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Cytokine Gene Polymorphism in Bahraini Patients with Coronary Heart Disease

الأنماط الجينية الوراثية للسيتوكينات لدى مرضى شرايين القلب التاجية من البحرين

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Abstract:There is increasing evidence that inflammatory processes play a central role in the pathogenesis of atherosclerosis. Studying the genetic variants of the inflammatory system components in atherosclerosis is very important because it might explain the diverse and variable inflammatory responses among individuals. We herein investigated the possible association between polymorphisms in the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6 and (interferon-gamma (IFN-γ), and the anti-inflammatory cytokines IL-10 and transforming growth factor-beta1 (TGF-β1), and correlated them with the severity of cardiovascular complications involving arterial coronary heart disease in 28 Bahraini patients, recruited from Bahrain Defense Forces Hospital (BDFH). Twenty nine asymptomatic subjects were randomly selected from blood donors, and were used as negative controls. For each study object (patients and controls), blood specimen collected and genomic DNA was extracted. Genotyping was performed by SSP method using Cytokine Genotyping Tray kit, followed by electrophoresis. The prevalence of the polymorphisms of all studied cytokines in these patients revealed no significant difference in comparison with the apparently healthy normal control subjects (TNF-α p<0.3735, IL-6 p<0.2863, IFN- γ p<0.4222, IL-10 p<0.462, TGF- β1 p<0.0741). Thus, cytokine genes may not play significant role in coronary heart disease process, as their actual effective protein.

Keywords: *Keywords: Atherosclerosis, TNF-α, IL-6, IFN- γ, IL-10, TGF- β1, SNPs*

المستخلص: توجد دلائل متزايدة على أن الإلتهاب له دور كبير في الكيفية التي يحدث بها مرض تصلب الشرايين. كما وان دراسة الاشكال الوراثية المختلفة (التراكيب الجينية المتعددة) للمواد والوسائط التي تساهم بطريقة أو اخرى في المراحل المختلفة لتطور هذا المرض كالسيتوكينات (Cytokines) مهمة جدا لعدة أسباب، فهي قد تساعد في تفسير التنوع والاختلاف في مدى الاستجابة الالتهابية للمرض بين الافراد. وبناءً على ذلك تم في هذا البحث دراسة إمكانية الارتباط والمزاملة بين تعدد الأشكال الجينية (Polymorphism) لبعض السيتوكينات المدعمة للعملية الالتهابية (TNF-α، IL-6، IFN- γ و أخرى مثبطة للعملية الالتهابية TGF-β1 ، IL-10 مع شدة المضاعفات القلبية المتعلقة بالأوعية الدموية المتضمنة لمرض واعتلال الأوعية التاجية القلبية لدى ثمانية وعشرين مريضاً بداء القلب الإكليلي (Arterial coronary heart disease) في مستشفى قوة دفاع البحرين. تسعة وعشرين شخصاً سليماً تم استخدامهم كمجموعة ضابطة. سحبت عينة دم لكل عنصر خضع للدراسة سواء من المرضى او الأصحاء ومن ثم تم استخلاص الحمض النووي الوراثي منها. بعد ذلك تم تحديد الطراز الوراثي (Genotyping) لكل سيتوكين بواسطة التفاعل التسلسلي البلمري (PCR) وتحديدا بواسطة (SSP) باستخدام مجموعة أدوات (Cytokine Genotyping Tray Kit) ثم تم تحليل ناتج التفاعل التسلسلي البلمري وقراءته. لوحظ انه لم يكن هنالك فرق معتبر في انتشار وتوزع الأشكال الجينية المتعددة لجميع السيتوكينات المدروسة بين المرضى مقارنة بالمجموعة الضابطة وكانت النتائج على النحو التالي:- (TNF-α p<0.3735، IL-6 p<0.2863، IFN- γ p<0.4222، IL-10 p<0.462، TGF- β1 p<0.0741) وعليه فإن التراكيب الجينية للسيتوكينات قد لا تلعب دورا مؤثرا في القابلية للإصابة بمرض الاوعية التاجية القلبية كما تفعل البروتينات الناتجة من هذه الجينات.

