Molecular Detection of Mutated Factor V (Leiden) and Factor XIII in Ischemic Heart Disease

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ABSTRACT

The present work was performed to evaluate the incidence of gene mutation affecting factor V Leiden (FVL) &factor XIII in cases of myocardial ischemia. Thirty cases of myocardial ischemia as well as ten age and sex matched healthy subjects were included in the study. DNA was extracted from blood samples drawn from all subjects included in the study and was subjected to PCR amplification for factors V (Leiden) and factor XIII genes, using biotinylated specific primers. The PCR products were hybridized using strip containing immobilized allele-specific oligonucliotide probe. The products of hybridization were visualized using striptavidin-alkaline pH and color substrate. LDL-c was significantly higher in heterozygous factor V leiden The results of the study showed that heterozygous mutations involving factor V Leiden cases were in a positive correlation with LDL-c results and homozygous mutations involving factor XIII cases showed a positive correlation with AST changes. These results gave an idea about the mutational changes involving factors V Leiden and XIII and may be considered as risk factors for the incidence of myocardial infarction.

INTRODUCTION

Factor V Leiden mutation was detected in 10% of patients with coronary artery diseases (CAD)[1]. The results suggest that FVL may be one of the important risk factors in developing CAD in northeast Turkey. Systematic review of prospective studies was conducted a considering the risk of recurrent venous thromboembolism among heterozygous carriers of FVL mutation. It was found that heterozygosity of FVL associated with increasing risk of recurrent thromboembolism [2]. In addition, the risk is lower with PTM.On the other hand, FXIII is a transglutaminase that is responsible for covalently cross-linking fibrin therapy stabilizing the fibrin clot In addition, FXIII val-34 Leu was claimed to protect against the occurrence of MI and stroke[3]. Further, **in (2004)** it was stated that val 34 Leu FXIII polymorphism is associated with increased activation of thrombosis[4]. There is an observed positive relationship between FXIII polymorphisms and ischemic stroke (IS) when assuming an additive model of inheritance effect [5&6]. In addition, IS risk was markedly higher in female smokers who had FVL. No such

interaction was present in men. It was concluded that there was an important interaction between the common risk factors and the polymorphism (FVII; FXIII) in the development of MI[(7).]

Auro et al. [(8)] detected that the variants in 4 thrombosis genes (FV, ICAM1, PC and THB1) contribute to arterial cardiovascular events in some Finland population. They stated that analyses comprising several genes belonging to the same pathway may reveal cumulative allelic effects. When acting together these gene variants may affect the disease risk more profoundly than do the single predisposing variants.The aim of the present study is to detect different mutations of factor V Leiden (FVL) and FXIII in patients with ischemic heart disease.

MATERIAL & METHODS

This study was carried on 30 patients who had suffered myocardial ischemia. The sex ratio was 8 females to 22 males. Their age range was (57.16 ±5.6) years. They were selected from October 6 University Hospital. 10 healthy subjects matched for sex and age were served as controls. The two groups were subjected to full clinical examination, electrocardiogram (ECG), measuring of arterial blood pressure. Serum cholesterol, High density lipoprotein cholesterol (HDL -c), low density lipoprotein cholesterol (LDL-c), prothrombin time, international normalized ratio (INR), serum alanine transaminase (ALT), serum aspartate transaminase (AST)[(9)] were determined. In addition, serum bilirubin[(10),] blood urea [(11),] serum creatinine[(12),] hemoglobin, serum glucose and blood film preparation for platelet count were performed.

Mutations of factor V Leiden (FVL) and factor XIII (FXIII) were detected according to the methodology of kit obtained from Vienna Lab diagnostics Gmb H Vienna, Austria. The procedure included three steps as follows: 1) DNA isolation; 2) PCR amplification using biotinylated primers; 3) hybridization of amplification products to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines. Bound biotinylated sequences were visualized and detected using streptavidin-alkaline phosphatase and color substrates. The genotype of the sample was determined using the enclosed collector sheet (Vienna Lab Diagnostics GmbH).

