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Analysis of SNP rs1800796 Association with Risk of Rheumatoid Arthritis in Pakistani Population: A Case Control Study

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ABSTRACT

Genetic polymorphisms in the interleukin 6 gene (*IL-6*) and its promoter regions have extensively been studied because of their potential role in the pathogenesis of rheumatoid arthritis (RA). Due to the confounding results, reported in several studies, we performed the systematic literature search on IL-6 -572 G/C polymorphism (rs1800796 SNP) and assessed its association with RA in the overall world population, and in the subgroup analysis of Asian and Caucasian populations. The results showed that in overall population, IL-6 -572 G/C shows a significant association towards increased risk of RA in allelic (OR=0.8130, 95%CI=0.708-0.932, p=0.003), co-dominant and dominant models; same hold true for the Asian population. Stratification by ethnicity revealed that in Asian population also, the association with RA manifestation is significant. However, in a cohort of 200 RA and 176 healthy subjects (n=376) from Pakistan, significant association between IL-6 -572 G/C polymorphism and progressive RA could not be established despite the fact that GG genotype exhibited higher susceptibility (p=0.088) towards RA in comparison with the CC counterpart (p=0.803). Further, using STRING software, we tried to elucidate the interconnection between different ILs and their combinatorial effect on the RA development and/or progression. In conclusion, our study suggests that the analyses of single- and multiple nucleotide polymorphisms in larger cohorts, originating from diverse ethnic groups, are imperative to ascertain the genetic contributors of RA in a specific population.

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Authors' Contribution
UP, KH and RA collected the samples and recorded the data. HN performed experiments and wrote the manuscript. RAK carried out statistical analysis while HK performed the data analysis. SS supervised the research and reviewed the manuscript.

Key words
Genetic polymorphism, Pathogenesis, Rheumatoid arthritis, Single nucleotide polymorphism, Stratification

INTRODUCTION

Rheumatoid arthritis (RA), a chronic autoimmune disorder, is affecting 0.5-1.0 % of the world population (Sokka, 2007). It is generally believed that an imbalance between the pro- and anti-inflammatory cytokines causes joints' inflammation, deformities, destruction, and the disability in the affected RA patients (Firestein, 2003); interaction of several genetic, epigenetic and environmental factors also contribute to this effect (Choi *et al*., 2006). Genetic factors that are involved in the disease pathogenesis include loci for genes of human leukocyte antigen (HLA) and non-HLA genes like TNF- α, IL-1 β, CTLA4, IL-23 receptor, PADI4 and STAT4 (Kurko, 2013). Amongst the non-HLA genes, interleukins (ILs, a subset of large cytokine family), appear to be essential in activating the various immune responses, such as inflammation. ILs, after their production, generally proceeds to the target cells with the help of IL-specific receptors and activate numerous signaling cascades including those related to the inflammatory responses (Assier *et al*., 2010).

Genetic predisposition of several pro-inflammatory cytokines has been reported to be associated with increased risk or pathogenesis of RA in different population groups. More importantly, single nucleotide polymorphisms (SNPs) in genes of IL-6 [a key mediator of inflammation in tissues and/or synovial fluids of RA patients] have been reported in British, Turkish, Polish, Spanish, Chinese and Iranian subjects in the individual retrospective studies. Some of the reported SNPs displayed protective role whereas others were found associated with increased risk of RA in different ethnic groups.

Since genetic predisposition is population dependent, the screening of population for identification of SNPs in RA-associated immune-modulatory cytokines and/or IL genes may serve as useful "genetic marks" for the early diagnosis of RA. However, substantial inconsistency in the SNPs data found due to different ethnic backgrounds,