كلمات مدخلية : *تصلب الشرايين، TNF-α، IL-6، IFN- γ، IL-10، TGF- β1، SNPs.*

Introduction

Atherosclerosis is a pathological process that takes place in the major arteries, clinically manifested as coronary heart disease, stroke and peripheral vascular disease, which are considered to be the commonest causes of morbidity and mortality in the industrialized world (Georg Noll, 1998). There is a general consensus that an inflammatory milieu is a key component in the development of atherosclerosis (Fazio, 2001). From the initial stages of leukocyte recruitment to diseased endothelium, till eventual rupture of unstable atheromatous plaque, pro-inflammatory mechanisms and thus cytokines mediate key steps in atherogenesis and its complications (Blake, 2001).

The expression of pro-inflammatory cytokines and their receptors has been demonstrated in atheromatous tissue, and the serum levels of several of these cytokines have been found to be positively correlated with coronary arterial disease and its sequelae (Berrahmoune *et al.*, 2005). Furthermore, studies in knock-out or transgenic mice for specific cytokines have revealed, that while some cytokines are indeed intrinsically pro-atherogenic, others may have anti-atherogenic properties (Von der Thüsen *et al.*, 2003). The net effect of an inflammatory response is ultimately determined by the balance between pro-inflammatory and anti-inflammatory cytokines. As inflammation is a key component of atherosclerotic disease, genes coding for inflammatory cytokines are therefore considered to be potential candidates for predisposition to risk of coronary heart disease (Olivieri *et al.*, 2006).

Common variations in genes, called polymorphisms, have been recently associated with the risk of ischemic heart diseases. More importantly, a growing body of evidence indicates that these genetic variations can modulate, by increasing or decreasing, the effect of environmental risk factors on the development of atherosclerosis (Fazio 2001). Studying the genetic variants of the inflammatory system in atherosclerosis is highly important due to

Firstly, differences in the genetic regulation of inflammatory processes might explain why some people but not the others develop the disease and why some develop a greater inflammatory response than the others (Anderiotti *et al.*, 2002).

Secondly, the origin of coronary artery disease is multifactorial, with complex interactions between genetic and environmental components and generally, the incidence of coronary artery disease increases additively as a function of the number of conventional risk factors. However, some individuals with coronary artery disease do not exhibit any conventional risk factors, which suggest the contribution of genetic factors (Mitsuhiro *et al.*, 2000).

Thus, elucidation of the molecular genetic basis of coronary atherosclerosis through identifying potential involved susceptibility genes could contribute for an early genetic diagnosis, and risk stratification, independent and prior to disease development. It may aid initiation of preventive measures aimed at specific targets in any stage of pathogenesis and can also provide an opportunity to individualized therapy based on genetic information (Amento *et al.*, 1991).

Materials and Methods

1. Study Population

Twenty eight patients (aged 31-80; 15 males and 13 females) with signs of severe coronary atherosclerosis confirmed by coronary angiography (>70% stenosis in one or more arteries, and clinical signs of stable or unstable angina pectoris, typical ECG abnormalities) were enrolled from Bahrain Defense Force (BDF) Hospital. Twenty nine asymptomatic and apparently disease free subjects (no symptoms or history of cardio-vascular diseases and normal blood pressure) randomly selected from blood donors and considered as negative controls. The study was approved by the medical ethical committee and all subjects gave informed consent form. For each patient, a questionnaire was filled with data including age, sex, ethnicity, height and weight in addition to attached medical laboratory report which contained the main lipid profile biochemical tests.

2. Genomic DNA Extraction

Venous blood was collected into EDTA coated tube and mixed thoroughly to enhance the

stability of DNA in the blood. Buffy coat was collected and pellet was lysed by adding 500μl of the digestion buffer 100mM NaCl, 10mM Tris, 25mM EDTA, 0.5% SDS and 10ul of proteinase K (20mg/ml) then incubated at 55 C° overnight. 500μl phenol-chloroform-isoamyl alcohol was added to the mixture, mixed for 30 sec then centrifuged for 10 minutes. The supernatant was transferred to a new 1.5 μl microcentrifuge-tube, and the DNA was precipitated by adding of 1/2 vol of 7.5M ammonium acetate and 2 vol. of iced-cold 100% ethanol. DNA pellet was washed with 1ml 70% ethanol solution, air-dried and then dissolved in 200-300μl nuclease-free distilled water and stored at 4C°. The concentration of DNA samples were measured by spectrophotometer.

