ROLE OF INTERLEUKIN-6 (-174 G/C) GENE PROMOTER POLYMORPHISM AND IL-6 LEVELS IN RISK PREDICTION OF CORONARY ARTERY DISEASE IN YOUNG PAKISTANI SUBJECTS

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Abstract: IL-6 is a pleiotropic cytokine which actively participates in the immune-inflammatory process underlying the pathogenesis of CAD.The most widely studied of these is the -174 G/C variant in the IL-6 gene promoter region. there is still paucity of studies reporting the effect of the IL-6 gene promoter SNP -174 G/C (Rs1800795) 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(Rs1800795) and IL-6 serum levels in the diagnosis and risk prediction of PCAD patients.

Keywords: Interleukin-6, Promoter Polymorphism, Coronary Artery Heart disease.

I. INTRODUCTION

Premature Coronary Artery Disease (PCAD) is one of the earliest manifestations of coronary atherosclerosis. 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 are involved in the pathogenesis of CAD having overlapping, antagonistic and synergistic effects. 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.

IL-6 is a pleiotropic cytokine which actively participates in the immune-inflammatory process underlying the pathogenesis of CAD**[1].** IL-6 levels in serum have been associated with the risk and mortality in coronary heart disease **[2].** The hereditability of IL-6 has been estimated to be about >60%[3] but still to date only a few genetic variants influencing 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 **[4][5]** others have demonstrated no association between the **them [6].**Moreover, there is still paucity of studies reporting the effect of the IL-6 gene promoter SNP -174 G/C (Rs1800795) 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(Rs1800795) and IL-6 serum levels in the diagnosis and risk prediction of PCAD patients.

II. DETAILS EXPERIMENTAL

The case-control study was conducted at the Clinical Pathology Laboratory (CPL), Army Medical College, Rawalpindi, in collaboration with the Cardiovascular Genetics Institute, University College London, UK. Ethical approval was duly sought from the Institutional Review Committee of Army Medical College (AM College) under the auspices of the National University of Science and Technology (NUST) Islamabad, Pakistan and the ethical review committee of the collaborating institution. Duration of the study was 6 months.

2.1.Subjects

Three hundred subjects aged <45 years scheduled to undergo coronary angiography were consecutively screened. Out of these 150 patients with > 70% stenosis in at least one coronary vessel on angiography which is the gold standard, were labeled to have PCAD. Those who were angio-graphically proven to be disease free were labelled as controls (n = 150) after informed consent. Patients with a history of hematological, neoplastic, renal, liver or thyroid disease were excluded. Furthermore, patients with infectious or autoimmune diseases, familial hyperlipidemia, congenital heart disease, hepatitis, valvular heart disease, asthma, rheumatoid arthritis or life expectance of less than 12 months and those unable to give informed consent were also excluded. In the controls subjects with any acute or chronic illness or those on anti- inflammatory drugs were excluded. Pregnant women and women on contraceptives were also excluded from the study. Demographic characteristics were noted. Medical examination by a general physician was conducted. Written informed consent was obtained from subjects.

2.2. Biochemical Analysis:

All laboratory investigations were carried out in the Clinical Pathology Laboratories of AM College, NUST Rawalpindi, Pakistan. Blood samples were taken in the morning on the day of angiography of the respective patient. 10 ml blood sample was obtained by veni-puncture. 7ml was transferred to a plain vacutainer tube for serum analysis and 3 ml transferred to EDTA tube for DNA extraction.

Enzyme Linked Immuno-sorbent Assay (ELISA) technique was used for measuring the concentrations of serum IL-6 (Invitrogen, USA) commercial kit with monoclonal antibodies. The calculated overall intra assay coefficient of variation (CV) for IL-6was 5.8% while the limit of detection was <2 pg/ml. respectively. DNA was extracted usingQIAamp DNA Blood Mini Kit (QIAGEN) according to the manufacturer's instructions. Genotyping was performed using TaqMan Assay using the standard assay protocol at the Cardiovascular Genetics Institute, University College London, UK.

