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POLYMORPHISM IN INTERLEUKIN-4 GENE AND THE RISK OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE IN A NORTH INDIAN POPULATION : A CASE-CONTROL STUDY

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ABSTRACT

The genetic variability (polymorphisms) can be a factor in COPD risk. We investigated the potential relationship between COPD and polymorphism of the interleukin 4 (IL-4 70 bp repeats) gene. In our study we observed the association between IL-4 (70 bp repeats) genotypes and COPD risk. A total of 400 subjects (200 cases and 200 controls). Using a case control design individuals were genotyped for IL-4 using polymerase chain reaction (PCR) and restriction fragment length polymorphism techniques. The data obtained were analyzed using multiple logistic regressions by the help of SPSS ver 11.5. The OR of combined genotype of IL-4(Rp1-Rp2+Rp1) was showed 2 fold increase risk of COPD. In smokers, 5 fold significantly increased risk of COPD was observed with Rp1 genotype of IL-4. The genetic polymorphism in cytokine and IL-4 (70 bp repeats) gene may play an important role in the development of COPD.

KEY WORDS: Chronic obustructive pulmonary disease (COPD), Interleukin-4 (IL-4), Polymorphism, Cytokine, 70 bp repeats (VNTR).

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by the presence of airflow obstruction that results from intrinsic airways disease (*e.g.,* chronic bronchitis or emphysema). In emphysema, the parenchymal destruction with associated loss of elastic recoil, leads to a reduction in the tethering or distending function of the lung parenchyma on airways. Chronic bronchitis is characterized by excessive secretion of bronchial mucus which is manifested by productive cough for 3 months or more in at least 2 consecutive years in the absence of any other disease. Most patients who have only chronic bronchitis do not have any substantive airflow limitation and should not be classified as having COPD (Bartolome, 2003).

Chronic obstructive pulmonary disease (COPD) includes chronic pulmonary emphysema and chronic bronchitis. It is the fourth leading cause of death in the world with an increase in prevalence and mortality (Pauwels *et al.,* 2001; Mannino *et al.,* 2002). It is generally accepted that cigarette smoking is the major risk factor for COPD. However, only 15% of smokers develop symptomatic COPD, implying undue susceptibility compared with the remainder of population at large (Burrow *et al.,* 1977; Sandford and Silverman, 2002). In developing countries, there are several other risk factors, including air pollution (particularly indoor air pollution from burning fuels, poor diet and occupational exposure to various dusts. COPD involves inflammation in the respiratory tract (Barnes, 2003). Cytokines play a critical role in the orchestration of chronic inflammation in all diseases, including COPD (Barnes, 2001 & 2003).

IL-4 is a prototypic member of Th2 cytokines. It is a potent anti-inflammatory cytokine. It reduces the production of proinflammatory cytokines and destructive enzymes by monocytes. The *IL-4* gene is located on the long arm of chromosome 5 (5q23–31) together with other Th2 cytokine genes. It is present in a cluster of cytokine genes (*IL-3, -5, -9, -13,* and *-15,* granulocyte colony-stimulating factor, and interferon regulatory factor) (Lebeau *et al.* 1989). This gene contains a variable number of tandem repeat polymorphism located in third intron. It consists of three 70-bp repeats in intron-3, a rare allele with two repeats and much rarer with four repeats (Mout *et al.,* 1991).

On the other hand, *IL-4* VNTR gene polymorphism has been associated with rheumatoid arthritis and IL-4 C-589T with asthma (Walley and Cookson, 1996; Buchs *et al.,* 2000). Not much information is available regarding the role of *IL-4* gene polymorphism in COPD. There are only a few published studies on SNPs cytokines in COPD patients at international as well as national levels and hence the present work was aimed to determine the impact of genotype on the risk of COPD by evaluating

genetic polymorphism in *interleukin- 4* gene. This may lead to detection of the host genotype susceptibility for COPD.

1. MATERIAL AND METHODS

1.1. Study Subjects

200 pathologically confirmed COPD patients have been recruited from Government Medical College and Hospital, Sector 32, Chandigarh, Government Ayurvedic College, Peprola (HP) and Government Ayurvedic Hospitals Hamirpur (HP). Detailed data regarding age, education, history of cigarette smoking, spouse's smoking history has been obtained from an interviewer administered questionnaire. Informed consent was also obtained from both the cases and the controls. The present study is an extension of the earlier one carried out in the laboratory by Dr. Hitender Thakur (2008) and the samples collected by him have been used in the present study. The study was approved by human subject's ethical committees of the involved hospitals/ institutions.

