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European Journal of Experimental Biology, 2013, 3(3):512-516

ISSN: 2248 –9215
CODEN (USA): EJEBAU

The study of effects common paraoxonase polymorphism (L55M) on atherosclerosis risk in diabetic patients by PCR-RFLP

Bayatmakoo Roshanak[1], Mobaiyen Haedeh[2], Monfaredan Amir[3], Katayoun Bahman Sofiani[4] and Ronak Benissy[5]

[1]*Department of Biochemistry, Faculty of Medicine, Tabriz branch, Islamic Azad University, Tabriz, Iran*
[2]*Department of Microbiology, Faculty of Medicine, Tabriz branch, Islamic Azad University, Tabriz, Iran*
[3]*Department of Hematology, Faculty of Medicine, Tabriz branch, Islamic Azad University, Tabriz, Iran*
[4]*Department of Immunology Faculty of Medicine, Tabriz Branch, Islamic Azad University, Tabriz, Iran*
[5]*Faculty of Medicine, Tabriz branch, Islamic Azad University, Tabriz, Iran*

ABSTRACT

Human serum paraoxonase1 (PON1) are proteins by molecular mass of 43 KDa(355 amino acids) that synthesis in liver and bind to high density lipoproteins (HDL). One of two polymorphism occurred in coding region of PON1 gene is substitution an amino acid of leucine (L) with methionine (M) at codon 55 which effects PON1 activities. On the other hand, most of diabetic patients' death is about atherosclerosis and its side effects. So, this study is conducted to study L55M polymorphism in type II diabetic patients referred to Shahid Madani Hospital, Tabriz, Iran. In this study, venous blood sampling was done on 95 diabetic (case group) and 105 non- diabetic (control group) with atherosclerosis patients. After DNA extraction and identification of quality of DNA, L55M mutation was assayed by using specific primers in PCR and byproduct of PCR was digested by Hinf1. From 200 samples that were taken from Iranian who lived in north-west of Iran, the 55LL, 55LM and 55MM genotypes in case group were 33.8%, 42.6% and 23.6% and in control group 29.9%, 48.3% and 21.8%, respectively. In several studies, relationship between presence of polymorphism and level of enzymatic variation were shown. These variations are introduced as a risk factor for heart and arterial disease and have an important role in incidence of atherosclerosis but in this study, there wasn't significant deference in frequency of polymorphism alleles by p value in 95% confidence index. These results showed that further studies must be done by more cases.

Key words: Paraoxonase gene (PON), PCR-RFLP, Frequency of polymorphism, Diabetes, Atherosclerosis.

INTRODUCTION

The oxidative variation with low density lipoproteins (LDL) in the vascular wall cells is believed to play key role in the pathogenesis of atherosclerosis [16, 6]. It is shown that the LDL oxidation can be controlled with high density lipoproteins (HDL) in the in-vitro [6, 7, 8, 17,]. In the co-culture setting, the HDL is protected by poorly produced oxidative LDL in the vascular wall cells [11]. This conservative effect of HDL depends on the enzymes such as Paraoxonase (PONs) [6] platelet activating factor acetyl hydrolyase [18], and lecithin and cholesterol acyl transferase HDL depending enzymes where LDL oxidation get delayed along with the prevention of lipid peroxide. Among these enzymes, paraxonase have been studied more than others [6].

It is believed that in human, PON1 plays a key role in the prevention of nourotoxic damages, atherosclerosis and even in Non-Houchkin lymphomas [3, 1]. PON1 has a medium length gene with 25.8 kb in down-stream of

initiation codon which codes 355 amino-acids by having five common one nucleotide polymorphism. Three of polymorphisms are located in promoter site and two of them in the coding site (Q 192 R, L55M) [1, 2]. According to the difference in presence of polymorphism in paroxanase genes in various areas, this study is to evaluate the polymorphism L55M in diabetic patients with atherosclerosis in East-Azerbaijan province, Iran.

MATERIALS AND METHODS

In order to determine the number of sample in two groups of control and case, the software of PS with applying α, R and M coefficients were used. Referral patients during 2010-2011 to the cardiovascular center of Shahid Madani Hospital in Tabriz, Iran were asked to give satisfaction consent under the supervision of moral committee with 9019 moral code issued at the date of 24/07/2011 in order to fulfill the related task. The type of the study is case-control with confidence level 95% to determine the sample size. 95 people with the background of coronary artery disease (CAD) who never had diabetic antecedent were considered as control group, 105 ones by certain diagnosis of CAD with diabetes background as case group were established in this regard. There have been considered some factors in the study as exclusion criteria:
A) Taking anti-pregnancy drugs in women,
B) Diabetic background more than 20 years,
C) More than 85 years old.

