A sequencing study of \textit{CTLA4} in Pakistani rheumatoid arthritis cases

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Abstract

Rheumatoid arthritis (RA) is a multifactorial autoimmune disease. The interaction of genetic and environmental factors is likely necessary for RA. Among potential genetic factors, many major histocompatibility complex (MHC) and non-MHC variants may be involved in RA susceptibility. \textit{CTLA4} is involved in the regulation of T-cell response during an immune reaction, and multiple \textit{CTLA4} single nucleotide polymorphisms (SNPs) have been associated with numerous autoimmune diseases, including RA. To our knowledge, the genetic association of \textit{CTLA4} with RA risk has not been examined previously in the Pakistani population. In this study, we sequenced the entire \textit{CTLA4} gene and flanking regions in 95 Pakistani RA cases followed the screening of identified variants in Study 1 sample consisting of 350 RA cases and controls. Four common significant variants identified in Study 1 sample were further examined in a larger Study 2 replication sample comprising 1,678 independent RA cases and controls. We report significant associations of three variants from the combined analysis: rs3087243 (OR = 1.26, \(p = 4.47\times10^{-3}\)), rs5742909 (OR = 1.78, \(p = 4.60\times10^{-3}\)), and rs11571319 (OR = 1.48, \(p = 6.64\times10^{-3}\)); the latter is a novel association in the Pakistani sample.

Introduction

RA is an inflammatory, chronic, autoimmune syndrome that causes articular damage, synovial joint destruction, and related comorbidities [1]. RA is characterized by increased levels of autoantibodies, inflamed bones and joints, synovitis, and destruction of bone and cartilage that lead to fatigue, chronic pain, and in the worst cases, permanent disability [2]. A complex network of immune cells (B-cells, T-cells, mast cells, plasma cells, and dendritic cells) and cytokines (pro-inflammatory and anti-inflammatory) are involved in the etiology of RA [3]. Globally, RA affects almost 0.5 to 1% of the general population [4]; in Pakistan, its prevalence is approximately 0.5% [5]. RA can affect both sexes at any age, but the prevalence of RA is higher in women than men [6]. The interplay of genetic and environmental factors (climate, diet,
geography, smoking, and microbiome) leads to the onset of RA [7, 8]. Class II major histocompatibility complex (MHC-II) is the most important genetic locus for RA susceptibility. Many genome-wide association studies (GWAS) and meta-analyses of GWAS have identified more than 100 single nucleotide polymorphisms (SNPs) loci associated with RA susceptibility. Most of the identified single-nucleotide polymorphisms (SNPs) in these loci are clustered around immune-related genes [9].

Cytotoxic T-lymphocyte antigen-4 (CTLA4) also known as CD152, is an inhibitory glycoprotein present on the surface of T-cells. It regulates the activation of autoreactive T-cells, tolerance against self-antigens, and inhibits the differentiation of monocytes into osteoclasts [10]. T-cells have a key role in RA derived autoimmune response, therefore mediator of T-cells such as CTLA4 has a regulatory role in RA pathogenesis [11]. The human CTLA4 gene is present on the long arm of chromosome 2 at position 33.2q. It belongs to the immunoglobulin superfamily and consists of four exons [12]. Exon one contains the sequence for extracellular leader peptide, exon two encodes extracellular ligand-binding site, exon three encodes the transmembrane domain and exon four encodes the cytoplasmic tail [13]. Several genetic studies have reported the association of CTLA4 SNPs with numerous autoimmune conditions, including RA [14]. CTLA4/rs231775 (+49A/G), rs3087243(CT60 G/A) and rs5742909 (-318 C/T) are most widely studied for their associations with RA susceptibility in different populations [15, 16].

Hitherto, most of the genetic studies on RA have been conducted on East-Asians, Europeans, or European-derived populations, with limited genetic data available in the Pakistani population. In an effort to comprehensively examine the role of CTLA4 genetic variation with RA susceptibility in Pakistanis, we re-sequenced the entire CTLA4 gene in 95 RA cases and then examined the identified variants in more than 2,000 cases and controls.