Exposure to passive smoking was assessed by questioning spouse's smoking history. 200 age and sex matched apparently healthy controls were recruited in the study. The following inclusion and exclusion criteria were kept in mind during blood sample collections.

Inclusion criteria:

a) Chronic respiratory symptoms and signs such as cough, breathlessness, wheezing.

b) Forced expiratory volume in 1 sec (FEV1) of less than 80% predicted.

c) Ratio of FEV1/forced vital capacity (FVC) =0.7.

d) Subjects in age range of 19yrs to 65 yrs were included as COPD primarily afflicts this age group.

e) All subjects irrespective of smoking, tobacco Intake, alcohol consumption status were included in the study after obtaining informed consent. Smoker healthy controls were asked to undergo chest radiography to rule out any abnormal pulmonary function.

Exclusion criteria :

Following parameters were included in exclusions criteria during our sample collections:

a) Subjects with history of asthma were excluded from study.

b) Subjects with malignancy like lung cancer or with autoimmune disease were excluded to avoid spurious results.

c) In the healthy smoking group individuals with either abnormal pulmonary function or abnormal findings on chest radiography were excluded.

2.3. Collection of blood samples

2 ml of peripheral blood were obtained both from cases and controls in EDTA coated vials and stored at -80^{0}C.

2.4. Isolation of genomic DNA from blood

Genomic DNA was isolated by SDS / proteinase K and phenol chloroform method (http:www.genome-ou-edu/protocols – book/ protocols- partII.html and Maniatis, 1989) and stored at -20^{0}C for further use.

2.5. Reagents used in study

1**. Tris-EDTA buffer (pH 7.6)-** The buffer was prepared by adding 1.214 g of Tris (hydroxy methyl amino methane) (Hi Media, India) in 10 ml of distilled water and taking it as stock (1M), its pH was adjusted to 7.0. 0.5M of EDTA (ethylene diamine tetraacetetic acid) (Hi Media, India) was prepared by dissolving 1.46g in 10ml of distilled water and its pH was adjusted to 8.0 with sodium hydroxide. Then 1ml of Tris stock (10mM) and 0.2 ml of EDTA stock (0.5 mM) were added to 98.8 ml of distilled water to make the working solution. The solution was sterilized by autoclaving.

2. **20X sodium citrate buffer (SSC)** – 17.53 g of NaCl (Hi Media, India) was dissolved in 80 ml of distilled water and then 10 ml of 0.3M sodium citrate (Hi Media, India) was added. The pH of the solution was adjusted to 7.0 with HCI. Then the final volume was made to 100ml with distilled water. To make the final working solution, 1 ml of 20X SSC buffer was added to 19 ml of distilled water. The solution was sterilized by autoclaving.

3. **Sodium acetate (0.2M) pH 7.2** –16.40g of sodium acetate (Hi Media, India) was dissolved in 800ml distilled water. Its pH was adjusted to 7.2 with acetic acid. Then the final volume was made to 1000ml with distilled water. The solution was sterilized by autoclaving.

4. **10% sodium dodecyl sulphate (SDS) pH 7.2** – The solution was prepared by dissolving 10g of SDS (sodium dodecyl sulphate) (Hi Media, India) in 100ml distilled water and stored at room temperature.

5. **50X Tris acetate buffer (TAE)** - 24.2 g of tris base was dissolved in 85 ml of distilled water. Then 10 ml of 0.5 M EDTA and 5.71 ml of glacial acetic acid were added. The solution was sterilized by autoclaving.

6. **Ethidium bromide (10mg/ml)** - 1 g of ethidium bromide (EtBr) (Hi Media, India) was dissolved in 100 ml of water. The solution was stirred on a magnetic stirrer. The bottle was wrapped around with aluminium foil and stored at 4°C.

2.6. Protocol for DNA Isolation:

1. Frozen blood samples were thawed.
2. 1ml of blood was added to 2.5ml eppendorf. Then 0.8ml IX SSC buffer was added and the contents were mixed. The mixture was centrifuged at 10,000 rpm for 5 minutes in a cooling centrifuge (4°C).
3. 1ml of supernatant was removed and discarded into

disinfectant.

