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CTLA-4 +49 Polymorphism and Susceptibility to Rheumatoid Arthritis in Pakistani Population

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Abstract.- Cytotoxic T lymphocyte Antigen 4 (CTLA-4), similar to co-stimulatory molecule CD28, plays an important role in down regulation of T cell activation. CLTA-4 binds to B7 with much greater affinity as compared to CD28, thus limiting the enhanced activation of T cells and preventing autoimmune diseases. CTLA-4 gene has an important variation rs231775 which interferes with the functioning of CTLA-4 causing uncontrolled activation of T cells. rs231775 may prone individuals to development of rheumatoid arthritis (RA), a severe autoimmune disease. This study aims to find the possible role of rs231775 polymorphism and the risk factors smoking, hypertension and diabetes in conferring susceptibility to RA in Pakistani population. Allele specific PCR based strategy was used to amplify rs231775 in samples of 100 RA patients and 100 age and sex matched healthy individuals. GG genotype of rs231775 polymorphism was found to increase the risk of RA development by 3.0186 times (OR 3.0186; 95% CI 1.6774-5.4322). Smoking increased the risk of RA development by 3.1672 times (OR or 3.1672; 95% CI 1.7766-5.6462). Diabetes was found to increase the risk of RA development by 2.172 times (OR 2.172; 95% CI 1.1897-3.9669). Hypertension also increased the risk of RA development by 2.280 times (OR 2.280; 95% CI 1.2676-4.1021). It can be concluded that smoking, diabetes, hypertension and rs231775 play an important role in development of RA in Pakistani population.

Key words: CTLA-4, rs231775, rheumatoid arthritis, Pakistani population.

INTRODUCTION

Rheumatoid arthritis (RA) is a severe autoimmune disease affecting 0.5-1% of the World population. RA being the common inflammatory disease affects multiple joints causing polyarthritis. This condition if not cured at earlier stages, results in major joint disability and impairment (Gibofsky, 2012; Scott *et al*., 2010; Wang *et al*., 2014).

Complex interaction between genetic and environmental factors decides the development of RA. Other risk factors for RA include high caffeine intake, red meat, alcohol, less use of antioxidant diet, high birth weight, infectious agents, occupation involving exposure to silica dust and low-grade socioeconomic status (Alamanos and Drosos, 2005; Arend and Firestein, 2012; Frisell *et al*., 2013; McAllister *et al.,* 2011). The conditions that influence hormones such as pregnancy, breastfeeding and the use of the oral contraceptive pills were reported to be the prime risk factors for RA in females (Liao *et al*., 2009a; Oliver and Silman, 2006).

Various genes were found to be associated with RA development. Human leukocyte antigen (HLA) is the most diverse region in the human genome. Contribution of HLA to RA heritability has been estimated to be 11-37 %. Among HLA alleles HLA-DRB1-01, DRB1-04, HLA-DRB1-13 and DRB1-15 have been found to be associated with RA. The most important non-HLA genes linked with RA include protein tyrosine phosphatase, nonreceptor type 22 (PTPN22), peptidyl arginine deiminase type IV (PADI4), signal transducer and activator of transcription 4 (STAT4), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), tumor necrosis factor-receptor associated factor 1/complement component 5 (TRAF1/C5), tumor necrosis factor (TNF), interleukin receptor 23 (IL23R), interferon regulatory factor 5 (IRF5) and cluster of differentiation 40 (CD40) (Ahmedullah *et al*., 2012; Colangelo and Zelenietz, 2011; Korczowska, 2014; Kurkó *et al*., 2013).

The CTLA-4 gene, consisting of four exons, is located on chromosome 2q33 (Dariavach *et al.*, 1988). Following activation a co-stimulatory molecule encoded by CTLA-4 gene covers CD4+

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and CD8+ T lymphocytes. Activating naive T cells involves an antigen and ligands B71 (CD80) and B72 (CD86) on antigen presenting cells. CTLA-4 is absent from resting T cells while activated T cells express CTLA-4 along with CD28. T cell activation is stimulated by CD28. CTLA-4 shows 500–2500x higher affinity of binding with B7 as compared to CD28. So, there is a vital role of CTLA-4 in down regulating T cells activation (Chan *et al.*, 2014).

CTLA-4 generates two major isoforms by alternative splicing, membrane bound (mCTLA-4) and soluble form (sCTLA-4). The sCTLA-4 form consists of three exons, lacking exon 3. Normally human serum possesses low sCTLA-4 levels (Pawlak *et al.*, 2005). High levels of sCTLA-4 enhance the susceptibility to autoimmune diseases as sCTLA-4 has ability to interfere the binding of CTLA-4 with B7. The result is malfunctioning of CTLA-4 and may affect the down regulation of T cells (Al Fadhli, 2013).

