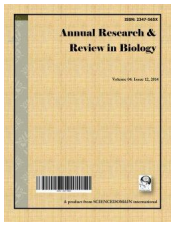

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Comparative Study between Greeks and Albanians with the Use of Restriction Fragment Length Polymorphisms of Apolipoprotein B Gene

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Authors' contributions

The work has been carried out in collaboration with all the authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of this study was to compare the genetic polymorphisms of apolipoprotein B gene between two different populations, Greeks and Albanians living in Greece and to investigate the possibility of discriminating the two populations by using these polymorphisms.
Methodology: Restriction fragment length polymorphisms at codons 2488 (XbaI) and 4154 (EcoRI) of the apolipoprotein B gene were investigated in the above populations, in order to determine if there are differences between them. Two specific DNA regions, each containing the polymorphic site, were amplified by polymerase chain reaction. The products were digested and electrophoresis on 2% agarose gel was followed. A total number of 160 unrelated individuals from each population were randomly collected.
Results: The allelic frequencies of the samples from Greeks and Albanians showed variability patterns for the XbaI and EcoRI Restriction Fragment Length Polymorphisms. For Greeks and

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Albanians the presence of E+/+ genotype was almost the same (67.5% and 70.6% respectively), without statistical significant differences and the E-/- genotype showed low common presence (6.3% and 2.5% respectively). The presence of X-/- genotype had almost the same ratio for the two populations (48.1% for Greeks and 39.4% for Albanians) and the presence of X+/+ genotype was low enough for both of them.

Conclusion: The study of the two populations (Greeks and Albanians) did not show any statistically significant differences concerning the frequency of the genotypes of XbaI and EcoRI polymorphisms of the *APOB* gene.

Keywords: Greece; Albania; restriction fragment length polymorphisms; restriction enzyme; apolipoprotein B.

1. INTRODUCTION

Restriction Fragment Length Polymorphism (RFLP) is a technique in molecular biology that exploits variations in homologous DNA sequences. In RFLP analysis, the DNA sample is digested by restriction enzymes and the restriction fragments are separated by gel electrophoresis according to their lengths. RFLP analysis was the first low priced DNA profiling technique with widespread applications. It is still an important tool in genome mapping, localizing genes for genetic disorders and determining the risk for a disease and paternity testing [1].

Restriction enzymes (or restriction endonucleases) are enzymes that cut DNA at or near specific recognition nucleotide sequences which are called restriction sites [2-4]. These enzymes are mainly found in bacteria and they can provide a defence mechanism against invading viruses [5,6]. Two processes can form the restriction modification system: Firstly, inside a prokaryote, the restriction enzymes selectively cut up foreign DNA (restriction) and secondly, the host DNA is protected by a modification enzyme (a methyltransferase) that transforms the prokaryotic DNA and blocks cleavage [7].

More than 3000 restriction enzymes have been studied and over 600 of these are obtainable commercially [8]. These enzymes are usually used for DNA modification in laboratories and are a vital tool in molecular cloning [9-11]. Restriction endonucleases are categorized into four groups (Types I, II, III and IV). This categorization is based on their structure and enzyme cofactor requirements, the character of their target sequence and the position of their DNA cleavage site relative to the target sequence [12-14].

Apolipoprotein B (Apo B) is a component of chylomicrons, low-density lipoproteins, very low lipoproteins and inter-mediate-density lipoproteins. Two RFLPs (XbaI rs693 and EcoRI rs1042031) detected with the restriction enzymes XbaI and EcoRI represent single base alterations in the coding region of the *APOB* gene. The *APOB* gene is located on the short (p) arm of chromosome 2 on position 2p23-p24 and precisely from base pair 21,001,428 to base pair 21,044,072. It is a large gene, which consists of 29 exons and 28 introns. Mutations in the *APOB* gene are known to cause many disorders such as famlial hypobetalipoproteinemia, inherited hypercholesterolemia or high level of heart disease risk. Despite the fact that the polymorphisms of apolipoprotein B have been extensively studied in many ethnic groups, there is little information about their distribution among populations from southeastern Europe.

The EcoRI polymorphism of the *APOB* gene appears in exon 29 (4154 G>A). This results in an amino acid change (Glu→Lys). A restriction site for the EcoRI enzyme is present with the guanine (E+ allele). Otherwise it is lost (E- allele). The XbaI RFLP in exon 26 of the *APOB* gene involves the 2488 nucleotide (C>T). The presence of thymine develops a restriction site for the XbaI enzyme characterizing the X+ allele, while its absence determines the X- allele. Both variations are synonymous and they do not affect the amino acid sequence of apo B [15].

