

RESEARCH ARTICLE

Lack of Association of a Common Polymorphism in the 3' -UTR of Interleukin 8 with Non Small Cell Lung Cancer in Kashmir

Imtiyaz Ahmad Bhat¹, Arshid A Pandith², Bashir A Bhat³, Niyaz A Naykoo⁴, Iqbal Qasim¹, Roohi Rasool¹, Sheik Aejez Aziz⁵, Zafar Amin Shah^{1*}

Abstract

Background: Chronic inflammation is considered as an important factor in the pathogenesis of lung cancer. The presence of inflammatory cells and higher levels of pro-inflammatory cytokines in the tumor microenvironment and their surrounding tissues is gaining much importance in research. **Materials and Methods:** One hundred ninety NSCLC cases and 200 age, smoking and sex matched controls were evaluated for association of *IL-8* -251 (rs4073) and *IL-8* -845 (rs2227532) in our population. Restriction fragment length polymorphism (RFLP) was used followed by direct sequencing for the detection of SNPs. **Results:** The *IL-8* -845 polymorphism was not found in our population. No significant association was observed between the *IL-8* -251 AT genotypes and *IL-8* -25 AA genotypes and NSCLC ($p=0.05$) in our population. The *IL-8* -251 A allele was also non-significant ($p=0.05$) in NSCLC patients. **Conclusions:** In conclusion, this report reveals lack of association between *IL-8* - 251 A/T polymorphism and NSCLC in our Kashmir Valley population.

Keywords: Interleukin 8 - restriction fragment length polymorphism - non small cell lung cancer (NSCLC)

Asian Pac J Cancer Prev, 14 (7), 4403-4408

Introduction

Lung carcinogenesis is a complex process requiring the acquisition of genetic mutations that confer the malignant phenotype as well as epigenetic alterations. Inflammatory signals in the lung cancer microenvironment can promote apoptosis, resistance, proliferation, invasion, metastasis, and secretion of proangiogenic and immunosuppressive factors (Peebles et al., 2007). Nearly 20% of cancer deaths are attributed to chronic infection and/or inflammation with gastrointestinal and lung cancer accounting for substantial portion of total burden (Balkwill, 2001; Aggarwal et al., 2009). The seventh proposed hallmark of cancer due to the genetic instability resulted from cancer related inflammation (Mantovani, 2009).

Chronic inflammation is considered an important factor in the pathogenesis of human carcinomas including lung carcinoma. Elevated number of inflammatory cells and higher levels of pro inflammatory cytokines are seen in the tumor microenvironment and their surrounding stromal tissues (Takizawa et al., 2000; Balkwill, 2001). The increased risk of lung cancer in patients with chronic inflammatory diseases such as chronic obstructive pulmonary disease, asthma, and chronic interstitial lung fibrosis have been observed (Cohen et al., 1997; Mayne et al., 1999; Boffett et al., 2001). The mechanisms that link

infection, inflammation, immunity and cancer has been shown by various studies, the cytokines being considered an important component in this linkage are produced by activated innate immune cells that stimulate tumor growth and progression (Wan et al., 2007). Interleukin-8 (*IL-8*) is involved in initiation and amplification of acute and inflammatory reactions as well as in the maintenance of chronic inflammatory response. *IL-8* is produced by wide range of normal cells and is well known for its tumorigenic, proangiogenic and leukocyte chemotactic properties (Du et al., 2002). Hypoxia, acidosis and nitric acid have been found to regulate the expression of *IL-8* in tumor microenvironment (Xie et al., 2001). In recent years, studies have shown that *IL-8* is closely associated with the occurrence of tumors. The secretion and expression of *IL-8* mRNA and protein have been detected in many tumor tissues and GA cell lines (Cohen et al., 1995; Brew et al., 1996; Yoshimura et al., 2002). Promoter regions of a number of cytokine genes contain polymorphisms that directly influence cytokine production (Bidwell et al., 2001). The *IL-8* gene is located on chromosome 4q13-21 and consists of four exons, three introns, and a proximal promoter region (Mukaida et al., 1989). Several polymorphisms -845 (T/C), -738(T/A), -353 (A/T), -251 (T/A) and +678 (T/C) have been reported in the *IL-8* gene. Interestingly *IL-8* production can be controlled by