CTLA-4 maintains T cell tolerance. When expressed on regulatory T cells (Tregs), it obstructs the activation of inappropriate naive T cells while in conventional T cells; it stops detrimental buildup of abnormal T cells which can destroy vital organs. In this way, CTLA-4 prevents disease development. Tregs having reduced CTLA-4 expression are unable to avoid activation of pathogenic T cells and thus become reactive to self or environmental antigens (Jain *et al*., 2010).

A single nucleotide variation at exon 1 of CTLA-4 (rs231775) occurs due to substitution of adenine by guanine at position 49 (A49G). As a result amino acid threonine is replaced by alanine at position 17 (T17A). This results in reduced expression of CTLA-4 on T cell surface (Lee *et al.*, 2012). Case control association studies were designed to access the role of susceptibility genes in disease development. These studies involve identification of single nucleotide polymorphisms (SNPs) in genes related to possible pathogenesis. Allele frequencies of targeted SNPs were analyzed in patients and controls. The association of SNPs with disease development is prescribed if significant difference exists between allele frequencies of cases and controls (Nawaz *et al.*, 2014). Association of CTLA-4 +49 polymorphism and RA remains unclear due to controversial reports and needs further investigation. Little work has been done to determine the possible role of rs231775 polymorphism and RA in Pakistani population. Present study aims to explore the allele frequency and any contribution of rs231775 polymorphism in the pathogenesis of RA in Pakistani population.

MATERIALS AND METHODS

Population studied

All procedures were in agreement with the declaration of Helsinki. Protocol of present study was approved by The Advance Study and Research Board, University of Sargodha. Ethical Committee, University of Sargodha granted permission for the start of the research work. Study comprised of two groups. First group named as RA group consisted of RA patients and the second control group included age and sex matched healthy individuals. A form was designed to keep record of name, age, gender, smoking, hypertension, diabetes and RF factor status related to individuals.

Sample collection

Samples were collected from September 2013 to February 2014. One hundred RA patients (confirmed by RF test) and 100 healthy volunteers were selected. Sterilized syringes (BD, USA) were used to collect 5ml blood from each individual and blood samples were shifted to EDTA coated vials (BD, USA). Before further analysis, the samples were kept at -20°C for further study. Data regarding smoking status, diabetes and hypertension is shown in Table I.

Genetic analysis

Genomic DNA was isolated by using GF-1 blood DNA extraction kit (Vivantis Cat. No. GF-BD-100 USA). Genomic DNA was used for PCR amplification of rs231775 using one reverse (R) and two forward primers (F1 and F2). Sequences of primers were following:

Forward primer 1 (F1)
5`AGGCTCAGCTGAACCTGGCTA3`,
Forward primer 2 (F2)
5`AGGCTCAGCTGAACCTGGCTG3`
Reverse primer (R)
5`CAGAGCCAGCCAAGCCAGAT3`

The PCR reaction was performed in a total volume of 50 µl, containing 25µl PCR Master Mix (Bio BASIC INC, Canada), 15 µl graded water, 4µl genomic DNA and 3 µl R1 mixed with 3µl of either F1 or F2 in separate PCR tubes. The thermal amplification program consisted of an initial denaturation at 94°C (1 min), followed by thirty two cycles at 94°C (30s), 66.5°C (1 min), and 68°C (1 min), and a final extension at 68°C (12 min).

In the presence of AA homozygous sample, bands (221bp) appear with F1 primer; in GG homozygous samples, bands (221bp) appear with F2 primer and in case of AG heterozygous samples, bands (221bp) appear with both F1 and F2 primers.

Statistical analysis

Chi square test was used for analysis of Hardy Weinberg Equilibrium (HWE). Gene frequencies, allele frequencies and difference in genetic and allelic frequencies were determined by chi square analysis. SPSS Software version 16 for windows (SPSS Inc., Chicago Illinios, USA) was used to apply chi square test and other non parametric tests. Odds ratio was calculated using an online calculator (Bland and Altman, 2000).

RESULTS

Table I describes the baseline characteristics of RA patients and control group. For age and gender, there was no significant difference between the groups ($p>0.05$). Table I also explains the association of RA with different risk factors which include smoking habit, diabetes and hypertension. Significant association between smoking and RA was observed ($p<0.01$). Analysis with odds ratio revealed that smoking increased the risk of RA development by 3.167 times (OR 3.167; 95% CI 1.7766-5.6462). Results indicated significant association between diabetes and RA ($p<0.05$). Diabetes increased the risk of RA development by 2.172 times (OR 2.172; 95% CI 1.1897-3.9669). Strong association existed between hypertension and RA ($p<0.01$). Results suggested that hypertension increased the risk of RA development by 2.28 times (OR 2.280; 95% CI 1.2676-4.1021).

