Identification of the Rare, Four Repeat Allele of *IL-4* Intron-3 VNTR Polymorphism in Indian Populations

Henu Kumar Verma[1], Aditya Nath Jha[1], Prafulla Kumar khodiar[1], Pradeep Kumar Patra[1,2], Lakkakula Venkata Kameswara Subrahmanya Bhaskar[1*]

[1]Sickle Cell Institute Chhattisgarh, [2]Department of Biochemistry, Pt. JNM Medical College, Raipur, Chhattisgarh, India

ABSTRACT

Background: Cytokines are cell signaling molecules which upon release by cells facilitate the recruitment of immune-modulatory cells towards the sites of inflammation. Genetic variations in cytokine genes are shown to regulate their production and affect the risk of infectious as well as autoimmune diseases. Intron-3 of interleukin-4 gene (*IL-4*) harbors 70-bp variable number of tandem repeats (VNTR) that may alter the expression level of *IL-4* gene. **Objective:** To determine the distribution of IL-4 70-bp VNTR polymorphism in seven genetically heterogeneous populations of Chhattisgarh, India and their comparison with the finding of other Indian and world populations. **Methods:** A total of 371 healthy unrelated individuals from 5 caste and 2 tribal populations were included in the present study. The *IL-4* 70-bp VNTR genotyping was carried out using PCR and electrophoresis. **Results:** Overall, 3 alleles of *IL-4* 70-bp VNTR (a2, a3 and a4) were detected. The results demonstrated the variability of the IL-4 70-bp VNTR polymorphism in Chhattisgarh populations. Allele a3 was the most common allele at the 70-bp VNTR locus in all populations followed by a2 allele. This study reports the presence four repeat allele a4 at a low frequency in the majority of the Chhattisgarh populations studied. Further, the frequency of the minor allele (a2) in Chhattisgarh populations showed similarity with the frequencies of European populations but not with the East Asian populations where the a2 allele is a major allele. **Conclusions:** Our study provides a baseline for future research into the role of the *IL-4* locus in diseases linked to inflammation in Indian populations.

Verma H, et al. Iran J Immunol. 2016; 13(2):124-131.

Keywords: Cytokine, IL-4, Indian Populations, VNTR

---

*Corresponding author: Dr. Lakkakula VKS Bhaskar, Department of Biochemistry, Sickle Cell Institute, Chhattisgarh Genetic Lab, Medical College, Raipur, Chhattisgarh, India, e-mail: lvksbhaskar@gmail.com

INTRODUCTION

Cytokines are cell-signalling molecules released by cells, which can stimulate the movement of immune modulatory cells towards the sites of infection and inflammation and plays central role in host inflammatory responses. It also plays the crucial role of coordination between cell-mediated and humoral immune responses. The outcome of inflammation is characterized by the complex interplay and balance between pro- and anti-inflammatory cytokines (1). Indeed, the level of cytokine, the nature of the target cell and their downstream response, the nature of produced cytokines as well as the temporal and sequential cytokine action greatly influences the outcome. In addition, the genetic variations in cytokine genes are known to modulate their differential expression and hence the balance between pro-inflammatory and anti inflammatory immune response (2).

Interleukin 4 (IL-4) is an anti-inflammatory cytokine produced by CD4+ Th2 cells, basophils and mast cells and regulates balance between TH1 and TH2 immune response, induces immunoglobulin class switching and humoral immunity. It promotes TH2 cell differentiation while inhibiting the TH1 cell differentiation and plays a dominant role in immunopathology of diseases (3-6). The gene which codes for human IL-4 has been mapped to chromosome 5 (5q31-33) (7). Studies have documented that the genetic variations in *IL-4* (-590C/T, -34C/T and a 70-base pair (bp) VNTR in intron-3: rs2243250, rs2070874 and rs79071878 respectively) regulate serum levels of IL-4 (8,9). Further, it has been well established that these three variations are in strong linkage disequilibrium (LD) (10,11), therefore, in the present study, we investigated the *IL-4* intron-3 VNTR polymorphism. Commonly, two and three copies of this VNTR have been observed in most of the world populations.