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varied number of sample size and the uncorrected physiological status (Dar *et al*., 2016; Zhai *et al*., 2012; Dai *et al*., 2014; HuaLee *et al*., 2015; Cai *et al*., 2014; Chen *et al*., 2012; Lee *et al*., 2012) has prompted us to perform systematic literature search on key activators/ mediators of inflammation in RA, such as IL-6. In the present study, we therefore have assessed the association of polymorphism in the promoter region of IL-6 (-572 G/C) with RA, firstly in overall population and later in Asian and Caucasian ethnicities; independent case control studies, recently added in the databases, were included in the analysis. The probable association of IL-6 -572G/C polymorphism in Pakistani RA patients versus healthy subjects (n=376) was thereafter evaluated using real-time PCR and restriction fragment length polymorphism (PCR-RFLP) analysis. Finally, using "STRING" online software, we have provided an analysis for the interconnection between different genetic polymorphisms of ILs and their combinatorial effect on the RA development and/or progression. This, to our knowledge, is the first study from Pakistan screening RA patients for genetic predisposition of IL-6 -572G/C polymorphism.

MATERIALS AND METHODS

Literature search-identification of eligible studies and data extraction

We performed a web-based search strategy using the PubMed, Springer-link and Google Scholar as databases, with keywords/terms: "genetic variation", "polymorphisms", "IL-6", "rheumatoid arthritis" and "arthritis", for the identification of the studies examining association between IL-6 polymorphism and RA. The studies containing the overlapping data and/or data of family-based linkage analysis were excluded while the one, meeting the following two-fold criteria were included: 1) original research articles (excluding reviews) reporting the gene polymorphism of IL-6 -572G/C (rs1800796), 2) Case control studies having sample size ≥100 each and odds ratio (OR) with confidence interval (CI) 95%, when used in research validations.

The information extracted from the research articles were: authors' name, year of publication, country of origin, ethnicity of the patients enrolled, number of reported cases vs controls, and the number of genotypes and allelic frequency.

Statistical analysis

All statistical analyses were performed using STATA software version 14.1 (USA). Two-sided p-values <0.05 were considered as statistically significant. The strength of the association between IL-6 572 G/C rs1800796 and RA risk was assessed by the ORs along with their corresponding 95% CI. Four genetic models (Allelic, Dominant, Recessive and Additive) were used while calculating the ORs. Subgroup analyses were also performed by ethnicity as either Asian or Caucasian. Additionally, variations between studies were determined using Chi-squared based Q statistic test (Wu and Li, 1999). The effect of heterogeneity was quantified using I^2 statistic test and was categorized as: high ($I^2 > 50\%$), middle ($25\% < I^2 < 50\%$) and low ($I^2 < 25\%$) (Higgins and Thompson, 2002). Random effect model was used when a p-value <0.10 and I> 50% were the outcome, indicating significant heterogeneity. Fixed effect model was used in the cases where no heterogeneity existed. Genotype distribution of each polymorphism in controls was checked for Hardy Weinberg equilibrium (HWE) by the chi-square test. Publication bias was investigated using both Funnel plot and Egger linear regression test. A p-value <0.05 indicated a significant publication biasness.

Subjects of case-control study

Two hundred patients (35 males and 165 females; mean age ±44.1 years) from Punjab, Pakistan diagnosed with RA, in accordance with the 2008 Classification Criteria of the American College of Rheumatology, along with 176 healthy subjects (55 males and 121 females; mean age±45.1) of the same geographic and ethnic background, were enrolled in this study. The patients were recruited from the Department of Rheumatology, Sheikh Zayed Hospital, Lahore, Pakistan and the blood samples were collected with the informed consent while maintaining the privacy and confidentiality of the study subjects. The study design was duly approved by the Ethical Review Board of the University of the Punjab, Lahore (Bioethics-138/17).

Genotyping

Genomic DNA from the collected blood samples was isolated using Gene Jet Genomic DNA Purification Kit (ThermoFisher Scientific) and its quantification was done on Nano Drop 2000 spectrophotometer. A pair of forward and reverse primers viz F-IL-6 5′-GGAGACGCCTTGAAGTAACTGC-3′,R-IL-6 5′-GAGTTTCCTCTGACTCCATCGCAG-3′ was used to amplify a 163 bp promoter region of IL-6, by performing real time-PCR on Bio-Rad CFX-96 system (USA) (Shahid *et al*., 2019). The amplicons were digested with *Bsr*BI restriction enzyme (recognition sequence: CCG↓CTC) in accordance with the manufacturer's instructions followed by analysis on 2 % agarose gel to identify the -572 G/C polymorphism in IL-6 promoter. Genotype and allele frequencies of RA patients, for IL-6 -572 G/C SNP, were compared with the control group using two-sided

Pearson's chi-square test on SPSS software for windows, version 16.0. (SPSS Inc. Chicago, IL). A p-value ≤ 0.05 and OR with 95% CI were considered as significant. In order to verify the HWE of genotypes, Chi-square test was performed.