¹Department of Immunology and Molecular Medicine, ²Advanced Center for Human Genetics, ³Department of Plastic Surgery, ⁴Department of Medical Oncology, Sher i Kashmir institute of Medical Sciences Srinagar, ⁵Sher-e- Kashmir University of Agricultural Sciences and Technology of Kashmir, India *For correspondence: imty82@gmail.com

the -251 A/T in the promoter region of this chemokine (Hull et al., 2000). Recent data revealed that the *IL-8* -251 A allele is associated with a high expression level of *IL-8* protein and a severe neutrophil infiltration (Araki et al., 2007). Elevated *IL-8* levels were associated with disease progression and recurrence in human prostate, breast, lung and gastric cancers (Taguchi et al., 2005; Ahmed et al., 2006; Lurje et al., 2008; Millar et al., 2008). Recently, genetic polymorphisms of the *IL-8* gene have been implicated in the susceptibility to a range of cancers, including Colon cancer (Ohyuchi et al., 2005), Gastric cancer (McCarron et al., 2002), Prostate cancer (Ben et al., 2007) and nasopharyngeal cancer (David et al., 2008). In the backdrop of above cited studies, we hypothesize that the *IL-8* promoter SNP which results in higher *IL-8* secretion, was associated with increased risk of non small cell lung carcinoma. To test this hypothesis, we evaluated the relationship between *IL-8* -251 A/T and *IL-8* -845 T/C polymorphism and non small cell lung cancer risk in a case control study.

Materials and Methods

This study included 190 lung cancer patients and 200 age, sex, dwelling and smoking matched controls. All the lung cancer cases selected for study were histologically confirmed as non small lung cancer cases. Patients were recruited from Out Patients Department, Department of Medical Oncology and Department of Cardiovascular and Thoracic Surgery, Sher i Kashmir Institute of Medical Sciences Srinagar between April 2010 and March 2012. All those patients who had prior history of cancer other than lung cancer and patients who had received any chemotherapy/radiotherapy were excluded from our study. All participants of the control group were selected from individuals receiving routine medical examinations in the same hospital, with no history of cancer, and no symptoms of other acute or chronic inflammation lung diseases (i.e. COPD, asthma, etc.), peritonitis, rheumatoid arthritis, or late stage kidney diseases. Ethical approval was taken from the ethical committee of SKIMS and World Medical Association (Declaration of Helsinki) protocol was followed. All the participants were informed and gave written consent for participation in the study.

Five milliliter peripheral blood samples were collected in EDTA vials from lung cancer patients and healthy controls and later stored at -80°C till further use. A detailed questionnaire was completed for each patient and control. The questionnaire included information on the number of cigarettes smoked daily/quantity of tobacco smoked every day and the number of years the subject had been smoking. For smoking status, a person who had smoked (Cigarette/Hokka) at least once a day for >1 year was regarded as smoker. The cases included 163 (85.79%) males and 27 (14.71%) females with age distribution of ≤50 years 59 (31.05%) and >50 years of age were 131 (68.95%) with mean age of 57.80±10.97. The control group had 170 (85%) males and 30 (15%) females with age distribution ≤50 years 65 (32.5%) and >50 years 135 (67.5%) with mean age of 56.69±12.22. Smokers in cases were 114 (60%) and non smokers were 76 (40%)

and in control group smokers were 116 (58%) and non smokers 84 (42%). Non small cell lung cancer included 130 (68.42%) cases who had stage I and stage II and 60 (31.58%) cases who had stage III and IV. Squamous cell carcinoma patients were 140 (73.69%) while others which included large cell carcinoma, bronchogenic carcinoma and adenocarcinoma were 50 (26.32%) (Table 1).

