Original Article

CD14 C-159T polymorphism and its association with chronic lung diseases: A pilot study on isocyanate exposed population of Central India

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CONTEXT: CD14 functions as a multifunctional receptor for bacterial cell wall components including endotoxin and lipopolysaccharide and is likely to influence the cytokine profile and subsequent immunoglobulin E production in response to antigen/allergen contact in allergic phenotypes.
AIMS: The present study was to investigate genetic polymorphism in CD14 gene - 159C/T, which may be one of the risk factor for increased prevalence of Chronic Lung Diseases in the Central India.
SETTINGS AND DESIGN: Survivors of Methyl isocyanates toxicity in Bhopal still suffering from various respiratory ailments were examined.
MATERIALS AND METHODS: Polymerase chain reaction-restriction fragment length polymorphism was performed to determine the polymorphism of C-159T. Statistical analysis used: All analysis was done using SPSS software, version 11.5 (SPSS, Chicago, IL, USA).
RESULTS: The genotype and allelic frequencies were in Hardy-Weinberg's equilibrium. Prevalence of CC, CT, and TT were 5.5%, 22.2% and 9.25% respectively in asthmatics; 16.6%, 20.3% and 5.5% respectively in chronic obstructive pulmonary disease (COPD) patients and 5.5%, 14.8% and 1.85 respectively among interstitial lung disorder (ILD) patients; whereas the control cohort with no methyl isocyanate exposure displayed (CC, CT, and TT) cytosine,thymine as 2%, 1.6% and 2% respectively. Increased risk of Asthma among those carrying TT genotype and T allele (odds ratio [OR] =2.61 and 2.02 respectively).
CONCLUSION: COPD risk significantly found among those with CC genotype and C allele (OR = 2.81 and 1.50 respectively), whereas ILD risk found significantly among CT genotype and C allele (OR = 1.75 and 1.40 respectively). Therefore, single nucleotide polymorphism (SNP) C-159T polymorphism in CD14 gene might be a risk factor for development of CLD in this population.

Key words: Asthma, Bhopal, CD14, chronic obstructive methyl isocyanate, pulmonary disease

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Introduction

The Industrial catastrophic aerosol spill of methyl isocyanate (MIC) on a sleeping city in Central India was the world's worst chemical disaster in 1984 with a death toll of approximately 3,000-6,000 people and leaving 200,000 injured.[1] A large number of the inhabitants in the township of Bhopal were exposed to different degrees, depending on their proximity to the plant along with various other atmospheric factors.

It was thought possible that a wide spectrum of lung diseases could result from this gas exposure.[2] Clinical studies have shown chronic illnesses such as pulmonary fibrosis, bronchial asthma, chronic obstructive pulmonary disease (COPD), emphysema, recurrent chest infections in the exposed cohorts. Survivors continue to experience a higher incidence of reported health problems including febrile illnesses, respiratory, neurologic, psychiatric and ophthalmic symptoms[3] and lungs were one of the most commonly affected organ. De reported the relative risk for pulmonary function abnormalities in gas victims as significantly more among those who were young at the time of disaster. Increased smoking habit among gas

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victims might have played an additive effect on the predominance of obstructive pattern in spirometry.[4]

Vijayan and Sankaran reported pulmonary function abnormalities which occurred in gas-exposed subjects as a consequence of an abnormal accumulation of lung inflammatory cells (lymphocytes and neutrophils), and that the intensity of lung inflammation and reduction in pulmonary function are greater in severely exposed subjects.[5]

We studied CD14 promoter region as it has an important role in innate immune defense molecules that mediate clearance of pathogens and apoptotic cells from the lung. CD14 is the most essential molecule known so far, playing a vital role against several enterotoxigenic bacteria. Its' pattern recognition receptor binds mainly with lipopolysaccharide (LPS), lipotechoic acid, arachidonic acid and thus releases various cytokines which act for body's defense. It was thought that increased activity of the CD14 promoter might be due to single nucleotide polymorphism in the 5' genomic region of CD14 at position- 260 (allele C and T) resulting in inflammation and increase risk of chronic lung diseases viz. asthma, COPD and interstitial lung disorder (ILD). The substitution of C T leads to an increased transcriptional activity, which is paralleled by a decreased affinity of DNA/protein interactions between the Sp1, 2, 3 proteins and the GC box in the CD14 promoter. This may be important for the pathogenesis of inflammatory diseases.[6]

