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Original article

Association study of *CCR6* rs3093024 with Rheumatoid Arthritis in a Pakistani cohort

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ABSTRACT

C-C Chemokine receptor 6 (CCR6), an important protein in inflammatory and immunological responses, has been previously reported to be associated with rheumatoid arthritis (RA). Therefore, in order to replicate these findings, a case-control study was conducted on 500 subjects (including 250 RA patients and 250 healthy controls) of Pakistani origin. The aim of this study was to determine the association of *CCR6* rs3093024 variant with RA and identify its role in splicing events using bioinformatics tools. The clinical and demographic characteristics of the patients were collected using a well-designed questionnaire. The genotype frequencies of *CCR6* rs3093024 variant were determined using tetra-primer ARMS-PCR (amplification of refractory mutation system-polymerase chain reaction) method. A significant difference was found between *CCR6* rs3093024 genotype frequencies [$P = 0.0016$, $\chi^2 = 12.915$]. Similarly, a significant difference in the allele frequencies between RA patients and healthy controls was also observed [$P = 0.0003$ and OR (95% CI) = 0.63 (0.49–0.80)]. The stratification of patients showed that there was a significant increase in AA genotype against AG + GG in patients [$P = 0.0014$, OR (95% CI) = 2.0 (1.32–3.02)]. Furthermore, using bioinformatics analysis, it was found that *CCR6* rs3093024 variant might create a potential splicing enhancer motif (SF2/ASF (IgM-BRCA1) with score of 77.92; Threshold 70.53), which might have important impact on the product of this gene. This study suggests that the A variant of *CCR6* rs3093024 variant is significantly associated with RA-risk and its G variant is protective in Pakistani population but a multi-cohort large sized population study is needed to elucidate these results. Moreover, functional studies are needed to highlight the effects of this polymorphism on the function of *CCR6* gene.

1. Introduction

Rheumatoid arthritis (RA), a chronic autoimmune disease, targets synovial joints and causes the inflammation of joints. Therefore, it is categorized as a chronic inflammatory disease. In North American and European populations, its prevalence is about 0.5–1.0%, but this frequency is quite lower (0.2–0.3%) in Southeast Asia (including China and Japan) (Silman and Pearson, 2002). With respect to men, the women are more effected with female to male ratio of 3:1. Furthermore, the age of onset in women is around 40–50 years and is later for men (Alamanos et al., 2006).

Although, the pathogenesis of RA has not been fully known but there are many factors, which contribute to its pathogenesis including multiple genetic and various environmental factors. With the advancements in technology, different study protocols like genome wide association study (GWAS), GWAS *meta*-analysis and candidate gene approaches have explored a large number of genes/loci and even some specific variants underlying RA in different populations (Kochi et al., 2010; Stahl et al., 2010; Jalil et al., 2013; Kim et al., 2016; Karami et al., 2019; Mohammadi et al., 2019). The *CCR6* gene, located on chromosome 6 (6q27), encodes a 374-amino acid long C-C chemokine receptor type 6 protein. It is one of those genes having promising role in the pathogenesis of RA. It is expressed on the cell surface and helps

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in differentiation of Th17 (T helper 17) cells from other $CD4^+$ (cluster of differentiation 4) cells (Zaballos et al., 1996; Hirota et al., 2007). Furthermore, this gene is expressed in memory T-cells and immature dendritic cells and also have important role in B-cell maturation and differentiation. Therefore, during immunological responses, it helps in the regulation of T-cell and the recruitment and migration of dendritic cell (Nanki et al., 2009).

The SNPs located in *CCR6* have been reported to be associated with RA (Kochi et al., 2010; Jalil et al., 2013) while our previous study has also showed its importance in autoimmune diseases including RA (Akhtar et al., 2019). The CCR6 protein acts as surface marker for Th17 cells and has important role in transcription. Any change in the cysteine residues that are highly conserved, results in the CCR6 intracellular influx which leads to reduction in receptor signaling and activity, which in turn effects the receptor-legend interaction (Ai and Liao, 2002). From the previous studies, it is evident that the A allele of *CCR6* gene is associated with RA-risk (Kochi et al., 2010; Stahl et al., 2010; Chang et al., 2012; Freudenberg et al., 2011; Teng et al., 2012).

