cytochrome P450 enzymes of hepatocytes, mitochondria, neutrophils and macrophages that have been activated with endotoxins [36]. The liver cell damage and formation of scar tissue in the development of cirrhosis ultimately leads to liver failure as a result of the abnormal activities of the liver enzymes that detoxifies the carcinogenic and toxic compounds [35]. It is almost certain that free radicals play a role in the pathogenesis of various hepatobiliary diseases. It is not clear how the kidney is affected because it has very effective antioxidant mechanisms. However results are promising with the use of agents that protect the hepatocyte and mitochondrial membranes which are attacked by the free radicals, from the lipid peroxidation. Paradoxically, experiments show that blockage of some systems which are thought to reduce free radical damage increases the damage. [34]. The PON enzyme system is thought to be a member of the enzymes which play an important role in protecting tissues from oxidative stress. This idea can be supported by the inactivation

of PON1 by H_2O_2 which is one of the reactive oxygen metabolites [8].

We therefore hypothesize that PON1 genotypes influence paraoxonase activity levels and increase the risk of the progression of chronic hepatitis to cirrhosis. PON1-192, PON1-55, PON2-148, PON2-311 polymorphisms were studied in the chronic hepatitis patients without and with cirrhosis and in the normal healthy volunteers to investigate this hypothesis.

Material and Methods

This study was carried out with the permission of the Ethics Committee of Scientific Research, Celal Bayar University, Faculty of Medicine. The patient population included 64 chronic hepatitis patients without cirrhosis, 30 patients with cirrhosis diagnosed by biopsy compared with a control group (n=68) of normal healthy volunteers with hepatitis negative markers. All patients were recruited from the outpatient clinic of Gastroenterology, Celal Bayar University Faculty of Medicine.

Genomic DNA of leukocytes was isolated for polymerase chain reaction (PCR) analysis by using a commercial isolation kit (Omega Bio-Tek Blood DNA isolation kit catalog no: DZ3392, Doraville, GA, USA). The mismatch primers for genotyping the polymorphisms of codon 192 and codon 55 in the PON1 gene were used as described by Motti et al. [30]. The primer sequences for PON1-192 and PON1-55 were as follows: 192-forward TTG AAT GAT ATT GTT GCT GTG GGA CCT GAG and 192-reverse CGA CCA CGC TAA ACC CAA ATA CAT CTC CCA GAA; 55-forward GAG TGATGT ATA GCC CCA GTT TC and 55-reverse AGT CCATTA GGC AGTATC TCC G; for genotyping the PON2 polymorphisms at codons 148 and 311, primers were designed as described by Ying Zhang et al. The sequences were as follows: 148-forward CAG AAA TAA CAC CAG ACG GAC AG (7088–7066, NT_007933) and 148-reverse TCA GAT GCA ACA GAG AAT TGA CT (483–461, L48513); 311-forward GGT TCT CCG CAT CCA GAA CAT TGA A (923–947, L48513) and 311-reverse AAT TGT TCA GTC ATC GCC CTT TCA T (1121–1097, L48513).

The multiplex-PCR was performed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA) in a 50 µl volume containing 0.8 mmol/l dNTPs, 10x PCR buffer, 1.5 mmol/L $MgCl_2$, 0.2 µmol/l of both PON1-192 primers, 0.2 µmol/l of both PON1-55 primers, 0.24 µmol/l of both PON2-148 primers, 0.3 µmol/l of both PON1-311 primers and 2.0U Taq DNA polymerase (Promega, Madison, WI, USA). After initial denaturation (5 min at 94 ºC), 35 cycles of 1 min at 95 ºC, 45 s at 60 ºC and 1 min at 72 ºC were run with a final extension time of 10 min at 72 ºC. The multiplex-PCR amplification products were digested with 10 U HinfI (New England Biolabs, Cambridge, UK) at 37 ºC for 1–2 h. The digested products were separated by polyacrylamide gel electrophoresis and silver stained [31].

The statistical package program SPSS 10.0 was used for assessment of the data. In the assessment of the data, the Chi-square test was used for all gene polymorphisms. Then logistic regression model was used for the gene polymorphisms which were significant in chi-square test with one variable. In this model, Univariate OR and Multivariate OR ratios were calculated in 95% confidence interval.