LPS is a principle mediator of bacterial pathogenesis and other inflammatory disorders. Exposure of host cells to LPS triggers the release a battery of host defense molecules including cytokines, chemokines, interferons, cell adhesion molecules, lipid mediators, reactive oxygen species, and nitrogen intermediates. Although these molecules are essential to host defense against bacterial infection, excessive production of these mediators can cause organ damage, leading to chronic respiratory disease (CRD). Cellular activation by LPS is mediated by signal transduction cascade initiated by the interaction between LPS and its receptor, CD14. CD14-mediated recognition and signaling require four proteins: LPS-binding protein, CD14, MD-2, and Toll-like receptor family proteins.[7]

The increase in sCD14 was suggested to be associated with the accumulation of neutrophils at inflammatory sites. However, the biological significance of this response has not been fully elucidated. Moreover, acquisition of LBP by sCD14 has been shown to transport LPS to high-density lipoprotein and lead to detoxification of LPS in plasma, resulting in reduced pathogenesis.[8]

Keeping the above facts in mind, the present investigation has been planned to study SNP in CD14-260C >T (rs2569190) promoter region to get an insight of this loci and correlate it to the significantly high percentage of lung related cases among the gas effected population of Bhopal.

Inter-(simple sequence repeat)-polymerase chain reaction (ISSR-PCR) simultaneously sample numerous regions of the genome, thus allowing us to readily measure the occurrence of genomic events in a patient with stressed respiratory parameters.

Materials and Methods

Subjects

In this study, molecular screening of 54 gas-victims was evaluated, suffering from mild to severe airway obstruction, medically diagnosed as suffering from asthma, COPD and ILD. Their mean age was 46 (range 25-85) years and forty patients were male [Table 1]. Smokers with airflow limitation ($n = 27$), and age-matched control smokers ($n = 12$) and control never-smokers ($n = 7$) without airflow limitation were included in the present study. Five four patients (40 males and 14 females) with a history of exposure to toxic gas in 1984, and presenting with respiratory symptoms were included in the study. The main presenting symptoms were cough and dyspnea on exertion. Patients were selected partly from visits to the out-patient department at the Department of Pulmonary Medicine, Bhopal memorial Hospital and Research Centre, Bhopal established for toxic gas victims. Each individual was examined by a physician along with a case history to follow-up the patient's progress. Our control population was of three types namely, Normal lungs-no isocyanate exposure, normal lungs and isocyanate exposed and COPD with no isocyanate exposure. Control patient sampling was done partly from the patient's homes which were visited by the investigators and those visiting Bhopal Memorial Hospital and Research Centre (BMHRC), Bhopal.

Table 1: Clinical Characteristics of the studied population of Bhopal

Clinical phenotypes*	Control subjects			Chronic lung diseases (CLD)		
	C1=10	C2=30	C3=10	Asthmatic subjects n=20	COPD subjects n=23	ILD subjects n=11
Sex (male/female)	5M/5F	18M/12F	7M/3F	14M/6F	17M/6F	9M/2F
Mean age (y)	53±9	56±3	58±5	45±12	53±16	40±4
Isocynate exposure †	22±5	20±6	4±3	2±2	3±1	2±2
Duration of disease (y)	NA	7±12	NA	17±15	15±5	6±3
Av.Total IgE (IU/mL)	89.6±11	412±26	93±34	954±164	242±144	111±74
FVC (%)	91±14	88±13	90±11	88±13	87±12	90±11
FEV1 (%)	91±13	82±10	89±14	86±14	81±12	90±14

*Mean±SD; †Distance of residence(kms.) from UCIL during 1984; FVC: Forced vital capacity, FEV1: Forced expiratory volume in 1 min, C1: -MIC-CLD, C2: -MIC+CLD, C3: +MIC-CLD

A piloted structured questionnaire would be administered in Hindi/English to enquire into a variety of potentially confounding factors including socioeconomic variables such as literacy, income, and employment. Literacy was defined as the completion of 8th level in school. Men with low incomes were those with no paid work or a monthly wage of less than 500 rupees.

Isocyanate exposure severity

A preliminary assessment of the area of exposure and the effects suffered was made by talking to people who lived at varying distances and directions from the plant. Place of residence at the time of the gas leak would be confirmed from official records held by each of those interviewed.