2.3. Statistical analysis:-

Statistical analysis was performed using SPSS-17 (SPSS Inc, Chicago) and MedCalc software version 9.6.4.0.Kolmogorov-Smirnov test was applied on data which revealed non-Gaussian distribution for cytokines (P<0.05). Mean, SD, median was calculated for descriptive statistics. Mann-Whitney U test was applied for comparison of PCAD patients and controls. Receiver operator characteristic curve (ROC) was constructed using MedCalc software in order to evaluate the diagnostic value including sensitivity, specificity, likelihood ratio and diagnostic odds ratio of IL-6. Genotype of -174G>Candallele frequencies were measured by the chi-square and Fisher exact test, respectively. To evaluate possible relationship of -174G>C polymorphism and increased IL-6 with PCAD binary logistic regression analysis was carried out. A p-value of <0.05 was considered to be significant.

Table-I:-Demographic Characteristics of Patients and Controls.

Parameters	Cases (n=150)	Controls (n=150)	p-value
Age (years)	40 ± 4.23	35 ± 7.55	0.76
Gender(M/F)	141 / 9	145 / 5	0.63
Weight (Kg)	74.1 ± 11.7 **	67.7±10.1	0.009
Height (m)	1.68 ± 0.06	1.69 ± 0.08	0.85
BMI (Kg/m²)	26.32 ± 3.7 **	23.6 ± 3.5	<0.002
Positive Diabetes (%)	(37) **	(4)	<0.01
Positive Premature CAD Family History (%)	(44)**	(3)	<0.01
Positive DM family history (%)	(37)**	(15)	<0.01
Smokers (%)	(61)*	(39)	<0.05
Total Cholesterol (mmol/l)	4.9 ± 3.77	4.5 ± 1.38	0.56
Serum IL-6 (ng/dl)	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 DeviationCategorical variables were compared using x^2test while continuous variables were compared using Welch's t-tests. .*p<0.05 ;**p<0.01.

Table-2:-Diagnostic performance of IL-6 at different cut offs for diagnosis of PCAD.

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

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

Table-3: Binary Logistic Regression Analysis for Prediction of Premature Coronary artery disease

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

Logistic regression, adjusted for age, sex, smoking, BMI and diabetes, was performed for each group; Exp(B):odds ratio; CI: Confidence interval; Sig: significance.

**p<0.01; *p<0.05

Table-4: IL-6-174G>C genotype and allele frequencies of study population n=300

IL-6 -174 G/C genotype	PCAD patients (n=150)	Controls (n=150)	p-value
GG	110 (36.6%)	147 (49%)	0.02*
GC	45 (15%)	50 (16.6%)	
CC	10 (3.33%)	2 (0.6%)	
IL-6-174G>C Risk allele frequency (RAF)			
C	0.197	0.136	

IL-6:Interleukin-6; PCAD: Premature Coronary Artery Disease; RAF: Risk Allele Frequency *p<0.05

III. RESULTS AND DISCUSSION

3.1. RESULTS

Total 300 subjects consisting of 150 PCAD patients and 150 angio-negative controls participated in the study. Mean ± SD age of PCAD patients was 40 ± 3.80 (142 males and 8 females) while in controls it was 38 ± 7.8 yrs (145 males and 5 females. Baseline characteristic data of the subjects is shown in **(Table-I)**.Serum IL-6 levels were significantly higher in PCAD patients as compared to controls ($p<0.01$). ROC analysis of biomarkers in PCAD patients and controls revealed that Area under curve (AUC) and 95% (CI) of IL-6 was 0.667 (0.592 - 0.737). **(Fig-I).** The diagnostic odds ratio of IL-6 was highest at a cutoff level of 3.7 ng/dL. The sensitivity and specificity at this cutoff for the diagnosis of PCAD was 77% and 81% respectively. (**Table-2)**.

In IL-6 -174 (G/C) polymorphism homozygous CC was significantly associated with occurrence of PCAD and serum IL-6 levels were significantly higher in CC genotype subjects when compared to GC and GG ($p<0.05$**) (fig-2).**Genotype andalleledistributionofIL-6-174G>C polymorphism is shown in**Table 3.**Binary logistic regression analysis for possible association of CAD with serum IL-6 and-174G>C polymorphism is shown in (**Table 4)**. 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.