Table II describes the genotype and allele frequencies along with the results of Hardy Weinberg Equilibrium (HWE) estimation. G allele was found to be higher in RA group. Results of HWE estimation indicate that allele frequencies were deviant from HWE in control group. Similar results were observed in RA group, nevertheless the difference was small. Table II also describes the association between rs231775 polymorphism and RA. Association was estimated in terms of Chi-square test (X^2) and odds ratio with 95% confidence interval (95% CI). The analysis indicated a strong association between rs231775 polymorphism and RA ($p<0.01$). GG genotype increased the risk of RA development by 3.0186 times (OR 3.0186; 95% CI 1.6774-5.4322). AG genotype did not show any association with RA development (OR 0.782; 95% CI 0.4224-1.4477). AA genotype was found to have protective effects against the disease development. It lowered the risk of RA development by 0.3598 times (OR 0.3598; 95% CI 0.1913-0.6766).

DISCUSSION

RA is influenced by genetic, environmental and many other factors. Genetic factors contribute 50-60% risk for developing RA, besides environmental and other non-genetic factors (Ahmedullah *et al.*, 2012). According to Gibofsky (2012), RA is more prevalent in women as compared to men. Findings of the present study are harmonious with Gibofsky (2012), as females contributed 59% of total RA patients. Rheumatoid arthritis is more common in women due to female sex hormones, oral contraceptives usage, menopause and breastfeeding history. These sex hormones have significant roles in regulation of the inflammatory response (Centers for Disease Control and Prevention, 2012)

With age, the number of immune cells is reduced in the body and the ability of immune system to produce normal immune response is lowered, making a person more susceptible to autoimmune diseases (Grolleau-Julius *et al.*, 2010). Chibnik *et al.* (2011), Kerr (2004) and Scott *et al.* (2013) found that peak prevalence of RA is at late 4^{th} or 5^{th} decade of life. RA onset at earlier ages develops more erosive and severe disease as compared to disease onset at 50s. Chibnik *et al.* (2011) proposed that genetically susceptible

Table I.- Baseline characteristics of RA patients and controls.

Characteristics	RA patients (n=100)	Control (n=100)	Total (n = 200)	p value	Odds Ratio	95%CI	X^2 (p-value)
Age (Years) [a]	48.97±12.397	46.94±12.685	47.95±12.551	0.835			
Gender (Female)[b]	59	63	122	0.717			
Smokers [b]	62	34	96	0.004	3.167	1.776-5.646	15.705 (0.000)
Diabetic [b]	42	25	67	0.037	2.172	1.189-3.966	6.486 (0.01)
Hypertensive [b]	47	28	75	0.028	2.280	1.267-4.102	7.701 (0.005)

[a] Data are shown as mean ± standard deviation. Students T test was used for comparison of groups of RA and Control.
[b] Chi square test of the difference between the two groups (RA patients and Control) defined in terms of disease presence.
Smokers were defined as individuals with history of smoking ≤15 cigarettes per day for five years. Individuals with glucose level of 120mg/dl (fasting) were considered diabetic. The patients were marked as hypertensive on basis of blood pressure with more than 140/90mmHg.

Table II.- Genotype and allele frequencies.

Genotype/Allele	RA patients (n=100)	Control (n=100)	Total (n=200)	Odds Ratio	95%CI	X^2 (p-value)
GG	54	28	82	3.018	1.677-5.432	15.912 (0.000)
AG	26	31	57	0.782	0.422-1.447	
AA	20	41	61	0.359	0.191-0.676	
G	0.67	0.44	0.55			
A	0.33	0.56	0.45			
HWE (p)	16.98 (0.000)	13.64 (0.000)	35.9 (0.000)			

HWE: Hardy–Weinberg equilibrium; p: statistical p-value

individuals are more likely to develop RA at earlier age. According to Bergstrom *et al.* (1986) prevalence of RA is higher in patients with age of 65-74 years, while 75 year or older patients have lower disease prevalence. Our results are in accordance with these findings as RA was found to be more prevalent in late forties of life (48.97±12.397).

Smoking increases RA risk by raising serum rheumatoid factor (RF) levels and subsequently RF stimulated bone erosion. Smoking has been reported to raise white blood cell count and abnormal level of circulating T lymphocytes (Saag *et al.*, 1997). Relative risk of developing RA in smokers varies with the number of cigarettes smoked per day. Smoking 6-9 cigarettes per day increases the risk for developing RA by 2.5 times (OR 2.5; 95% CI 1.3 to 4.7), while smoking 10-19 cigarettes daily increases the risk by 3 times (OR 3; 95% CI 0 2.0 to 4.6) (Stolt *et al.*, 2003). Smoking is significantly associated with RA in present study ($p<0.01$). Significant value may be due to large number of smoker RA patients in our study but earlier reports of RA association with smoking confirm that significant results are due to smoking itself. Analysis with odds ratio revealed that smoking increased the risk of RA development by 3.167 times (OR 3.167; 95% CI 1.7766-5.6462). These findings are in accordance with Saag *et al.* (1997) and Stolt *et al.* (2003) who found smoking as an important risk factor for RA development.