IL-8 genotyping

DNA extraction was performed according to the manufacturer's protocol for Qiagen DNA extraction kits (Qiagen, Hilden, NRW, Germany). DNA content was quantified by spectrophotometric absorption (Nanodrop Spectrophotometer, BioLab, Scoresby, VIC, Australia). Polymerase chain reaction (PCR) was performed using an iCycler Thermal Cycler (Bio Rad, Hercules, CA, USA). *IL-8* -251 A/T (rs4073) and *IL-8* 845C/T (rs2227532) genotypes were determined using PCR-RFLP method followed by sequencing. Primers were designed and selected using Primer3, version 0.4.0 software. For *IL-8* -251 A>T, the primers were forward 5'-CAC TGG AAT TAA TGT CTT AGT ACC A-3' and reverse-5'-AAG CTT GTG TGC TCT GCT GTC TCT-3'. For *IL-8* -845 C/T, the primers were forward 5'-AACCCAGCAGCTCCAGTG-3' and reverse 5'-AGA TAA GCC AGC CAA TCA TT -3'. The PCR reaction mixture consisted of DNA taq polymerase (1.5 U), sense and antisense primers (0.5 μmol/l), MgCl₂ (50 mmol/l), dNTP (0.2 mmol/l), and DNA template (1 μg). The thermal conditions were an initial denaturing step of 4 min at 95°C, then 35 cycles of denaturing for 30s at 95°C, annealing for 30 s (at 60°C for 251 A>T and 61°C for -845), extension for 30s at 72°C, and a final extension step of 10 min at 72°C. The amplified products were digested with 10 units of *Mun* I (Ferments, USA) and *Vsp* I (Ferments, USA) for *IL-8* -251 and *IL-8* -845 T/C respectively and incubated at 37°C for 16 hours. The digested products were checked on 3% agaroses gel, the RFLP picture for *IL-8* -251 genotype was identified as (A/A 500 bp/298bp, T/T 798 bp, A/T 798 bp/500 bp/298 bp) and *IL-8* -845 was identified as T/T (341 bp/193), C/C (534 bp) T/C (534 bp/341 bp/193 bp) (Figure 1). The results obtained by RFLP were later

Table 1. General Characteristics of the Study Population

Variables		Cases N (%)	Controls N (%)	p value
Age years	<50	59 (31.05)	65 (32.5)	0.8
	>50	131 (68.95)	135 (67.5)	
Sex	Male	163 (85.79)	170 (85)	0.88
	Female	27 (14.21)	30 (15)	
Smoking status	Non Smoker	76 (40.0)	84 (42)	0.76
	Smoker	114 (60.0)	116 (58)	
Dweller	Rural	127 (66.80)	129 (64.5)	0.67
	Urban	63 (33.15)	71 (35.5)	
Stage	I & II	130 (68.42)		
	III & IV	60 (31.58)		
Histology	Sq.Cell C ^a	140 (73.69)		
	Others ^B	50 (26.32)		

^aAdjusted for age, sex, dwelling and smoking. ^bOthers include adenocarcinoma, large cell carcinoma and bronchogenic carcinoma

confirmed by direct sequencing Figure 2.

Statistical analysis

Odds ratios (ORs) and their 95% confidence intervals (CIs), with adjustments for age, sex dwelling and smoking were calculated by Fisher's exact test/Chi square test as appropriate. Fisher's exact test/Chi square test was used for calculating p values. Significance level was taken at $p < 0.05$. Statistical tests were performed using the software SPSS 16.0 (SPSS Inc., Chicago, Illinois).

Results

Two SNPs *IL-8* -845 C/T(rs2227532) and *IL-8* -251 A/T (rs4073) were successfully evaluated in 190 lung cancer patients and 200 controls. *IL-8* -845 SNP was not present in our studied group and it was excluded for further analysis. The genotype frequencies of -251TT, -251AT and

251AA was 19.47%, 35.26% and 45.26 in cases while in controls frequencies were 27.5%, 33.0% and 39.5% in cases and controls respectively (Table 2) but variant allele