Today, it is well established that each of the Indian populations has their own unique genetic architecture due to the long time isolation and endogamy practice. Previous studies also demonstrated that some alleles are confined to the populations of Chhattisgarh and genetic isolation of these populations (12). Although, several studies have documented the distribution of *IL-4* polymorphisms in various ethnicities of India (10,11), to the best of our knowledge, no studies have investigated the *IL-4* variants in Chhattisgarh populations. Further, *IL-4* acts as an anti-inflammatory mediator and is shown to regulate many chronic and inflammatory diseases, in the present study we analyzed the *IL-4* VNTR in the populations of Chhattisgarh.

MATERIALS AND METHODS

Chhattisgarh is situated between 17 to 23.7 degree North latitudes and 8.40 to 83.38 east longitude. Chhattisgarh is inhabited by a diverse population of tribes, castes, religious and migrant populations. Three ml of venous blood samples were collected from 371 individuals belonging to 7 Chhattisgarh ethnic populations (5 castes and 2 tribes) to analyse the variations in the allele frequency spectrum of *IL-4* VNTR. All individuals were normal and healthy. Informed written consent was obtained from each individual at the time of blood collection.

Genomic DNA was isolated by standard protocols using phenol-chloroform extraction and ethanol precipitation method (13). Polymerase chain reaction-electrophoresis method was adopted for genotyping *IL-4* 70-bp VNTR (10). Briefly, primers flanking

IL-4 70-bp VNTR resulted in a 389-bp (a2 allele; 2 repeats of 70 bp), 459-bp (a3 allele; 3 repeats of 70 bp) and 529-bp (a4 allele; 4 repeats of 70 bp) (Figure 1). All amplifications were performed in a 10-µl reaction volume comprising 2x Emerald Amp GT PCR master mix, 1 pmol of forward primer, 1 pmol of reverse primer, and 40 ng of genomic DNA. To score the genotypes, amplified PCR products were resolved through 2% agarose gel electrophoresis and visualized using gel documentation instrument (Omega 16vS Molecular Imaging and Analysis System). Allele frequencies in each population were determined by direct counting. Hardy-Weinberg equilibrium (HWE) proportions were calculated by chi-square goodness of fit test with 2 degrees of freedom. The genotypes of *IL-4* 70-bp VNTR were compared with world populations.