Polymorphism of microsatellites was determined in CRD patients using $(CA)_8RG$ (Cytosine Adenine,any purine base any pyrimidine base) primer. Control group included age-matched non-exposed ($n = 40$) and control exposed but with no CRD ($n = 10$).

DNA was isolated from 5 ml blood collected in ethylene diamine tetraacetate (EDTA) vacutainer, using DNA isolation kit for mammalian blood (QIAmp DNA Blood Maxi and Mini kits; QIAGEN, Inc., Valencia, CA, USA). DNA samples were stored at – 20°C.

ISSR-PCR

The ISSR-PCR method for microsatellite polymorphism was studied using polymerase chain reaction (PCR-peltier thermal cycler (PTC)-200 thermal cycler, M.J. Research, Inc., Watertown, MA, USA) as described elsewhere.[9] The PCR products were analyzed on non-denaturing 7% polyacrylamide gel electrophoresis (PAGE) at 120 V for 20 min. The gels were then viewed under ultra violet (UV) illumination at $\lambda_{260\ nm}$. The genomic instability index was computed by dividing the number of altered bands seen in the PCR products amplified from patients by the total number of products generated from the corresponding normal subjects.

CD14 genotyping

Genomic DNA from MIC-exposed as well as non-exposed subjects was used to screen for genetic variants in the promoter region of the CD14 gene. Two overlapping PCR products based on the reported sequence were amplified. The first product (P1) encompassed the interval between base pair (bp) –513 and bp – 61 from the transcription start site, and was amplified using primers 5'-GTGCCAACAGATGAGGTTCAC-3' and 5'-CCTCTGTGAACCCTGATCAC-3'. The second product (P2), including the interval between bp – 151 and bp + 291 from the transcription start site, was amplified using primers 5'-CCTGAAACATCCTTCATTGC-3' and 5'-CGCAGCGGAAATCTTCATC-3'.

Both PCR was carried out in a volume of 25 μl containing 50 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 0.8 unit of Taq DNA polymerase supplied by (Platinum, Invitrogen) and 5 pmol of each primer. The DNA was denatured at 96°C for 3 min, and temperature cycling was set at 96°C for 40 s, 56°C (P1) or 58°C (P2) for 40 s, and 72°C for 50 s for 38 cycles, followed by a final extension at 72°C for 10 min. The sizes of the generated PCR products were 479 (P1) and 442 bp (P2).[10]

PCR products were electrophoresed on 2% agarose gels and visualized with ethidium bromide staining and ultraviolet illumination.

Total serum immunoglobulin E (sIgE)

Total sIgE levels were measured with the ELISA test using commercially available kits obtained from Demeditec Diagnostics Enzyme Immunoassay kit, Germany. The assay threshold was set at 0.1 IU/ml as described elsewhere.[11]

Ethics

Institutional Research Ethics Committee of the hospital approved the present study and patients gave their informed consent through structured questionnaires comprising of Patient medical history, family background, occupational details etc.

Statistical analysis

All the statistical analyses were conducted using the SPSS software, version 11.5 (SPSS, Chicago, IL, USA). Chi-square test was used to estimate significant differences between the three CLD groups in the study, to compare differences (asthmatics, COPD, and ILD with normal subjects). Pearson's correlation coefficient and *P* value was used to assign significant relationships. Linear Regression analysis was performed in order to examine the significance of the effect of distance of residence from the Union carbide plant (degree of isocyanate exposure). In the genotypic analysis, each individual contributed two allele (one from each chromosome) to the analyses and the frequency and estimated counts of each genotype were then assessed against all others using standard procedures for odds ratio (OR) and confidence interval (CI) calculations.

Results

A total of 54 MIC exposed patients suffering from asthma/COPD/ILD was recruited in our study. Of them, only 46% were literate, and 21% of the sampled population belonged to low-income level. Only men were smokers consuming approximately ten cigarettes per day, comprising of 48% of the sampled population. The duration of diagnosed bronchial Asthma, COPD or ILD was on an average 17 years ± 15, 15 ± 5 and 6 ± 3 years respectively. Wide variations were observed in the serum total immunoglobulin E (IgE) levels with very high level of 959.6, 242 and 111 IU/ml among Asthma, COPD and ILD patients respectively whereas, control group without toxic insult and normal lung functioning had an average serum IgE level of 89.6 IU/ml FVC and FEV1 were slightly more hampered in case of asthmatic subjects [Table 1].