For genetic associations, the independent replication studies on known genes or variants are important for validation. The association of *CCR6* rs3093024 with RA has been reported in many populations including European and Asian population, but it has not been reported in Pakistani population yet. Therefore, a detailed case-control study on *CCR6* variant rs3093024 in a sample set of Pakistani origin was conducted. Furthermore, the possible impacts of *CCR6* variant rs3093024 on splicing were also examined in this study.

2. Methodology

2.1. Subjects

In this study, a total of 500 subjects were included containing 250 RA cases and 250 matching healthy controls. A well-designed questionnaire was used for the collection of clinical and demographic data of each subject. After proper diagnosis by certified rheumatologist at Rheumatology Department, Lady Reading Hospital (LRH) Peshawar, Pakistan and satisfying 2010 American college of rheumatology (ACR) criteria (Kay and Upchurch, 2012), the patients were included in the study. The individuals having no immunological disease or symptoms were included in matching control group (Table 1). An informed consent form was signed from each subject included in the study, and the study was approved by the Ethical committee of Department of Biotechnology, Abdul Wali Khan University Mardan, Pakistan.

2.2. DNA extraction and genotyping

A venous blood sample was taken from each RA patient and healthy control in a 10-ml EDTA (ethylene diamine tetra acetic acid) tube and genomic DNA was extracted through phenol–chloroform method (Sambrook et al., 1989). For genotyping of rs3093024, a tetra-primer ARMS-PCR method was used. All the four primers were designed manually by taking FASTA sequence from dbSNP database (https://www.ncbi.nlm.nih.gov/snp/rs3093024). The primer used were Forward outer (F_O) TGTATTGCTGCCAGCCTTCC, Reverse outer (R_O) CTATGTACAGCACACAAGCTAC, Forward A allele (F_A) TGTGAACTGGACGCTCGCA and Reverse G allele (R_G) GATCAGTGAAGAAATGCCACC. All the four primers, which were F_O, R_O, F_A and R_G, were used in a single PCR reaction mixture. For initial denaturation, the temperature was set to 95 °C for 3 mins, which was followed by 30 cycles of denaturation at temperature of 95 °C for 30 s, annealing at temperature of 57 °C for 35 s and extension at temperature of 72 °C for 1 min. The final extension was performed at 72 °C for 5 min. The intended product length was 447bps (F_O-R_O; Common in all), 302 bps (F_O-R_G; unique to heterozygous AG allele and homozygous G allele) and 184 bps (F_A-R_O; unique to heterozygous AG allele and homozygous A allele). The PCR amplifications were done on 96-well plate in thermocycler (T100, BioRad). The PCR products were then loaded and visualized on 1.5% agarose gel.

Table 1
Clinical and demographic characteristics of RA patients and healthy controls.

Variable	Cases (n = 250)	Controls (n = 250)	*P* value
Gender (male/female)	63/187	69/181	0.6121
Age in years Mean (±SD)	43.5 (±14.5)	42 (±12.6)	0.217
Disease duration in years Mean (±SD)	4.1 (±3.7)	–	–
Sero-positive antibody Mean (±SD)	100% (RF positive)	–	–
ESR Mean (±SD)	40.60 (±15.8)	–	–

2.3. In-silico analysis

The *CCR6* SNP rs3093024 is an intronic SNP, therefore it may play an important role in splicing and may be responsible for alternative splicing. For this purpose, different bioinformatics tools, including SpliceView (http://bioinfo.itb.cnr.it/oriel/splice-view.html), ASSP (http://wangcomputing.com/assp/), NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/), ESEfinder (http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process = home) (Smith et al., 2006) and Human Splicing Finder Ver3.1 (http://www.umd.be/HSF/) (HSF3.1) (Desmet et al., 2009) were used. Default conditions were used in all the tools. Being the most advanced tool (Abramowicz and Gos, 2019), HSF3.1 (Human Splicing Finder 3.1) compares both the mutated and wild sequence and gives prediction about potential splicing site, ESE (Exon Splicing Enhancers) and ESS (Exon Silencing Enhancers).

2.4. Statistical analysis

The genotypic data of both the RA patients and controls were statistically checked for HW (Hardy-Weinberg) equilibrium. The association of the variant was assessed through Chi-square (χ^2) and Fisher's exact tests by calculating Odd Ratio (OR) at 95% CI, following different statistical models (co-dominant, homozygous dominant, homozygous recessive and additive). If the frequency of an allele in the cases were low than that in controls, then it was considered as low-risk allele and vice-versa for the high-risk allele. Both the homozygote and heterozygote genotype frequencies in cases and controls were compared with low-risk allele in order to calculate the *P*-values for each genotype. Moreover, the *P*-value for all the three genotypes of the SNP was calculated by comparing these genotypes together. The *P*-value of ≤ 0.05 was used for the establishment of statistical significance.

