INFLUENCE OF INTERLEUKIN-6 (-174 G/C) SINGLE NUCLEOTIDE POLYMORPHISM ON SERUM IL-6 LEVELS IN PREMATURE CORONARY ARTERY DISEASE

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ABSTRACT

OBJECTIVES: To evaluate role of Interleukin-6 (-174 G/C) gene promoter polymorphism and the serum level of (IL-6) in the identification of premature coronary artery disease (PCAD).

METHODS: The case-control study was carried out at Army Medical College, Rawalpindi, Pakistan, from July, 2014 to Jan, 2015 in collaboration with University College London, UK. One hundred and fifty patients, <45 years of age with greater than seventy percent blockade in at least one major coronary artery on angiography, were labeled as cases. While 150 subjects who were declared negative for coronary artery disease on coronary angiography were taken as controls. Genotyping was performed using Taqman Assay while serum IL-6 was measured using Enzyme Linked Immuno-sorbent Assay (ELISA).

RESULTS: Total 364 subjects participated in the study. Mean±SD age of PCAD patients was 41±3.80 while in controls it was 36±7.8 years. Serum IL-6 levels were high in the cases (p<0.01). In IL-6 -174(G/C), polymorphism homozygous CC demonstrated significant association with occurrence of PCAD. Serum IL-6 levels showed a significant rise in CC genotype subjects when compared to GC and GG (p<0.05). The serum IL-6 levels showed significant correlation with the atherosclerotic burden (p<0.05). Sensitivity-specificity of IL-6 at cut off value of 3.7 ng/dl was 77% and 81% respectively.

CONCLUSION: IL-6 has significant diagnostic potential for PCAD with moderately high sensitivity and specificity. IL-6 (-174 G/C) polymorphism CC genotype can aid in the risk prediction of PCAD.

KEY WORDS: Gene promoter polymorphism (Non-MeSH); Interleukin-6 (MeSH); Coronary Artery Disease (MeSH), Premature Coronary Artery Disease (Non-MeSH); Risk prediction (Non-MeSH); Polymorphism, Single Nucleotide (MeSH).

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INTRODUCTION

Premature Coronary Artery Disease (PCAD) occurs as the foremost effect of coronary atherosclerosis. Premature CAD is defined as >70% stenosis in at least one vessel on coronary angiography in patients <45 years of age.[1] Timely detection and effective management are the key factors for the effective management of PCAD. One of the major events in the pathogenesis of atherosclerotic plaque formation and progression in CAD is inflammation. Multiple pro-inflammatory and anti-inflammatory cytokines play an active role in early atherosclerosis. Interleukin-6 (IL-6) is one of the key players in the modulation of immunological and inflammatory processes during various stages of premature coronary artery disease.[2]

IL-6 is an inflammatory cytokine which actively takes part in the immune-inflammatory process underlying the pathogenesis of CAD.[3] The hereditability of IL-6 has been estimated to be about >60%[4] but still to date only a limited number of gene polymorphisms affecting IL-6 levels are known. The most widely studied of these is the -174 G/C variant in the IL-6 gene promoter region. While some studies have reported significant association of -174 G/C single nucleotide polymorphism with CAD,[5,6] others have demonstrated no association between them.[7] Keeping in view the role of IL-6 in the pathogenesis of CAD, it is being considered as a potential target for drug therapy because it has been seen that individuals with variants in IL-6 gene that hampers the IL-6 pathway were found to be at a reduced risk for coronary heart disease.[8]

There is still paucity of studies reporting the potential effect of the IL-6 gene promoter SNP -174 G/C (Rs. 1800795) on the serum IL-6 levels and the risk prediction of CAD in Premature coronary artery disease patients of Pakistan. Hence, we sought to assess the role of the IL-6 gene promoter SNP -174 G/C (Rs. 1800795) and IL-6 serum levels in the diagnosis and risk prediction of PCAD patients.

METHODS

This was a case-control study carried out at the Chemical Pathology Laboratory (CPL), Army Medical College, Rawalpindi, along with the Cardiovascular Genetics Institute, University College London, UK. Ethical approval was duly sought from the Institutional Review Committee of both the concerned institutions. Duration of the study was from July, 2014 to Jan, 2015.