The Microsatellite banding pattern with $(CA)_8$RG primer when compared to that of the control population revealed an altered banding profile in these patients [Figure 1]. On statistical analysis of the existing data, there existed a positive correlation +0.37 and +0.11 ($P < 0.05$) between age, smoking status and the percent genomic instability respectively. The average genomic instability using microsatellite primers of COPD patients were found to be 1.82 and 0.78 times more than that found in case of control population and patients suffering from bronchial asthma. On statistical analysis of cohort exposed to MIC/not MIC with respect to smoking status resulting in asthmatic state, we found highly statistically significant association (t value 3.6329; $P < 0.0067$) and an OR,1.6829 (95% CI, 0.5417-5.2288).

Regression analysis of the sIgE level displayed a negative slope (y = –59.535× +842.12; slope = 0.4875). Thus, indicating the effect of MIC exposure on the inhabiting population as a measure of distance of residence from the union carbide india limited (UCIL) plant that is high values of total serum IgE and inhaled MIC by the population [Figure 2]. A significant increase in IgE levels was observed in the MIC effected asthmatic population when compared with normal control subjects (*r*, –0.698, $P < 0.0018$). The odds of occurrence of Asthmatic condition in the population were found to be 3.86 times more in an isocyanate exposed population than a not-exposed population (95% CI 1.2507-11.8952). The elevated sIgE level, versus MIC and non-MIC hit population also displayed extremely statistically significant data (*t* value, 7.6493; *P*, 0.0001). The odd of elevated sIgE level was 1.43 times more among the MIC exposed population than among the not-exposed population (95% CI, 0.14-14.4).

PCR-RFLP analysis obtained genotypes for 104 subject enrollees. Frequencies of CC, CT, and TT genotypes were 5.5%, 22.2% and 9.25%, respectively among screened asthmatics. Allele frequency for C was estimated at 45%, and distribution was very similar to the

expected Hardy-Weinberg equilibrium [Table 2]. Patients with COPD condition genotypic frequency of CC, CT, and TT were 16.6%, 20.3% and 5.5% respectively. T allele frequency was highest in case of asthma patients (63%); C allele frequency was 55% and 58.3% in case of COPD and ILD patients. CD14/–159 T was associated with increased risk of bronchial asthma (OR = 2.02, 95%CI: 1.25-3.26). Genotype frequency also did not deviate from HWE [Table 3]. The frequency of CC and CT genotypes showed increased risk for COPD and ILD respectively (OR = 2.81 and 1.75; 95% CI, 1.14-6.91 and 0.65-4.73 respectively).

The screening of the promoter region (rs2569190) of the CD14 gene revealed the presence of polymorphism, a C-to-T transition at bp – 159 (CD14/–159) from the major transcription start site in approximately 20% of the studied population with MIC exposure.

Discussion

This study is the first demonstration of association between CD14 polymorphism (CD14/–159) and serum total IgE level in patients exposed to MIC and suffering from CLD viz. bronchial asthma, COPD and ILD in the city of Bhopal (India). The study revealed robust associations of TT, CC and CT genotype of CD14 with the cohort suffering from bronchial asthma, COPD and ILD respectively. The odds of bronchial asthma occurring in T allele were 2.02 times more than in C allele; whereas the odds of COPD and ILD occurring was 1.5 time and 1.4 times in C allele than T allele respectively. The presence of T allele of CD14 gene was found to be a greater risk factor in asthmatic patients than in the healthy controls. Our results were consistent with studies on Northern and western India[12] reporting of suggestive evidence of association of CD14 gene locus with atopic asthma and its association at genotypic as well as the allelic level with observed differences in IgE level. Similar findings have been cited from populations of Brazil,[13] Tunisia[14] and Poland.[15] On the other hand, studies on Pakistanis adults[16] clearly show C-159T polymorphism to be associated with allergic rhinitis, whereas A-1145G polymorphism has been found associated with allergic asthma. Zhang *et al.* performed a meta-analysis and found – 159C/T polymorphism might be a protective factor for atopic asthma in the Asian population.[17] Common variants in genes in the pathway of pathogenesis may alter protein function and individual's susceptibility to disease.[18-21]

