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Susceptibility of CTLA-4 gene with development of Rheumatoid Arthritis

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ABSTRACT
The study was conducted to identify the susceptibility of *CTLA-4* gene in Rheumatoid Arthritis patients of Pakistani population. Case-control study was conducted based on 30 patients and 30 control. Blood samples were collected from Mayo Hospital Lahore, Punjab Pakistan. According to the World Health Organization criteria all patients were clinically diagnosed with Rheumatoid Arthritis and assessed for clinical parameters. Patients were with mean age of 52.4 and 51 years for males and females respectively. DNA was isolated from blood through phenol-chloroform method of extraction. Primers were optimized and genotyping was done by Polymerase Chain Reaction which was followed by DNA-sequencing and restriction fragment length polymorphism. As a result of polymorphism of A into G was identified on rs231775 polymorphic site on *CTLA-4* gene. Male patients were overweight having 29.86 BMI value and female patients were obese having 30.1 BMI Indicate that they were at high risk of disease development. The mean age of male patients was 50.57 years and that of females was 45.69 years and among them 9 patients have positive family history of arthritis. A significant association was detected between allelic and genotypic frequencies of rs231775 and onset of RA (p=0.001). In conclusion rs231775 was significantly associated with onset of Rheumatoid Arthritis in Pakistan population and females are at higher risk. Further studies should be conducted on large scale to evaluate the association of *CTLA-4* polymorphism with Rheumatoid Arthritis.

Key Words: Rheumatoid Arthritis, Pakistan, Population, Polymorphism, Genotyping

INTRODUCTION
Rheumatoid Arthritis (RA) is an autoimmune disorder that is described by the pain, inflammation, joint stiffness, damaged of synovial joints, cartilage as well as bones (Alamanos and Drosos 2005). It influences approximately 1% of the world population (Moreland L 2005). According to the pathophysiology of RA, damage began at the synovial membrane of the joint, characterized by the complex interaction of immune modulators like effector cells and cytokines (Smolen and Steiner 2001). The later inflammatory changes lead to destruction of bone and cartilage (McCain 2009). With respect to gender, women effected by rheumatoid arthritis three times more commonly than men (Baker et al. 2014). The risk of RA is more among those who aged between 40 and 65 years (Alamanos et al. 2006). Both genetic as well as environmental factors are responsible for onset of RA (Smolen and Steiner 2006). Higher intake of caffeine, alcohol, red meat, obesity, less use of antioxidant diet, and cheap socioeconomic status are the other factors associated with the risk of RA (Arend and Firestein 2012). In female's hormones influenced during pregnancy and breastfeeding were reported to be the primary risk factors associated with RA (Liao et al. 2009). There are almost one hundred genes that are linked with the risk of RA (Smolen et al. 2016). Some of these genes are HLA class II histocompatibility antigen, DRB1 beta chain (*HLA-DRB1),* Peptidyl arginine deiminase, type IV *(PADI4),* Protein tyrosine phosphatase, non-receptor type 22 (*PTPN22)*, cytotoxic T-lymphocyte-associated protein 4 (CTLA*4)* (Hughes et al. 2010). *CTLA-4* (Cytotoxic T Lymphocyte Associated Protein-4) gene is located on chromosome 2q33 (Blomhoff et al. 2004). *CTLA-4* is a 40- KDa protein and belongs to the immunoglobulin superfamily. It encodes a receptor protein which is expressed only on the surface of activated T cells to down regulates the autoimmune process and transfer inhibitory message to T cells (Ostrov et al. 2000). In the present work, we have targeted the single nucleotide polymorphisms (SNPs) in the *CTLA-4* gene, because of its possible role on expression level or function of the T cells. With this hypothesis, our goal was (i) to explore whether rs231775 polymorphism is related to the development of RA and (ii) and the association of risk factors including smoking, hypertension and diabetes with the development of this disease

MATERIALS AND METHODS
Blood samples were collected from 30 rheumatoid arthritis (7 males, 23 females) and 30 healthy subjects (7 males, 23 females) from the Mayo Hospital Lahore Punjab, Pakistan. The inclusion and exclusion criteria for the selection of arthritis among the patients included: BMI, Family History, Hand and Feet involvement, Stiffness, Positive Rheumatoid Factors, Age. Blood samples were collected from patients and control in EDTA coated tubes and stored at 4°C for DNA extraction. DNA isolation was done by using phenol-chloroform extraction method. After the isolation of genomic DNA, the extracted genomic DNA was confirmed by the gel electrophoresis. For the quantification (concentration) and qualification (purity) of all the DNA samples, nano dropping was done.