Materials and methods

Subjects

A total of 2,028 subjects comprising 1,291 RA cases (mean age ± SD = 41.8 ± 12.36, 76.2% women), and 737 controls (mean age ± SD = 40.7 ± 12.49, 39.5% women) were derived from our two published studies [2, 17]. While Study 1 comprised 239 cases and 111 controls collected from October 2009 to December 2012 and Study 2 consisted of 1,222 cases and 737 controls collected from September 2015 to May 2017. After obtaining Institutional Review Board (IRB) approvals, blood samples were collected from five rheumatology facilities located in two adjacent cities in Pakistan: Pakistan Institute of Medical Sciences, Military Hospital, Fauji Foundation Hospital, and Rehmat Noor Clinic in Islamabad, and Kahota Research Laboratories Hospital in Rawalpindi. All recruited RA cases were diagnosed by rheumatologists following the 1987 ACR (American College of Rheumatology) classification criteria [18]. All control subjects were recruited from the general population and had no history of any autoimmune disease at the time of enrollment. Written informed consent was taken from all study participants at the time of recruitment. Blood samples were collected in EDTA coated tubes to avoid coagulation and processed shortly after the collection. This study was approved by the IRB of the University of Pittsburgh, USA (IRB no. PRO12110472).

Genomic DNA extraction

Standard phenol-chloroform extraction method or GeneJET Whole Blood Genomic DNA Purification (Thermo Scientific USA) was used to extract genomic DNA from whole blood and NanoDrop™ 2000 spectrophotometer (Thermo Scientific USA) was used for quantification.
**CTLA4 sequencing**

The entire CTLA4 gene (all four exons and introns) and flanking regions on chromosome 2 (hg19, chr2: 203866788–203874960) were polymerase chain reaction (PCR)-amplified using nine sets of primers in 95 RA cases from Study 1. PCR primer sequences are available in Table S1. All primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/). Automated DNA sequencing of PCR products was performed in a commercial lab (Beckman Coulter Genomics, Danvers, MA) using the Sanger method on ABI 3730xl DNA Analyzers. The sequences were aligned against a reference sequence (NM_005214) by Variant Reporter™ Software v1.0 (Thermo Scientific USA) to identify variants.

**Genotyping**

Follow-up genotyping of sequence variants was performed on 350 subjects from Study 1 using iPLEX® Gold (Sequenom). Genotype call rate ≥98%, concordance with Hardy-Weinberg Equilibrium (HWE), and discrepancy rate <1% were used as QC measures. The iPLEX® Gold (Sequenom) genotyping was performed in the Core Laboratories of the University of Pittsburgh, Pittsburgh, USA. Follow-up genotyping of 4 selected SNPs from Study 1 was conducted on Study 2 samples (1,052 RA cases, 626 healthy controls) using TaqMan® (Applied Biosystems, ThermoFisher Scientific) genotyping assays (C___2415786_20, C__30981401_10, C__30981401_10 and C___3296043_10) following manufacturer's guidelines. The 384-wells plates containing dried DNA were used in both genotyping methods. After thermal cycling of functionally tested TaqMan® assay, QuantStudio™ 12K Flex system (Applied Biosystems, ThermoFisher Scientific) was used for the end-point fluorescence reading of 384-wells DNA plates. Sequences of iPLEX® Gold (Sequenom) genotyping primers are available upon request.

**Statistical analysis**

Haploview 4.0 [19] (www.broadinstitute.org/haplovlew) was used to analyze variants identified through sequencing for their minor allele frequency (MAF) and linkage disequilibrium (LD) patterns. A chi-square goodness of fit test was used to check the concordance with Hardy-Weinberg Equilibrium (HWE). Logistic regression was employed for case-control association analysis using sex and age as covariates. Association of significant variants with anticyclic citrullinated peptide (anti-CCP) and rheumatoid factor (RF) seropositivity was also examined using logistic regression with age and sex as covariates. p<0.05 was considered as suggestive evidence of association. All association analyses were implemented in R version 3.4.4 (http://www.r-project.org).

**Results**

**CTLA4 sequencing**

Sequencing of the entire CTLA4 gene and flanking regions in 95 Pakistani RA patients identified 30 variants, including two novel variants (GRCh38: 203869988 and GRCh38: 203870218). Most of the variants were in intronic regions and only one coding variant (rs231775) was identified. Four variants (rs231774, rs231773, rs11571317, and rs5742909) were in the 5′ upstream region, one was in 3′UTR (rs11331867) and three (rs231721, rs11571319, and rs3087243) were in the downstream region.