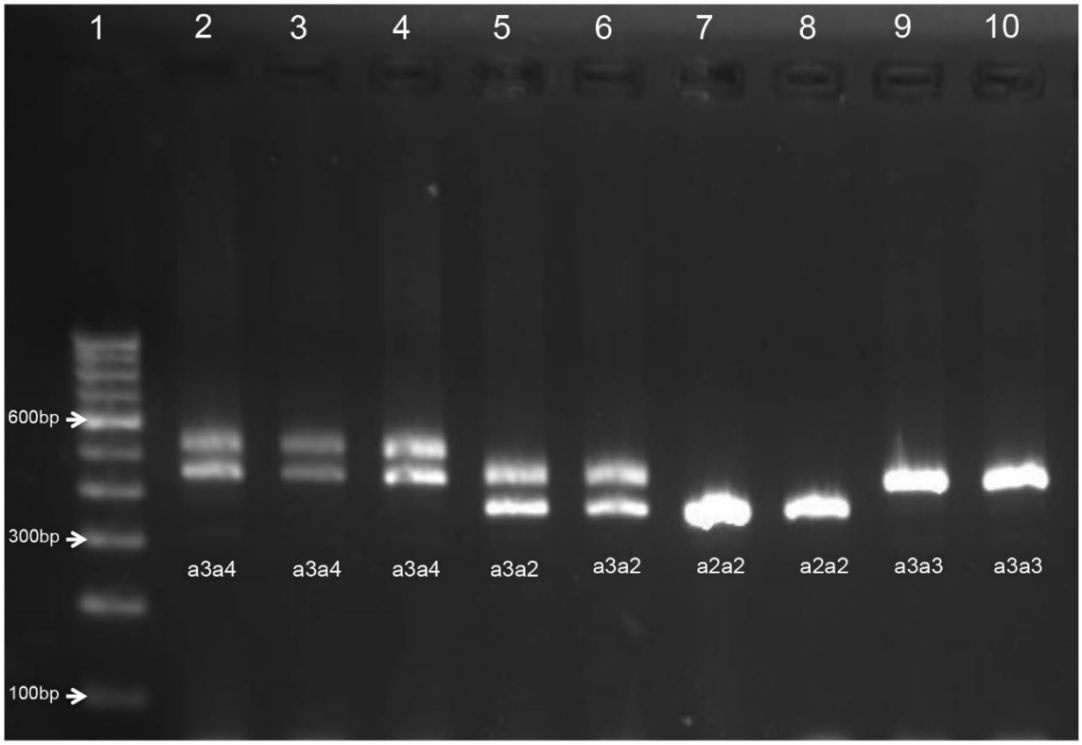

Figure 1. Agarose gel showing PCR products of different alleles of IL-4 70-bp VNTR. Homozygote alleles are characterized by one band, heterozygotes by two bands. From left: lane 1: 100 bp DNA ladder; lane 2, 3, 4: a3a4; lane 5, 6: a2a3; lane 7, 8: a2a2 and lane 9, 10: a3a3.

RESULTS

The polymorphic *IL-4* 70-bp VNTR was examined by PCR in 7 Chhattisgarh populations. The distributions of allele frequencies and genotype frequencies are shown in Figure 1 and Table 1, respectively. We observed *IL-4* 70-bp VNTR, allele a3 was the most frequent allele followed by a2 in all populations (Figure 2 and Table 1). Interestingly, allele a4 was found at a low frequency in the form of heterozygotes (Figure 1), in five of the seven populations studied (Figure 2 and Table 1). None of the populations showed deviation from Hardy-Weinberg equilibrium for *IL-4* 70-bp VNTR genotypes (Table 1).

Table 1: Genotype frequencies of *IL-4* VNTR polymorphism in populations of Chhattisgarh.

Population	Baniya (n=51)	Ganda (n=32)	Gond (n=31)	Kurmi (n=57)	Ravat (n=30)	Satnami (n=25)	Teli (n=146)	Total (n=371)
a3a3	36 (70.6)	23 (71.9)	18 (60.0)	25 (43.9)	21 (70.0)	15 (60.0)	76 (52.1)	214
a2a3	10 (19.6)	8 (25.0)	9 (30.0)	30 (52.6)	8 (26.7)	8 (32.0)	55 (37.7)	128
a2a2	3 (5.9)	0 (0.0)	2 (6.7)	2 (3.5)	0 (0.0)	2 (8.0)	14 (9.6)	23
a3a4	2 (3.9)	1 (3.1)	1 (3.3)	0 (0.0)	1 (3.3)	0 (0.0)	1 (0.7)	6
HWE-p	0.189	0.698	0.811	0.052	0.674	0.538	0.647	0.634