Genotyping

For genetic analysis, genes and single nucleotide polymorphism (SNP's) were selected to determine whether the genes or SNP's were associated with rheumatoid arthritis by using NCBI website https://www.ncbi.nlm.nih.gov/snp/. Genotyping of *CTLA-4* with variants +49(A/G) (rs231775) was done by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using BbvI enzyme. For Polymerase chain reaction (PCR), primers **F:** CCACGGCTTCCTTTCTCGTA; **R:** AGTCTCACTCACCTTTGCAG were examining for their validity with sequences by using the website https://www.ncbi.nlm.nih.gov/. With the help of Gradient PCR primers were optimized with an annealing temperature range of 49°C to 52°C and the annealing temperature for *CTLA-4* was found to be 50°C. The 12µl of reaction mixture for primer optimization consists of 1.50 µl genomic DNA, 3 µl master mix, 0.70 µl of each primer, and 6 µl of DEPC water. PCR was done following protocol: denaturation at 95°C for 5 minutes, 35 cycles of 45 seconds at 94°C, 45 seconds at 55°C, and 45 seconds at 72°C and then 10 minutes at 72°C. In order to confirm the PCR product gel electrophoresis was done on 1.2% of agarose for 30min at 110V. The gel was then observed on benchtop UV Trans-illuminator. Each PCR product was then purified and for this purpose, 20µl of each sample was mixed with 80µl of 100% chilled ethanol. The eppendorfs were placed in dark for 40-45 minutes and centrifuged for 25 min at 13500rpm. Supernatant was discarded and 2 µl of DEPC water was added in each eppendorf after drying of pellets. After purification sequencing of PCR products were done at the Center of Applied Molecular Biology (CAMB). Then PCR products were precipitated by the use of isopropanol. Then samples were loaded on ABI PRISM genetic analyzer 3130XL for sequencing the fragments of interest. The sequences were seen on BioEdit software and then blast on NCBI and UCSC. After PCR RFLP was performed to determine the *CTLA-4* single nucleotide polymorphism at location (rs231775). RFLP reactions were performed with mixtures consisting of 5 µl PCR product, 9 µl nuclease free water, 1ul buffer, and 1 ul BbvI enzyme. Reaction mixture in RCR tubes were first incubate at 37°C for 5 hours and then at 65°C in water bath for 5-10 minutes. The mixture containing 5ul reaction mixture and 3ul loading dye, of each tube as run against ladder on 2% agarose gel. Then gel was observed under UV on trans illuminator.

Statistical Analysis

Hardy Weinberg Equilibrium was applied. The demographic data was presented in percentage form. Genotype and allelic frequencies were calculated by SHEsis available online (http://analysis.bio-x.cn/SHEsisMain.htm). Chi2, Pearson's and Fisher's tests were also applied.

RESULTS

Blood samples and demographic data were collected from 30 rheumatoid arthritis (7 males, 23 females) and 30 healthy subjects (7 males, 23 females). Demographic data of controls and diseased were shown in **Table 1.** It was observed that out of 30 patients, 5 females and 3 males were with positive family history of rheumatoid arthritis whereas all controls were with negative family history of arthritis.

Table 1: Demographic data of patients and controls

Characters	Patients		Control	
	Male (n=7)	Female (n=23)	Male (n=7)	Female (n=23)
Age (Years)	52.42	51	46.42	37.52
BMI (kg/m^2)	29.86	30.1	26.98	23.68
Age of diagnose (years)	50.57	45.69	----	----
Duration of disease (years)	5.6	6.1	----	----
Positive family history	6	3	----	----

DNA Isolation

After DNA isolation its presence was confirmed by running it on 0.8% gel electrophoresis as shown in **Figure 1**. By nondrooping each DNA sample was quantified.