**Genotyping of CTLA4 variants in Study 1 sample**

Of 30 sequence variants identified in the discovery phase, 24 were successfully confirmed/genotyped in 350 individuals. The genotype call rate for all SNPs was ≥98% and they were in...
concordance with HWE. Out of 24 variants, 12 were rare (MAF < 1%), 6 were uncommon (MAF between 1 to 5%) and 6 were common (MAF > 5%) (Table 1, Fig 1). Among those only observed in cases, eight variants (rs231781, GRCh38: 203869988, GRCh38: 203870218, rs500168522, rs231774, rs231780, rs231721 and rs231773) were singleton, two variants (rs231776 and rs231778) were observed in two individuals. We did not observe any rare or less common variant in more than two independent cases. Case and control allele frequencies were similar for most variants with >1% MAF in the Study 1 sample, except for six most common SNPs (rs231775, rs231779, rs231777, rs11571319, rs5742909, and rs3087243) that showed a trend for the association (p-range = 0.2 to 0.8). There was a strong linkage disequilibrium (LD) between rs231775 and rs231779 (r² = 0.95), and between rs231777 and rs11571319 (r² = 0.92) (Fig 2).

Table 1. 24 SNPs identified by sequencing and successfully genotyped in extended samples.

| SNP Name | SNP ID | Position | Variant Type | Minor Allele | MAF  | p-value  |
|----------|--------|----------|--------------|--------------|------|----------|
| CTL4p5377 | rs231781 | 203872164  | Intron       | A            | 0.14 | 9.88E-01 |
| CTL4p3201 | GRCh38: 203869988 | 203869988  | Intron       | G            | 0.14 | 9.88E-01 |
| CTL4p3495 | rs500168522 | 203870282  | Intron       | T            | 0.14 | 9.88E-01 |
| CTL4p713  | rs231774 | 203867500  | Upstream     | T            | 0.14 | 9.88E-01 |
| CTL4p5187 | rs231780 | 203871974  | Intron       | G            | 0.14 | 9.88E-01 |
| CTL4p7215 | rs231721 | 203874002  | Downstream   | C            | 0.14 | 9.88E-01 |
| CTL4p3431 | GRCh38: 203870218 | 203870218  | Intron       | G            | 0.15 | 9.88E-01 |
| CTL4p162  | rs231773 | 203866949  | Upstream     | G            | 0.15 | 9.88E-01 |
| CTL4p3691 | rs55657178 | 203870478  | Intron       | T            | 0.29 | 9.82E-01 |
| CTL4p1340 | rs231776 | 203868127  | Intron       | A            | 0.29 | 9.82E-01 |
| CTL4p2311 | rs231778 | 203869098  | Intron       | G            | 0.29 | 9.82E-01 |
| CTL4p3079 | rs1048095615 | 203869866  | Intron       | A            | 0.29 | 5.97E-01 |
| CTL4p5477 | rs114352937 | 203872264  | Intron       | T            | 1.01 | 4.04E-01 |
| CTL4p4351 | rs41265961 | 203871138  | Intron       | A            | 1.01 | 3.98E-01 |
| CTL4p1429 | rs540826181 | 203868216  | Intron       | C            | 1.29 | 6.12E-01 |
| CTL4p498  | rs11571317 | 203867285  | Upstream     | T            | 1.15 | 3.52E-01 |
| CTL4p7008 | rs113318671 | 203873795  | 3’ UTR       | T            | 1.44 | 5.34E-01 |
| CTL4p3154 | rs148575091 | 203869941  | Intron       | T            | 1.89 | 5.52E-01 |
| CTL4p837  | rs5742909 | 203867624  | Upstream     | T            | 5.76 | 5.30E-01 |
| CTL4p2078 | rs231777 | 203868865  | Intron       | T            | 9.97 | 8.63E-01 |
| CTL4p7428 | rs11571319 | 203874215  | Downstream   | A            | 10.93 | 7.67E-01 |
| CTL4p1204 | rs231775 | 203867991  | Exon         | G            | 32.41 | 2.00E-01 |
| CTL4p2977 | rs231779 | 203869764  | Intron       | T            | 32.46 | 2.05E-01 |
| CTL4p7409 | rs3087243 | 203874196  | Downstream   | G            | 43.93 | 4.07E-01 |