Subjects:- Three hundred and sixty four subjects aged <45 years reporting to the cardiac care centre with chest pain and scheduled to have angiography were recruited consecutively. Out of these 165 patients who had greater than 70% blockade in at least one coronary artery diagnosed angiographically, were selected as cases. Those who were angiographically free from the disease or had <10% stenosis in a single coronary artery were selected as controls (n=199). Cases who had previously been diagnosed with any blood disorder, carcinoma, renal, congenital heart disease, hepatitis, liver or thyroid disease or any acute infection were excluded. Demographic characteristics along with ethnicity were noted. Subjects were explained the process in detail and informed consent was taken in written form. The procedures adopted comply with the ethical standards of Helsinki Declaration of 1975.

Biochemical Analysis:

Blood samples were taken on the day which was scheduled for angiography of the respective patient preferably early in the morning. Ten millilitre blood sample was obtained by venipuncture. 7ml was transferred to a red top serum tube for serum analysis and 3 ml transferred to EDTA tube for DNA extraction.

Enzyme Linked Immunosorbent Assay (ELISA) technique was used for measuring the concentrations of serum IL-6 (Duo set kit-R&D systems) commercial kit using human monoclonal antibodies. The coefficient of variation (CV), intra assay, for IL-6 was 5.8% while the limit of detection was <2 ng/dl respectively. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN). Genotyping was carried out using Taqman Assay using the standard assay protocol at the Cardiovascular Genetics Institute, University College London, UK. For each Taqman genotyping assay there is a forward primer and a reverse primer which bind sequences either side of the SNP to be genotyped allowing amplification of the region during PCR. There are two allele specific probes labeled with a different fluorophore VIC or FAM at the 5' end. At the 3' end is a quencher which prevents fluorophore from fluorescing. During the process if only one fluorophore fluoresces the sample is homozygous for the corresponding allele. If a signal from both fluorophores is present the sample is heterozygous. The components when using Taqman buffer are shown in Table 1. 2ul of the reaction mix was added per well. The plate was then centrifuged and the PCR performed using the Taqman genotyping assays as below.

- 500C for 2 minutes
- 950C for 10 minutes
- 950C for 15 sec
- 600C for 1 minute

The intensity of each single nucleotide polymorphism (SNP) was based on the criteria of three clear clusters in two scales generated by Sequence Detection Systems (SDS) software version 2.3 Applied Biosystems Inc (ABI).

Statistical analysis:-

Statistical analysis was done by SPSS-22 (SPSS Inc, Chicago) and MedCalc software version 9.6.4.0. Mean, SD, median was calculated for descriptive statistics. Mann-Whitney U test was applied for comparison of PCAD patients and controls. MedCalc software was used to construct Receiver operator characteristic curve (ROC) to estimate the diagnostic value and diagnostic odds ratio of IL-6. Genotype of -174G>C and allele frequencies were measured by the Chi-square and Fisher exact test, respectively. To evaluate possible relationship of -174G>C polymorphism and increased IL-6 levels with PCAD binary logistic regression analysis was carried out. A p-value of <0.05 qualified as significant.

RESULTS

Total 364 subjects consisting of 165 PCAD patients and 199 angio-negative controls participated in the study. Age of PCAD patients was 41 ±3.80 years while in controls it was 36±7.8 years. After applying the Kolmogorov-Smirnov test on data non-Gaussian distribution for cytokines was seen (P<0.05). Demographic data of the subjects is demonstrated in table-II. Serum IL-6 levels were highly raised in PCAD patients (p<0.01). Area under curve (AUC) and 95% (CI) of IL-6 was 0.667 (0.592 - 0.737) [Fig-I]. A serum level of 3.7 ng/dL had the highest odds ratio of serum IL-6 for PCAD with the maximum sensitivity and specificity is shown in Table III.

