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Short Communication

Polymorphism in *CD40* Gene Associated with the Prevalence of Rheumatoid Arthritis in Pakistan

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NR has conducted the experiments and wrote the manuscript. MA supervised the research work. FZ help in data analysis. R analyzed the data and wrote the manuscript.

Key words
Rheumatoid arthritis, CD40, Polymorphism, SNP, PCR

ABSTRACT

The polymorphism in *CD40* gene have been associated with rheumatoid arthritis (RA). A single SNP rs1535045 was genotyped in 100 RA patients and 100 healthy controls. Some risk factors linked with RA like hypertension, diabetes and smoking were also assessed in the local population of Pakistan. Study subjects included both RA patients and age co-related healthy controls. A total of 100 RA patients with mean age of 51.26 years and individuals for control group were selected in accordance with RA patients' age. Databases of SNPs provide a powerful resource for association studies that try to establish a relationship between a phenotype and regions of the genome. Total genomic DNA was separated from the blood samples of studied individual. Allele specific PCR based technique was used to study the target SNP for genetic analysis. The SNP, rs1535045 was found to be strongly linked with RA ($p<0.01$). Genotype and allele counts were assessed by using Chi square analysis. The study suggested that the genotype AT increases the chances of RA by 6.334 times (OR: 6.334, 95% CI: 2.3141–17.333). The risk factors like smoking, diabetes and hypertension were also found to be significantly associated with RA.

Rheumatoid arthritis (RA) is an autoimmune inflammatory disorder, in which immune system is affected in such a way that finally leads the destruction of joints cartilage. Its prevalence in world was reported as 0.5- 1% (Naqvi *et al*., 2017). The disease is more dominantly affecting females in comparison to males reported by Khalil *et al*. (2017) in Rawalpindi city of Pakistan. In Pakistan, 0.142% of females living in Karachi city are the patients of RA (Naqvi *et al*., 2017). Affected males have lower concentration of testosterone as compared to normal males while the affected females show no difference (Silman and Hochberg, 2001). Various environmental and genetic factors are playing a role in pathophysiology of RA. Many studies have documented the relationship between RA and risk factors like smoking, obesity, diabetes, cardiovascular disorders, hypertension, some types of bacteria and viruses.

There are number of genes associated with RA like MHC class II genes, *PTPN22, CTLA4, STAT4, IRF5, FCGR3A, IL6ST, IL2RA, IL2RB, CCL21, CCR6, NCF-1 TRAF1-C5* and *CD40* among others according to GWAS (Genome wide association studies) (van der Woude *et al*., 2009, 2010).

Cluster of differentiation 40 (CD40) is one of the contributing agents for RA development. CD40 signaling is known to play pivotal function in the formation of chronic inflammatory and autoimmune diseases (Peters *et al*., 2009). CD40 is a member of tumor necrosis factor (TNF) receptor superfamily. It is a type I membrane glycoprotein of 45–50 KDa comprising on 277 amino acids (Huang *et al*., 2021). CD40 is located on chromosome 20q12- q13.2, spans 11 kb, and has nine exons and eight introns of between 29 and 412 bp in length (Huang *et al*., 2021). Immune and non- hematopoietic cells, such as B cells, macrophages, dendritic cells, fibroblasts, and endothelial cells represents that gene under certain pathogenic conditions (Elgueta *et al*., 2009; Karnell *et al*., 2019). When CD40 combine with its ligand CD154 on T cells, the intracellular kinases and transcriptional factors are activated. It leads towards inflammatory responses (Kawabe *et al*., 2011). CD40 gene react with TNF receptor associated factor (TRAF) proteins for its signaling pathway (Brown *et al*., 2001).

When CD40 interacts with CD154, ultimately causes the CD40 exposure in to the cholesterol-rich membrane micro domains and then TRAF binds with the short cytoplasmic tail of CD40 consisting only of 64 amino acids (Bishop *et al*., 2007). Because of these interactions, mitogen and stress-activated protein kinase (MAPK/SAPK) cascades, transcription factors are expressed and it also causes the secretion of cytokines and triggers

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the B cells differentiation and stimulation of humoral immune response (Bishop, 2004). It may also affect on endocrine tissues and thus contributes in the development of autoimmune diseases like Grave's disease, type-1 Diabetes, Systemic Lupus Erythramatosis (SLE) and RA among others (Jacobson *et al*., 2007). *CD40* gene is one of the risk factors for RA so any polymorphism in its locus increased the severity of RA (van der Linden *et al*., 2009; Bax *et al*., 2011; Scott *et al*., 2011). Its role has also been described in Graves's disease and multiple sclerosis as well (Tomer *et al*., 2002; Mukai *et al*., 2005).