Genotyping of selected CTLA4 variants in Study 2 sample

Four of the six SNPs that showed the smallest p-values in the Study 1 samples were examined in the Study 2 sample. Because of the strong LD between rs231775 and rs231779 and between rs231777 and rs11571319, only one SNP from each pair (rs231775 and rs11571319) was selected for follow up genotyping. The genotype call rate in Study 2 sample was >90% for all four SNPs and they were in concordance with HWE. Statistically significant associations of rs3087243 (p = 4.47E-03, OR = 1.26), rs11571319 (p = 6.64E-03, OR = 1.48) and rs5742909 (p = 4.60E-03, OR = 1.78) were observed with RA risk (Table 2).
Association with anti-CCP and RF

Anti-CCP and RF data were available on 1,010 RA patients; of which, 877 were positive for anti-CCP and 914 for RF. In order to examine if RA-associated SNPs also affect seropositivity
of anti-CCP or RF, we performed logistic regression analyses, but found no significant associations (Table 3).

**Discussion**

Rheumatoid arthritis is a multifactorial, inflammatory autoimmune disease [20]. Many MHC and non-MHC genetic variants are associated with RA susceptibility [21]. CTLA4 regulates the T-cell response in an immune reaction and genetic variation CTLA4 has been reported to be associated with RA susceptibility [22]. To further explore the role of CTLA4 genetic variation with RA susceptibility, we sequenced the entire CTLA4 gene and flanking regions in Pakistani RA patients. A total of 30 variants were identified, including two novel ones; the latter were observed in singleton. Twenty four of the 30 variants were successfully genotyped in Study 1 sample of 350 RA cases and controls. Six of them (rs231775, rs231779, rs231777, rs11571319, rs5742909, and rs3087243) were selected for follow-up. After considering high LD between two sets of SNP pairs (rs231775 with rs231779, and rs231777 with rs11571319), four SNPs were moved forward in a larger replication sample of 1,678 RA cases and controls (Study 2 sample) where 3 showed statistically significant associations (rs3087243, rs11571319, rs5742909). Previously, rs3087243 has been reported to be associated with multiple diseases [23–25] and autoimmune conditions such as type 1 diabetes [26] and RA [27]. Similarly, rs5742909 has been reported to be associated with RA susceptibility in Canadians [15] and Egyptians [28]. In our study, rs3087243/G was associated with an increased risk of RA, as has also been reported in Europeans and Asians [27, 29]. However, in Mexicans rs3087243/G was associated with decreased RA risk [30], but it showed no association in the Polish population [31]. Two RA GWAS-implicated CTLA4 SNPs (rs11571302, a downstream variant, and rs231735, an upstream variant), which were not covered in our sequencing sample, are also in LD with rs3087243 (Fig 3) [32, 33]. We observed a novel association of rs11571319 with RA in the Pakistani sample. To our knowledge, rs11571319 has not been reported previously to be associated with RA risk, although its association has been reported with Graves’ disease [34] and asthma [35]. Anti-CCP and RF seropositivity confer acute disease activity in RA patients

### Table 2. Common CTLA4 SNPs genotyped in 1,678 RA case-control subjects.

| SNP     | Position       | Variant Type | Minor Allele | MAF  | OR (95% CI) | p-value |
|---------|----------------|--------------|--------------|------|-------------|---------|
| rs231775 | 203867991      | Exon         | G            | 0.32 | 1.11 (0.94, 1.29) | 2.21E-01 |
| rs3087243 | 203874196     | Downstream   | G            | 0.43 | 1.26 (1.02, 1.38) | 4.47E-03 |
| rs11571319 | 203874215    | Downstream   | A            | 0.10 | 1.48 (1.11, 1.94) | 6.64E-03 |
| rs5742909 | 203876724      | Upstream     | T            | 0.05 | 1.78 (1.18, 2.60) | 4.60E-03 |

OR = Odds ratio; CI = Confidence interval

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### Table 3. Association results of tested CTLA4 SNPs with anti-CCP and RF seropositivity.

| SNP     | Minor Allele | MAF  | OR (95% CI) | p-value | OR (95% CI) | p-value |
|---------|--------------|------|-------------|---------|-------------|---------|
| rs11571319 | A            | 0.10 | 1.28 (0.71, 2.05) | 0.37 | 0.95 (0.55, 1.52) | 0.85 |
| rs5742909  | T            | 0.05 | 1.18 (0.57, 2.49) | 0.66 | 0.97 (0.47, 2.05) | 0.95 |
| rs231775   | G            | 0.31 | 0.96 (0.66, 1.21) | 0.80 | 1.30 (0.90, 1.81) | 0.18 |
| rs3087243  | G            | 0.44 | 1.00 (0.63, 1.12) | 1.00 | 1.19 (0.74, 1.38) | 0.33 |

OR = Odds ratio; CI = Confidence interval

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[36] and may explain the observed genetic associations with RA risk. However, in this study, we could not establish this link, indicating that the association of CTLA4 variants with RA risk is independent of anti-CCP and RF status, as has also been shown for other RA-associated genetic markers [37].