Results

The configuration of Paraoxonase gene products obtained by PCR amplification and restriction is shown in Figure 1. Frequencies of the distribution findings of PON gene polymorphisms of individuals are shown in Table 1. There was no statistically significant difference between groups for PON1-192, PON1-55, and PON2-148 polymorphisms. But, differences between PON2-311 genotype frequencies were found as statistically significant.

In the patient group which had Hepatitis and cirrhosis (0.6702, n=94) SS genotype frequency were significantly higher than in the control group (0.4853, n=68). Odd's ratio was found as 3.855 (95% CI=1.514-9.814). With these results, SS genotype may be regarded as a candidate risk factor for development of the disease. CC genotype frequency in the patient group (0.1064, n=94) occurred significantly lower than the control group (0.2647, n=68) (Table 2).

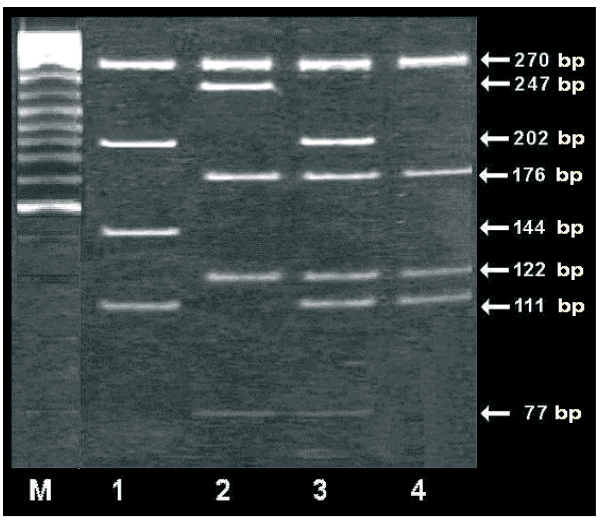

Figure 1. Paraoxonase gene amplification and restriction products; M marker DNA sequence; Sequence No. 1; from top to bottom (during the actual cutting with restriction enzyme) before amplification products: PON2-148 (270 bp), PON2-311 (199 bp), PON1-55 (144 bp) and PON1-192 (111 bp); Sequence No. 2; from top to bottom: PON2-148 AG (270 and 247 bp 2 lines), PON2-311 SS (176 bp), PON1-55 LL (122 bp), PON1 -192 RR (77 bp); Sequence No. 3 from top to bottom: PON2-148 AA (270 bp), PON2-311 SC (176 and 202 bp 2 lines), PON1-55 MM (144 bp), PON1 - 192 QR (77 and 111 bp 2 lines) from top to bottom: PON2-148 AG (270 and 247 bp 2 lines), PON2-311 SS (176 bp), PON1-55 LL (122 bp), PON1 -192 RR (77 bp); Sequence No. 4 from top to bottom: PON2-148 AA (270 bp), PON2-311 SS (176 bp), PON1-55 LL (122 bp), PON1 -192 QQ (111 bp).

SS genotype frequency has been lower in Non-cirrhotic hepatitis (0,60, n=60) when compared with the cirrhotic patients (0,7941, n=34). It was determined to be statistically significant. Besides of this, CC genotyped individuals in the cirrhotic patient group (n = 34) were seen at a significantly lower frequency than the Non-cirrhotic hepatitis group (n=60). It can be suggested with these results that the SS genotype may be progressive (OR=3.436; %95 CI=1.425-8.286) and the CC genotype may be protective factors for the development of the cirrhosis (Table 3).