The present study was designed to find out the role of *CD40* gene mutation in RA. The selected SNP was rs1535045 (A/C/T polymorphism). *CD40* gene has two blocks of linkage disequilibrium (LD) and the selected SNP (rs1535045) is in the region between the two blocks. The purpose of the present study was to find out the unexplored role of mutation in *CD40* gene with RA in Pakistani population. This study would be very helpful to find the genetic predisposition of RA.

Materials and methods

Blood samples were collected in EDTA coated vials (BD, USA) from 100 RA patients confirmed by RF test (Hinkle *et al*., 2014) as well as from 100 healthy individuals and stored at -20 °C for further use. Age, gender and other data related with risk factors of RA (like hypertension, smoking and diabetes) was also recorded.

Genomic DNA was extracted by using standard protocol of Vivantis DNA extraction kit (Cat# GF- BD-100). Primers used in the study were designed by invitrogen, USA (Forward: 5'-AGA AGC CTA CAC TTG ACT CAC-3', Reverse I: 5'-CTT TAC CTC TTT CCA GCT CCA -3', Reverse II: 5'-CTT TAC CTC TTT CCA GCT CCG-3', Reverse III: 5'-CTT TAC CTC TTT CCA GCT CCT-3'). All primers were mixed with nuclease free water for making final concentration of 100 pmol/µl. A portion of these primers were prepared and saved at -20°C. The PCR mix and primers were stored at -20°C and thawed on ice just before use. Gene was amplified by thermal cycler (Bioer technology), with annealing temp 61.9 °C.

Sample size was calculated by using online calculator provided by Creative Research Systems (http://www.surveysystem.com/sscale.htm). Hardy Weinberg equilibrium was analyzed using the chi-square test. Gene frequencies, allele frequencies and difference in genotype and allele frequencies between different groups were also examined. Chi-square test and other non-parametric tests were applied by SPSS® Software version 18 for windows (SPSS Inc., Chicago Illinois, USA 1989-2003) and MINITAB student version, release 12 for Windows (Minitab Inc.). Odds ratio were calculated using an online calculator.

Results

Supplementary Table I shows the baseline characteristics. It depicts that there is no considerable difference between the RA patients and healthy controls regarding age and gender ($p>0.05$). There is a significant difference between the two groups on the basis of their smoking habits, diabetes and hypertension ($p<0.01$).

Table I shows the genotype frequency in RA and control group, frequencies of A, C and T alleles in both groups and results of HWE. AA, TT, CT and AT genotype was higher in RA patients as compared to CC and AC genotype. The results indicated that T allele frequency was higher in RA group while A and C allele frequency was more in control group.

Table I. Genotype and allele frequencies and association of genetic polymorphism and RA.

Allele	Control group (N=100)	RA (N= 100)	Odds ratio	95% CI	Chi -square (p- value)
AA	3	8	2.8116	0.72 -10.924	33.92
CC	2	2	0.2063	0.0434 - 0.9803	(0.000)
TT	1	8	2.8116	0.7236-10.924	
AC	75	38	0.2043	0.1114-0.3747	
AT	5	25	6.334	2.3141 -17.333	
CT	14	19	1.4409	0.6778 -3.063	
A	0.43	0.395			
C	0.465	0.305			
T	0.105	0.3			

Table I also shows the results for association between rs1535045 polymorphism and RA ($p<0.01$). It was noticed that a strong association was present between the polymorphism and RA. It indicates that AC genotype acts as a strong protective factor while the genotype AA, TT, AT and CT acts as a weak protective factor. The AT genotype increased the chances of RA by 6.334 times (OR: 6.334, 95% CI: 2.3141–17.333). The CC and AC genotypes decreased the chances of RA by 0.2063 and 0.2043 times, respectively (OR: 0.2063, 95% CI: 0.0434–0.9803 and OR: 0.2043, 95% CI: 0.1114 – 0.3747, respectfully).

