The Professional Medical Journal
www.theprofesional.com

DOI: 10.17957/TPMJ/16.3282

ORIGINAL PROF-3282

PREMATURE CORONARY ARTERY DISEASE;

ASSOCIATION OF IL-6 RECEPTOR GENE POLYMORPHISM ASP358ALA (A>C RS8192284) WITH PRO-INFLAMMATORY CYTOKINES AND CYTOKINE MEDIATOR SERUM LEVELS

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Article received on:
06/02/2016
Accepted for publication:
21/06/2016
Received after proof reading:
08/08/2016

ABSTRACT… Objectives: Interleukin-6 receptor (IL-6R) gene (A>C, rs8192284) polymorphism has been associated with inflammatory biomarkers. We sought to investigate the association of IL-6R gene (A>C, rs8192284) polymorphism with IL-6, IL-18 and hS-CRP levels in PCAD. **Study Design:** Case control study. **Setting:** Army Medical College, National University of Sciences and Technology, Islamabad, Pakistan **Methods:** Total 520 subjects were recruited. 281 PCAD patients aged ≤45 years with >70% stenosis in at least one major coronary vessel along with 239 age and sex matched controls were recruited. IL-6R polymorphism was determined by TaqMan genotyping while IL-6 and IL-18 levels were measured using ELISA technique and hS-CRP on Immulite 2000. **Results:** The genotype distribution of IL-6R(rs8192284) in the cases and controls was: AA-56%(n=143);AC-36% (n=102); and CC-13% (n=36) and AA-59%(n=139); AC-33% (n=80); CC-8% (n=20) respectively. The risk allele frequency was significantly different between the cases and controls (p=0.038) .IL-6 levels were significantly high (p<0.01) while hS-CRP levels were significantly low (p<0.05) in subjects with IL-6R (rs8192284) CC genotype as compared to the AC and AA genotypes. Multivariate logistic regression analysis revealed that IL-18, IL-6 and hS-CRP still remained significant in the prediction of PCAD with a high odds ratio OR (p<0.05). **Conclusions:** Our study is the first to show that the presence of the IL-6R rs8192284 is associated with significantly high IL-6 level and significantly low hS-CRP levels in Pakistani Premature Coronary Artery Disease Patients.

Key words: Interleukin-6 Receptor; Polymorphism; hS-CRP; Interleukin-18 and Premature Coronary Artery disease; Interleukin-6

Article Citation: Ansari WM, Naveed AK, Khan MN, Khan OJ, Khan DA. Premature coronary artery disease; association of Il-6 receptor gene polymorphism Asp358ala (A>C rs8192284) with pro-inflammatory cytokines and cytokine mediator serum levels. Professional Med J 2016;23(8):912-917. **DOI:** 10.17957/TPMJ/16.3282

INTRODUCTION

Coronary Artery Disease (CAD) is one of the foremost reasons of morbidity and mortality in the developed and developing countries such as Pakistan.[1] Recent studies have shown that CAD is a multifactorial pathology and it involves an interaction between the immuno-inflammatory pathway with a special focus on the role of pro- and anti-inflammatory cytokines.[2] One of the important pro-inflammatory cytokines in this pathway is Interleukin-6 (IL-6) which acts through the Interleukin-6 receptor (IL-6R).[3] Since IL-6 acts through the IL-6R which in turn activates an intracellular signalling cascade leading to the inflammatory response, IL-6R can potentially become a therapeutic target for CAD. Although a number of studies have been carried out to study the association of IL-6 and IL-6 variants with CAD[4], however there is still a paucity of studies investigating the role of the IL-6R variants on the IL-6 levels and their association with CAD especially in subjects <45 years of age where the genetic predisposition is significant.[5] Human IL6R is present on 1q21, a vulnerable locus for CAD. IL6R variations have been associated with CAD[6] but it may not hold true for other populations due to the diverse ethnic backgrounds. Studies have shown that the IL-6R rs8192284 alters the expression of IL-6R which further affects the regulation of IL-6 signalling[7] However, the effect of the IL-6R variants on the pro-inflammatory cytokines and cytokine mediators especially in the pathogenesis PCAD is still not clear. Hence, we sought to investigate the association of (IL-6R) gene Asp358Ala (A>C, rs8192284) polymorphism with IL-6, IL-18 and hS-CRP levels in PCAD.

SUBJECTS AND METHODS

The case-control study was carried out at Army Medical College, National University of Sciences and Technology, Islamabad, Pakistan in collaboration with the Cardiovascular Genetics Institute, University College London, UK after taking approval from the Institutional review Committees of both the participating institutions. The study is in accordance with the Declaration of Helsinki and Higher Education Commission (HEC) protocol.