In IL-6 -174 (G/C) polymorphism homozygous CC was significantly related to the occurrence of PCAD and serum IL-6 levels were highly raised in CC genotype subjects in contrast to subjects with GC and GG genotypes (p<0.05) (Fig 2). Genotypic and allelic distribution of IL-6-174G>C polymorphism is shown in Table IV. Results of binary logistic regression analysis are shown in Table V. Significant correlation was found among serum IL-6 levels, BMI, LDL, total cholesterol, cigarettes smoked per day and the extent of blockade in PCAD patients (Table VI).

DISCUSSION

We observed that the sensitivity and specificity of IL-6 was high for the diagnosis of PCAD. This is in agreement to studies done previously showing

TABLE I: REAGENT VOLUMES FOR IL-6 RS1800795 TAQMAN GENOTYPING ASSAY USING TAQMAN BUFFER

Reagent	Volume
Taqman master mix (Life Technologies)	410ul
Nuclease free water (Sigma)	389.5/399.9
40x/80x SNP Specific Assay (Life Technologies)	20.5/10.3

The volume of SNP specific assay depends on whether it is supplied as 40x or 80x. Therefore; the volume of water also varies accordingly.

TABLE II: DEMOGRAPHIC CHARACTERISTICS OF PATIENTS AND CONTROLS

Variables	Cases (n=165)	Controls (n=199)	p-Value
Age (years) (Mean ± SD)	41±4.23	36±7.55	0.76
Gender(M/F)	155/ 10	194 / 5	0.63
Weight (Kg) (Mean ± SD)	73.1±11.7**	66.7±10.1	0.0009
Height (m) (Mean ± SD)	1.68±0.06	1.69±0.08	0.85
BMI (Kg/m^2) (Mean ± SD)	26.32±3.7**	23.6±3.5	<0.002
Positive Diabetes n (%)	61 (37)**	7(4)	<0.01
Positive Premature CAD Family History n (%)	73 (44)**	6 (3)	<0.01
Positive DM family history n (%)	61 (37)**	30 (15)	<0.01
Smokers n (%)	101 (61)*	78 (39)	<0.05
Total Cholesterol (mmol/l) (Mean ± SD)	4.9±3.77	4.5±1.38	0.56
Serum IL-6 (ng/dl) (Mean ± SD)	3.8±1.5	2.9±1.9	<0.01

PCAD: Premature Coronary Artery Disease; BMI: Body Mass Index; CAD: Coronary Artery Disease: DM: Diabetes Mellitus; SD=Standard Deviation. Categorical variables were compared using x2test while continuous variables were compared using Welch's t-tests. .*p<0.05 ;**p<0.01.

TABLE III: DIAGNOSTIC PERFORMANCE OF IL-6 AT DIFFERENT CUT-OFFS FOR DIAGNOSIS OF PREMATURE CORONARY ARTERY DISEASE

Biomarker	SN (%)	95%CI	SP (%)	95%CI	LR+	LR-	DOR
IL-6 (ng/dl) > 3.2	70.27	58.5-80.3	82.65	73.7-89.6	4.05	0.36	11
> 3.7*	77.03	65.8-86.0	80.61	71.4-87.9	3.97	0.28	14
> 3.4	78.38	67.3-87.1	78.57	69.1-86.2	3.66	0.28	13

*p<0.05; IL-6: Interleukin-6; SN: Sensitivity; SP: Specificity; LR+: Positive likelihood ratio; LR-: Negative likelihood ratio; DOR: Diagnostic Odds Ratio; CI: Confidence Interval

TABLE IV: BINARY LOGISTIC REGRESSION ANALYSIS FOR PREDICTION OF PREMATURE CORONARY ARTERY DISEASE

Variables	B	Exp (B)	(95%CI)	Sig.
IL-6 (ng/dl)	0.020	1.030	(1.003-1.018)**	0.005
IL-6 -174 (G/C) Genotype: CC versus CG+GG	0.320	1.072	(0.852-2.047)*	0.04

**p<0.01; *p<0.05; Logistic regression, adjusted for age, sex, smoking, BMI and diabetes, was performed for the subjects; Exp(B):odds ratio; CI: Confidence interval; Sig: significance.