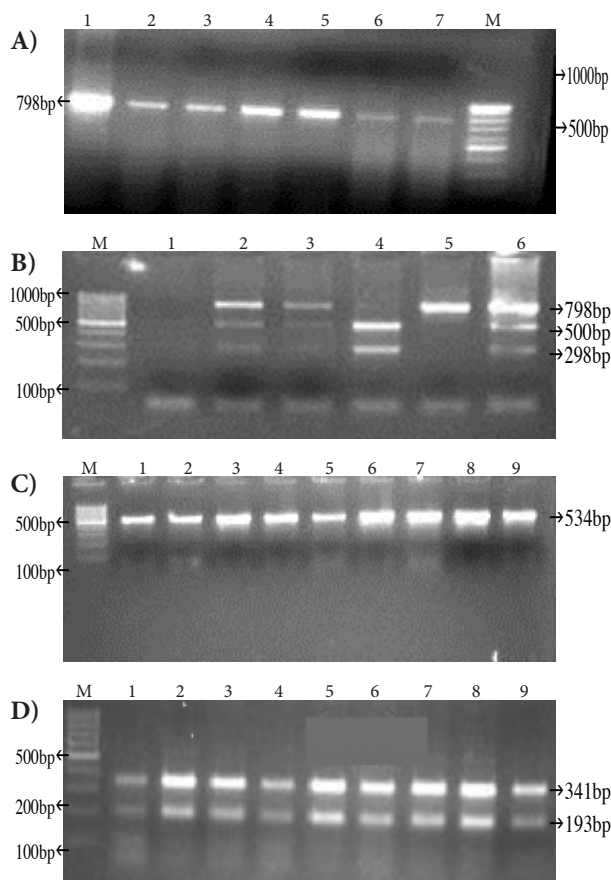


Figure 1. Amplification and Restriction Fragment Length Polymorphism Picture of *IL-8* -251T/A and *IL-8* -845T/C. **A)** Representative PCR amplification gel picture of *IL-8* -251 T/A. Lanes 1-7: 798bp amplified PCR product of *IL-8* -251 T/A. Lane M: 100 bp DNA Marker. **B)** RFLP picture of *IL-8* -251 T/A after restriction digestion with *Mun* I. (2%) agarose gel electrophoresis. Lane 5: Homozygous wild TT (798bp); Lane 4: Homozygous variant AA (500bp+298bp); Lanes 2, 3, 6: Heterozygous TA (798bp, 500bp+298bp); Lane M: 100bp DNA Marker. **C)** Representative PCR amplification gel picture of *IL-8* -845 T/C. Lanes 1-9: 534bp amplified PCR product of *IL-8* -845 T/C. Lane M: 100 bp DNA Marker. **D)** RFLP picture of *IL-8* -251 T/A after restriction digestion with VSPI (3%) agarose gel electrophoresis. Lanes 1-9, 4: Homozygous wild TT (341bp+193bp)

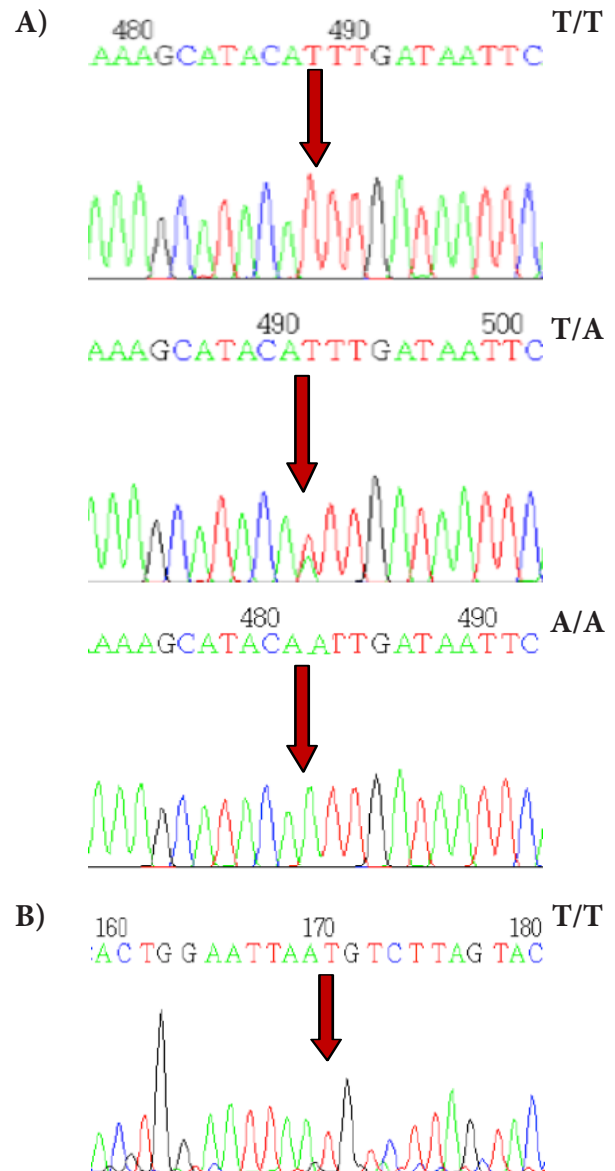


Figure 2. Partial Electropherograms. **A)** *IL-8* -251 T>A polymorphism showing wild TT, Heterozygous TA and AA variant genotypes. **B)** *IL-8* -845 C/G, showing wild TT genotype