1) DNA extraction:

5 ml blood were withdrawn from each subject and collected on EDTA containing tubes. Blood samples were pipetted into a 1.5 ml microtube, 1 ml lysis solution 1 was added and let to stand for 15 min. at room temperature, then centrifuged for 5 min. at 3000 rpm. 1 ml lysis solution 2 was added and centrifuged for 5 min. at 12000 rpm. The supernatant was removed except 50 μl of the visible soft pellet. 200 μl of GENXTRACT Resin was added to the pellet and vortex for 10 sec. Samples were incubated for 10 min. at 98°C and vortex for 10 sec, centrifuged for 5 min. at 12000 rpm. The resulting supernatant contains DNA template suitable for immediate use of PCR. The quality and quantity of DNA was analyzed by measuring the OD of the extracted DNA at 260

nm. Isolated DNA samples were stored at -20˚C till used for PCR and typing.

2) In vitro amplification:

Genotyping for FVL and FXIII polymorphism was performed as follows using biotinylated primeres into two reaction tubes for each amplification samples were prepared and placed on ice: 1)15 µl amplification for FVL; 2) 5 µl diluted Taq DNA polymerase; 3) 5 µl DNA template.

Primer sequence for FVL is

Forward primer:	**GCTTCCTCGCTCACTGAGTC**
Reverse primer:	**CTGGCCTTTTGCTCACATG**
PCR product size:	136 (bp), *Homo sapiens*

Primer sequence for FXIII is

Forward primer:	**GCAGTCCTGTCTGGGTCTTC**
Reverse primer:	**CTGAGATGAACCCTGGCATT**
PCR product size:	106 (bp), *Homo sapiens*

Tubes were copped tightly and run the following thermocycling programs:

1. Pre PCR: 94°C/2 min.
2. Denaturation and thermocycling at 94°C/15 sec. annealing at 58°C/30 sec. then elongation at 72°C/30 sec. (for 35 cycles).
3. Final extension: 72°C/3 min.

3) Hybridization:

Wash solution A, wash solution B, hybridization buffer, DNA template (DNAT) and conjugated buffer were prewarmed to 45°C.

20 µl DNAT were pipetted into the corresponding drop of DNAT.

10 µl amplification product was added into the same drop. Contents were mixed with a pipette and let to stand for 5 min. 1 ml of hybridization buffer was added into each tube.

4. Stringent wash: 1 ml wash solution A was added and washed 3 times.
5. Color development: 1 ml conjugate solution was added and incubated for 15 min.

One ml wash solution B was added and removed 3 times and then 1 ml color developer was added and incubated for 15 min. at room temp. in dark. hybridized products were added to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines. Bound biotinylated sequences were detected using streptavidin-alkaline phosphatase and color substrates. The genotype of the sample was determined using the enclosed Collector sheet (Vienna Lab Diagnostics GmbH).

Statistical methods

Statistical package of social science (SPSS) version 9.0 was used for analysis of data. Data was summarized as mean, SD and percentage. T-test was used for analysis of quantitative data, while

Chi square test was used for analysis of qualitative data. One way ANOVA test was done for analysis of more than 2 quantitative data followed by post HOCC test for detection of significance. P-value is considered significant if < 0.05 *.

RESULTS

The study was done on 22 male & 8 female, *patients* and 6 male to 4 female,*control*. The prevalence of diabetes mellitus was significantly higher in IH cases. (12/30). FVL was normal (wild) in 28 IH patients (93.33%) and 9 (90%) in control group. It was heterozygous in 2 cases only which represented 6.67% of the studied cases versus 1 case (10%) for the controls.there is a decrease in LDL-c of patients who have heterozygous alleles for FVL (87.5mg\dl vs.102.7 mg/dl, P < 0.02).

Concerning FXIII, the laboratory data of the studied patients showed wild type in 50% of IH versus 80% for the accompanied controls. Homozygosity was detected in 6.67% of the studied samples.