A total of 520 unrelated individuals were consecutively recruited by non-probability convenience sampling from the pre-angiography unit of National Institute of Heart Disease, Rawalpindi, Pakistan with a recent history of chest pain. Out of these, 281 CAD patients aged ≤45 years with >70% stenosis in at least one of the major coronary vessels were recruited as Premature coronary artery disease (PCAD) (Dhillon et al.,2006) sufferers while 239 age and sex matched subjects proven to be disease free on angiography were taken as controls. We excluded patients with other co-morbid conditions like infectious or autoimmune diseases, familial hyperlipidemia, congenital and valvular heart disease, arthritis, previous history of IHD or MI and those unable to give informed consent were also excluded. Among the controls those with acute or chronic illness or those on anti- inflammatory drugs or statins were excluded. Medical examination was conducted by a general physician and demographics were duly noted. Written informed consent was obtained from subjects.

Blood samples were obtained when the subjects were being prepared for angiography early in the morning. 10 ml blood sample was obtained by veni-puncture. 6ml was transferred to a red top serum tube for serum analysis and 4 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 (Duo set kit-R&D systems) commercial kit using human monoclonal antibodies. The intra assay coefficient of variation (CV) for IL-6 was 5.8% while the limit of detection was <2 ng/dl respectively. Sandwich enzyme immunoassay was performed on Enzyme Linked Immunosorbent Assay (ELISA) for measuring the concentrations of serum IL-18 using human IL-18 (Bendermed Systems, Austria) commercial kits. The calculated overall intra-assay coefficient of variation(CV) for IL-18 6.5% while the limit of detection was 9pg/ml. Serum hS-CRP was analyzed by chemiluminescent immunometric assay kit (Seimen, LA, California, USA) on Immulite 1000 (Immulite, Diagnostic Product Corporation, USA) with manufacture reagent as directed (Roberts et al., 2000). The analytical sensitivity was 0.1mg/L.

Pure Gene Gentra DNA Blood Kit (QIAGEN) was used for genomic DNA extraction. Genotyping for IL-6R rs8192284 was carried out using TaqMan Assay according to the standard assay protocol at the Cardiovascular Genetics Institute, University College London, UK. The genotypes were identified based on the criteria of three distinct clusters generated by Sequence Detection Systems (SDS) software version 2.3 (ABI).

Statistical analysis was performed using SPSS-22 (SPSS Inc, Chicago) Hardy-Weinberg equilibrium (HWE) was done. Continuous data were shown as mean ± SD and Student □-test was applied to analyze differences between cases and controls. Categorical variables were compared using □2 analysis. Genotype distribution of IL-6R rs8192284 and allele frequencies were measured by the chi-square and Fisher exact test, respectively. Linear-regression was performed based on an additive model to determine association of IL-6R variant with hs-CRP and IL-6 levels. Multivariate linear regression models with age, sex, and body mass index (BMI) as confounding variables, was done to evaluate possible potential of IL-6R rs8192284 C-allele and IL-6, IL-18 and hS-CRP levels along with the traditional risk factors in the risk prediction of PCAD. A p-value of <0.05 was considered significant.

RESULTS

Baseline characteristics of the study subjects are shown in Table-I. Genotype and allele distribution

of IL-R (rs8192284) was in Hardy-Weinberg Equilibrium in both cases and controls. The genotype distribution in the cases and controls was: AA-56% (n=143); AC-36% (n=102); and CC-13% (n=36) and AA-59% (n=139); AC-33% (n=80); CC-8% (n=20) respectively. The allelic distribution particularly the risk allele frequency was significantly different between the cases and controls (p=0.038) (Table-II).

IL-6 levels were significantly high (p<0.01) while hS-CRP levels were significantly low (p<0.05) in subjects with IL-6R (rs8192284) CC genotype as compared to the AC and AA genotypes. IL-18 levels were not significantly different amongst the three groups (p=0.15) (Table-III). On performing multivariate logistic regression analysis, serum IL-18, IL-6 and hS-CRP levels remained significant in the prediction of premature atherosclerosis with a high odds ratio (Table-IV).