Table 2. Association *IL-8* -251 Polymorphisms and Non Small Cell Lung Cancer

Variables	Cases N=190%	Controls N=200%	^a p value Odds ratio (95%CI)
<i>IL-8</i> -251 A/T			
TT	37 (19.47)	55 (27.5)	Ref
AT	67 (35.26)	66 (33)	0.1, 1.5 (0.88-2.59)
AA	86 (45.26)	79 (39.5)	0.06, 1.63 (0.97-2.74)
Allele			
T	141	176	0.05, 1.33 (0.99-1.77)
A	239	224	
<i>IL-8</i> -845 C>T			
TT	190 (100)	200 (100)	
CT	0	0	
CC	0	0	

^a*IL-8* -251: TT homozygous wild, TA heterozygous, AA homozygous variant, *IL-8* -845 TT homozygous wild, CT heterozygous, CC homozygous variant. ^aAdjusted for age, sex, dwelling and smoking

Table 3. Clinicopathological Relevance of *IL-8* -251 Polymorphisms in Non Small Lung Cancer

Parameters		Genotype (<i>IL-8</i> -251 A/T) N=190			Chi Square	p value
		TT	AT	AA		
Age	≤50 years	11	25	23	2	0.3
	>50 years	26	42	63		
Gender	Female	7	11	9	1.93	0.38
	Male	30	56	77		
Smoking status	Non Smoker	18	27	31	1.72	0.4
	Smoker	19	40	55		
Dweller	Rural	24	47	46	0.51	0.77
	Urban	13	20	30		
Histology	Sq.CC	29	52	59	3.32	0.19
	Others ^a	7	13	27		
Pathological stage	I & II	28	47	55	1.78	0.4
	III & IV	9	20	31		

^aOthers include Adenocarcinoma, large cell carcinoma, bronchogenic carcinoma

frequency was implicated more in cases than in controls with AT genotypes as 81.52% vs 72.5% respectively. When allele frequency was evaluated, the distribution of rare allele 'A' in cases was found higher in frequency 0.63 as against 0.56 in controls. However the difference was observed to be statistically nearly significant (p=0.05). There was no significant association of genotypes -251 AT and -251 AA between cases and controls (OR-1.5, (0.88-2.59) and (OR- 1.63 (95%CI 0.97-2.74) (Table 2) respectively. No significant distribution of -251 A allele was reported between cases and controls with odds ratio 1.33 (95%CI 0.99-1.77) (Table 2). The *IL-8* 251 A>T also did not show any significant association with age, gender, smoking status, histology, dwelling and stage disease of the patients with lung cancer (p>0.05) (Table 3).

Discussion

Interleukin-8 is a proinflammatory CXC chemokine associated with the promotion of neutrophil chemotaxis and degranulation. This chemokine activates multiple intracellular signalling pathways downstream of two cell-surface, G protein coupled receptors (CXCR1 and CXCR2). Increased expression of *IL-8* and/or its receptors has been characterized in cancer cells, endothelial cells, infiltrating neutrophils, and tumor-associated macrophages, suggesting that *IL-8* may function as a significant regulatory factor within the tumor microenvironment (Balkwill, 2004). The role of cytokines secreted by various inflammatory cells present in the tumor microenvironment are critical for tumor process (Vicari and Caux, 2002; Aamir et al., 2010). Interleukin 8 is a promising marker for many clinical conditions and currently being applied by various subspecialties of medicine either for the purpose of rapid diagnosis or as a predictor of prognosis (Xie, 2001). *IL-8* has angiogenic, mitogenic and motogenic activities (Yuan et al., 2005). *IL-8* expression control may be a valuable tool in designing new therapeutics for control of cancer growth and metastasis. Polymorphisms in pro-inflammatory factors could affect the body's inflammatory response by directly impacting

the expression and function of certain inflammatory cytokines. Ohyauchi et al. (2005) demonstrated that *IL-8* -251 A is associated with higher *IL-8* gene transcription and thus we evaluated this polymorphism in NSCLC patients of our population.