RESULTS

rs1800796 polymorphism and RA susceptibility in reported literature

The literature search, based on title and abstract details, identified a total of 62 articles. After critical analysis, 6 studies (published between 2006-2017) involving 1185 RA patients and 1119 controls, met our inclusion criteria whereas 56 studies were excluded being reviews, meta-analysis or due to the SNPs other than rs1800796 (Fig. 1). The ethnicity-based analysis was thereafter performed for Caucasians and Asian populations; the list of studies along with details of study subjects is summarized as Table I. The genotype frequencies of both cases and controls were extracted from each study and are listed in Table II. The genotype distribution of controls met the HWE. The results of meta-analysis revealed association between RA and rs1800796 polymorphism (IL-6 -572 G/C) in allelic (OR=0.8130, 95CI=0.708-0.932, p=0.003), co-dominant (OR=0.797, 95%CI=0.667-0.952, p=0.013) and dominant model (OR=0.784, 95%CI=0.617-0.997, p=0.048) in all study subjects. Furthermore, in subgroup analysis significant association was found in Asian population under the allelic and co-dominant model (Table II).

Heterogeneity and publication bias

A significant degree of heterogeneity (p<0.1) was noticed in most of the comparison models (Table II) and in order to identify the heterogeneity source, we performed meta-regression by ethnicity, year of publication and sample size. Publication bias could be a problem in this analysis because of the false number of positive studies. Generally, funnel plots are used to detect the publication bias but due to small number of studies included in our study, it was difficult to correlate. Egger's regression test, however, showed no evidence of publication bias in any of the genetic models (P>0.05).

Genotyping analysis

Prior to genotyping, the laboratory parameters for each patient such as rheumatoid arthritis factor (RA Factor), erythrocyte sedimentation rate (ESR), nodules formation (NF) and Disease Activity Score 28 (DAS28) were calculated and the results are summarized as Table III. 82 % of the enrolled patients were females with mean age 44 years, reflecting at least 4-times high burden of disease in elderly women than men.

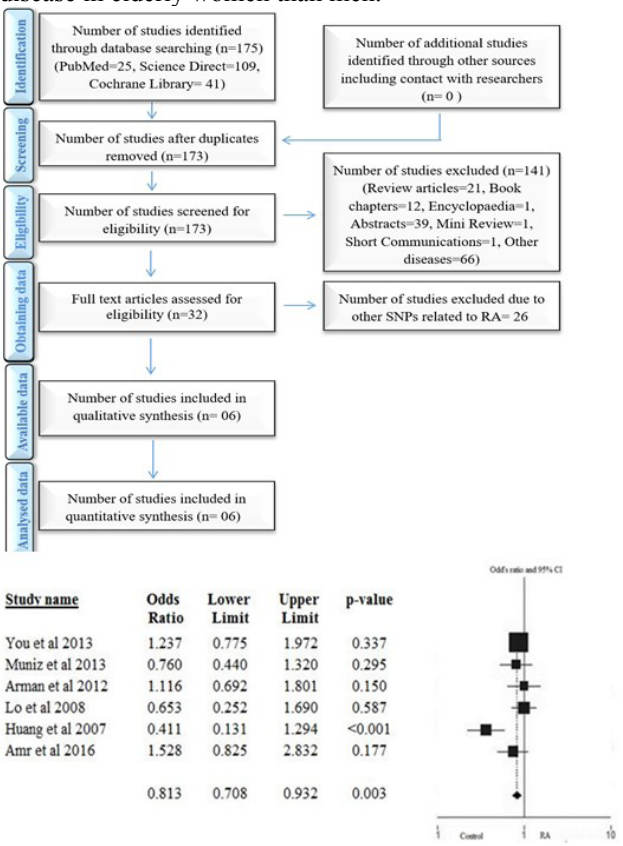

Fig. 1. Schematic representation of the steps followed to search, screen and include the previous research in this study. Lower panel shows the odd's ratio and p-values of the studies included in analysis.