There is possible gene-environment interaction, in which the SNP acts as a modifier of asthma risk in individuals with different degrees of environmental endotoxin exposure. Carriers of the TT genotype have been found to have higher serum levels of CD14 than carriers of the CT or CC genotypes.[22] An antagonistic interaction has been demonstrated between CD14 and endotoxin exposure:

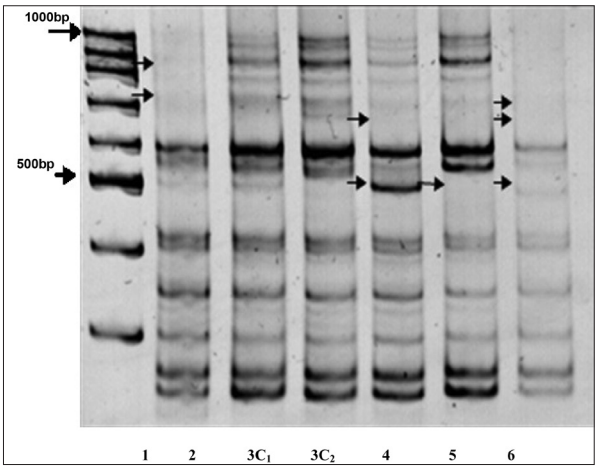

Figure 1: Regression analysis of total serum IgE level in isocyanate exposed patients and their radial distance of residence from UCIL plant

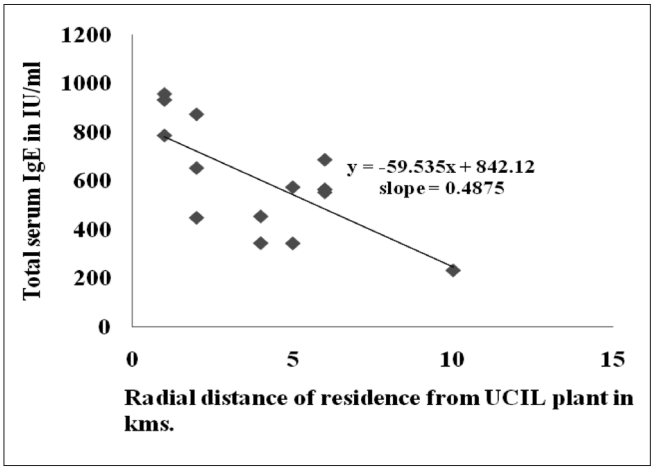

Figure 2: Microsatellite instability displayed using $(CA)_8RG$ microsatellite primer among methyl isocyanate gas victims suffering from chronic obstructive pulmonary disease, asthma and interstitial lung disorder (lane 2, 4-6 respectively) as compared with non-gas exposed with normal lung functioning (lane $3c_1$) and gas victims with normal lung functioning (lane $3c_2$) ; lane 1 is 100 bp marker. Arrows showing altered bands

Table 2: Allele and genotype frequencies and mean log-serum total IgE for C-159T polymorphism studied in the CD14 gene. Numbers in parentheses indicate the frequency (percentage). N Number of individuals in each group

Allele and Genotype frequency	Chronic Lung Diseases (CLD)			Control subjects (n=50)			Patient average total serum IgE, IU/ml (n=54)	Control average Total serum IgE, IU/ml (n=50)
	Patients (+MIC) n=54			1 (n=10)	2 (n=30)	3 (n=10)		
	Asthma (n=20)	COPD (n=23)	ILD (n=11)					
C	18 (45)	29 (63)	14 (58.3)	10 (50)	27 (45)	8 (40)	935±6.2	111±1.2
T	22 (55)	17 (36.9)	10 (41.6)	10 (50)	33 (55)	12 (60)	219±4.1	93±1.5
CC	3 (5.5)	9 (16.6)	3 (5.5)	1 (2)	6 (12)	1 (2)	959.6±2	100.1±1.1
CT	12 (22.2)	11 (20.3)	8 (14.8)	8 (1.6)	15 (30)	6 (12)	450.5±3.4	116.8±1.2
TT	5 (9.25)	3 (5.5)	1 (1.8)	1 (2)	9 (18)	3 (6)	213.2±6.9	102.6±1.7