With the exception of AST value, the readings are found to be within the normal levels. Subjects with wild type and/or heterozygous genes have normal values of AST. Meanwhile, homozygous patients have serum level of AST greater than 3.5 times of the normal value (133.0 U/L vs. 38.8 U/L, P < 0.00001).

Table 1: Characteristics of the studied patients and control subjects and the frequencies of the analyzed allele of FXIII and FVL

Variable	Controls n=10		Patients n=30	
	No.	%	No.	%
Sex				
Male	6	60	22	73.3
Female	4	40	8	26.7
Diabetes mellitus				
Negative	10	100	18	60
Positive	—	—	12	40
FXIII				
Wild type	8	80	15	50
Heterozygous	2	20	13	43.33
Homozygous	—	—	2	6.67
FVL				
Wild type	9	90	28	93.33
Heterozygous	1	10	2	6.67

Table 2: Comparison between demographic and laboratory data of Ischemic heart patients included in the study in relation to factor XIII

Variables	*Wild type Mean ± SD N = 15*	*Heterozygous type Mean ± SD N = 13*	*Homozygous type Mean ± SD N = 2*	*P-value*
Age of patients(yrs)	58.3 ± 6.4	57.7 ± 6.3	50.5 ± 13.4	0.3
Hb (g/dl)	13.1 ± 1.7	14.5 ± 1.5	14.0 ± 1.4	0.06
Platelets (10^3/UL)	203.4 ± 28.7	209.9 ± 33.4	160.0 ± 0.0	0.1
Cholesterol (mg/dl)	242.0 ± 35.8	257.5 ± 35.3	243.0 ± 32.5	0.5
Triglyceride (mg/dl)	192.2 ± 49.0	179.6 ± 18.4	210.0 ± 0.0	0.5
HDL-c (mg/dl)	32.0 ± 5.1	29.5 ± 6.0	32.0 ± 4.2	0.5
LDL-c (mg/dl)	105.1 ± 12.5	100.2 ± 12.4	101.0 ± 11.3	0.6
INR	1.1 ± 0.1	1.2 ± 0.1	1.3 ± 0.2	0.6
Prothrombin concentration (%)	86.5 ± 11.0	88.7 ± 8.8	74.0 ± 9.9	0.2
Prothrombin time (sec)	15.4 ± 2.9	16.2 ± 5.2	16.0 ± 2.8	0.9
Total billirubin (mg/dl)	0.9 ± 0.2	1.0 ± 0.2	1.2 ± 0.1	0.1
AST (U/L)	30.2 ± 15.7^{a}	34.2 ± 11.5^{a}	133.0 ± 80.6^{b}	0.0001*
ALT (U/L)	28.7 ± 20.1	32.9 ± 11.4	31.0 ± 14.1	0.8
Urea (mg/dl)	34.3 ± 15.2	35.0 ± 12.0	35.0 ± 9.9	1.0
Creatinine (mg/dl)	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.1	0.9

*P-value is significant if < 0.05**

Table 3: Comparison between demographic and laboratory data of Ischemic heart patients included in the study in relation to factor V

Variables	*Wild type Mean ± SD N = 28*	*Heterozygous type Mean ± SD N = 2*	*P-value*
Age of patients(yrs)	57.3 ± 7.0	60.0 ± 4.2	0.6
Hb (g/dl)	13.7 ± 1.7	14.5 ± 0.7	0.5
Platelets (10^3/UL)	203.6 ± 31.0	200.0 ± 56.6	0.9
Cholesterol (mg/dl)	248.7 ± 35.4	250.0 ± 42.4	0.9
Triglyceride (mg/dl)	188.5 ± 38.3	180.0 ± 14.1	0.8
HDL-c (mg/dl)	31.1 ± 5.5	29.0 ± 8.5	0.6
LDL-c (mg/dl)	103.8 ± 11.9	107.5 ± 3.5	0.02*
INR	1.2 ± 0.1	1.3 ± 0.1	0.4
Prothrombin concentration (%)	86.8 ± 10.1	85.0 ± 18.4	0.8
Prothrombin time (sec)	15.7 ± 4.1	16.5 ± 2.1	0.8
Total billirubin (mg/dl)	1.0 ± 0.2	0.9 ± 0.5	0.5
AST (U/L)	38.8 ± 33.7	38.5 ± 3.5	0.9
ALT (U/L)	31.1 ± 16.4	25.0 ± 12.7	0.6
Urea (mg/dl)	34.9 ± 13.4	31.0 ± 14.1	0.7
Creatinine (mg/dl)	1.1 ± 0.2	1.1 ± 0.2	0.9