4. Again 1ml of 1X SSC buffer, was called vortexed and centrifuged as above for 5 minutes. The supernatant was removed taking care of the pellet.
5. 375ml of 0.2M sodium acetate was added to each pellet and vortexed briefly. It was followed by the addition of 25ml of 10% SDS and 5ml of proteinase K (Bangalore Genei, India) (20mg/ml H_2O), vortexed briefly and incubated for 1 hour at 55^0C.
6. After checking that pellet was dissolved we added 120ml of phenol (60ml)/chloroform (57.6ml)/isoamyl alcohol (2.4ml) (Bangalore Genei, India) to each eppendorf and vortexed for 30 seconds. The samples were centrifuged for 5 minutes at 10,000 rpm.
7. The upper layer was carefully transferred to 1.5ml microcentrifuge tube, and 1ml of cold 100% ethanol was added. The mixture was mixed gently to precipitate the DNA.
8. The tube was kept at -20^0C overnight and then centrifuged at 5,000 rpm for 2 min in a cooling centrifuge (4^0C).
9. The supernatant was drained and the pellet was dissolved in 100ml of Tris-EDTA.
10. It was stored at –20°C after checking in 0.8% agarose gel (Hi Media, India) (Fig.1).

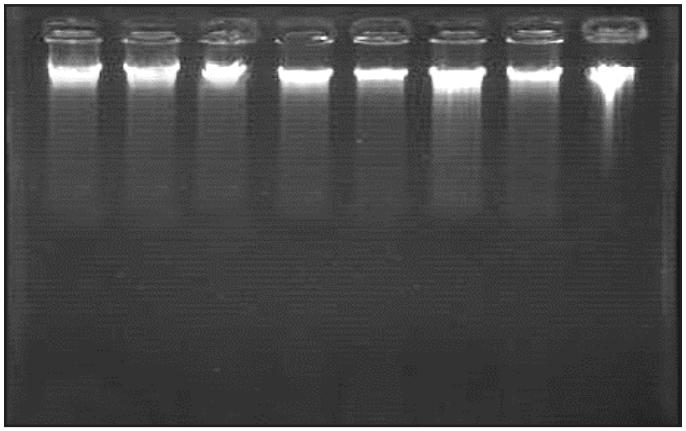

Fig. 1: Extracted Genomic DNA from the blood samples.

2.7. Estimation of isolated genomic DNA

The concentration of the isolated DNA was calculated by spectrophotometric method using Beckmann, UV-visible scanning spectrophotometer.

- 10µl of DNA was transferred to 990 ml Tris – EDTA buffer (pH 7.5) in a 1ml quartz cuvette.
- The contents were mixed well and the absorbance was noted at 260 nm on spectrophotometer.
- The quantity of DNA was calculated using the standard that 1ml of a solution with an A260 nm of 1.0 is equivalent to 50mg of double stranded DNA.

2.8. PCR- RFLP analysis

The region containing 70 bp VNTR within intron 3 of the *IL-4* gene was amplified using the primers (Sigma Aldrich, India) given in Table 1 (Wu *et al.*, 2003).

Table 1: Primer sequence used for amplification of *IL-4* VNTR (Wu *et al.*, 2003)

Gene	Primer	Primer sequences
IL-4	Forward	5′-AGGCTGAAAGGGGGAAAGC-3′
	Reverse	5'- CTGTTCACCTCAACTGCTCC -3'

PCR reactions were carried out in Bio-Rad Thermo cycler using the conditions given in Table 4.8. 100ng of genomic DNA was amplified in a 25 ml final volume reaction mixture containing 10X KCl buffer (2.5mL), 25mM $MgCl_2$ (2.5mL), 10 mM of each dNTP (0.5mL) (MBI Fermentas, US), 10mM of each primer (0.5mL), 5.0mg/ml BSA (2.5mL) (Bangalore Genei, India), 5U/1mL *Taq* DNA polymerase (0.3mL) (MBI Fermentas, US) and make volume to 25.0mL by adding double distilled water (Fig. 2).

