

## RESEARCH ARTICLE

# Effects of Tobacco Habits on the Polymorphism of NFKB1 and NFKB1A Gene of Head and Neck Squamous Cell Carcinoma in Indian Population

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### Abstract

**Background:** Polymorphism of NFKB1 and NFKB1A are highly associated with cancer. We have assessed polymorphism in the promoter region of NFKB1 -94 del/ins ATTG (rs28362491) and NFKB1A -826 C/T (rs2233406) with the risk of HNSCC in Indian population. **Methods:** Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method was used for the genotyping NFKB1 -94 del/ins ATTG and NFKB1A -826 C/T. Sequencing was done to validate the results of PCR-RFLP. Statistical analysis of data was done by Stata/SE-14.0 software. **Results:** ins/ins genotype was observed to be a risk factor of HNSCC as compared del/del genotype of NFKB1 -94 ATTG. Interactive effects of smoking and chewing on ins/ins genotype showed 13.96 and 10.92 fold increased risk of HNSCC. NFKB1A -826 C/T polymorphism, TT genotype showed no association with the risk of HNSCC as compared to wild type CC genotype. **Conclusion:** Our results showed NFKB1 -94 del/ins ATTG with smoking and tobacco chewing may increase the risk of HNSCC while NFKB1A -826 C/T plays a protective role in Indian population.

**Keywords:** HNSCC- NFKB1- NFKB1A-PCR-RFLP- polymorphism

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### Introduction

Head and Neck Squamous Cell Carcinoma (HNSCC) is the most prominent cancer in developing countries (Joshi et al., 2014). Over 200,000 cases of HNSCC occur each year in India and 30,000 in the United States (Guru K et al., 2012). HNSCC is a complex process in which multiple genes of the cells are altered and influenced by genetic and environmental factors. Alteration of genes occurred by a single-nucleotide polymorphism (SNP) located within the promoter or other regulatory regions of the gene are found to be associated with the development of various cancers (Shastry, 2002). Cigarette smoking and tobacco chewing are the leading causes of death from HNSCC, and at least 40% of all cancers are estimated to be attributable to tobacco habit in India (Prasad R, 2016). Prolonged environmental exposure like tobacco, alcohol habit and genetic alterations have important relationship with cancer incidence.

Nuclear factor of  $\kappa$ B (NF- $\kappa$ B) is a small menagerie of closely related proteins (p50, p52, Rel-A, Rel-B, and c-Rel) that form a different types of heterodimers and bind to common motif sequences known as  $\kappa$ B site. Several genes that mediate tumorigenesis and metastasis are regulated by nuclear transcription factor NF- $\kappa$ B complex.

In normal cells, NF- $\kappa$ B complex exists in an inactivated state by I $\kappa$ B, while in a cancer cell, this NF- $\kappa$ B complex is activated by various signals which degrade I $\kappa$ B $\alpha$  inhibitory protein through a cascade of reactions. After activation, NF- $\kappa$ B complex is translocated to the nucleus, binds the  $\kappa$ B element (at the promoter region), and activates gene that is responsible for the development of cancer (Karin et al., 2002). Based on other prior research, it is revealed that NF- $\kappa$ B complex is activated by carcinogens, tumor promoters which alter the genetic information and inflammatory cytokines. The activation of NF- $\kappa$ B complex can promote chemoresistance and tumorigenesis by suppressing apoptosis. It has been reported that the promoter region of the NFKB1 (which encoded protein NF- $\kappa$ B p50) and NFKB1A (which encodes protein I $\kappa$ B) genes are associated with a risk for Hodgkin's lymphoma (Chang et al., 2009), multiple myeloma (Spink et al., 2007), breast cancer (Curran et al., 2002), prostate cancer (Zhang et al., 2009), gastric cancer (Lo et al., 2009), colorectal cancer (Gao et al., 2007), melanoma (Bu et al., 2007), and oral cancer (Lin et al., 2012).