In this case control study we studied the association of *IL-8* -845C/T and *IL-8* -251A/T polymorphism in lung cancer patients. The *IL-8* -845 C/T SNP was not found in our study group. This is inconsistent with previous reports from Chinese and Korean populations, (Lee et al., 2007; Ye et al., 2007; Keshen et al., 2009; Hongxia et al., 2010). Therefore, this SNP was excluded for further analysis. The distribution of the genotypes -251TT, -251 AT and -251AA was 19.47%, 35.26% and 46% in cases and 27.5%, 33% and 39.5% controls respectively. We did not find any significant association between *IL-8* 251 T>A polymorphism and non small cell lung cancer. The frequency of *IL-8* -251 A allele was same in non small cell lung cancer and control group. We did not find any association between various clinic pathological characteristics and *IL-8* -251 A/T polymorphism in non small cell lung cancer. The various studies carried out to find the role of *IL-8* -251 SNP in lung cancer in different populations have shown the similar results which are in complete accordance with our study. The study carried out by Campa et al. (2005) comprised 2,144 cases and 2,116 controls patients and controls from six different countries Czech Republic, Hungary, Poland, Romania, Russia and Slovakia recruited between 1998 and 2002 yielded similar results. Another study carried out to study the role of *IL-8* -251A/T also reported insignificant association of *IL-8* -251A/T with non small cell lung cancer (Campa et al., 2004). Recently a meta analysis observed a significant association between *IL-8* -251T/A polymorphism and various cancers, while no associations was observed between this polymorphism and lung cancer (Na et al., 2012). A meta analysis carried out by Lin et al. (2010) has found that individuals carrying the *IL-8* -251 AA genotype were associated with a higher tumour risk in African population but not in Asian and European populations. The role of *IL-8* -251 T/A has also been studied in various malignancies and the results have been controversial. A study conducted in gastric cancer showed significant association of *IL-8* -251 AA with the susceptibility of gastric cancer and suggested *IL-8* -251 AA could become a biomarker for Asians (Huiping et al., 2012). However the role of *IL-8* -251 has remained inconsistent in the predisposition of colorectal cancer. Mohd et al. (2012) reported a significant association of *IL-8* -251 AA and colorectal cancer while as a meta analysis showed no association between *IL-8* -251AA and colorectal cancer (Li et al., 2012). A recent meta analysis has shown AA and AT genotypes of *IL-8* -251A>T polymorphism were associated with increased risk of oral cancer (Zhiming et al., 2013). Similarly subjects with the *IL-8* -251 A allele appeared to have lower susceptibility to liver cirrhosis than those with the *IL-8* -251T allele (Xue et al., 2012). The role of -251T/A in regulating the expression of *IL-8* -251 has been studied. A study carried out to study the effect of *IL-8* -251A/T reported that *IL-8* -251T allele is responsible in up regulating the expression of *IL-8* in gastric cancer

instead of *IL-8* -251A allele (Song et al., 2010). Another study carried out by Wei et al. (2005) reported that -251T allele possessed transcriptional activity 2 to 5 folds stronger than the -251A counterpart. While as the study conducted in Brazilian population showed *IL-8* -251 T/A genotype was associated with increased levels of *IL-8* mRNA transcripts (Denise et al., 2011). All these studies point out to the observation that the role *IL-8* -251 T/A is controversial in determining its role in the carcinogenesis of any histological type.

In conclusion, our results reveal that *IL-8* -251A/T polymorphism do not play a significant role in predisposition to non small cell lung carcinogenesis in our population.

Acknowledgements

The authors gratefully acknowledge the financial support provided by the Sher-i-Kashmir Institute of Medical Sciences, Kashmir for this work. Our thanks are also due to the Technical Staff of the department of medical Oncology & CVTS SKIMS Srinagar who helped us in collection of samples samples.

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