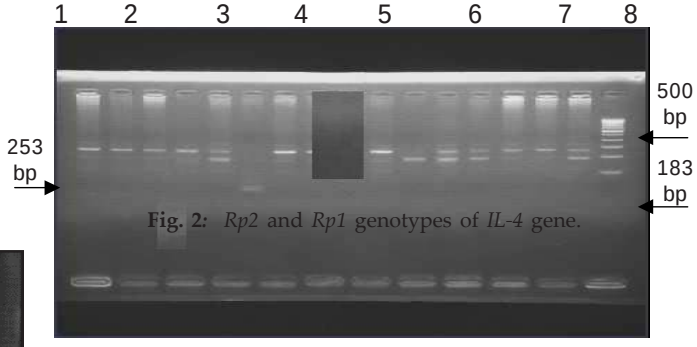

Fig. 2: *Rp2* and *Rp1* genotypes of *IL-4* gene.

Table 2: PCR conditions for *IL-4* VNTRs

S. N.	Steps	Temperature	Duration	No. of cycles
I	Denaturation 1	94°C	5 min	1
II	Denaturation 2	94°C	30 s	35
III	Annealing	55°C	30s	
IV	Extension 1	72°C	30s	
V	Extension 2	72°C	10 min	1

The *IL-4* intron 3 polymorphism PCR products, including 70 bp VNTR, were directly visualized on 2.5% agarose gel (Hi Media, India), and each allele was recognized according to its size. The *Rp1* and *Rp2* alleles were of 183 and 253 bp, respectively (Fig.2).

Lane 1,5 and 6: *Rp2* 253bp

Lane 2: *Rp1-Rp1* 183 bp

Lane 8: 100 bp DNA marker (Bangalore Genei)

Lane 3,4 and 7 : *Rp1- Rp2* 183, 253bp

3. STATISTICAL ANALYSIS

Age, smoking status and genotypes of all genes were tabulated for cases and controls. T-tests and $\times^2$ square tests were performed to find out any difference between cases and controls according to age, smoking status *etc*.

The association between polymorphisms in all genes with the risk of COPD was estimated by computing ORs

and 95%CI using a Multivariate Logistic Regression Analysis which included several potential confounding variables (*e.g.* age, gender and smoking status). Statistical analysis was performed using SPSS, version 11.5 and Epical Version 3.2.

Correlation analysis was done to establish correlation between smoking in COPD and the various cytokine genes.

4. RESULTS

4.1. Characteristics of Study Sample

The study included 200 COPD cases and 200 controls. The cases and controls were well matched according to age and sex. The mean age (±SD) was found to be 52.10±15.50 for cases and 44.26±14.42 for controls. Individuals were divided into three habit groups: smokers and non-smokers, users and non-users of tobacco and users and nonusers of alcohol. In present study smokers and non-smokers were used. Baseline characteristics such as age, smoking status *etc*. for cases and controls have been summarized in Table 3. 63% of the cases and 50.5% of the controls were smokers. There was a significant increase in the risk of COPD (OR 1.67, p=0.012) amongst smokers. Males were observed to have an increase in the risk of COPD rather than females (OR 1.53, p=0.039).

Table 3: Baseline characteristics of all COPD cases and controls

Cat.	*Variables*	Cases (%)	Control (%)	OR 95 (%CI)	p-valu
1.	**Sample size**	200	200		
2.	**Mean ±SD**	52.10±15.50	44.26±14.42	-	-
3.	**Gender**				
	Female	66 (33%)	86 (43%)	1	-
	Male	134 (67%)	114 (57%)	1.53(1.02-2.29)	0.039
4.	**Smoking status**				
	Never smokers	74 (37%)	99 (49.5%)	1	-
	Smokers	126 (63%)	101 (50.5%)	1.67(1.12-2.48)	0.012

Age is reported as mean =± SD, p-value of less than 0.05 was considered significant.

4.2. DISTRIBUTION OF *IL-4* (70 bp repeats) POLYMORPHISM AMONG CASES AND CONTROLS

The frequency of homozygous genotype (*Rp2*) in *IL-4* (intron 3, 70 bp repeats), of IL-4 was more in controls (66%) as compared to that COPD patients (48%). The frequency of heterozygous genotype (*Rp1-Rp2*) was marginaly higher in COPD patients (29.5%) as compared to controls (27%). In case of homozygous genotype (*Rp1*) the frequency was higher in COPD patients (22.5%) (Table 4).