1= -MIC-CLD, 2= -MIC+CLD, 3= +MIC-CLD

Table 3: Statistical relevance of Genetypic and Allelic frequency among MIC exposed cohort suffering from various Chronic Lung Diseases (CLD); *X^2 Chi square (*P* value)

CLD	Genotype			Allele	
	CC OR (95%CI)	CT OR (95%CI)	TT OR (95%CI)	C OR (95%CI)	T OR(95%CI)
Asthma	0.34 (0.12-0.95); 4.45(0.03)*	1.26 (0.55-2.86); 0.31(0.5)*	2.61 (0.89-7.6); 3.24(0.07)*	0.49 (0.30-0.79); 8.4(0.003)*	2.02 (1.25-3.26)
COPD	2.81 (1.14-6.91); 5.25(0.02)*	0.54 (0.24-1.21); 2.19(0.13)*	0.64 (0.21-1.96); 0.6(0.4)*	1.50 (0.92-2.48); 2.64(0.10)*	0.66 (0.40-1.09)
ILD	0.85 (0.29-2.5); 0.08(0.7)*	1.75 (0.65-4.73); 1.26(0.26)*	0.39 (0.07-1.97)	1.4 (0.86-2.27); 1.86(0.17)*	0.71 (0.43-1.16)

Homozygotes for the T allele appear to be protective for asthma at low levels of endotoxin exposure, but may increase asthma risk at high levels of endotoxin exposure which is slightly different in our screened population as CT genotype reported high sIgE level. Heterozygous CT appeared to offer more protective effects against endotoxin exposure. Based on studies on other research data and our findings, we hypothesized that higher CD14 expression in CT heterozygote increased sensitivity to the protective effects of low level endotoxin exposure compared to carriers of other genotypes. However, at higher levels of endotoxin exposure, induced CD14 expression could be increased in carriers of the T allele, for Asthma and C allele for COPD and ILD conditions.

We analyzed our data in various ways to test for association with various confounding factors such as age ($P < 0.0017$), smoking status ($P < 0.0067$), literacy ($P < 0.05$), occupation, percent genomic instability ($P < 0.0001$), serum IgE level ($P < 0.0001$) and spirometric paramerers such as FEV1 and FVC measurements ($P < 0.0001$). In the present study, normal levels of serum IgE itself were relatively higher than those values from western population studies.[23,24] The higher IgE levels in the normal control group may be explained by the higher incidence of parasitic infection and infestation in patients from this part of the world.

We found highly significant association of prevalence asthma in the existing population with exposure to MIC in terms of distance of residence from the UCIL plant ($P < 0.0018$). A potential mechanism for the worsening in lung function observed by is the possibility that active cigarette smoking impairs the efficacy of short-term inhaled corticosteroid treatment in mild asthma.[25] On the other hand, smoking along with age, health condition, eating habits, socio-economic status is to blame for COPD in the majority of cases.

Thus, in addition to smoking, exposure to exogenous factors, like air pollution and work conditions several host factors such as health status, eating habits, socio-economic conditions are also beneficial in determining the development of lung disease.

The instability of the microsatellite sequences investigated indicates a destabilization of the genome in the studied cohort suffering from CLD.

In addition to the promoter, many additional regulatory elements are necessary to influence gene expression, particularly for genes like CD14, which exhibit highly complex expression patterns. Regulatory elements, such as enhancers and repressors, may reside in intronic regions or up- and down-stream of the transcriptional unit.[26] A risk variant with no obvious and no known function may regulate a gene at a considerable genomic distance

from the location of the SNP. Therefore, it is vital to study the influence of gene-gene interaction as well as other polymorphisms in CD14 on the effects of this locus on CRD susceptibility. Therefore, results of this study corroborated with the findings of several other studies performed on different ethnic groups at the same time contradict some other studies. In conclusion, we found suggestive evidence of an association of the CD14 C-159T polymorphism with prevalence of CLD and high serum IgE in the population afflicted of isocyanate poisoning that may intensify further research in the 5q31-33 chromosomal region. We anticipate these data along with other studies reported in the literature would help to design better approaches in risk assessment of occupational and accidental exposure to isocyanates as the presence of a past MIC exposure has potential to be a useful and efficient predictor of various lung related disorders in the existing population.

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