The aim of the present study was to investigate if the above polymorphisms could be used as a discrimination method, when comparing different populations.

2. MATERIALS AND METHODS

2.1 Subjects

Samples of blood from 160 unrelated Greek individuals from Attica-Greece (74 males and 86 females) and 160 individuals from Albania living in Attica-Greece (119 males and 41 females), whose autopsy was performed in the Athens morgue, were collected. The information needed

about the individuals was taken from their medical files, reports and relatives (informed consent was acquired from relatives). The blood samples were obtained from femoral artery. The mean age for Greek males was 51.68 (range 16-90) and for Greek females 42.36 (range 17-88). The mean age for Albanian males was 51.97 (range 20-80) and for Albanian females 49.17 (range 20-79).

2.2 DNA Extraction

DNA material was extracted by standard techniques (QIAamp DNA mini blood kit) from 200 µl of whole venous blood collected with EDTA.

The extracted DNAs were quantified at 260 and 280 nm and stored at 4°C until analysis.

The desired segments were amplified by PCR using the apoB EcoRI and apoB XbaI protocols with the use of the necessary primers (New England BioLabs Inc). For EcoRI F 5'-CTGAGAGAAGTGTCTTCGAAG-3' and R 5'-CTCGAAAGGAAGTGTAATCAC-3' and for XbaI F 5'-GGAGACTATTCAGAAGCTAA-3' and R 5'-GAAGAGCCTGAAGACTGACT-3'.

The polymerase chain reaction was performed in a Thermocycler for EcoRI at 95°C for 8min, followed by 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min in 30 cycles. A final extension was conducted for 10 min at 72°C. For XbaI at 95°C for 5 min, followed by 95°C for 45 sec, annealing at 58°C for 45 sec and extension at 72°C for 45 sec in 33 cycles. A final extension was conducted for 10min at 72°C [16].

After the amplification of the products, they were submitted to digestion with the respective restriction enzymes. For the restriction protocol we used 1 µl -10 units of enzyme-, 31 µl pcr product (1 mg DNA) and 5 µl NE Buffer in a total volume of 50 µl in 37°C for one hour, according to the manufacturer's instruction (New England Biolabs).

The restriction products were separated and variations were visualized after electrophoresis on 2% agarose gel with ethidium bromide under UV light (Vilber Lourmat, Fluo-Link TFL-35M). The size of the fragments of DNA for EcoRI is 227 bp (base pairs), 253 bp and 480 bp and for XbaI 260 bp, 415 bp and 675 bp. The procedure was completed by photographing the results.

2.3 Statistical Analysis

Statistical analyses were conducted using IBM SPSS Statistics for Windows (Version 22.0. Armonk, NY: IBM Corp.) software package. Genotype and allelic frequencies were calculated by gene counting. Comparison of observed and expected genotypes under Hardy-Weinberg equilibrium was made using Chi-squared test (x^2 test). In addition, x^2 test was used to compare genotype and alleles frequencies between the two sexes and between the two population groups. x^2-values with $P<0.05$ were considered statistically significant.

3. RESULTS AND DISCUSSION

In Figs. 1 and 2 profiles of *APOB* gene digested by XbaI and EcoRI respectively are presented. The observed genotype frequencies of *APOB* did not significantly differ from expected values according to the Hardy-Weinberg equilibrium for both populations *(P-value>0.05)* (Tables 1 and 2). Table 3 presents the genotype distribution of EcoRI and XbaI RFLPS of *APOB* gene for Greek males and females. XbaI and EcoRI polymorphisms were compared between the two sexes by x^2 analysis. In addition the genotype distribution of EcoRI and XbaI RFLPS of *APOB* gene for Albanian males and females is presented in Table 4. The frequency of the *APOB* X+ and E+ alleles was higher in Greek males than Greek females, in contrast with Albanian population group, where females presented higher frequencies (Table 5). Nevertheless, no statistically significant differences of genotype and allele frequency distribution for either the XbaI or the EcoRI polymorphism at the *APOB* gene were observed between the two sexes in both population groups *(P>0.05)*.