TABLE V: IL-6-174G>C GENOTYPE AND ALLELE FREQUENCIES OF STUDY POPULATION (N=300)

IL-6 -174 G/C Genotype (percentage)	PCAD patients (n=165)	Controls (n=199)	P-value
GG n (%)	110 (67)	147 (74)	0.04*a
GC n (%)	45 (27)	50 (25)	
CC n (%)	10 (6)	2 (1)	
IL-6-174G>C allele count			0.027*b
G	265	344	
C	65	54	

IL-6:Interleukin-6; PCAD: Premature Coronary Artery Disease; RAF: Risk Allele Frequency *p<0.05 a) p-value was calculated by chi-square test; b)p-value was calculated by Fisher's exact test.

association of IL-6 with the degree of calcified coronary stenosis.[9] This might be because IL-6 overexpression leads to plaque instability and progression.[10] Ferroni et al., 2007 demonstrated that serum IL-6 had significant diagnostic potential in enzyme negative patients with chest pain.[11] The sensitivity and specificity of IL-6 has been shown to be 100% and 66% respectively for the prognosis of morbidity in acute coronary syndrome at a cut off value of 41pg/ml.[12]

In the current study, IL-6 promoter region polymorphism -174 G/C was significantly related to PCAD, and it remained significant in multivariate analysis, even after accounting for the confounding variables. Minor allele C at -174G>C was associated with high serum IL-6 levels.[13] To date, allele frequency information across Pakistan and even in South Asia is limited for the SNPs. Moreover, the information that is available has conflicting results and there is marked variation in the risk allele frequencies of these risk SNPs in different populations. While some studies have marked C as the risk allele,[14] others have demonstrated GG to be the genotype conferring the risk of CAD.[12] This is likely caused by the presence of population sub-structure.[15] Another probable explanation may be that the functional SNP in the IL-6 is yet to be identified with -174 G/C being the tagging SNP in certain populations where significant association has not been observed. On performing logistic regression analysis after adjusting for the confounding variables an independent association was found between PCAD and raised serum IL-6 levels and CC genotype of -174G>C polymorphism. This is an important observation because a previous study demonstrates that IL-6 may have a limited role in predicting early mortality in CAD and its performance may be limited by age related factors.[16] Whereas other studies have shown that interleukin-6 concentrations and IL-6 gene promoter SNP -174 G/C aid in the prognostic risk stratification of these CAD patients.[17]

We observed significant correlation of serum IL-6 levels with the extent of blockade in PCAD patients. This has been previously observed in a study where there was a positive association between the atherosclerotic burden

TABLE VI: CORRELATION OF SERUM INTERLEUKIN-6 LEVEL WITH DEGREE OF STENOSIS AND RISK FACTORS FOR PREMATURE CORONARY ARTERY DISEASE

Variables	IL-6 ng/dl) r	P-value
Body Mass Index (kg/m2)	0.216**	p<0.01
Triglycerides (mmol/l)	0.189	p=0.32
Total Cholesterol (mmol/l)	0.349*	p<0.05
High Density Lipoprotein (mmol/l)	-0.258	p=0.15
Low Density Lipoprotein (mmol/l)	0.272**	p<0.01
Cigarettes per day	0.192*	p<0.05
Gensini score	0.160*	p<0.05