In rare cases, mutations in a single gene such as MEFV (heterozygous mutations in exon 2 and exon 3) can also lead to the onset of RA as a consequence [38]. In these circumstances, population-based preventive genomic sequencing (PGS) for the genomic screening can help to identify the genetic health risk in the general population [39].

To the best of our knowledge, this is the first study of the genetic association of CTLA4 with the risk of RA in the Pakistani population where we found three significant associations, including one novel association. Our findings may have clinical implications with RA treatment outcome if confirmed in independent and larger studies; similar to those investigating the pharmacogenomics of drug therapy in RA [40, 41].

Supporting information
S1 Table. The sequence of CTLA-4 primers in 5’ to 3’ direction.

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References

1. McInnes IB, Schett G. Pathogenetic insights from the treatment of rheumatoid arthritis. The Lancet. 2017; 389(10086):2328–37.

2. Jalil F, Arshad M, Bhatti A, Jamal M, Ahmed M, Malik JM, et al. Progression pattern of rheumatoid arthritis: A study of 500 Pakistani patients. Pakistan journal of pharmaceutical sciences. 2017; 30(4):1219–23. PMID: 29039317

3. Siebert S, Tsoukas A, Robertson J, McInnes I. Cytokines as Therapeutic Targets in Rheumatoid Arthritis and Other Inflammatory Diseases. Pharmacological Reviews. 2015; 67(2):280. https://doi.org/10.1124/pr.114.009639 PMID: 25697599

4. Kaminsky ZA, Tang T, Wang S-C, Ptak C, Oh GHT, Wong AHC, et al. DNA methylation profiles in monozygotic and dizygotic twins. Nature Genetics. 2009; 41:240. https://doi.org/10.1038/ng.286 PMID: 19151718

5. Akhter E, Bilal S, Haque U. Prevalence of arthritis in India and Pakistan: a review. Rheumatology International. 2011; 31(7):849–55. https://doi.org/10.1007/s00296-011-1820-3 PMID: 21331574

6. Ortona E, Pierdominici M, Maselli A, Veroni C, Aloisi F, Shoenfeld Y. Sex-based differences in autoimmune diseases. Annali dell'Istituto superiore di sanità. 2016; 52(2):205–12. https://doi.org/10.4415/ANN_16_02_12 PMID: 27364395

7. Plant D, Flynn E, Mbarek H, Dieude P, Cornelis F, Arlestig L, et al. Investigation of potential non-HLA rheumatoid arthritis susceptibility loci in a European cohort increases the evidence for nine markers. Annals of the rheumatic diseases. 2010; 69(8):1548–53. https://doi.org/10.1136/ard.2009.121020 PMID: 20498205

8. Aslam MM, John P, Bhatti A, Jahangir S, Kamboh M. Vitamin D as a Principal Factor in Mediating Rheumatoid Arthritis-Derived Immune Response. BioMed Research International. 2019; 2019:1–12.

9. Firestein GS, McInnes IB. Immunopathogenesis of Rheumatoid Arthritis. Immunity. 2017; 46(2):183–96. https://doi.org/10.1016/j.immuni.2017.02.006 PMID: 28228278

10. Romo-Tena J, Gómez-Martín D, Alcocer-Varela J. CTLA-4 and autoimmunity: New insights into the dual regulator of tolerance. Autoimmunity reviews. 2013; 12(12):1171–6. https://doi.org/10.1016/j.autrev.2013.07.002 PMID: 23851140

11. Munoz-Valle JF, Valle Y, Padilla-Gutierrez JR, Parra-Rojas I, Rangel-Villalobos H, Vazquez del Mercado M, et al. The +49A>G CTLA-4 polymorphism is associated with rheumatoid arthritis in Mexican population. Clinica chimica acta; international journal of clinical chemistry. 2010; 411(9–10):725–8.

12. Scalapino KJ, Daikh DJ. CTLA-4: a key regulatory point in the control of autoimmune disease. Immuno-logical Reviews. 2008; 223(1):143–55.