Table 4: Distribution of *IL-4* (intron 3, 70 bp repeats) genotypes

IL-4 Genotypes	CASES(200)	CONTROLS(200)
Rp2-Rp2	96(48%)	132(66%)
Rp1-Rp1	45(22.5%)	14(7%)
Rp1-Rp2	59(29.5%)	54(27%)

The frequency of *Rp1* allele was found to be high in the cases (0.37), but lower in controls (0.21) reverse was the use for *Rp2* allele (Table 5).

Table 5: Observed allelic frequencies

Alleles	CASES	CONTROLS
Rp1	0.37	0.21
Rp2	0.63	0.79

4.3. ASSOCIATION BETWEEN *IL-4* (70 bp repeats) GENOTYPES AND COPD RISK

Positive association was observed between *IL-4* genotypes and overall risk of COPD. In case of *Rp1*, 4 fold increased risk of COPD was observed (OR=4.42, 95% CI=2.20-8.99, p=0.000). No significant association was observed *Rp1-Rp2* with (OR=1.57, 95% CI=0.97-2.54) genotype. When combined genotypes of *Rp1-Rp2+Rp1* were taken into account highly significant risk of COPD was observed (OR=2.10, 95%CI=1.38-3.21, p=0.000) (Table 6).

Table 6: Odds ratio of *IL-4* and COPD risk

Genotypes	CASES	CONTROLS	*OR (95% CI)	p-value
***Rp2* (ref.)**	96	132	1 (reference group)	
Rp1	45	14	4.42(2.20-8.99)	0.000
Rp1-Rp2	59	54	1.57(0.97-2.54)	0.067
Rp1-Rp2 +Rp1	104	68	2.10(1.38-3.21)	0.000

*OR adjusted for age and smoking status. OR, odds ratio; 95%CI, 95% confidence interval.

4.4. Effect of interaction between *IL-4* (70 bp repeats) genotypes and cigarette smoking on risk of COPD

Smoking status in *IL-4* genotypes

5.38 fold (95% CI = 2.16 –13.85, p=0.001) elevated risk of COPD was observed for smokers who had *Rp1* genotypes of *IL-4*. In case of non smokers, no significant association was found between any genotypes of *IL-4* and non smokers on the risk of COPD (Table 7).

5. DISCUSSION

Relationship between Cytokine gene polymorphism and risk of COPD

COPD is characterized by a slowly progressive irreversible airflow obstruction. Many inflammatory cells, mediators and enzymes are involved, but their importance is not well understood yet.

There is probably a complex interplay between genetic and environmental factors and many different genes are involved. The genes which have been implicated in the pathogenesis of COPD are involved in the metabolism of toxic substances in cigarette smoke, airway hyper-responsiveness, anti-proteolysis and the inflammatory response to cigarette smoke. The present study is based on the impact of *IL-4* (70 bp repeats) gene polymorphism in COPD.

IL-4 (70 bp repeats) showed a significant relation with COPD as well as smoking habit. *Rp1* alleles showed

Table 7: Assessment of interactions between *IL-4* genotypes and smoking habits in COPD cases and controls

Genotypes	Smokers		*OR (95% CI)	p-values	Non-smokers		*OR (95% CI)	p-values
	Cases (n=126)	Controls (n=101)			Cases (n=74)	Controls (n=99)		
***Rp2* (ref.)**	52	64	1 (reference group)		44	68	1 (reference group)	
Rp1	35	8	5.38 (2.16-13.85)	0.001	10	6	2.58 (0.79-8.68)	0.136
Rp1-Rp2	39	29	1.66 (0.87-3.17)	0.136	20	25	1.24 (0.58-2.64)	0.677
Rp2-Rp1 +Rp1	74	77	1.18 (0.71-1.98)	0.579	30	31	1.50 (0.76-2.95)	0.273

*OR adjusted for age, gender and alcohol consumption status. OR, odds ratio; 95%CI, 95% confidence interval.

4 fold increase risk of COPD, but combined genotype of *Rp1-Rp2+Rp1* resulted in only 2 fold. The smokers carrying *Rp1* had 5 fold increased risk of disease. Hegab *et al.* (2005) demonstrated that the distributions of the haplotypes (*IL-4* - 589 *C/T*: *IL-4* VNTR; *IL-4* - 33 *C/T*: *IL-4* VNTR) in the Japanese show differences between the COPD and the control groups. The present results are in consonance with this study.

There have been limitations like (1) small sample size, (2) complicated etiology of COPD cannot be explained with only cigarette smoking as it is a multifactorial disease, (3) fickle epidemiological criteria, and (4) quantification the levels of cytokines, in our study.