Following amplification and digestion of 163 bp fragment, we were able to see the two bands of 102- and 61 bps in case of dominant (GG) genotype; 163-, 102- and 61 bp fragments for heterozygous (GC); and a single 163 bp fragment for the recessive (CC) genotype (Fig. 2 right panel). The IL-6 -572 G/C SNP genotype distribution followed the HWE in the control group. Frequency of G-allele distribution in the patient and the control groups was 75 and 70 % whereas that of C- allele was 25 and 30 %, respectively (P=0.803, OR=0.880, 95% CI= 0.323-2.396). Significant association was not observed in the genotype frequencies when the cases and the control groups were compared, as shown in Table IV. The CC genotype, however, has relatively less OR (1.136 with lower bound of CI at 0.417) compared to the other homozygous GG group (1.423 with lower bound of CI at 0.948), suggesting that the subjects with CC genotype might have lesser susceptibility towards RA. As expected, the association was significant (P=0.038, OR=1.050, 95%

CI=1.019-1.081), with reference to the gender.

Table I. Studies included in the meta-analysis.

Authors	Country	Ethnicity	Genotyping method	Subjects enrolled		Genotype of cases*			Genotype of controls*			Association (P-value)
				Cases	Controls	GG	GC	CC	GG	GC	CC	
You *et al*., 2013	China	Asian	PCR-HRM	452	373	38	191	222	39	166	168	0.337
Muniz *et al*., 2013	Mexico	Caucasian	PCR-RFLP	137	102	74	58	05	62	37	03	0.295
Arman *et al*., 2012	Turkey	Caucasian	PCR-RFLP	178	247	143	31	04	194	52	01	0.150
Huang *et al*., 2007	China	Asian	PCR-SSP	120	168	04	15	101	13	50	105	< 0.001
Lo *et al*., 2008	Taiwan	Asian	PCR-RFLP	199	130	09	69	121	06	45	79	0.587
Amr *et al*., 2016	Egypt	Caucasian	PCR-RFLP	99	99	26	64	09	36	58	05	0.177
This study	Pakistan	Asian	PCR-RFLP	200	176	108	84	08	80	89	08	0.803

* Number of cases and control in each study depicting the homozygous (GG, CC) and heterozygous (GC)

Table II. Meta-analysis of the association between IL-6 rs1800796 polymorphism and RA.

Genetic model (IL-6 -572 G/C)	Comparison	Group & Subgroup	Test of association			Test of heterogeneity			Publication bias
			OR	95 % CI	P value	Model	I^2	P value	P value
Allele	6	Total	0.813	0.708-0.932	0.003	R	61.119	0.025	0.870
	3	Asian	0.802	0.680-0.946	0.009	R	83.108	0.003	0.786
	3	Caucasian	0.836	0.655-1.069	0.154	F	0.000	0.608	0.139
Codominant	6	Total	0.797	0.667-0.952	0.013	R	72.826	0.002	0.770
	3	Asian	0.689	0.554-0.856	0.001	R	81.818	0.000	0.895
	3	Caucasian	1.072	0.786-1.461	0.660	F	19.350	0.290	0.622
Dominant	6	Total	0.784	0.617-0.997	0.048	F	0.000	0.535	0.418
	3	Asian	0.697	0.471-1.032	0.072	F	0.000	0.581	0.912
	3	Caucasian	0.842	0.621-1.142	0.270	F	20.940	0.282	0.387
Recessive	6	Total	1.043	0.847-1.285	0.689	R	90.160	0.000	0.503
	3	Asian	0.993	0.799-1.233	0.951	R	95.810	0.000	0.213
	3	Caucasian	2.001	0.885-4.520	0.095	F	0.000	0.530	0.561