The genotype and allele frequencies of *APOB* gene polymorphisms XbaI and EcoRI, in the two population groups are shown in Tables 6 and 7 respectively. The frequency of the *APOB* X+ and E+ alleles was higher in the Albanians than in Greeks. In addition the *APOB* X+/+ and E+/+ genotype frequencies were higher in the Albanian population group than in Greek. However, in both cases, these differences can not be considered statistically significant *(P>0.05)*.

Table 1. Comparison of observed and expected genotype frequencies of the *APOB* polymorphisms XbaI and EcoRI in Greek population sample according to Hardy-Weinberg equilibrium

APOB	XbaI Genotype			x^2 (P-value)*	EcoRI Genotype			x^2 (P-value)*
	X++	X+-	X--		E++	E+-	E--	
Observed frequencies (%)	11.9	40.0	48.1	0.629	67.5	26.3	6.3	2.590
Expected frequencies (%)	10.2	43.4	46.4	(0.427)	65.0	31.3	3.8	(0.107)

** x^2 test was used to compare the two groups. P-value<0.05 indicates statistical significant differences between the two groups*

Table 2. Comparison of observed and expected genotype frequencies of the *APOB* polymorphisms XbaI and EcoRI in Albanian population sample according to Hardy Weinberg equilibrium

APOB	XbaI Genotype			x^2 (P-value)*	EcoRI Genotype			x^2 (P-value)*
	X++	X+-	X--		E++	E+-	E--	
Observed frequencies (%)	12.5	48.1	39.4	0.137	70.6	26.9	2.5	0.001
Expected frequencies (%)	13.4	46.4	40.3	(0.711)	70.6	26.8	2.5	(0.974)

** x^2 test was used to compare the two groups. P-value<0.05 indicates statistical significant differences between the two groups*

Table 3. Genotype frequencies of XbaI and EcoRI (*APOB* gene) for males and females in Greek population group

APOB genotype	Males		Females		x^2 (P-value)*
	N	Frequence (%)	N	Frequence (%)	
XbaI total	74	100.0	86	100.0	2.629 (0.269)
X++	12	16.2	7	8.1	
X+-	27	36.5	37	43.0	
X--	35	47.3	42	48.8	
EcoRI total	74	100.0	86	100.0	0.215 (0.898)
E++	51	68.9	57	66.3	
E+-	19	25.7	23	26.7	
E--	4	5.4	6	7.0	

** x^2 test was used to compare the two groups. P-value<0.05 indicates statistical significant differences between the two groups*

Table 4. Genotype frequencies of XbaI and EcoRI (*APOB* gene) for males and females in Albanian population group

APOB genotype	Males		Females		x^2 (P-value)*
	N	Frequence (%)	N	Frequence (%)	
XbaI total	119	100.0	41	100.0	1.855 (0.396)
X++	13	10.9	7	17.1	
X+-	56	47.1	21	51.2	
X--	50	42.0	13	31.7	
EcoRI total	119	100.0	41	100.0	0.692 (0.707)
E++	82	68.9	31	75.6	
E+-	34	28.6	9	22.0	
E--	3	2.5	1	2.4	

** x^2 test was used to compare the two groups. P-value<0.05 indicates statistical significant differences between the two groups*

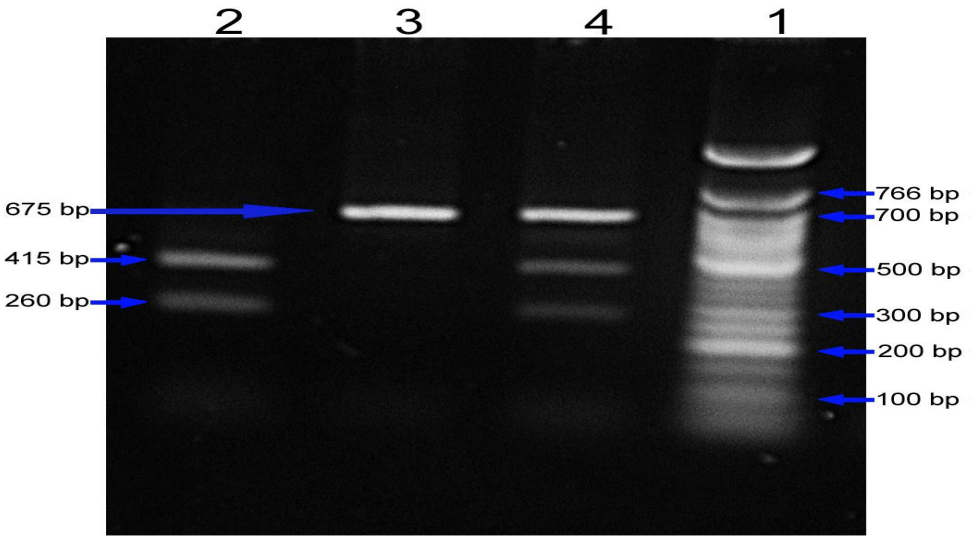

Fig. 1. Profile of *APOB* gene digested by Xbal . 1: Biolabs 50bp DNA ladder. 2: X+/+. 3: X-/-. 4: X+/-