3. Cytokine Genotyping Tray Method
Cytokine Genotyping Tray kit (Cat# CYTGEN, One Lambda, Inc. CA, USA) used to detect genetic variations in TNF-α -308(A/C), IL-6 -174(G/C), IFN-γ +874(T/A), IL-10 -1082(G/A), -819(T/C) and -592(A/C), TGF-β1 at 10(T/C) and 25(C/G) according to manufacturer>s instructions. The volume tube of D-mix (180μl /tube, One Lambda, Inc. CA, USA) for cytokine Genotyping primer set tray, the primer set tray were thawed at room temperature (20C° - 25C°), then D-mix was pulse-centrifuged in a micro-centrifuge (Eppendorf 5415 D). This was followed by adding 1μl of recombinant Tag Polymerase enzyme (Promega, USA, 5μl /unit) to each D-mix tube using single-channel pipette with 10μl fixed volume). To each negative well, 10 μl of the mixture (D-mix with Taq DNA Polymerase) are added. 19μl volume of the pre-prepared thawed and well-mixed DNA samples of either patients or controls were added to the mixture of (D-mix with recombinant Taq DNA Polymerase enzyme). Then, 10μl -13μl of this master mixture were aliquoted to each of the rest of 15 PCR reaction tubes for each patient or control using 100μl pipette (Eppendorf, Germany). After gentle mixing, the reaction tubes were completely well sealed by a special provided tray seal to prevent evaporative loss during PCR. The primer set tray was placed in a thermocycler (Thermal Cycler, BIO-RAD, USA) and a pressure pad was placed on the top of the tray before closing the thermocycler. PCR cycling conditions consisted of 1 cycle of denaturation (96C°;130 sec) and annealing (63C°;1 min), followed by 9 cycles of denaturation (96C°; 10 sec) and annealing (63C°;1 min). The third round included 20 cycles of denaturation (96C°; 10 sec), annealing (59C°; 50 sec), and extension (72C°; 30 sec). The reaction was then terminated by indefinite cooling at 4C°. PCR products in cytokines genotyping tray were stored at -20C° for later analysis by gel electrophoresis.

4. Gel Electrophoresis and Procedure
Gel electrophoresis and procedure for Gel System PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide.

5. Statistical Analysis
Statistical analysis was performed using Chi-square test of independence to examine the significant difference in overall number of genotypes distribution for each studied cytokine between the patient group subjects and control subjects. The null hypothesis (H0) indicates non-significant relation while the alternative hypothesis (H1) point out to significant relation. P-value for each test was determined. P-value > 0.05 implies no significant difference whereas P-value < 0.05 represents a significant difference.

Results and Discussion

The studied cytokines were particularly selected in view of their potential role as pro-inflammatory (TNF-α, IL-6 and IFN-γ) and anti-inflammatory (IL-10 and TGF-β1) mediators in several diseases including atherosclerosis (Auer *et al.*, 2003; Leon and Zuckerman, 2005; Nashed *et al.*, 2005). Values of several risk factors in patients and control subjects were determined and demonstrated in Table. 1. The mean age among patients was 54.6 year, controls mean age was 46.1. The control subjects included 22 (75.9%) males and 7 (24.1%) females whereas the patients consisted of 15 (53.57%) males and 13 (46.43%) females. In addition, the body mass index was relatively similar (29.66 in patients and 26.46 in controls).