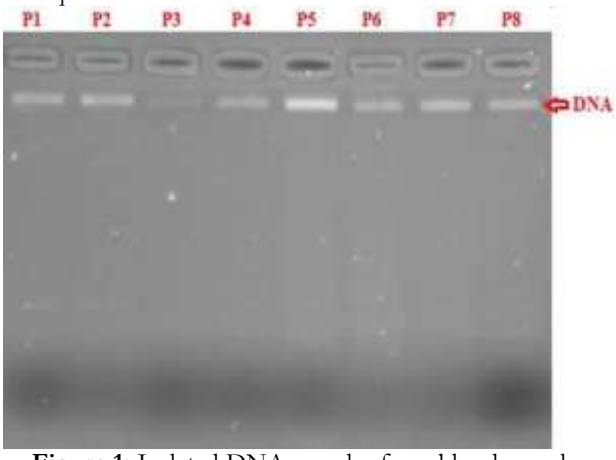

Figure 1: Isolated DNA samples from blood samples

PCR (Polymerase Chain Reaction)

The 327bp of PCR product was prepared by single PCR and presence of PCR product was confirmed by running it on 1.2% agarose gel as shown in **Figure 2**.

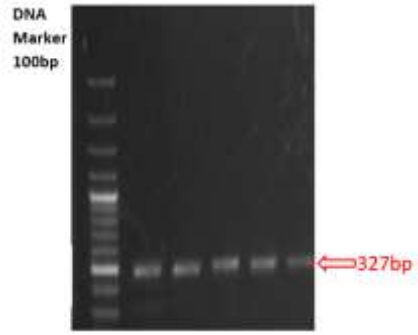

Figure 2: PCR product of different DNA samples

DNA Sequencing

The selected SNP was sequenced and then on NCBI its sequences was BLAST. The chromatograph of control and patients were given in **Figure 3** and the results of BLAST of SNP on NCBI were shown in **Figure 4**.