13. Teft WA, Kirchhof MG, Madrenas J. A MOLECULAR PERSPECTIVE OF CTLA-4 FUNCTION. Annual Review of Immunology. 2006; 24(1):65–97.
14. Kristiansen OP, Larsen ZM, Pociot F. CTLA-4 in autoimmune diseases—a general susceptibility gene to autoimmunity? Genes and immunity. 2000; 1(3):170–84. https://doi.org/10.1038/sj.gene.6363655 PMID: 11196709

15. Walker EJ, Hirschfield GM, Xu C, Lu Y, Liu X, Lu Y, et al. CTLA4/ICOS gene variants and haplotypes are associated with rheumatoid arthritis and primary biliary cirrhosis in the Canadian population. Arthritis and rheumatism. 2009; 60(4):931–7. https://doi.org/10.1002/art.24412 PMID: 19333938

16. Tsukahara S, Iwamoto T, Ikari K, Inoue E, Tomatsu T, Hara M, et al. CTLA-4 CT60 polymorphism is not an independent genetic risk marker of rheumatoid arthritis in a Japanese population. Annals of the rheumatic diseases. 2008; 67(3):428–9. https://doi.org/10.1136/ard.2007.079186 PMID: 18292106

17. Aslam MM, John K-H, Bhatti A, Jahangir S, Feingold E, et al. Exploration of shared genetic susceptibility loci between type 1 diabetes and rheumatoid arthritis in the Pakistani population. BMC Research Notes. 2019; 12(1).

18. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis & Rheumatism: Official Journal of the American College of Rheumatology. 1988; 31(3):315–24.

19. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 2005; 21(2):263–5. https://doi.org/10.1093/bioinformatics/bth457 PMID: 15297300

20. Taylor PC, Keystone EC, van der Heijde D, Weinblatt ME, del Carmen Morales L, Reyes Gonzaga J, Barrett JC, Fry B, Maller J, Daly MJ. Haploview : analysis and visualization of LD and haplotype maps. 2005; 21(2):263–5. https://doi.org/10.1093/bioinformatics/bth457 PMID: 15297300

21. Fan Q, Zhang J, Cui Y, Wang C, Xie Y, Wang Q, et al. The synergic effects of CTLA-4/Fo xp3-related genotype s and chromosomal aberrations on the risk of recurrent spontaneous abortion among a Chinese Han population. Journal of human genetics. 2018; 63(5):579–87. https://doi.org/10.1038/s10038-018-0414-2 PMID: 29476189

22. Wang K, Zhu Q, Lu Y, Lu H, Zhang F, Wang X, et al. CTLA-4 +49 G/A Polymorphism Confers Autoimmune Disease Risk: An Updated Meta-Analysis. Genetic Testing and Molecular Biomarkers. 2017; 21(4):222–7. https://doi.org/10.1089/gtb.2016.0335 PMID: 28384040

23. Fan Q, Zhang J, Cui Y, Wang C, Xie Y, Wang Q, et al. The synergic effects of CTLA-4/Fo xp3-related genotype s and chromosomal aberrations on the risk of recurrent spontaneous abortion among a Chinese Han population. Journal of human genetics. 2018; 63(5):579–87. https://doi.org/10.1038/s10038-018-0414-2 PMID: 29476189

24. Li F, Yuan W, Wu X. Association of CTLA-4 polymorphism s with increased risks of myasthenia gravis. Annals of human genetics. 2018; 82(6):358–69. https://doi.org/10.1111/ahg.12262 PMID: 30009380

25. Nasr SA, Arce M, Khaja A, Fernandez M, Naser N, Elwasila S, et al. Role of ATG16L, NOD2 and IL23R in Crohn's disease pathogenesis. World journal of gastroenterology. 2012; 18(5):412–24. https://doi.org/10.3748/wjg.v18.i5.412 PMID: 22346247

26. Barrett JC, Clayton DG, Concannon P, Akolkar B, Cooper JD, Ericb HA, et al. Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. Nature genetics. 2010; 42(6):508–14. https://doi.org/10.1038/ng.582 PMID: 20453842

27. Stahl EA, Raychaudhuri S, Remmers EF, Xie G, Eyre S, Thomson BP, et al. Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. Nature genetics. 2010; 42(6):508–14. https://doi.org/10.1038/ng.582 PMID: 20453842