The prevalence of the polymorphisms of all studied cytokines in patients with coronary heart disease revealed no significant difference in comparison with apparently healthy normal control subjects. P-value of TNF-α was = 0.374 presenting non-significant relation (Figure 1). P-value of IL-6 was = 0.2863 (Figure 2). This is the first study that investigated IFN- γ polymorphism in coronary heart disease and demonstrated no association within the selected subjects (P-value was = 0.422). The difference of the percentage fraction for each genotype of IFN- γ is illustrated in Figure 3. In addition, for IL-10, P-value= 0.462 indicating non significant difference. The difference of the percentage fraction for each genotype of IL-10 is illustrated in Figure 4. Finally, TGF- β1 P-value was = 0.074 implying non-significant difference. The difference of the percentage fraction for each genotype of TGF- β1 is illustrated in Figure 5.

The type of cytokines selected for this study may permit us to have a better correlation capability in light of their inverse action as pro- and anti-inflammatory agents. Hence, a patient having more inflammatory disease alleles is expected to have less anti-inflammatory ones and vise versa. The studied single nucleotide polymorphisms were located mostly in coding and promoter regions rather than taken randomly. These variants have more priority, as they are most likely to be of functional significance and to influence directly the traits under study (associated with functional effects and phenotypic outcomes) (Risch, 2000). Actually, for a single mutation to influence the outcome of an inflammatory response, the mutation must markedly alter the production or function of a critical inflammatory protein. Although possible, a more likely scenario is the inheritance of multiple mutations in multiple proteins, each leading to small changes in production or function, but with a serious deleterious effect (Water and Wunderink, 2003). The available evidence indicates that common diseases are due at least in part to genes with a small number of disease-associated alleles (Smith and Lusis 2002). Moreover, even the causative variant may not exist in the selected gene; such single nucleotide polymorphisms are as likely (or more likely) to be in linkage disequilibrium with the causative allele as are randomly placed single nucleotide polymorphisms (SNPs; Risch, 2000).

Association of cytokine gene variants with atherosclerosis has been controversial. There have been a number of reports of gene variants being associated with susceptibility to coronary artery disease. However, other studies revealed non-association relation. The present study showed non-association relation which may be due to the following:

Firstly, ultimate conclusion regarding individual cytokine could not be reached through the investigation of only one single genetic variation. For example, only TNF-α –308 G /A mutation has been tested here while other 11 single nucleotide polymorphisms described located in the promoter region such as –238, and –376 have not been subjected to study yet. Nevertheless, TNF-α –308 G/A locus accounts for approximately 50 percent of TNF-α based disease association studies, consequently, the association of TNF-α with a given condition is often based on the analysis of a single locus that may not be the susceptibility locus for the disease of interest or is in linkage disequilibrium with such a susceptibility locus. This principle is true in a number of well-studied cytokine genes including IL-10 (-1082 A/G) and IL-6 (-174 C/G) (Von der Thüsen Jan *et al.*, 2003).

Secondly, although common alleles are more likely to be found globally than rare variants and hence, a causal association between a candidate single nucleotide polymorphism and trait outcome should be reproducible in many ethnically diverse populations, the absence of association in a certain population does not necessarily negate it in others and vise versa. It is well known that the same mutation can cause a major disease phenotype in one strain of mice while no phenotype change detected in other genetically distinct strain. Thus, background factors differentiating populations can modify the expression of a gene and lead to different levels of association (Nashed, *et al.*, 2005).

Recently, the studies of cytokine polymorphismin genetically diverse populations have highlighted inherent ethnic variations and inter population discrepancies particularly between Caucasian and non-Caucasian sample cohorts are

often huge. Good examples of this occur for the –1082G allele of IL-10 (40%-60% in Caucasian but 0%-5% in some Asiatic populations) and the TNF-308A allele (15%-25 % in Caucasian but 2%-5% in Asiatic populations). This can be verified from our study results as some cytokines like IL-10 showed one totally new variant from polymorphisms stated in the kit. Even though it was only a single case, extended population number might reveal extra supportive evidence.