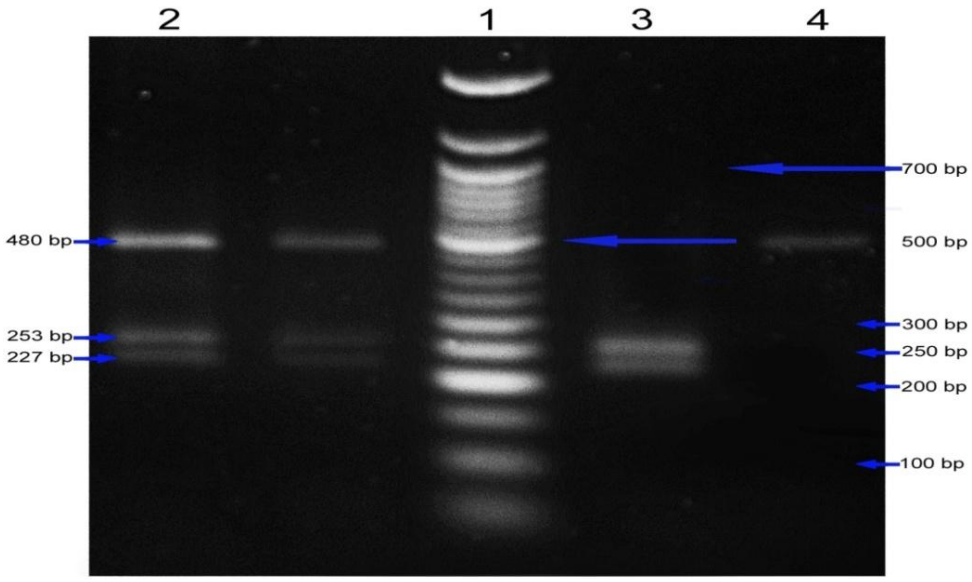

Fig. 2. Profile of *APOB* gene digested by EcoRI. 1: Biolabs 50bp DNA ladder. 2: E+/- . 3: E+/+. 4: E-/-

The purpose of this study was to compare the allelic frequencies of apo B RFLPs between samples of Greeks and Albanians living in Greece. During the last twenty years in Greece, many people from Albania have immigrated in Greece. They have settled in different cities, however, the majority of them lives in Athens and its suburbs and have created families and have given birth to children. Consequently, the study of genetic variations of this specific population is considered as an important matter, given they can be used as a valuable tool for identification and clinical purposes.

In our study the frequency of the genotypes in the two populations for both polymorphisms of apo B was almost the same. For EcoRI polymorphism the percentage of homozygotes

(E-/-) was quite similar and low enough in both populations. The same was observed for the homozygotes (X+/+) of polymorphism XbaI. At XbaI polymorphism the X-/- and X+/- genotype show an equilibrium in both populations, whereas at EcoRI polymorphism the three genotypes have a similar analogy.

Comparison between sexes led us in different conclusions. Greek males and females had almost the same frequencies with the exception of X+/+ (Greek males 16.2% and Greek females 8.1%, P=0.269). Albanian males and females presented quite similar results concerning the two polymorphisms. For both polymorphisms, Albanian and Greek males featured common frequencies. On the contrary, Greek and Albanian females presented alike frequencies for EcoRI polymorphism, but for XbaI polymorphism had differences (X+/+ 8.1% for Greek females and 17.1% for Albanian females, P=0.117). However, the differences observed in the genotype and alleles frequencies of the *APOB* gene polymorphisms XbaI and EcoRI between the two populations can not be considered statistically significant (*as P>0.05*).