3.2.DISCUSSION

We observed that the sensitivity and specificity of IL-6 was high for the diagnosis of PCAD. Positive correlation was also demonstrated between serum IL-6 levels and degree of stenosis in PCAD patients. This is in agreement to studies done previously showing association of IL-6 with the extent of calcified coronary atherosclerosis[7]**.**This is probably because IL-6 overexpression induces plaque instability and progression[8]**.Ferroni et al.,2007** demonstrated that serum IL-6 had significant diagnostic potential in enzyme negative patients with chest pain[9].

In the current study, IL-6 promoter region polymorphism -174 G/C was significantly associated with 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[10]**.**To date, allele frequency information across Pakistan and even in South Asiais 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[11] 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 [13] 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[14]**.**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 [15]**.**

One of the major strengths of our study is that we have included angio-negative individuals as healthy controls thus allowing for a better comparison between patients and controls. It is also the first kind of its study to the best of our knowledge which has studied the diagnostic accuracy of IL-6 in Premature Coronary artery disease patients who are young (< 45 years) and whose number is increasing alarmingly in Pakistan and South Asia. The major limitation of our study is its relatively small sample size. We therefore recommend that future studies be carried out in multiple centers on larger patient populations and 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.

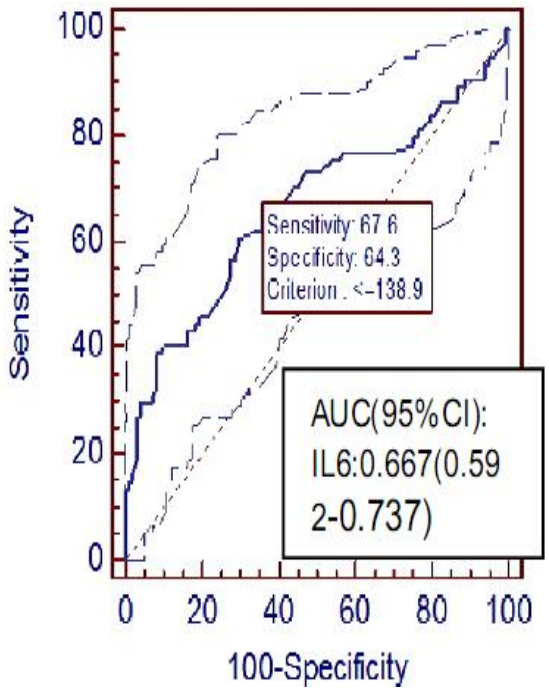


Fig-1: The ROC for IL-6 in the diagnosis of PCAD. PCAD=Premature Coronary Artery disease; AUC=Area under curve; ROC=Receiver operating curve; IL-6=Interleukin-6.

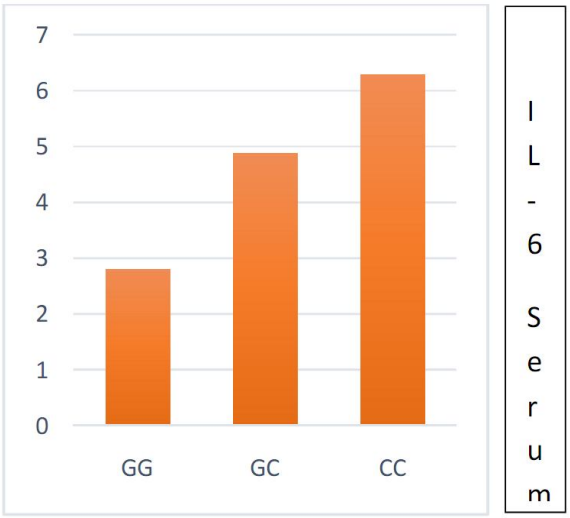


Fig-2: Influence of IL-6-174G>C polymorphism on the circulating levels of IL-6 in premature coronary artery disease patients (n=150).
Data represents mean ± SE. Serum IL-6 levels, < 0.01 in each genotype compared by 1-way-analysis of variance.

CONCLUSIONS

We have seen that while IL-6 has good diagnostic performance for diagnosis of PCAD and may be used in combination with clinical assessment for better therapeutic management. We also identified a significant association of IL-6-174G>Cpromoter regionpolymorphismwith PCAD in Pakistani patients with the CCgenotype contributing to raised serum levelsIL-6 in patients.

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