Besides above limitations, the present study has several important strengths too. These include: (1) adjustment of age, sex and alcohol consumption, (2) recruitment of sex and age matched controls to negate the effect of these parameters on results, (3) the controls and cases were drawn from same ethnic population randomly, (4) interviewing was blind to case status.

6. Conclusion

Following points are apparent from the present study:

- Polymorphic gene *IL-4* showed a significant increase in the risk of COPD.
- A highly significant 4 fold risk of COPD was observed with *Rp1* genotype of *IL-4* gene.
- The combined genotype of *IL-4(Rp1-Rp2+Rp1)* was showed 2 fold increase risk of COPD.
- In smokers, 5 fold significantly increased risk of COPD was observed with *Rp1* genotype of *IL-4*.
- 2 fold but (not significantly) increased risk was observed in non smokers with genotype *Rp1* of *IL-4*.

This case-control study reports for the first time in north Indian population. Case control association studies offer a potentially powerful approach to identify common genetic variants that influence susceptibility to disease, but are impaired by the impression that are not consistently reproducible. Besides this, present study acts as a platform for the future studies to be conducted on COPD. The facts noted in this study will be used by the researchers in the prognosis and diagnosis of the disease. A small sample size is the limitation of this study. Further studies are required with large numbers of sample sizes to evaluate the exact role of *IL-4* (70 bp repeats) gene in north Indian population for the development and progression of COPD.

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REFERENCES

1. Barnes P.J. (2001). Cytokine modulators as novel therapies for airway disease. Eur. Resp. J. 18: 67S–77S.
2. Barnes P.J. (2003). New concepts in chronic obustructive pulmonary disease. Annu. Rev. Med. 54: 113-29.
3. Bartolome Celli (2003). Medical therapy for COPD and emphysema. Lung Volume Reduction Surgery for Emphysema Published by Informa Health Care. Chapter 5: 599-122.
4. Burrows B., Knudson R.J., Cline M.G. and Lebowitz M.D. (1977) Quantitative relationships between cigarette smoking and ventilatory function. Am Rev Respir Dis.115: 195-205.
5. Hegab A. E., Sakamoto T., Saitoh W., Nomura A., Ishii Y., Morishima Y., Iizuka T., Kiwamoto T., Matsuno Y., Massoud H. H., Massoud H. M., Hassanein K. M., Sekizawa K. (2005). Polymorphisms of TNF alpha, IL-1beta, and IL-1RN genes in chronic obstructive pulmonary disease. Biochem Biophys Res Commun. 329(4):1246-52.
6. Lebeau M., Lemons R., Espinosa R., Larson R., Arai N., Rowley J. (1989). Interleukin-4 and interleukin-5 map to human chromosome 5 in a region encoding growth factors and receptors and are deleted in myeloid leukemias with a del (5q). Blood. 73(3): 647–650.
7. Mannino D.M. (2002). COPD: epidemiology, prevalence, morbidity and mortality, and disease heterogeneity. Chest. 121: 121S-126S.
8. Mannino D.M., Homa D.M., Akinbami L.J. (2002). Chronic obstructive pulmonary disease surveillance: United States, 1971–2000. MMWR Surveill Summ. 51:1–16.

9. Mout R., Willemze R., Landegent J. (1991). Repeat polymorphisms in the interleukin-4 gene (IL4). Nucleic Acids Res. 19: 3763.

10. Pauwels R. (2001). Global initiative for chronic obstructive lung diseases (GOLD): time to act (editorial). Eur Respir J. 18(6):901–902.

11. Pauwels R.A., Buist A.S., Calverley P.M.A., Jenkins C.R. and Hurd S. S. (2001). Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 163: 1256-1276.

12. Sandford A.J., Silverman E.K. (2002). Chronic obstructive pulmonary disease. 1: Susceptibility factors for COPD the genotype-environment interaction. Thorax. 57(8): 736-41.

13. Thakur H. (2008). Genetic epidemiology of chronic obstructive pulmonary disease (COPD) in north Indian population. Ph.D. Thesis.

14. Wu M.C., Huang C.M., Tsai J.J.P., Chen H.Y. and Tsai F.J. (2003). Polymorphisms of the interleukin-4 gene in Chinese patients with systemic lupus erythematosus in Taiwan. Lupus. 12: 21-25.

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