HWE-p: Hardy-Weinberg equilibrium p value

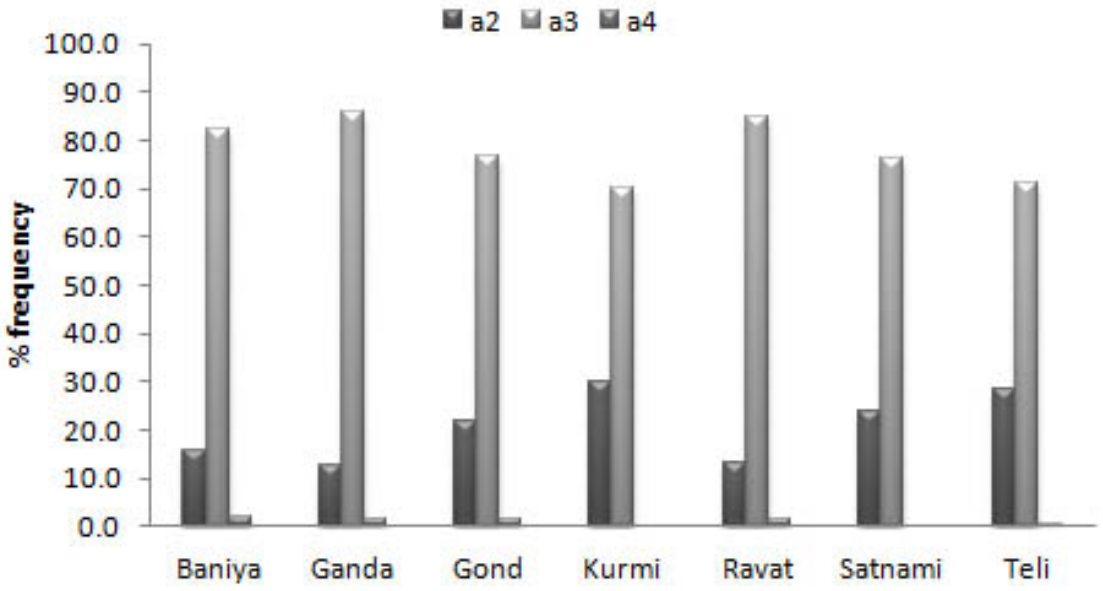

Figure 2. Frequency distribution of *IL-4* VNTR alleles in seven ethnically different populations of Chhattisgarh.The length of each bar represents the percent frequency of respective allele. a2, a3 and a4 are *IL-4* VNTR alleles.

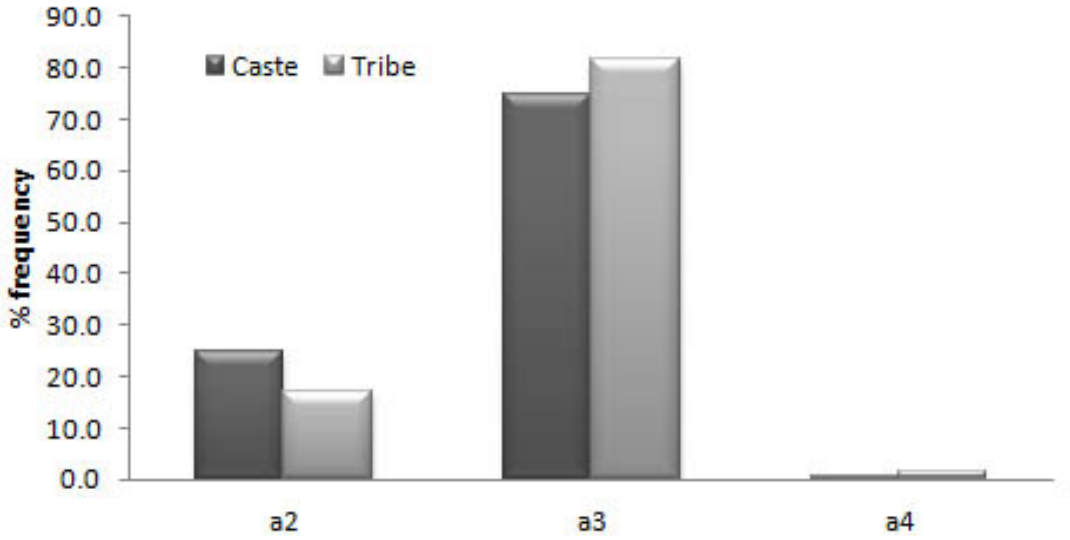

Figure 3. Frequency distribution of *IL-4* VNTR alleles in caste and tribal populations of Chhattisgarh. The length of each bar represents the percent frequency of respective allele. a2, a3 and a4 are *IL-4* VNTR alleles.