P-value is significant if < 0.05

Table 4: Comparison between LDL-c in relation to factor V

Variables	*Factor V Wild Mean ± SD N = 27*	*Factor V Hetero Mean ± SD N = 27*	*P -value*
LDL	103.8 ± 11.9	107.5 ± 3.5	0.02*

P-value is significant if < 0.05

Table 5: Comparison between AST in relation to factor XIII

Variables	*Factor XIII Wild Mean ± SD*	*Factor XIII Hetero Mean ± SD*	*Factor XIII Homo Mean ± SD*	*P -value*
LDL	30.2 ± 15.7 [a]	34.2 ± 11.5[a]	133.0 ± 80.6[b]	0.0001*

P-value is significant if < 0.05
Different symbol indicate significance

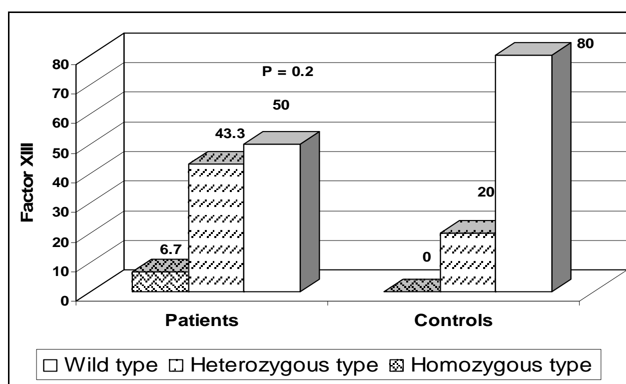

Fig. (1): Comparison between factor XIII in Ischemic heart patients and controls included in the study

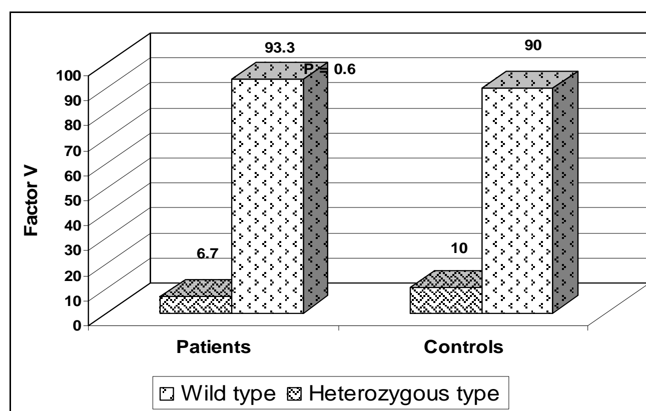

Fig. (2): Comparison between factor V in Ischemic heart patients and controls included in the study

DISCUSSION

The present study was conducted to evaluate the development of ischemic heart diseases.This study included 10 control and 30 patients with history of ischemic heart disease.The present findings are consistent with those recorded by ***Demirer et al.,*** [13] who observed no association among FVL mutation and metabolic control parameters

The present results pointed to 93.33% of MI patients with normal phenotype for FVL versus 90% for the controls. Heterozygous carriers represented 6.7% of the patients and 10% for the control subjects.