3. Results

3.1. Clinical and demographic characteristics of RA patients and healthy controls

The demographic and clinical characteristics of RA patients and healthy controls are listed in Table 1. For RA patients, the mean age was 43.5 (±14.5) with 74.8% females. For controls, the mean age was 42 (±12.6) with 72.4% females. The mean age of disease for

RA patients was 4.1 (±3.7) years. The clinical characteristics showed that the RA patients were having higher RA Factor (RF) and ESR (Erythrocyte sedimentation rate) levels when compared to healthy controls.

3.2. Association of CCR6 rs3093024 with Rheumatoid Arthritis

The distribution of *CCR6* rs3093024 genotypes in RA cases and controls was determined using tetra-Primer ARMS-PCR method. After completing the genotyping of cases and controls, 10% of the samples were repeated in order to record the rate of genotyping error, which was 0%. Significant differences in the genotype frequencies of cases and controls were observed. The genotype frequencies for RA cases were 24% (60) for homozygous G, 31.6% (79) for homozygous A and 44.4% (1 1 1) for heterozygous AG while for healthy controls, the genotype frequencies were 34.4% (86) for homozygous G, 18.8% (47) for homozygous A and 46.8% (1 1 7) for heterozygous AG. The co-dominant genotyping model showed a significant distribution of genotypes between cases and controls (χ^2 = 12.915; *P*-value = 0.0016). Similarly, both the homozygous dominant and recessive models showed significantly positive trend in the association of rs3093024 with RA [GG vs AG + AA; *P* = 0.0138, OR (95%CI) = 0.6 (0.41–0.89) and AA vs GG + AG; *P* = 0.0014, OR (95% CI) = 2.0 (1.32–3.02)].

The allele frequencies between cases and controls also showed a significant difference with *P* = 0.0003 and OR = 0.63 (95% CI; 0.49–0.80). The *P*-values, chi-square test and OR (95% CI) values for the genotype and allele frequencies between RA cases and controls are listed in Table 2. This study demonstrates that the A allele of *CCR6* rs3093024 is significantly associated with RA-risk in Pakistani population.

3.3. In-silico analysis

For the prediction of potential role of SNP rs3093024 in splicing, several bioinformatics tools, including SpliceView, ASSP, NetGene2, ESEfinder and HSF3.1 were used. According to SpliceView, ASSP and NetGene2, rs3093024 does not affect splicing process. On the other hand, ESEfinder and HSF3.1 came up with about same results. HSF3.1 showed that this SNP could lead to break one potential splice site and also could create another potential splice site. About Exonic Splicing Enhancers, two new splicing enhancer sites were predicted to be created while three sites were broken. As for Exonic Splicing Silencers, four new ESS sites were predicted to be created and only one site was predicted to be broken. One hnRNP (hetero nuclear ribonucleoprotein) silencing site were also predicted to be broken. The ESEfinder results also came up with potential splicing alterations. It predicted the creation of three new sites for splicing proteins while also predicted the loss of two sites. Details of splicing proteins and results of both the tools are listed in Table 3.

4. Discussion

Rheumatoid Arthritis is an inflammatory disease in which joints damage and disability occur due to synovial joint destruction. The heritability of RA has been reported to be about 60% (Karami et al., 2019), whereas the remaining 40% could be due to environmental and other factors. GWAS is a popular method for the detection of genetic risk factors. The MHC (major histocompatibility complex) and non-MHC loci have been reported to account for about 23% of the genetic risk alleles for RA, which implies that there are still undiscovered risk alleles (Stahl et al., 2010). The association studies conducted so far only provide a glimpse of the genetic factors (Stahl et al., 2010; Klareskog et al., 2009; Kochi et al., 2010). Many studies have reported greater than 150 SNPs located at more than 70 gene loci (Stahl et al., 2010; Kochi et al., 2010; Kim et al., 2016; Mohammadi et al., 2019). In this study, the association of *CCR6* rs3093024 variant with RA was studied.