Table 1. Genotype frequencies according to Groups

Polymorphism		Group 1 Non-cirrhotic hepatitis Freq .	%	Group 2 Cirrhosis Freq .	%	Group 3 Control Freq .	%
PON1-192	QQ	11	0.1833	8	0.2353	16	0.2353
	QR	33	0.55	18	0.5294	37	0.5441
	RR	16	0.2667	8	0.2353	15	0.2206
Total		**60**	**1.00**	**34**	**1.00**	**68**	**1.00**
PON1-55	LL	46	0.7667	29	0.8529	37	0.5441
	LM	12	0.20	5	0.1471	12	0.1765
	MM	2	0.0333	0	0,00	19	0.2794
Total		**60**	**1.00**	**34**	**1.00**	**68**	**1.00**
PON2-148	AA	42	0.70	27	0.7941	41	0.6029
	AG	14	0.2333	7	0.2059	22	0.3235
	GG	4	0.0667	0	0,00	5	0.0735
Total		**60**	**1.00**	**34**	**1.00**	**68**	**1.00**
PON2-311	**SS**	36	0.60	27	0.7941	33	0.4853
	SC	16	0.2667	5	0.1471	17	0.25
	CC	8	0.1333	2	0.0588	18	0.2647
Total		**60**	**1.00**	**34**	**1.00**	**68**	**1.00**

Table 2. PON2 - 311 gene polymorphism distribution between hepatitis and cirrhosis (all patients) and control group

Polymorphism		Group 1 - 2 Hepatitis and Cirrhosis Freq .	%	**Group 3 Control** Freq .	%	Multivariate OR (95% confidence interval)
PON2–311	**CC**	10	0.1064	18	0.2647	1
	SC	21	0.2234	17	0.25	2.404 (0.832-6.948)
	SS	63	0.6702	33	0.4853	3.855 (1.514-9.814)

Table 3. PON2 - 311 gene polymorphism distribution between Non-cirrhotic hepatitis and patients with cirrhosis

Polymorphism		Group 1 Non-cirrhotic hepatitis Freq .	%	Group 2 Cirrhosis **Freq .**	**%**	**Multivariate OR** (95% confidence interval)
PON2–311	**CC**	8	0.1333	2	0.0588	1
	SC	16	0.2667	5	0.1471	2.223 (0.815-6.063)
	SS	36	0.60	27	0.7941	3.436 (1.425-8.286)

Discussion

Chronic hepatitis, cirrhosis, and even primary HCC can develop after hepatitis B and C infections. Hepatitis also may occur as a result of autoimmune mechanisms. Developing from hepatitis to cirrhosis, or response to treatment can be different from person to person. These differences may be originated from the nature of the disease or the individual properties of the patient which are related with proteins and play role in pathogenetic and/or drug metabolism pathways. All of these individual properties are responsible for genetic predisposition to the disease. The occurrence and progression of hepatitis may be due to a person's genetic susceptibility. The percentage of the difference in the progression of the disease is due to the personal differences is not known [32, 33].

Paraoxonase has been suggested to contribute the progression of hepatitis to cirrhosis because of its capability of hydrolyzing lipid peroxides and antioxidant functions. Ferre et al. had shown that there is an increase of the fibrosis markers and the concentration of PON1 along with a decrease of PON1 activity in patients with liver failure. They thought that PON1 plays an active role in the development of chronic liver failure and formation of oxidative stress, fibrosis and apoptosis in liver cell [43].

In this study, a statistically significant difference was found in frequencies of PON2-311 polymorphisms between control and patients groups. Although there were differences in results of PON1-192, PON1-55 and PON2-148 genes, the univariate OR was statistically insignificant at 95% confidence interval.

Paraoxonase enzyme polymorphisms are probably related with the development of cirrhosis by playing a role with other known antioxidant mechanisms at different levels which is related with different structures of enzyme caused by polymorphic changes .

Especially, the "S" allele of the PON2 gene affects the progression of the disease and so it could be necessary to determine the patients in risk groups. The existence of serine (Ser) amino acid instead of cysteine at the 311th position of the PON2 protein as a result of the "S" polymorphism can affect the enzyme activity negatively. When we consider that 3 cysteine residues in the PON2 protein play a very important role in enzyme activity, substitution of cysteine amino acid instead of serine amino acid can affect the activity of the PON2 enzyme positively.

Definition of the relationship between polymorhisms of the genes whish code enzymes that are attendant in detoxification mechanisms is important for determination the high risk population for varied chronic diseases. This study is important to demonstrate whether PON enzyme systems, like other enzyme systems are important parameters that play a role in oxidative stress. We thought that by increasing the number of the samples in patient and control groups, the results can be statistically significant for the other gene polymorphisms of the PON gene. Therefore, studies of the enzyme activity of these genes in serum or tissues can support the results of polymorphism studies.

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