Our results were very close with those of Hovarth et al. [17] in their study on Bulgarians (Bulgarians X+/+ 21.8%, X+/- 42.9%, X-/- 35.4% and E+/+ 68.7%, E+/- 25.9%, E-/- 5.4%). In a comparative study between Greeks and Southern Italian people [18], the frequencies concerning the two polymorphisms of EcoRI and XbaI were found to be appreciably different between the two populations. As far as it concerns the frequencies of the Greek sample in our study versus the sample of Greeks mentioned above, the results for EcoRI are quite similar (E+/+ 69.1%, E+/- 25.5%, E-/- 5.3%) but the results for XbaI polymorphism showed different distribution (X+/+ 21.2%, X+/- 57.4%, X-/- 21.3%).

Table 5. Alleles frequencies of XbaI and EcoRI (*APOB* gene) for males and females in Greek and Albanian population groups

Alleles	**Males**		**Females**		**x^2 (P-value)***
Greeks	**N**	**Frequence (%)**	**N**	**Frequence (%)**	
X+	51	34.5	51	29.7	0.847 (0.400)
X-	97	65.5	121	70.3	
Total	148	100.0	172	100.0	
E+	121	81.8	137	79.7	0.226 (0.672)
E-	27	18.2	35	20.3	
Total	148	100.0	172	100.0	
Albanians	**N**	**Frequence (%)**	**N**	**Frequence (%)**	
X+	82	34.5	35	42.7	1.781 (0.187)
X-	156	65.5	47	57.3	
Total	238	100.0	82	100.0	
E+	198	83.2	71	86.6	0.524 (0.600)
E-	40	16.8	11	13.4	
Total	238	100.0	82	100.0	

**x^2 test was used to compare the two groups. P-value<0.05 indicates statistical significant differences between the two groups*

Table 6. Genotype frequencies of the *APOB* gene polymorphisms XbaI and EcoRI in Greek and Albanian population groups

APOB genotype		**Greeks**		**Albanians**		**x^2 (P-value)***
		N	**Frequence (%)**	**N**	**Frequence (%)**	
XbaI	**Total**	160	100.0	160	100.0	2.624 (0.269)
X++		19	11.9	20	12.5	
X+-		64	40.0	77	48.1	
X--		77	48.1	63	39.4	
EcoRI	**Total**	160	100.0	160	100.0	2.696 (0.260)
E++		108	67.5	113	70.6	
E+-		42	26.3	43	26.9	
E--		10	6.3	4	2.5	

** x^2 test was used to compare the two groups. P-value<0.05 indicates statistical significant differences between the two groups*

Table 7. Alleles frequencies of the *APOB* gene polymorphisms XbaI and EcoRI in Greek and Albanian population groups

Alleles	Greeks		Albanians		x^2 (P-value)*
	N	Frequence (%)	N	Frequence (%)	
X+	102	31.9	117	36.6	1.562
X-	218	68.1	203	63.4	(0.243)
Total	320	100.0	320	100.0	
E+	258	80.6	269	84.1	1.300
E-	62	19.4	51	15.9	(0.300)
Total	320	100.0	320	100.0	

** x^2 test was used to compare the two groups. P-value<0.05 indicates statistical significant differences between the two groups*

In recent years many studies have used RFLPs in order to compare populations taking into account factors like hypercholesterolemia or arteriosclerosis [15,16]. For example, the X-/- genotype was more frequent in Brazilian women with coronary artery disease (CAD) than in control women [19]. Studies on Caucasians from Europe [20] and Chinese subjects [21] did not detect significant associations between XbaI variability and CAD. In another research [22], the comparison between Italians from South and North Italy using EcoRI-RFLP showed quite different results between the two population samples (the frequency of E- is much higher in South Italians than that observed in North Italians). Similar studies present results from populations like Kuwait, Egypt, China and India [23-26].

It is worth mentioning that Rosser et al. [27], on the basis of their study of Y-chromosomal diversity, suggested that populations in Europe are related fundamentally on the basis of geography, rather than on the basis of linguistic affinity. Indeed Serbia, Croatia, Greece and Albania do not differ much in terms of their locations in relation to Bulgaria. Our study, concerning the specific polymorphisms of apolipoprotein B, supports the above observation, which means that they could not be used as a discrimination tool between the two populations.

4. CONCLUSION

The study of the two populations (Greeks and Albanians) did not show any statistically significant differences concerning the frequency of the genotypes of XbaI and EcoRI polymorphisms of the *APOB* gene and consequently they cannot be used for discrimination purposes.

ETHICAL APPROVAL

The study design was approved by the Ethics Committee of the School of Medicine of National and Kapodistrian University of Athens, Greece.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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