RESEARCH ARTICLE

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

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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)

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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 tumorogenic, 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

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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 (Ohyauchi 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 -251A/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 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). 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 ACC 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’-AACCCAGCAGTCCAGTGT-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 MunI (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% agarose 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 | Controls | 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      | Stage I & II | 130 (68.42) |         |
|            | III & IV | 60 (31.58) |         |
| Histology  | Sq.Cell C+ | 140 (73.69) |         |
|            | Othersa | 50 (26.32) |         |

*Adjusted for age, sex, dwelling and smoking. aOthers 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% | p value | Odds ratio (95%CI) |
|-----------|-------------|----------------|---------|-------------------|
| AIL-8 251 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 | | |

| IL-8 -845 C>T | | | |
| TT | 190 (100) | 200 (100) | | |
| CT | 0 | 0 | | |
| CC | 0 | 0 | | |

*IL-8 -251: TT homozygous wild, TA heterozygous, AA homozygous variant, IL-8 -845 TT homozygous wild, CT heterozygous, CC homozygous variant. *Adjusted for age, sex, dwelling and smoking
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 metal 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 -251AA 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 -251T/A reported that IL-8 -251T allele is responsible in up regulating the expression of IL-8 in gastric cancer

| Parameters       | Genotype (IL-8 -251 A/T) | Chi Square | p value |
|------------------|--------------------------|------------|---------|
|                  | TT | AT | AA      |
| Age ≤50 years    | 11 | 25 | 23 2 0.3 |
| Age >50 years    | 26 | 42 | 63    |
| Gender Female    | 7  | 11 | 9 1.93 0.38 |
| Gender 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           | 7  | 13 | 27    |
| Pathological stage | I & II | 28 | 47 | 55 1.78 0.4 |
| Pathological stage | III & IV | 9  | 20 | 31    |

*Others include Adenocarcinoma, large cell carcinoma, broncogenic carcinoma*
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 TA 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.

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