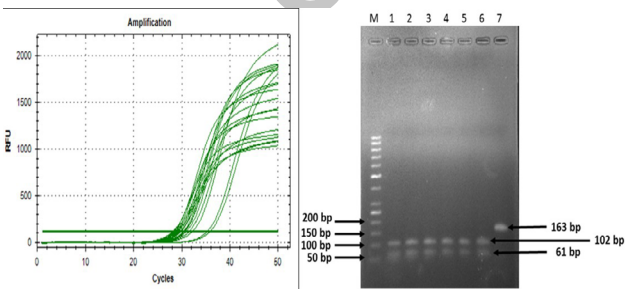

Fig. 2. Amplification of 163 bp fragment of IL-6 promoter using real-time PCR system (left panel) and its restriction digestion to identify the rs1800976 SNP genotype in Pakistani subjects (right panel). A 2% agarose gel stained with ethidium bromide after restriction digestion of amplicon is shown. Lanes M, 50 bp DNA size marker; Lanes 1-7, PCR-RFLP of RA samples showing GG (lanes 1-6) and CC (lane 7) genotypes, respectively.

DISCUSSION

IL-6 (MW: approx. 26 kDa), a pleiotropic cytokine, is involved in the regulation of T- and B-cells' proliferation, differentiation and/or maturation and is responsible for activating the host immune system. Owing to the involvement of IL-6 in diverse repertoire of biological functions, we predicted the functional protein networks of IL-6 by the STRING database, which revealed its interconnectivity with other inflammatory cytokines viz. IL-17A, 1L-17F, IL-18 and IL-22 etc., via NF-κB1 and STAT1, STAT3 proteins (Fig. 3, upper panel). Earlier studies have documented that IL-6 differentiates the T-lymphocytes into T_H17 cells, which in turn produce IL-17 (Firestein, 2003). Since T_H17 cells are considered to be the key players in inducing damage to the tissues during the course of inflammatory and other autoimmune

disorders (such as RA), the role of IL-6 in the development and/or progression of RA seems vital (Fig. 3, lower panel). This further suggests that the genetic and/or epigenetic alterations affecting the *IL-6* expression may contribute in dysregulating the functions of STAT1, STAT3 and NFKB proteins, which directly/indirectly are involved in stimulating the expression of a wide variety of genes linked with inflammatory responses.

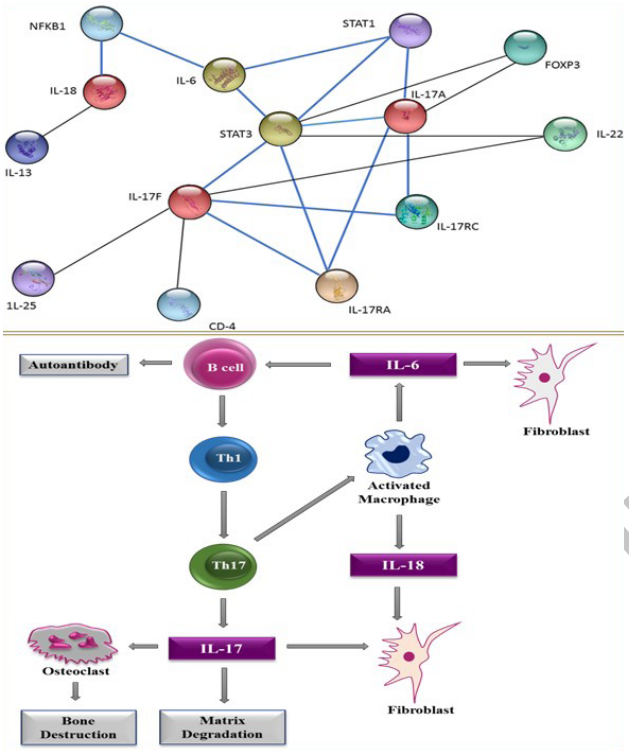

Fig. 3. Interactive partners of IL-6 predicted using the STRING software (top panel) and their involvement in bone destruction in RA patients (lower panel).