Segregation of study populations into caste and tribes also showed a similar trend of allelic distribution (Figure 3). Further, *IL-4* 70-bp VNTR genotypes collected from published papers (11,14,10,15-17,9,18-23) were categorised as 22, L2 and LL wherein 2 represent a short allele (two repeats; a2 allele), L represents long allele (three and four repeats; a3 and a4 allele) and plotted as a forest plot (Figure 4). Comparison of the *IL-4* 70-bp VNTR a2 allele among global populations revealed that East Asian and Southeast Asian populations have very high a2 allele frequency (65 to 80%), followed by populations of north-west coast of Africa and Europe (Figure 4). The frequencies of a2 allele in Chhattisgarh populations showed similarity with the frequencies of European populations.

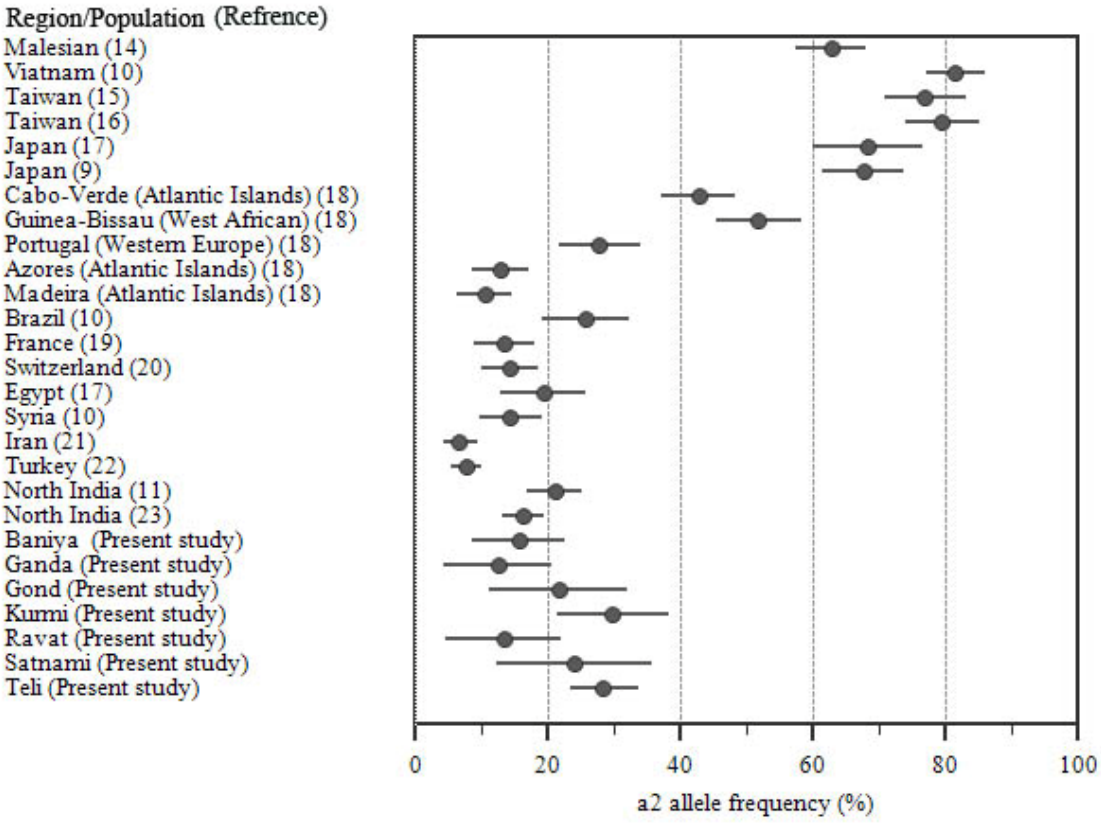

Figure 4. The frequency of IL-4 70-bp VNTR in the current study compared to the world populations. The horizontal whiskers in the forest plot show a2 allele frequency with 95% confidence intervals.