Alam *et al.* (2011) and Al-Bishri (2013) found hypertension to be the most common co-morbidity among RA patients. According to Panoulos *et al.* (2008), prevalence of hypertension in most RA populations lies between 52% and 73% in mean age, ranging from 51 to 66 years. The results of present study revealed significant association of hypertension with RA ($p<0.01$). Hypertension increased the risk of RA development by 2.28 times (OR 2.280; 95% CI 1.2676-4.1021). The results of present study are in accordance with Panoulos *et al.* (2008).

Al-Bishri (2013) reported diabetes to be second most common co-morbidity with RA. Type 2 diabetes is more prevalent in RA patients (Doran, 2007). Liao *et al.* (2009b) reported that diabetes increased the risk of RA development by 1.4 times (OR 1.4; 95% CI 2.0–2.2). Significant association of diabetes with RA also exists in the present study.

Cytotoxic T lymphocyte associated antigen 4 (CTLA-4) has a key role in developing susceptibility to autoimmune disorders. Function of CTLA-4 is to down regulate T cell activation. CTLA-4 gene polymorphisms were known to confer risk for RA development (Krummel and Allison, 1995). rs231775 polymorphism in exon 1 of CTLA-4 causes reduced expression of CTLA-4 on T cell surface, thus interfering with its normal function of T cell down regulation (Anjos *et al.*, 2002). Present study explores any association between rs231775 SNP and RA.

The CTLA-4 +49 polymorphism has been studied broadly for conferring susceptibility to autoimmune diseases. Previous works came up with controversial conclusions regarding rs231775 involvement in RA development.

Barton *et al.* (2000) did not find any link of RA and CTLA-4 +49 polymorphism in UK and Spanish population. Findings of present study are contrary to Barton *et al.* (2000) as significant difference (p 0.001) was found in genotype and allele frequencies of RA patients and healthy controls. Our findings are also in disagreement to Milicic *et al*. (2001), Lee *et al*. (2002) and Sfar *et al.* (2010) who found that CTLA-4 +49 polymorphism has no role in susceptibility to RA in UK, Korean and Tunisian populations, respectively.

Lee *et al.* (2003) revealed positive association between CTLA-4 +49 polymorphism and RA in Chinese people from Taiwan. The genotype GG and allele G were found to impart greater risk for RA development. Genotype AG and allele A conferred protection against RA. Present study describes the same results as Lee *et al.* (2003); the only exception was that no role for AG was found in RA development in our study. Lei *et al.* (2005) found CTLA-4 +49 polymorphism to be the susceptibility factor for RA in Chinese Han population, as significant difference was observed regarding genotype frequencies in RA patients and healthy controls. rs231775 was considered to be a susceptibility factor for RA in western Mexican population by Muñoz-Valle *et al.* (2010). Results of present study also depict significant association (p 0.001) of CTLA-4 +49 polymorphism and RA. The present findings are also in agreement with Lei *et al*. (2005) and Muñoz-Valle *et al.* (2010). Association of +49 polymorphism in CTLA-4 gene with RA risk in European and Asian populations was investigated by Han *et al.* (2005), Li *et al.* (2012) and Lee *et al.* (2012). The results indicated +49 polymorphism as a risk factor for RA in Asians but not in Europeans. Results of present study are analogous to Han *et al.* (2005), Li *et al.* (2012) and Lee *et al.* (2012), as CTLA-4 +49 polymorphism has been found to impart risk for RA development in Asian (Pakistani) population. Tang and Zhou (2013) determined that CTLA-4 +49 G allele increases the risk of RA in Chinese Han population. Our findings are in accordance with Tang and Zhou (2013), as G allele frequency was higher in RA patients (0.67) as compared to controls (0.33).

The inconsistency of results among different populations enlightens the role of genetic background in development of RA in various populations. Limitations of this study involve small sample size, use of single technique for SNP detection, lack of detailed data about risk factors and consideration of just one SNP. Future studies involving large sample size and other SNPs in CTLA-4 gene will reveal greater detail of CTLA-4 gene and RA development. However, the present findings may be useful for future investigations.

CONCLUSIONS

Present findings conclude that rs231775 polymorphism of CTLA-4 gene has considerable role in development of RA. GG genotype increased the risk of RA development. AA genotype showed protective effects against the disease development while AG genotype had no significant effect on disease development. Smoking, hypertension and diabetes also increased the risk of RA development. So it can be concluded that rs231775 polymorphism, smoking, diabetes and hypertension determine the chances of RA development in Pakistani population.

Conflict of interest statement

The authors have no conflict of interest to declare.

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