Our findings agree with ***Lalouschek et al.*** [7] ***(2005)*** who found that the prevalence of the FVL did not differ significantly between ischemic stroke patients and controls***Emmerich et al.*** [14]. came to a conclusion that heterozygosity for FVL yields a lifelong hypercoagulable state associated with approximately 5 fold increased risk of venous thrombosis ,the risk is increased with homozygous gene to 50 foldsOn contrast ***Gurlertop et al.,*** [1] observed FVL mutation in 10% of coronary arterial diseases (CAD) and were totally absent in control individualsThey suggested that FVL mutation may be one of the important risk factors in developing CAD. This opinion is in concert with that of ***Endler and Mannhalter***[15]. who stated that FVL mutation is most common genetic risk factors of venous thrombosis (VTE) On the other hand, ***Marchiori et al.,*** [2] concluded that in patients with VTE heterozygous carrier of FVL is clearly associated with increased risk of recurrent thrombo-embolism

The present study revealed significant increase in heterozygosity of FVL and LDL-c value compared to wild gene of FVL. This result is in agreement with that of ***Avellone et al.*** [16] who detected that patients suffered of MI and activated protein C resistance displayed significantly higher level of LDL-c. It is also run in full agreement with that of ***Ganong***[17]. that LDL plasma concentration correlates positively with myocardial infarction and ischemic stroke Biochemical studies have revealed that FXIII is a key regulator of fibrinolysis and in addition to its role in haemostasis, it has also been implicated in the pathology of arterial and venous thrombosis [18].

The present results revealed that the wild type of FXIII was detected in 50% of the examined patients and 80% for the controls. Whereas 43.33% of patients are heterozygous carriers versus 20% for the control subjects. Homozygous alleles of FXIII are represented in 6.66% of the patients. In this respect ***Catto et al. (1999)*** stated that homozygosity for FXIII, mutation is associated with increased enzyme activity and heterozygous exhibit intermediate activity when compared with non-carriers[19]. FXIII val-34 Leu was claimed to protect against the occurrence of MI and stroke [3]***).*** It displayed a heterozygous ethnic distribution and a protective effect against venous thrombosis was also proposed [20].

A common polymorphism of the FXII A subunit (FXIII val 34 Leu) has

been identified as a protective factor against both arterial and venous thrombosis. ***Hancer et al.*** [(21)] in MI patients study supported the hypothesis that val 34 Leu polymorphism in FXIII gene has a protective effect against MI. Mainly the genotype 34 Leu of FXIII showed significantly protective effect in MI patients [(22)].

The present data reflected high level of aspartate transaminase (AST) in FXIII homozygous patients. The value was 3.5 times greater than normal. It is well known that liver, kidney, myocardium, haematopoietic tissues erythrocytes are richer in transaminases than other body tissues. Consequently, destruction or necrosis of any of these tissues - as a result of pathological process, hypoxia state in these active cells or environmental factors, lead to release of a large amount of these enzymes into the serum [(17, 23,24, 25&26)].

Finally,it could be concluded that the concept that ischemic heart disease is attributed to genetic and environmental factors interactions, is generally approved. So, further studies are necessary using more patients and analysis of different predisposing variants to know more about the risk factors causing MI.

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تقييم دور الطفرات الجينية لمعامل تجلط الدم رقم V ليدن ومعامل رقم XIII فى حالات نقص الدم لعضلة القلب

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أجريت هذه الدراسة لتقييم دور الطفرات الجينية لمعامل تجلط الدم رقم V ليدن ومعامل رقم XIII فى حالات نقص الدم لعضلة القلب ,. و أجريت هذه الدراسة على ٣٠ حالة مصابة بنقص الدورة الدموية لعضلة القلب مع مقا رنتها ب١٠ اشخاص اصحاء .تم فحص DNAمن عينات الدم موقع الدراسة و ذلك لعمل PCRلكل من معامل تجلط الدم رقم V ليدن ومعامل رقم XIII اوضحت النتائج ما يلى ان المرضى ذوى الاختلاف الجينى لمعامل رقم v كانت لهم علاقة ذات دلالة احصائية مباشرة مع تغيرات انزيم الكبد AST و المرضى ذوى الاختلاف الجينى لمعامل رقم XIII كانت لهم علاقة ذات دلالة احصائية مباشرة مع تغيرات LDL-c وهذه الدراسة تعطى فكرة عن اهمية متابعة التغيرات فى هذين العاملين مع باقى المتغيرات لحماية مرضى القلب