Table II shows the association of RA with risk factors like smoking, hypertension and diabetes. The data showed the strong association of RA with smoking ($p<0.01$), diabetes (<0.05) and hypertension (<0.05). Smokers have 2.8421 times the increased chances of RA as compared to non-smokers. Diabetes was found to increase the chances of RA by 2.5977 times and hypertension acts as a risk factor for RA and increases the chances of RA by 2.8674 times.

Discussion

The present study explored the association of CD40

gene polymorphism (rs1535045) with RA. This gene is involved in cell mediated as well as humoral immune response. Severity of RA symptoms increases in patients with AA, TT, AT and CT genotypes of rs1535045. The same SNP rs1535045 was associated with other autoimmune disorders like coronary artery disease (CAD) and increases blood lipid levels as reported by Zhou *et al*. (2016). García-Bermúdez *et al*. (2012) found that rs1535045 increases the chances of arthrosclerosis in RA patients. The results indicated that T allele frequency was higher in RA group as compared to normal. Similarly, the high frequency of T allele was observed in CAD patients by Zhou *et al*. (2016). The present SNP was not studied previously in Pakistani population in relation with RA.

Table II. Association of RA with smoking, diabetes and hypertension.

Status	RA	Control group	Odds ratio	95%CI	Chi-square (p-value)
Smokers	40	19	2.8421	1.4985-5.3903	10.602 (0.000)
Non smokers	60	81			
Diabetic	49	27	2.5977	1.4393-4.6883	10.272 (0.0013)
Non diabetic	51	73			
Hypertensive	37	17	2.8674	1.4804-5.554	10.147(0.001)
Normal	63	83			

More women are affected with RA as compared to males. The present study reveals that 57% females are affected with RA then males. Similar results were observed by Erum *et al*. (2017) where she observed 88.5% female RA patients in Karachi, while 76.4% and 74% females were reported in Lahore and Rawalpindi, respectively (Jalil *et al*., 2017) The mean age of RA subjects was 51.26±1.234 years in the present study. The results are in accordance with the findings of Khalil *et al*. (2017) and Masood *et al*. (2017), as they stated the mean age of 50±12.96 years and 51.75±9.25years in RA patients, respectively.

Smoking is one of the factors that have been proven to raise the possibilities ofRA (Malattia and Luca, 2006) as it increases the serum RF level. Smoking plays a major role in the onset of RA. Present study also showed strong association of smoking with RA. Our results match with the observation of Sugiyama *et al*. (2010), who also founds a significant association of smoking with RA. The present study was also in accordance with the findings of Kallberg *et al*. (2010).

Diabetes and RA, as both are autoimmune diseases and they are also interconnected with each other. As RA affects joint tissues and results in inflammation thus contributing in insulin resistance. Due to insulin resistance, RA patients have an increased risk of developing type 2 diabetes (Bolen *et al*., 2008). The present results are also in accordance with the observations of Bolen *et al*. (2008) and Lu *et al*. (2014) and found a significant role of RA in developing diabetes. About 8.21% of RA patients were reported to be suffering with RA by Alam *et al*. (2011). He also reported 13.74% hypertension in RA patients and also noted 6.63% ischemic heart disease in RA. The present SNP (rs1535045) was also found to bea cause of cardiovascular events in RA and diabetes mellitus (Burdon *et al*., 2006). The present results found a highly significant association of hypertension with RA. Panoulas*etal*. (2008) also reported the association of hypertension with RA. Due to RA pain, more adrenaline is released which cause an increased heart rate and finally raises the blood pressure. Panoulas *et al*. (2007) reported a highly significant relationship of hypertension with RA.

Conclusion

The frequencies of AA, TT, AT and CT genotypes vary among RA and normal individuals. The AT genotype of rs1535045 is more prevalent among RA patients according to our results. It can be concluded that rs1535045 polymorphism of *CD40* geneis associated with RA in local population of Pakistan. Smoking, diabetes and hypertension also increase the chances of RA in local population of Pakistan.

IRB approval

The study design followed the institutional guidelines for the animal care and experiment and was duly approved by concerned institutional Research Body vide reference No.UOS/Acad/477 dated: 20/04/2013.

Ethical statement

The study was approved by Ethical Committee of University of Sargodha.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20191230091215

Statement of conflict of interest

The authors have declared no conflict of interest.

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