This is a case-control study, in which a difference in allele frequency is investigated between affected individuals and unrelated unaffected controls. From epidemiological perspectives, a major limitation in this approach is the potential for confounding (that is, spurious association resulting from correlation with the true risk factor) leading to artefactual as opposed to causal association (Risch, 2000). In addition, a positive association does not establish causality and often indicates linkage disequilibrium with the actual mutation. Consequently, the results of genetic association studies are considered provisional and require pending confirmation through in vitro and in vivo experimentation (Amento *et al.*, 1991). In conclusion, this study revealed no significant association between genetic variants of certain cytokines and susceptibility to coronary heart disease in a sample of Bahraini population. Thus, future studies, which may include extended population number is needed. Also, polymorphisms of cytokines along with their receptors may elaborate a better image if they are investigated simultaneously.

Table 1. Characteristic of Patients and Controls (the table illustrates values of lipids as major risk factors in patients and control subjects).

Clinical and biological Feature	**Patients (n=28)**		**Controls (n=29)**	
Age(Years)	**54.6**		**46.1**	
Gender (male, female)	**15 (53.57%)**	**13 (46.43%)**	**22 (75.9%)**	**7 (24.1%)**
Body Mass Index (kg/m²)	29.66		26.46	
Cholesterol (3.8 -5.2 Mmol/L)	4.24±0.24		3.83±0.20 (p<0.15)	
Triglyceride (0.1-2.3 Mmol/L)	1.93±0.14		1.44±0.05 (p<0.23)	
LDL-Chol. (0 - 3.4 Mmol/L)	2.76±0.15		2.12±0.17 (p<0.11)	
HDL-Chol. (0 - 1.7 Mmol/L)	0.88±0.012		1.27±0.11 (p<0.14)	

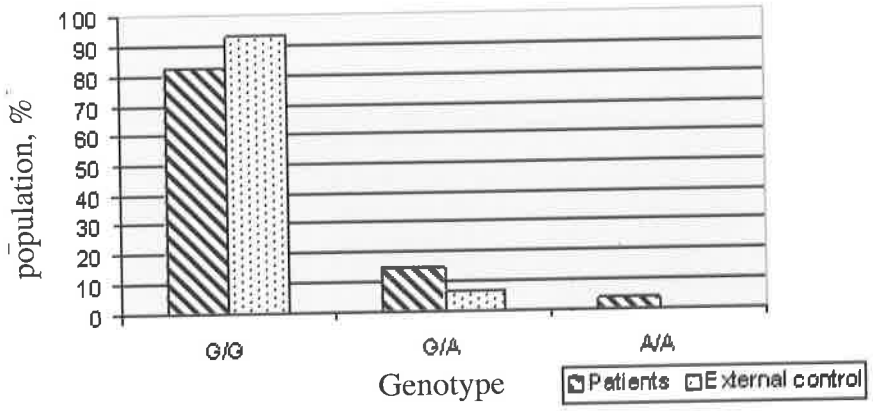

Fig. 1. The micrograph demonstrates TNF-α genotypes distribution. Note lack of significant association (p<0.3735).

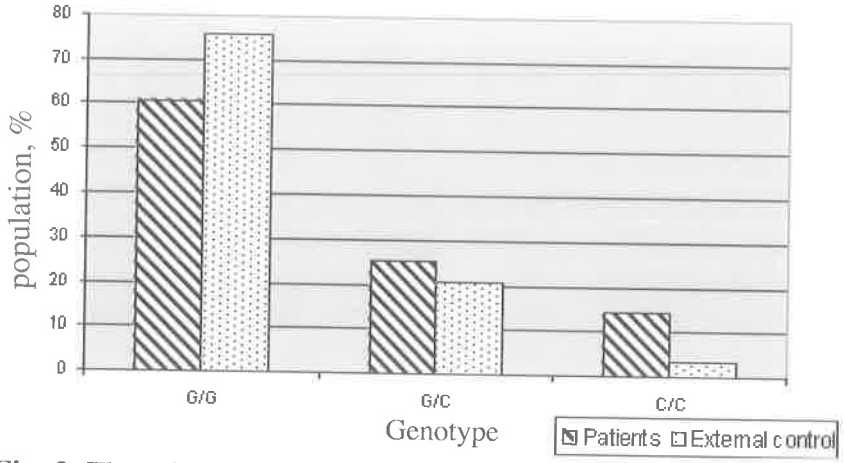

Fig. 2. The micrograph shows IL-6 genotypes distribution. Note lack of significant association ($p<0.2863$).