CCR6 rs3093024 is a deep intronic SNP located in intron 10 of 12 introns of *CCR6* gene transcript variant 1 with ensemble ID ENST00000609590.1 and intron 1 of 2 introns of *CCR6* gene transcript variant 2 with ensemble ID ENST00000400926.2. Its position in intron 10 is 74729bps from 5′ end of the intron having 90357bps length with splice distance of −15629bps from 3′ end (nearest splice site) while in variant 2 it is located at 7291bps from 3′ end of intron having 24023bps with the splice distance of 7291bps from the 3′ end (nearest splice site). Therefore, it is neither located in the coding region nor near any splice site in both variants but many studies have proved its association with many autoimmune diseases including Rheumatoid Arthritis (Kochi et al., 2010; Stahl et al., 2010; Chang et al., 2012; Freudenberg et al., 2011; Teng et al., 2012).

Besides RA, *CCR6* rs3093024 has also been reported to be associated with psoriasis (Matoshvili et al., 2015), autoimmune thyroid disease (Kunisato et al., 2018), systemic sclerosis (Ochoa et al., 2015), and lupus nephritis (Zhou et al., 2015). Many studies have shown the positive association of other variants of *CCR6* with RA but very few studies have been conducted so for on the association of *CCR6* rs3093024 with RA. *CCR6* rs3093024 was reported for the first time to be associated with RA by Kochi et al. in Japanese population (Kochi et al., 2010). Another study carried out on Korean population also showed a positive association of this SNP with RA (Freudenberg et al., 2011). Chang et al showed positive association of *CCR6* rs3093024 with RA in Taiwanese population (Chang et al., 2012) and Teng et al. showed its positive association with RA in Asian population (Teng et al., 2012). All these studies have shown that the A allele of the *CCR6* rs3093024 was significantly associated with RA. In the present study, the *CCR6* rs3093024 was genotyped using tetra-primer ARMS-PCR. Their genotypic and allelic frequencies were calculated using chi-square test. The heterozygous genotype AG frequencies were found to be higher in both cases and controls, which were 44.4% and 46.8% in cases and controls, respectively. Homozygous genotype GG frequency was lower in cases (24%) as compare to controls (34.4%) while the homozygous A frequency was higher in cases (31.6%) as compared to controls (18.8%). Similarly, the allele frequency of G-allele was lower in cases (46%) than controls (57.6%) while the A-allele was higher in cases (54%) as compare to controls (42.4%). This study suggests a significant association between *CCR6* rs3093024 with RA and the A-allele is a risk-factor for Pakistani population. *P*-values for all the tests were found to be significant. The genotype frequencies showed a significant association with *P*-value of 0.0016, while the allele frequencies also showed a significant association with *P*-value of 0.0003 (Table 2). These results are in close agreement with the previous studies, which have reported the association of *CCR6* rs3093024 with RA (Kochi et al., 2010; Chang et al., 2012; Freudenberg et al., 2011; Teng et al., 2012).

Bioinformatics analysis of this SNP showed that it may affect the splicing process in many ways, such as the creation of silencer or enhancer motifs and abrogation. The *in-silico* analysis of rs3093024 was done to find out its significance. This study suggested some interesting results. *CCR6* rs3093024 could create a new splicing enhancer motif SF2/ASF (IgM-BRCA1) with score of 77.92 (Table 3). Other potential enhancer and silencer motifs were also created but with less score (within + 10 score of threshold), which suggests that apart from SF2/ASF (IgM-BRCA1), rs3093024 have no effect on the splicing mechanism of *CCR6*. Literature study showed that the *CCR6* rs3093024 may have effect on regulation of *CCR6* gene but it is not known that which allele may up regulate or down regulate the gene (Deng et al., 2013). The deep intronic SNPs

Table 2
Genotype and Allele frequencies of RA cases and healthy controls.

Models	Genotype	Cases	Controls	OR (95% CI)	χ^2 -value	P-value
Co– Dominant Genotyping	G/G A/G A/A	60 (24%) 111 (44.4%) 79 (31.6%)	86 (34.4%) 117 (46.8%) 47 (18.8%)	–	12.915	0.0016
Dominant	G/G A/G + A/A	60 190	86 164	0.6 (0.41–0.89)	–	0.0138
Recessive	A/A G/G + A/G	79 171	47 203	2.0 (1.32–3.02)	–	0.0014
Additive	G A	230 (46%) 270 (54%)	288 (57.6%) 212 (42.4%)	0.63 (0.49–0.80)	–	0.0003

Table 3
HSF3.1 and ESEfinder results for rs3093024 to predict potential splice sites.