These patients with their descriptions were asked to give venous blood sampling by the use of vacuum system made of Trimo (Japan). The genomic DNA from the white blood cells directly extracted using QIAamp DNA Blood Mini Kit (QIA gene) according to the manufacturer's instructions. The applied materials in the polymerase chain reaction and its amount have been shown in table 2-1. All above mentioned materials were taken and bought from Japanese Takaray Company. The primers sequence applied in the study are as following: 55F GAGTGATGTATAGCCCCAGTTTC and 55R AGTCCATTAGGCAGTATCTCCG (12). The applied method for the analysis of L55M mutation is PCR-RFLP. The temperature program in PCR for the evaluation of polymorphism L55M is shown in table 2-2. After the completion of polymerase chain reaction and polymerization of expected gene by 397 bases pair byproduct; it was digest by Hinf1 restriction enzyme. They were electrophoresis along with the 50 bases pair size-marker on the 3% agarose gel. In the mutation conditions, the site of restriction enzyme is being eliminated for the enzyme. The obtained pattern was as following after digestion withHinf1:
1) Normal position (nucleotide T at codon 163): two byproduct with the size of 119 and 25 base pairs.
2) Mutated position (nucleotide A at codon163): a piece with 144 base pairs due to the action of Hinf1 enzyme.
3) Heterozygote mutated position (nucleotides T and A in two alleles at codon 163): three byproduct by the sizes of 119, 144, 23 base pairs

RESULTS

Table 1. Characteristic pattern of PCR reaction in evolution of L55M in Paraoxonase gene

Reaction Ingredients	PCR Standard	Amount(µl)
ddH20	X	17.8
10X PCR Buffer	1X	2.5
Mgcl2 (50mM)	1-5mM	1.5
dNTP(10mM)	0.2mM	1
Forward primer (10pmol/µl)	0.1-1 pmol/ µl	0.1
Reversed primer(10pmol/µl)	0.1-1 pmol/ µl	0.1
Taq DNA polymerase	0.5-2.5 unit	1
Template DNA	100-500ng/ µl	1
Final volume		25

Table 2. PCR temperature program for the analysis of polymorphism L55M paraoxonase gene

Step	Temperature (◦C)	Time (S)	Number of cycle
First denaturation	95	300	1
Cyclic denaturation	95	30	32
Annealing	62	30	
Extension	72	30	
Final extension	72	600	1

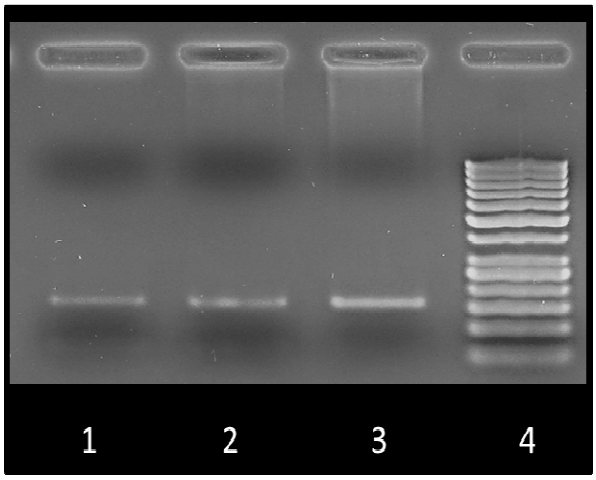

Figure 1. Column 1-3, the byproduct of L55M polymorphism with 144 base pairs, Column 4: Size marker (50 base pairs)

In this study the most common mutated genes of paraoxonase (L55M) using PCR-RFLP have been evaluated. During the observation of the mutation in each of these samples, the related sample is being studied to get confident for the certain existence of the mutation. The polymerization of some pieces of genes by the length of 144 base pairs after the reaction PCR and electrophoresis of them is shown in figure1.

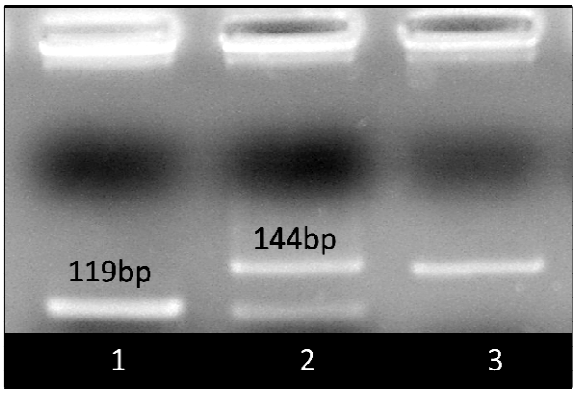

Figure 2. Column 1, the product from the polymerization site of polymorphism L55M and digest with Hinf1 by the size of 119 base pairs (normal), column 2, the product from the polymerization site of polymorphism L55M and digest with Hinf1 by the size of 144 and 119 base pairs (heterozygote), column 3, the product from the polymerization site of polymorphism L55M and digest with Hinf1 by the size of 144 base pairs (homozygote).