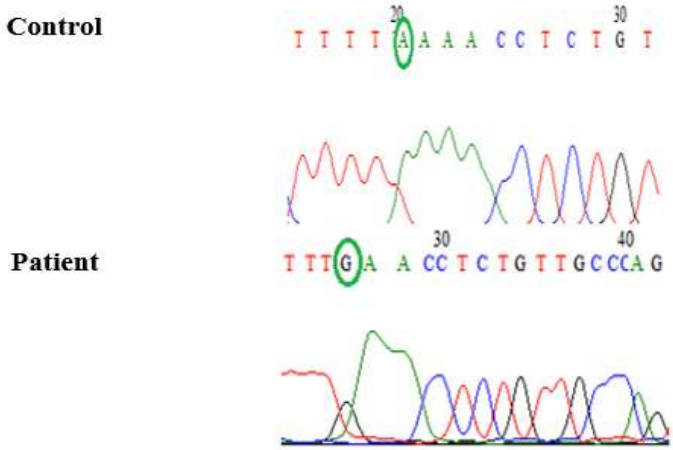

Figure3: Chromatographic representation of rs 231775 of *CTLA-4* gene (Control Genotype AA and Patient Genotype GG)

```
Query  131        aaa-aaaaaGGCTTTCTATTC-AGTGCCTTCTGTGTGTGCACATGTGT-ATACATATCTG  187
                   ||| ||||||||||||||||| |||||||||||||||||||||||| ||||||||||
Sbjct  204739039  AAACAAAAAGGCTTTCTATTCAAGTGCCTTCTGTGTGTGCACATGTGTAATACATATCTG  204739098

Query  188        GGATC-AAGCTATCTATATAAAGTCCTTGATTCTGTGTGGGTTCAAACACATTTCAAAGC  246
                   ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  204739099  GGATCAAAGCTATCTATATAAAGTCCTTGATTCTGTGTGGGTTCAAACACATTTCAAAGC  204739158

Query  247        TTCAGGATCCTGAAAGGTTTTGCTCTACTTCCTGAAGACCTGAACACCGCTCCCATAAAG  306
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  204739159  TTCAGGATCCTGAAAGGTTTTGCTCTACTTCCTGAAGACCTGAACACCGCTCCCATAAAG  204739218

Query  307        CCATGGCTTGCCTTGGATTTCAGCGGCACAAGGCTCAGCTGAACCTGGCTGCCAGGACCT  366
                   ||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||
Sbjct  204739219  CCATGGCTTGCCTTGGATTTCAGCGGCACAAGGCTCAGCTGAACCTGGCCACCAGGACCT  204739278

Query  367        GGCCCTGCACTCTCCTGTTTTTTCTTCTCTTCATCCCTGTCTTCTGCAAGGTTGAGTGAG  426
                   |||||||||||||||||||||||||||||||||||||||||||||| | ||||||||
Sbjct  204739279  GGCCCTGCACTCTCCTGTTTTTTCTTCTCTTCATCCCTGTCTTCTGCAAAGGTGAGTGAG  204739338

Query  427        ACT  429
                   |||
Sbjct  204739339  ACT  204739341
```

Figure 4: Representation of BLAST sequence on NCBI rs 231775. Absence of bond (|) indicates point of mutation. Subject= Reference sequence; Query= Patient Sequence; Box represent mutation (A/G)

Restriction Fragment Length Polymorphism (RFLP)

The Digestion of PCR product with BbvI endonuclease enzyme produces two different sized bands i.e. 244bp and 83bp were visualized on 2% agarose gel. In **Figure 5** the two bands produced by the activity of enzyme showed homozygous mutation.

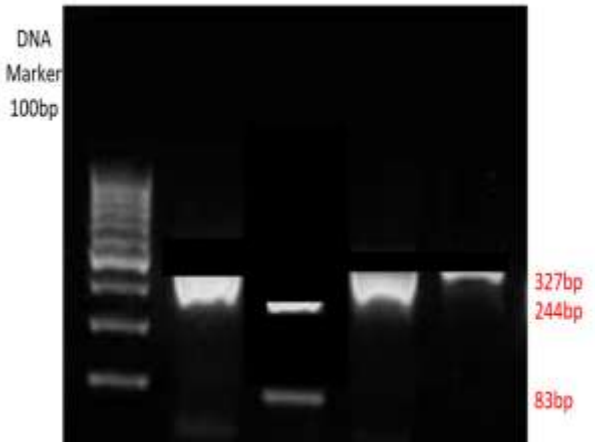

Figure 5: PCR-RFLP digestion product

Association of RA with genetic polymorphism

The genetic polymorphism association of RA with *CTLA-4* was summarized in Table 1. Summary of allelic and genomic frequency were shown in **Table 2 & 3** respectively. Sequencing of *CTLA-4* SNP identified genotype G/G of variant rs231775 in patients and A/A in controls. All SNPs followed Hardy Weinberg Equilibrium and variant rs231775 was significantly associated with onset of RA in Pakistani population at genotypic and at allelic level ($P<0.01$). Similarly, single site test at allelic level showed that at rs231775 (allele G) had a significantly increased risk of RA onset as compared to those carrying A allele since the frequency of G and A allele was 0.800 and 0.200 in patients whereas 0.000 and 1.000 in controls. The *P* values were calculated from Fisher's test.

Table 2: Single site analysis of genetic variants in RA patients and controls

SNP ID	Allele	Frequency in Patients (n)	Frequency in Controls (n)	Fisher's *p*-value	Pearson's *p*-value	χ^2
rs 231775	G	0.800 (48)	0.000 (0)	5.55e-016	3.97e-019	80.000000 df=1
	A	0.200 (12)	1.000 (60)			

Table 3: Genetic frequencies distribution in RA patients and controls

SNP ID	Genotype	Frequency in Patients (n)	Frequency in Controls (n)	Fisher's *p*-value	Pearson's *p*-value	χ^2
rs 231775	G/G	0.800 (24)	0.000 (0)	2.76e-010	2.62e-010	40.000000 df=1
	A/A	0.200 (6)	1.000 (30)			

DISCUSSION

RA, a long-time autoimmune disorder that mainly affect joints and results in swollen, warm and painful joints. The prevalence of RA varied from population to population (Majithia and Geraci SA 2007; Pedersen et al. 2007). In Pakistan cousin marriages occur at the risk of 52-54% due to this the risk of RA increases day by day among the Pakistani population (Altschuler J 2016). Therefore, the current study was accompanied to assess the genetic susceptibility of RA in Pakistan population.