28. Fatthah SA, Ghattas MH, Saleh SM, Abo-Elmatty DM. Cytotoxic T-lymphocyte-associated protein 4 gene polymorphism is related to rheumatoid arthritis in Egyptian population. Archives of Physiology and Biochemistry. 2017; 123(1):50–3. https://doi.org/10.1080/13813455.2016.1230135 PMID: 27808571

29. Okada Y, Wu D, Trynka G, Raj T, Terao C, Ikari K, et al. Genetics of rheumatoid arthritis contributes to biology and drug discovery. Nature. 2014; 506(7488):376–81. https://doi.org/10.1038/nature12873 PMID: 24390342

30. Torres-Carrillo N, Ontiveros-Mercado H, Torres-Carrillo NM, Parra-Rojas I, Rangel-Villalobos H, Ramirez-Duenas MG, et al. The -319C/+49G/CT60G haplotype of CTLA-4 gene confers susceptibility to rheumatoid arthritis in Mexican population. Cell biochemistry and biophysics. 2013; 67(3):1217–28. https://doi.org/10.1007/s12013-013-9640-6 PMID: 23703680

31. Luterek-Puszynska K, Malinowski D, Paradowska-Gorycka A, Safranow K, Pawlik A. CD28, CTLA-4 and CCL5 gene polymorphisms in patients with rheumatoid arthritis. Clinical Rheumatology. 2017; 36(5):1129–35. https://doi.org/10.1007/s10067-016-3496-2 PMID: 27988912

32. Gregersen PK, Amos CI, Lee AT, Lu Y, Remmers EF, Kastner DL, et al. REL, encoding a member of the NF-kB family of transcription factors, is a newly defined risk locus for rheumatoid arthritis. Nature Genetics. 2009; 41(7):820–3. https://doi.org/10.1038/ng.395 PMID: 19503088
33. Eyre S, Bowes J, Diogo D, Lee A, Barton A, Martin P, et al. High-density genetic mapping identifies new susceptibility loci for rheumatoid arthritis. Nature Genetics. 2012; 44(12):1336–40. https://doi.org/10.1038/ng.2462 PMID: 23143596

34. Chen X, Hu Z, Liu M, Li H, Liang C, Li W, et al. Correlation between CTLA-4 and CD40 gene polymorphisms and their interaction in Graves’ disease in a Chinese Han population. BMC Medical Genetics. 2018; 19(1).

35. Choi H, Tabashidze N, Rossner P, Dostal M, Pastorkova A, Kong SW, et al. Altered vulnerability to asthma at various levels of ambient Benzo[a]Pyrene by CTLA4, STAT4 and CYP2E1 polymorphisms. Environmental Pollution. 2017; 231:1134–44. https://doi.org/10.1016/j.envpol.2017.07.057 PMID: 28807506

36. Sulaiman FN, Wong KK, Ahmad WAW, Ghazali WSW. Anti-cyclic citrullinated peptide antibody is highly associated with rheumatoid factor and radiological defects in rheumatoid arthritis patients. Medicine (Baltimore). 2019; 98(12):e14945-e.

37. Elshazli R, Settin A. Association of PTPN22 rs2476601 and STAT4 rs7574865 polymorphisms with rheumatoid arthritis: A meta-analysis update. Immunobiology. 2015; 220(8):1012–24. https://doi.org/10.1016/j.imbio.2015.04.003 PMID: 25963842

38. Migita K, Abiru S, Sasaki O, Miyashita T, Izumi Y, Nishino A, et al. Coexistence of familial Mediterranean fever and rheumatoid arthritis. Modern Rheumatology. 2012.

39. Cho MK. Preventive Genomic Sequencing in the General Population: Do PGS Fly? Am J Bioeth. 2015; 15(7):1–2. https://doi.org/10.1080/15265161.2015.1054160 PMID: 26147253

40. Umicɛev Mirkov M, Cui J, Vermeulen SH, Stahl EA, Toonen EJM, Makkinkie RR, et al. Genome-wide association analysis of anti-TNF drug response in patients with rheumatoid arthritis. Annals of the rheumatic diseases. 2013; 72(8):1375. https://doi.org/10.1136/annrheumdis-2012-202405 PMID: 23233654

41. Cui J, Stahl EA, Saevarsdottir S, Miceli C, Diogo D, Trynka G, et al. Genome-wide association study and gene expression analysis identifies CD84 as a predictor of response to etanercept therapy in rheumatoid arthritis. PLoS Genet. 2013; 9(3):e1003394-e.