Parameters	PCAD patients n=281 Mean ± SD	Controls n=239 Mean ± SD
Age (y)	43 ± 3.80	39 ± 7.8
Sex (m/f)	270/11	227/12
Height (m)	1.69 ± 0.07	1.68 ± 0.07
Weight (kg)	73.9 ± 4.0*	70.6 ± 13.6
BMI (kg/m2)	25.9 ± 4.36*	23.7 ± 5.15
Systolic BP(mm of Hg)	124.7 ± 12.3**	114.4 ± 6.0
Diastolic BP(mm of Hg)	81.4 ± 8.08**	70.6 ± 4.0
Smokers n (%)	145 (52%) **	62 (26%)
HTN self n (%)	94 (33%) **	26 (11%)
Family history PCAD n (%)	77 (27%) **	22(9%)
Family history HTN n (%)	79 (28%) **	28 (12%)
Family history DM n (%)	48 (17%)*	19 (8%)

Table-I.
*PCAD: Premature Coronary Artery Disease; BMI: Body Mass Index; CAD: Coronary Artery Disease; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein. DM: Diabetes Mellitus; SD=Standard Deviation.**p<0.01. Categorical variables were compared using a χ^2 test while continuous variables were compared using Welch's t-tests.*

Genotypes	Cases n (%)	Controls n (%)	Risk Allele	p-value
CC **AC** **AA**	36 (13) 102 (36) 143 (56)	20 (8) 80 (33) 139 (59)	C 0.038*	
RAF	0.310	0.251		

Table-II. Genotypic distribution of IL-6R (rs8192284) in PCAD cases (n=281) and controls (n=239).
*RAF= Risk Allele Frequency; n=Individual gene count. *p<0.05*

Parameter	AA (n=143) Median (IQR)	AC (n=87) Median (IQR)	CC (n=25) Median (IQR)	p-value
IL-6 (ng/dl)	2.31 (0.9-2.42)	2.9 (1.9-3.2)	3.8 (2.6-4.1)**	0.001
IL-18 (pg/ml)	230 (80-240)	260 (182-300)	290 (258-300)	0.09
hS-CRP(mg/l)	6.0 (5.0-12.0)**	4.0 (3.1-7.0)	2.9 (1.9-3.1)	0.001

Table-III. Comparison of cytokine levels and cytokine mediator levels according to strata of the genotypes ofIL6Rvariant rs8192284 in the PCAD cases (n=281)
*IL-6=Interleukin-6; IL-18=Interleukin-18; hS-CRP=High sensitivity C-Reactive Protein; IQR=Interquartile range.**p<0.01*

Variables	B	S.E	Exp(B) (95%CI)	Sig.
Premature CAD family history	1.977	1.034	0.147(0.019-1.07)	0.057
BMI (kg/m^2)	0.109*	0.73	1.29 (0.131-2.58)	0.041
Smoking (number of cigarettes smoked per day)	1.837	0.628	4.470(1.819-23.0)**	0.004
IL-6 (ng/dl))	0.606	0.182	1.832(1.282-2.618)**	0.001
hS-CRP(mg/dl)	0.495	0.135	1.641(1.261-2.137)**	0.000
IL-18 (pg/ml)	0.012	0.005	1.011(1.005-1.019)**	0.007
IL-6R (rs8192284) CC vs AC+ AA	0.023	0.014	0.997(0.992-1.002)	0.170

Table-IV. Multivariate analyses showing odds ratio for the prediction of premature atherosclerosis.
*CAD=Coronary Artery Disease; BMI=Body Mass Index; IL-18=Interleukin-18; hS- CRP=High sensitivity C-Reactive Protein; S.E.=Standard Error; Exp(B)=Odds ratio; CI=Confidence Interval; Sig=significance. **p<0.01. B=beta coefficient, BMI=Body Mass Index; SE=Standard error; CI=Confidence Interval *p<0.05*

Parameters Degree of stenosis p-value
IL-18 (pg/ml) 0.578** 0.0001
IL-6 (ng/dl) 0.351** 0.0001
hS-CRP(mg/dl) 0.633** 0.0001
IL-6R rs8192284 C-allele 0.121 0.11

Table-V. Spearman's correlation between cytokine levels and degree of coronary stenosis in PCAD patients.
*IL-18: Interleukin-18; IL-6: Interleukin-6; hS-CRP: High-sensitivity C-reactive protein. r=Spearman's correlation rank coefficient **p<0.01*

This difference remained significant (p<0.05) even after adjustment for confounding variables (age, gender, hypertension, diabetes, statins and anti-hypertensive medications). Serum pro-inflammatory cytokine levels and hS-CRP correlate with the degree of stenosis while the IL-6R (rs8192284) did not show any significant association with the degree of atherosclerotic blockade (Table-V).