Current study revealed the risk of RA in females was higher as compared to males. Women were at higher risk due to female sex hormones, menopause, oral contraceptives usage and breastfeeding history (Center of disease control and prevention 2012). A study also reported that women suffering from RA showed more swear symptoms as compared to men, even they had same level of disease (Baskin and Wilson CB 1984).

In current study it was observed that women affected by RA at younger age as compared to men. Study similar to this reported that RA tend to smack women at younger age as compared to men and can blow harder, too (Wahidin 2004). The number of immune cells present in our body reduces with age and also lowered the capability of immune system to yield normal immunity. This makes a person more susceptible to autoimmune diseases (Grolleau-Julius et al. 2010). Current study demonstrated that the mean age of diagnosis of RA in male and female were 50.57 and 45.69 years respectively. Similar to current study it was found that in late forties of life RA was more prevalent (Sameem et al. 2015).

Existing study also confirmed that the mean duration of disease in male and female were 5.6 and 6.1 years respectively. In contrast to this study, it was reported that the mean duration of disease in both genders was 9.4 years (Zavaleta-Muniz et al. 2013).

Current study demonstrated that positive family history was an important risk factor in the beginning of RA. Similar study reported that having a family history of rheumatoid arthritis ~3–5 times increases the risk of developing RA (Grant et al. 2001). In contrast to this RA with negative family history may be due to bad health, obesity, smoking and other risk factors (Perricone et al. 2011).

Present study demonstrated significant risk of RA related with high BMI. Mean BMI in male and females were 29.86 and 30.1 respectively. There is no nonlinear confirmation of relationship between the BMI and threat of RA was reported. A major positive relationship was detected when linear relationship was molded for every 5kg/m^2 increase in BMI risk of developing

RA increases (Feng et al. 2016). Similarly, females whose BMI is greater than 30 have 26% increase the risk of RA (Alamanos and Drosos 2005).

Current study demonstrated that CTLA-4 polymorphism was considerably connected with the onset of RA at both genotypic and allelic level. Similar to current study the relationship of CTLA-4 (A/G) with RA was studied in many populations likewise in Asian and European population, a number of studies have been conducted in which CTLA-4 A49G polymorphism is associated with rheumatoid arthritis (Han et al. 2005). In UK CTLA-4 (A/G) was not linked with RA (Barton et al. 2000).

Current finding accomplishes that rs231775 polymorphism of CTLA-4 gene has a significant role in the onset of RA. GG genotype increased the possibility of RA development. AA genotype showed defensive effects against the development of disease while obesity and positive family history also increased the risk of RA. So, it can be concluded that rs231775 polymorphism, obesity and positive family history determine the risks of RA development in population of Pakistan.