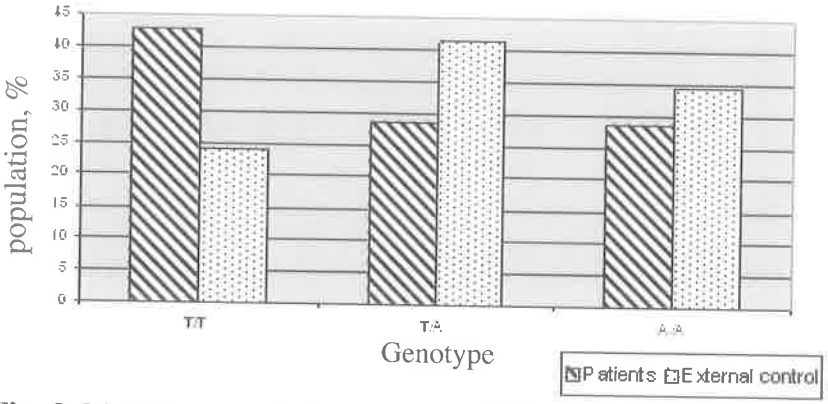

Fig. 3. The micrograph demonstrates IFN-γ genotypes distribution. Note lack of significant association ($p<0.4222$).

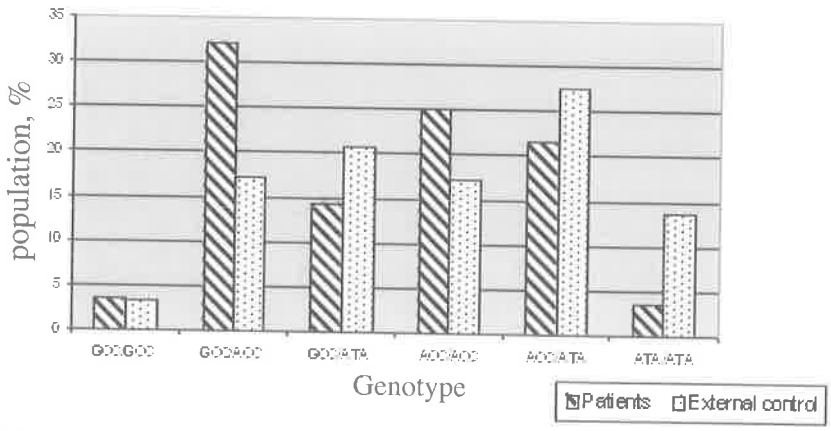

Fig. 4. The micrograph represents IL-10 genotypes distribution. Note lack of significant association ($p<0.462$).

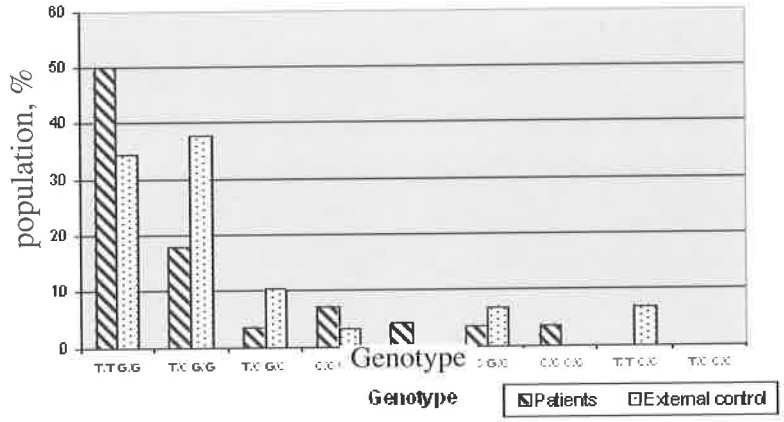

Fig. 5. The micrograph shows TGF- β1 genotypes distribution. Note lack of significant association (p<0.0741).

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