DISCUSSION

To the best of our knowledge, there were no reports regarding the population specific allele frequency distribution for *IL-4* 70-bp VNTR in the Chhattisgarh populations. Analysis of *IL-4* 70-bp VNTR revealed the polymorphic nature of this VNTR in study populations. Allele a3 was the most common allele at the 70-bp VNTR locus in all

populations followed by a2 allele. This study also reports the presence of a four repeat allele, a4, in the majority of the Chhattisgarh populations studied.

The human IL-4 is a strong anti-inflammatory agent and is known to induce differentiation and expansion of Th2 cytokine-producing eosinophils (24). The *IL-4* 70-bp VNTR polymorphism is determined by the number of repeats ranging from 2 to 4 copies. The frequent allelic form consists of 3 repeats (a3) followed by 2 repeat (a2) allele. Sequence alignment of intron-3 region revealed that a2, a3 and a4 alleles are present only in humans, whereas a1 allele has been observed in other primates (10). However, in humans, the a1 allele has been reported very rarely (17). In the present study we observed that a3 is the major allele followed by a2 and a4. Measurement of IL-4 production in peripheral Th cells in individuals has previously revealed that the two repeat (a2) allele is a high producer of IL-4 compared to a3 allele (9). The a2 allele frequency among world populations revealed wide variations and a2 allele distribution in Chhattisgarh populations showed close proximity with European populations. The four repeat allele is very rare and has been reported only in few populations (8). In the present study, we report the presence of this rare a4 allele in 5 out of seven populations of Chhattisgarh, India. A previous study with a large number of samples (3452 samples from 76 distinct populations) across India failed to detect the a4 allele (10). Populations used in their study represented only one population from Chhattisgarh (10). This finding, again, supports the uniqueness of each Indian populations and their heterogeneous genetic architecture.

Despite the extensive debate regarding the ethnic and geographic differences in genetic studies, it seems evident that different allelic variants may contribute to disease susceptibility in different populations. This may reflect linkage with other variants in the same gene rather than association with these VNTR per se. Previous studies report that IL-4 70-bp VNTR is in strong LD with its other promoter polymorphisms (-590C/T and -34C/T) (10). Our study adds to the knowledge on the distribution of *IL-4* 70-bp VNTRs among the Indian populations.

ACKNOWLEDGMENTS

The authors acknowledge funding from the Sickle Cell Institute Chhattisgarh, Government of Chhattisgarh and CCOST, Government of Chhattisgarh (Project Ref. No. 2740/CCOST/MRP/2015).

REFERENCES

1. Tayal V, Kalra BS. Cytokines and anti-cytokines as therapeutics--an update. Eur J Pharmacol. 2008; 579:1-12.
2. Ollier WE. Cytokine genes and disease susceptibility. Cytokine. 2004; 28:174-8.
3. Wurtz O, Bajenoff M, Guerder S. IL-4-mediated inhibition of IFN-gamma production by CD4+ T cells proceeds by several developmentally regulated mechanisms. Int Immunol. 2004; 16:501-8.
4. Murphy KM, Reiner SL. The lineage decisions of helper T cells. Nat Rev Immunol. 2002; 2:933-44.
5. Guo L, Hu-Li J, Zhu J, Watson CJ, Difilippantonio MJ, Pannetier C et al. In TH2 cells the IL-4 gene has a series of accessibility states associated with distinctive probabilities of IL-4 production. Proc Natl Acad Sci U S A.. 2002; 99:10623-8.