Amongst the several polymorphisms of *IL-6* gene, the two most commonly reported (i.e., -174 and -572 G/C) lie in the promoter region (Muniz *et al*., 2013; Arman *et al*., 2012). This region gives us an opportunity to predict the RA related loci. Preliminary review of the previous research studies revealed contradictory data relating to the role of rs1800796 SNP in RA development and/or progression. Huang *et al*., 2007, for instance, reported significant association of IL-6 -572 G/C polymorphism with RA whereas others (Muniz *et al*., 2013; Arman *et al*., 2012; Li *et al*., 2014; Lu *et al*., 2009; Srirangan and Choy, 2010; Fishman *et al*., 1998; Terry *et al*., 2000) described insignificant (Table I). We, therefore, performed critical assessment of the impact of proposed association in these case-control studies. Interestingly, the association between RA and IL-6 -572G/C polymorphism was found in all study subjects cumulatively, in the allelic, co-dominant and dominant models. In the subgroup analysis, the allelic and co-dominant models displayed significant association in the Asian ethnic group but not in the Caucasians. Overall, the results identified the IL-6 -572 G/C polymorphism as the hotspot region to be involved in the disease activity.

Table III. Demographic/clinical information of the Pakistani RA patients enrolled in this study.

Parameters	RA subjects (n=200)
Gender	
Male	35 (18 %)
Female	165 (82 %)
Mean age (years)	44.15±10.91
Elderly (<50 years)	
Male	16
Female	114
Elderly (≥50 years)	
Male	19
Female	51
RA factor	
Positive (%)	154 (77 %)
Negative (%)	46 (33 %)
Mean disease duration (years)	07.43±6.18
Mean ESR (mm/h)	39.73±22.01
Mean DAS28	05.94±1.003

Table IV. Comparison of genotype and allelic frequencies of -572G/C polymorphism in Pakistani population.

SNP rs1800796		RA Patient n=200 (%)	Control n=176 (%)	OR	95% CI	P-value
Geno-type	GG	108 (54)	80 (45)	1.423	0.948-2.136	0.088
	GC	84 (42)	89 (50)	1.397	0.930-2.098	0.107
	CC	08 (4)	08 (05)	1.136	0.417-3.093	0.803
Allele	G	300 (75)	249 (70)	0.880	0.323-2.396	0.803
	C	100 (25)	105 (30)			

When analyzed in the Pakistani RA patients and the controls (n=376), enrolled in this study, the differences in the genotype and allelic frequencies were found insignificant. Though our findings are in good agreement with those reported in case of Turkish and Mexican populations (Table I and references therein) but are in conflict with overall results of meta-data. Of note, varying

genotypes predominated in different population groups; genotype CC in the Chinese and Taiwan populations; GC in Egyptian; and GG in Turkish, Mexican and Pakistani populations. This heterogeneity advocates the importance of registering patients from different ethnic backgrounds along with their detailed clinical information in genome-wide association studies of RA.

The systematic literature search followed by case-control experimentation is of great significance as adding up new research data from Pakistan, for the first time. However, more research with significantly large number of patients originating from diverse ethnicities needs to be performed on *IL-6* SNPs. More so, one or two SNPs cannot qualify as casual for polygenic complex diseases or be considered fully predictive of RA, primarily because this complex disease involves a large number of genes and environmental factors as potential risk factors. Nonetheless, we have provided estimation of SNP effect sizes with 95 % CI that may be used in developing polygenic risk scores for RA.

CONCLUSION

While earlier studies demonstrated that IL-6 rs1800796 polymorphism is associated with RA susceptibility in different genetic models, our case-control study involving Pakistani subjects (Asian ethnicity) revealed non-significant association between IL-6 rs1800796 gene polymorphism and RA risk; the GG genotype group, however, was found more susceptible to RA than the counterpart CC group. Further studies on next generation sequencing data along with detailed functional characterization of this variant seems imperative.

Compliance with ethical standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was supported by a grant from Higher Education Commission, Government of Pakistan (Grant No. 8488/2017). Authors are thankful to all RA patients who volunteered to provide the blood samples for this study. Informed consent was obtained from all individual participants included in the study.

Conflict of interest statement

Authors of this manuscript declare no financial / competing conflicts of interest.

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