DISCUSSION

We observed in our study that the risk allele frequency for IL-6R rs8192284 was significantly different between the cases and controls. This is probably due to the reason that IL-6R mediates its action through the pro-inflammatory cytokine IL-6 so the primary effect is on the IL-6 levels which then contributes to the immune-inflammatory process in the pathogenesis of early atherosclerosis.[8] While some studies have shown the association of the IL-6R rs8192284 with CAD[9] other have denied any such association and are in agreement with our study.[10] Previous studies have shown that IL-6R rs8192284 is a functional polymorphism which alters the IL-6 levels and IL-6 receptor signalling.[11] The IL-6 levels have shown a significant fall with increasing copies of the A-allele in metabolic syndrome.[12]

IL-6 levels were significantly high while the hS-CRP levels were significantly low in subjects carrying the IL-6R rs8192284 risk allele-C. This was a very interesting observation as studies have shown previously that IL-6 may stimulate the production hS-CRP in the liver.[13] The probable reason for this is that IL-6 and hS-CRP may be working in collaboration under the effect of the IL-6R rs8192284 polymorphism rather than having a sequential effect on one another in PCAD patients. Similar observation has been seen in another study.[14] Certain studies also suggest that hS-CRP levels are associated with IL-6R rs8192284 independent of the IL-6 levels.[15] Although the IL-6R rs8192284 risk allele frequency was not significantly different between the cases and controls in our study we observed

that the odds ratio of IL-6R (rs8192284) C-allele for the risk prediction of PCAD showed a rising trend with increasing quintiles IL-6 level (p for trend = 0.15) and a became significant with increasing quintiles of hS-CRP level (p for trend = 0.02). This again emphasizes the observation that IL-6R rs8192284 plays a role in premature atherosclerosis by altering the serum levels of pro-inflammatory cytokine IL-6 and cytokine mediator hS-CRP primarily.IL-18 levels were however not significantly different between the different genotypes of IL-6R rs8192284 probably because they act independently of the IL-6R and IL-6 signaling pathway.

In order to further assess the role of cytokines in the prediction of atherosclerosis multivariate logistic regression analysis was performed keeping in view the classical risk factors. This revealed that IL-18, IL-6 and hS-CRP still remained significant in the prediction of premature atherosclerosis with a high odds ratio. HS-CRP particularly has been shown to have a pivotal role in identifying the subjects at the risk of CAD.[16] IL-18 and IL-6 are pro-inflammatory cytokines which play a vital role in the pathogenesis of premature atherosclerosis leading to PCAD. They not only provide the link between the immune-inflammatory network operating in PCAD but also affect the entire course of the disease including morbidity and mortality.[17] The pro-inflammatory cytokines and the cytokine mediator levels also correlated with the degree of stenosis in PCAD patients unlike the IL-6R rs8192284 C-risk allele which did not show any correlation. This is in agreement with a previous study.[18,19] So it is basically the modulation in the pro-inflammatory cytokine and cytokine mediator level which leads to premature atherosclerosis under the effect of the IL-6R polymorphism rs8192284.

The major strengths of our study are that it is the first of its kind to be carried out on premature coronary artery disease patients of Pakistan studying the relationship between IL-6R rs8192284 and the IL-6, IL-18 and hS-CRP levels in CAD. The controls are angiographically proven to be disease free minimizing the chance of including false negatives in the study as controls. The limitation might be the small sample size which should be increased in future studies. Future studies should also be targeted towards studying how the anti-inflammatory cytokine levels get altered with the IL-6R rs8192284 polymorphism.

CONCLUSION

Our study is the first to show that the presence of the IL-6R 358Ala allele is associated with significantly higher serum pro-inflammatory cytokine (IL-6) level and a significantly lower cytokine mediator, HS-CRP, levels in PCAD patients but it is not significantly associated with the degree of atherosclerotic blockade.

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AUTHORSHIP AND CONTRIBUTION DECLARATION

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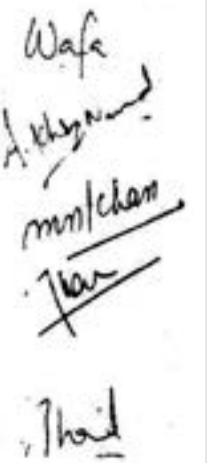
1	Dr. Wafa Munir Ansari	Concept, design of study, sample collection, data entry, data collection, data analysis, manuscript drafting, revision	
2	Prof. Dr. Abdul Khaliq Naveed	Data analysis, critical review, drafting and revision of manuscript	
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4	Dr. Omer Jamshed Khan	Sample collection, Data entry, Data analysys, Maniscript revision	
5	Prof. Dr. Dilshad Ahmed Khan	Concept, Design of study, Sample collection, data entry, Data collection, Data analysis, Manuscript drafting revision	