6. Banchereau J, Briere F, Galizzi JP, Miossec P, Rousset F. Human interleukin 4. J Lipid Mediat Cell Signal. 1994; 9:43-53.
7. Sutherland GR, Baker E, Callen DF, Hyland VJ, Wong G, Clark S, et al. Interleukin 4 is at 5q31 and interleukin 6 is at 7p15. Hum Genet. 1988; 79:335-7.
8. Mout R, Willemze R, Landegent JE. Repeat polymorphisms in the interleukin-4 gene (IL-4). Nucleic Acids Res. 1991; 19:3763.
9. Nakashima H, Miyake K, Inoue Y, Shimizu S, Akahoshi M, Tanaka Y et al. Association between IL-4 genotype and IL-4 production in the Japanese population. Genes Immun. 2002; 3:107-9.
10. Jha AN, Singh VK, Kumari N, Singh A, Antony J, van Tong H et al. IL-4 haplotype -590T, -34T and intron-3 VNTR R2 is associated with reduced malaria risk among ancestral indian tribal populations. PloS one. 2012; 7:e48136.
11. Mishra A, Jha AN, van Tong H, Singh VK, Gomes CE, Singh L, et al. Analysis of genetic variants in the IL-4 promoter and VNTR loci in Indian patients with Visceral Leishmaniasis. Hum Immunol. 2014; 75:1177-81.
12. Kashyap VK, Guha S, Sitalaximi T, Bindu GH, Hasnain SE, Trivedi R. Genetic structure of Indian populations based on fifteen autosomal microsatellite loci. BMC Genet. 2006; 7:28.
13. Sambrook J, Russell DW. Molecular Cloning: A Laboratory Manual. vol v. 1. Cold Spring Harbor Laboratory Press; 2001.
14. Vasudevan R, Norhasniza MN, Patimah I. Association of variable number of tandem repeats polymorphism in the IL-4 gene with end-stage renal disease in Malaysian patients. Genet Mol Res. 2011; 10:943-7.
15. Wu MC, Huang CM, Tsai JJ, Chen HY, Tsai FJ. Polymorphisms of the interleukin-4 gene in chinese patients with systemic lupus erythematosus in Taiwan. Lupus. 2003; 12:21-5.
16. Tsai FJ, Chang CH, Chen C, Hsia TC, Chen HY, Chen WC. Interleukin-4 gene intron-3 polymorphism is associated with transitional cell carcinoma of the urinary bladder. BJU Int. 2005; 95:432–5.
17. Hegab AE, Sakamoto T, Saitoh W, Massoud HH, Massoud HM, Hassanein KM et al. Polymorphisms of IL-4, IL13, and ADRB2 genes in COPD. Chest. 2004; 126:1832-9.
18. Berenguer AG, Camara RA, Brehm AD, Oliveira S, Fernandes AT. Distribution of polymorphisms IL-4-590 C/T and IL-4 RP2 in the human populations of Madeira, Azores, Portugal, Cape Verde and Guinea-Bissau. Int J Mol Epidemiol Genet. 2012; 3:179-83.
19. Buchs N, Silvestri T, di Giovine FS, Chabaud M, Vannier E, Duff GW et al. IL-4 VNTR gene polymorphism in chronic polyarthritis. The rare allele is associated with protection against destruction. Rheumatology (Oxford). 2000; 39:1126-31.
20. Genevay S, Di Giovine FS, Perneger TV, Silvestri T, Stingelin S, Duff G et al. Association of interleukin-4 and interleukin-1B gene variants with Larsen score progression in rheumatoid arthritis. Arthritis Rheum. 2002; 47:303-9.
21. Salimi S, Mohammadoo-Khorasani M, Yaghmaei M, Mokhtari M, Moossavi M. Possible association of IL-4 VNTR polymorphism with susceptibility to preeclampsia. Biomed Res Int. 2014; 2014:497031.
22. Inanir A, Tural S, Yigit S, Kalkan G, Pancar GS, Demir HD et al. Association of IL-4 gene VNTR variant with deep venous thrombosis in Behcet's disease and its effect on ocular involvement. Mol Vis. 2013; 19:675-83.
23. Shukla RK, Kant S, Bhattacharya S, Mittal B. Association of cytokine gene polymorphisms in patients with chronic obstructive pulmonary disease. Oman Med J. 2012; 27:285-90.
24. Chen L, Grabowski KA, Xin JP, Coleman J, Huang Z, Espiritu B et al. IL-4 induces differentiation and expansion of Th2 cytokine-producing eosinophils. J Immunol. 2004; 172:2059-66.