r: Spearman Correlation coefficient. **p<0.01; *p<0.05

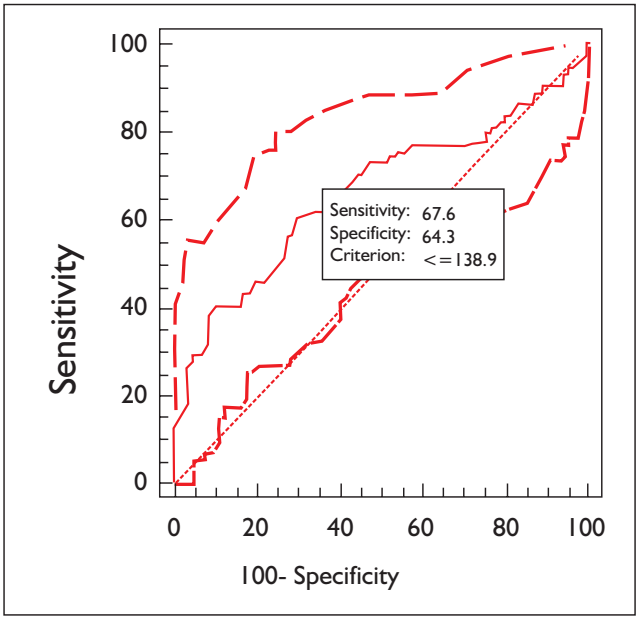

Fig 1: The ROC for IL-6 in the diagnosis of PCAD

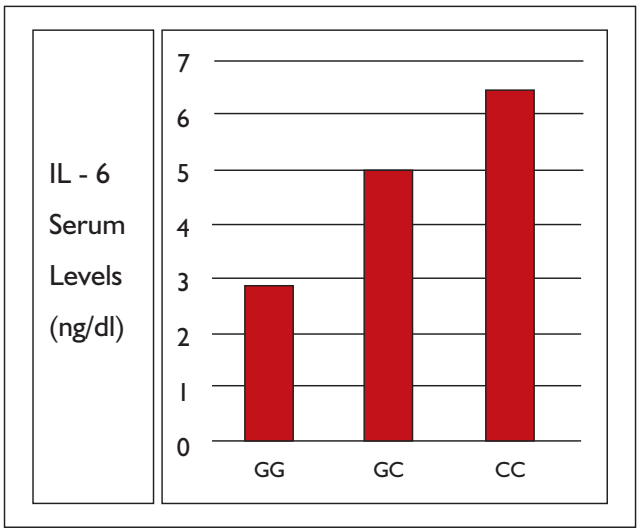

Fig 2: Influence of IL-6-174G>C polymorphism on the circulating levels of IL-6 in premature coronary artery disease patients (n=150)

and serum IL-6 level.[18] This is probably because of the pro-atherogenic nature of IL-6 and its distinctive role in macrophage polarization and other stages of inflammation in atherosclerosis.[19] Serum IL-6 levels also correlated significantly with the BMI in PCAD patients. This may be due to IL-6 altering the resistance to insulin in an individual. This correlation has been reported in a previous study in overweight, post-menopausal women.[20] Another study suggests that IL-6 may be secreted in relation to the increase in fat deposit resulting in a positive correlation with BMI and total cholesterol.[21] As far as the correlation between smoking and IL-6 is concerned a moderate positive correlation between IL-6 and smoking load has been demonstrated earlier.[22] One of the probable explanations for this may be that the cigarette smoke activates alveolar macrophages and epithelial cells to release inflammatory mediators leading to increase in the oxidative stress through stimulatory effects on the acute phase response.[23]

The strengths of our study are based on the factors that we have included angio-negative subjects as disease free controls for a better comparison. It is also the first kind of its study which has studied the diagnostic accuracy of IL-6 in PCAD patients who are young and whose number is increasing alarmingly in Pakistan and South Asia. Moreover, we have identified a cut off value for serum IL-6 level with the best possible sensitivity and specificity for the diagnosis and risk stratification of PCAD. The possible limitation of our study is its relatively limited sample size. Future research endeavors should also include acute coronary syndrome cases of PCAD for a better assessment of the diagnostic efficacy of IL-6 and the role of IL-6 -174 G/C SNP in PCAD patients.

CONCLUSION

We have seen that IL-6 has good diagnostic performance for diagnosis of PCAD and may aid clinical assessment for better therapeutic protocol. We also identified a significant association of IL-6-174 G>C promoter region polymorphism with PCAD in Pakistani patients with the CC genotype contributing to raised serum levels IL-6 in patients.

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CONFLICT OF INTEREST

Authors declared no conflict of interest

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AUTHOR'S CONTRIBUTION

Following authors have made substantial contributions to the manuscript as under:

WMA: Concept & study design, acquisition of data, drafting the manuscript, final approval of the version to be published.

AKN: Analysis &interpretation of data, critical revision, final approval of the version to be published.

DAK: Acquisition of data, drafting the manuscript, final approval of the version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.