Then, byproduct of PCR including exon from paraoxonase gene was digested by using Hinf1in 37◦C. When thymine existed in the exon at codon 163 in both chromosomes of the studied sample, the digestion site is never provided for Hinf1 and after the electrophoresis of the product on the 1.5% agarose gel, only one piece of 144 bp was observed. While thymine were located in one strand of chromosome and adenine in another strand, digestion site of Hinf1 were establish. After the electrophoresis of by product, the pieces 144, 119 and 25 base pairs were seen on the gel. If the studied sample of DNA includes nucleotide adenine on two chromosomes, we will have two pieces of 119 and 25 bp after the electrophoresis. Therefore, it could understand that the allelic changes of L55M (Figure 2).

Table 3. The frequency of mutation in L55M paraoxanase gene in control and case groups

Genotype	Cardiovascular patient		P Value with confidence 95%
	with diabetes	without diabetes	
55LL	33.8%	29.9%	p>0.05
55LM	42.6%	48.3%	p>0.05
55MM	23.6%	21.8%	p>0.05

The mutation of L55M (163T>A) was evaluated in 400 chromosomes related to 200 people with the background of diabetic and non-diabetic coronary-vascular disease in the North-west of Iran. The results of the study were shown that there is no significant difference in both groups of CAD patients (first group: diabetic, second group: non-

diabetic) in terms of allelic polymorphism in genotypic L55M. The interesting point of this study is that the frequency of this type of polymorphism is fairly high (Table 3).

Table 4. Intervention and predisposal parameters involved in coronary artery diseases with the frequency of genotype L55M in control and case groups, simultaneously

Clinical signs	Test group			Control group		
Genotype	**55LL**	**55LM**	**55MM**	**55LL**	**55LM**	**55MM**
Frequency (%)	33.8%	42.6%	23.6%	29.9%	48.3%	21.8%
Present and past smoking background	67%			59%		
Period of diabetes diagnosis(year)	12.9 %±6.6%			0		
Systolic blood pressure(mmHg)	140±93			128±67		
Diastolic blood pressure(mmHg)	72±23			70±14		
Total cholesterol (mMol/l)	5.1 ±1			4.9±1.8		
LDL (mMol/l)	3.2±1.34			2.7±2		
HDL (mMol/L)	1.4±0.3			0.98±0.1		
Triglyceride(mMol/l)	2.57±1.7			1.98±0.9		

In this study, other interventional and predisposal parameters involved in the cardiovascular diseases were evaluated that have been shown in table 4.

DISCUSSION AND COCLUSION

The diabetes particularly type II is the most proven risk factor in the progression of cardiovascular diseases especially cardiac failure [14].The environmental and genetically disorders in lipids metabolism are inevitable factors in the pathogenesis of myocardial infarction [15]; moreover, high level lipoproteins such as low density lipoprotein (LDL) or cholesterol, glycosylated LDL, the level of paraoxanase also plays a key role in this pathogenesis [15].The genetically controlling factors of metabolism and variation in lipoproteins is mostly getting sophisticated in the diabetic patients. The gene that is responsible for the pathogenesis of cardio-artery diseases is subjected to paraoxanase gene where it is coded on the chromosome 7 [10]. There are two polymorphic loci on PON 1 gene in the human. The first locus is at codon 192 which substitute amino acid glycine (Q) to arginin (R) and the second one is related to locus 55 instead of methonine (M) to leucine (L). Some studies considered that this type of polymorphisms with varition in level enzymatic related together and these changes have been introduced as the risk factors of the cardiovascular diseases and the appearance of atherosclerosis [5, 12]. In the field of coronary artery diseases, the presence of these polymorphisms particularly 55LL have been specified as the progressive factors in the other atherosclerotic conditions such as retinopathy and its affection in diabetic patients in comparisons with control group [4]. The vascular disorders in the pre-diabetic conditions such as the lack of glucose tolerance and progression of the atherosclerosis in diabetic patients have been fairly proven [4].

The frequency of this polymorphism is different in different populations. In the study of Lakshmy et al (2010), the genotypes of LL with 80 people(64.2%), LM with 41 one (33.1%) and MM 3 ones (2.4%) were reported but there is no found significant difference between control and two experimental groups in different genotypes [6]. In the study of Chen et al, the frequency of polymorphism in genotypes LL, LM, MM is subjected to the number of studied people and percent of the distribution in Caucasians is 26 (33%), 33 (42%) and 19 (24%), respectively. In Afro-Americans, this number is 65 (58%), 44 (39%) and 3 (3%); in Spanish people it is reported as following: 101 (51%), 85 (43%), 14 (7%), respectively and of course there is found significant difference in three related groups [2]. The results of this study in the atherosclerotic with diabetes and non-diabetes patients have been shown in table 1, but no any significant differences found in this regard.

Acknowledgement
This study has been carried out by the financial support of Islamic Azad University, Tabriz Branch; in this case, all of authors appreciate warmly the kind efforts of the vise-president of Islamic Azad University research center as well as the clinical laboratory of Plasma for providing necessary facilities in fulfilling our experimental plan.

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