REFRENCES

1. Alamanos Y, Drosos AA (2005) Epidemiology of adult rheumatoid arthritis. Autoimmun Rev 4: 130-136.
2. Alamanos Y, Voulgari PV, Drosos AA (2006) Incidence and prevalence of rheumatoid arthritis, based on the 1987 American College of Rheumatology criteria: a systematic review. Semin Arthritis Rheum 36:182–8.
3. Altschuler J (2016) Migration, Illness & Healthcare: A Journey Through Migration and its Impact on Health. Biosocieties 12:568-587.
4. Arend WP, Firestein GS (2012) Pre-rheumatoid arthritis: predisposition and transition to clinical synovitis. Nat Rev Rheumatol 8: 573.
5. Baker JF, Ostergaard M, George M (2014) Greater body mass independently predicts less radiographic progression on X-ray and MRI over 1–2 years. Ann Rheum Dis 73:1923–8.
6. Barton A, Myerscough A, John S, Gonzalezgax M, Ollier W, Worthington J (2000) A single nucleotide polymorphism in exon 1 of cytotoxic T-lymphocyte-associated-4 (CTLA-4) is not associated with rheumatoid arthritis. J Rheumatol 39: 63-66.
7. Baskin DS, Wilson CB (1984) Transsphenoidal treatment of non-neoplastic intrasellar cysts. J Neurosurg 60: 8-13.
8. Blomhoff A, Lie BA, Myhre AG, Kemp EH, Weetman AP, Akselsen HE, Huseby ES, Undlien DE (2004) Polymorphisms in the cytotoxic T lymphocyte antigen-4 gene region confer susceptibility to Addison's disease. J Clin Endocrinol Metab 89: 3474-6.
9. Centers for Disease Control and Prevention (2012).
10. Feng M, He J, Wang Y, Zhang X, Jin YB, Li X, Wu LJ (2016) Dietary intake and risk of rheumatoid arthritis—a cross section multicenter study. J Clin Rheumatol 35: 2901-2908.
11. Grant SF, Thorleifsson G, Frigge ML, Thorsteinsson J, Gunnlaugsdóttir B, Geirsson ÁJ, Jónsson H (2001) The inheritance of rheumatoid arthritis in Iceland. Arthri Rheumatol 44: 2247-2254.
12. Grolleau-Julius A, Ray D, Yung RL (2010) The role of epigenetics in aging and autoimmunity. Clin Rev Allergy Immunol 39: 42-50.
13. Han S, Li Y, Mao Y, Xie Y (2005) Meta-analysis of the association of CTLA-4 exon-1 +49A/G polymorphism with rheumatoid arthritis. J Hum Genet 118: 123-132.
14. Hughes LB, Reynolds RJ, Brown EE, Kelley JM, Thomson B, Bridges SL (2010) Most common single-nucleotide polymorphisms associated with rheumatoid arthritis in persons of European ancestry confer risk of rheumatoid arthritis in African Americans. Arthritis Rheum 62: 3547-53.
15. Liao KP, Alfredsson L, Karlson EW (2009) Environmental influences on risk for rheumatoid arthritis. Curr Opin Rheumatol 21:279-283.
16. Majithia V, Geraci SA (2007) Rheumatoid arthritis: diagnosis and management. Am J Med 120: 936-939.
17. McCain JJ (2009) Unmet needs in the treatment of rheumatoid arthritis. Am J Manag Care 5: 1-6.
18. Moreland L (2005) Unmet needs in rheumatoid arthritis. Arthritis Res Ther 7: 2-8.
19. Ostrov DA, Shi W, Schwartz JC, Almo SC, Nathenson SG (2000) Structure of murine CTLA-4 and its role in modulating T cell responsiveness. Science 290: 816–819.
20. Pedersen J K, Svendsen AJ, Horslev-Petersen K (2007) Incidence of rheumatoid arthritis in the southern part of Denmark from 1995- 2001. J Rheumatol 1: 18.
21. Perricone C, Ceccarelli F, Valesini G (2011) An overview on the genetic of rheumatoid arthritis: A never ending story. Autoimmunol Rev 10: 599-608.
22. Sameem M, Rani A, Bashir R, Riaz N, Batool SA, Irfan S, Arshad M, Nawaz SK (2015) Ctla-4 +49 Polymorphism and Susceptibility to Rheumatoid Arthritis in Pakistan Population. Pak J Zoo 47: 1731-1737.
23. Smolen JS, Aletaha D, Mclnned IB (2016) Rheumatoid arthritis. Lancet 388:2023-2038.
24. Smolen JS, Steiner G (2006) Therapeutic strategies for rheumatoid arthritis. Nat Rev Drug Discov 2: 473-88.
25. Smolen JS, Steiner G (2001) Therapeutic strategies for rheumatoid arthritis. Nat Rev Drug Discov 2: 473-88.
26. Wahidin A (2004) Older women in criminal justice system: Running out of time. Jessica Kingsley Publisher.
27. Zavaleta-Muniz SA, Martin-Marquez BT, Gonazalez-Lorez L, Flores-Martinez SE (2013) Autoimmune disease genetics. J Rheum 40:30-34.