Method and Silencer/ Enhancer Protein	A allele (Value 0–100)	Motifs G Allele (Value 0–100)		Threshold	Result
Potential Splice sites	Gg**a**cgctcgcagtg (79.19)	ggacgctcgcggTG (50.25)			Site broken
−36.55 (Position −10bp)					
	Gc**a**gtggca (59.06)	GCGgtggca (69.64)			New site
+17.91 (Position −2bp)					
SF2/ASF (IgM-BRCA1)	cgctcgc**a** (76.31)	cgctcgcG (76.58) (Linked SR Protein SC35) (Position −7bp)		70.51	+0.36%
		ctcgcGg (77.92)			New site
	cgc**a**gtg (83.15)				Site broken at Position −3bp
SF2/ASF	cgc**a**gtg (77.23)			72.98	Site broken at position −3bp
		cGgtggc (71.77) (linked SR Protein SF2/ASF (IgM-BRCA1)			New site
EIEs (Zhang et al)	Gc**a**gtg				Site broken at position −2bp
	c**a**gtgg				Site broken at position −1bp
	agtggc				Site broken at position of SNP
ESE motifs from HSF		Linked ESE protein 9G8		59.245	New site at SNP position
Silencer motifs (Sironi et al)	Sironi	Motif 3 - TCTCCCAA	Motif 3 - TCTCCCAA	60	−17.69% at −6bp position
	Silencer	gctcgc**a**g (76.97)	gctcgcGg (63.35)		
	Sironi		Motif 1 - CTAGAGGT	60	New site
−10.61 at −5bp position					
	Silencer		ctcgcGgt (68.80)		
	Sironi		Motif 2 - [T/G]G[T/A]GGGG	60	New site
−20.95 At −3bp position					
	Silencer		cgcGgtg (60.84)		
	Sironi	Motif 2 - [T/G]G[T/A]GGGG		60	Site broken 1.02 at −2bp position
	Silencer	gcagtgg (60.23)			
	Sironi	Motif 2 - [T/G]G[T/A]GGGG	Motif 2 - [T/G]G[T/A]GGGG	60	+5.72% at SNP position
	Silencer	agtggca (65.59)	Ggtggca (69.35)		
Silencer IIEs motifs (Zhang et al)		Ggtggc		New site at SNP position	
Silencer hnRNP motifs	c**a**gtgg (66.67) hnRNP A1		65.47	Site Broken at −1bp position	
ESEfinder	SRSF1	Cgcagtg (2.69)		1.95	Site broken at position −3bps
	SRSF2 (IgMBRCA1)		ctcgcGg (2.81453)	1.867	New site at Position −5bps
			cGgtggc (2.02498)		New site at Position −1bp
		Cgcagtg (3.51253)			Site broken at position −3bp
	SRSF2		cgctcgcG (2.63255)	2.383	New site at position −7bps

doesn't have reported role in disease pathogenesis but since they are continuously being reported to be associated with risk factors of many diseases including autoimmune diseases, their pathogenicity mechanism should be explored.

This study has some limitations too. First, this study was performed on a single cohort. This SNP needs to be studied in many cohorts in order to elucidate its full association pattern. Sex-specific studies are also needed in order to study its association in males and females as females are more commonly affected with RA. Although this study showed significant association of this SNP with RA, more replication studies are needed to further strengthen these results. Furthermore, in vitro studies are needed to underlie

the exact mechanism of association of *CCR6* rs3093024 with autoimmune diseases by studying its effect on up regulation and down regulation of this gene.

5. Conclusions

In conclusion, it was the first association study of *CCR6* rs3093024 with RA, conducted on Pakistani cohort. A significant association of this SNP was found with RA. It was observed that the A allele of this SNP may be associated with RA-risk while its G-allele may be protective in Pakistani population. This SNP was also characterized in order to underline its functional effects using an *in-silico* approach. It was found that it may affect splicing mechanism of the *CCR6* mRNA, which may ultimately lead to regulation of this gene. A multi-cohort study involving large number of individuals is needed for the association study of *CCR6* with RA. Furthermore, the physiological relevancy of *CCR6* rs3093024 in pathogenesis of RA are needed to be studied in a functional study.

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