It has been reported that combined effect of NFKB1 -94 del/ins ATTG and NFKB1A -826 C/T polymorphism with betel nut and smoking habit associated with increased risk of oral cancer in Taiwanese population (Lin et al.,

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2012). NFKB1 -94 del/ins ATTG and NFKB1A -826 C/T may play an important role in the expression of NF- $\kappa$ B p50 and I $\kappa$ B proteins, respectively. Over expression of NF- $\kappa$ B p50 protein found in non-small cell lung carcinomas (Mukhopadhyay et al., 1995), human breast carcinomas, colon, lung, breast tumor cell lines (Dejardin et al., 1995) and in mouse skin carcinogenesis (Budunova et al., 1999). The present study aims to explore the association of NFKB1 -94 del/ins ATTG (rs28362491) and NFKB1A -826 C/T (rs2233406) polymorphism with the risk of HNSCC and correlate the polymorphism with environmental risk factors in Indian population.

## Materials and Methods

### Study group selection

The participants of this study included 312 HNSCC patients recruited at the time of diagnosis from the Institute Rotary Cancer Hospital, All India Institute of Medical Sciences, New Delhi and 312 healthy controls resided in the same geographic area. The study was approved by the Institution Ethics Subcommittee of All India Institute of Medical Sciences, New Delhi (Ref. No. IESC/T-60/01.02.2013). Written informed consent was obtained from all participants involved in this study. The demographic data and clinical characteristics of the study group were collected. Controls with any disease or medical history of disease were excluded. Patients with any other chronic diseases and previously exposed to radiotherapy, chemotherapy and surgery were excluded from the study group. Patients were clinically staged at the time of diagnosis, according to the TNM staging system of the American Joint Committee on Cancer (AJCC) Staging Manual.

### Genomic DNA extraction

Genomic DNA was extracted from 200  $\mu$ l EDTA-anticoagulated peripheral blood using mdi G Blood Genomic DNA (gDNA) Miniprep Kit and dissolved in a TE buffer (10 mM Tris, 1 mM EDTA; pH 7.8), which was subsequently quantified by measuring the OD at 260 nm and used for the Polymerase Chain Reaction- Restriction Length Fragment Polymorphism (PCR-RFLP).

### Genotyping

Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method was used for genotyping of NFKB1 -94 del/ins ATTG (rs28362491) and NFKB1A -826 C/T (rs2233406). A forward primer, TGGGCACAAGTCGTTTATGA, and a reverse primer, CTGGAGCCGGTAGGGGAAG, for NFKB1 -94 del/ins ATTG and a forward primer, GGTCCTTAAGGTCCAATCG and a reverse primer, GTT GTGGATACCTTGCACTA, for NFKB1A -826 C/T were used for the experiment (He et al., 2009). PCR program was applied as follows: 95°C for 3 min initial denaturation, followed by 34 cycles of 95°C for 30 sec denaturation, annealing at 56.2°C (NFKB1-94 del/ins ATTG) and 60.5°C (NFKB1A -826 CT) for 30 sec, 72°C for 1 min extension and a final extension at 72°C for

10 min. The PCR product of NFKB1-94 del/ins ATTG was digested with 1 unit of restriction enzyme PflMI (10 U/ $\mu$ l, Fermentas) and incubated at 37°C for 20 min and subsequently run on 2.5% agarose gel, whereas, the PCR product of NFKB1A -826 C/T was digested with restriction enzyme Bfal (10 U/ $\mu$ l, NEB) at 37°C for 1 h, analysed by 10% polyacrylamide DNA gel.

### Statistical evaluation

Statistical analysis of the data was done by using Stata/SE-14.0 software. Chi square test was used to compare the differences in demographic characteristic distributions between the control group and HNSCC patients. Hardy-Weinberg equilibrium (HWE) was tested using a goodness-of-fit  $\chi^2$  test. The adjusted odds ratios (AORs) and 95% confidence intervals (CIs) of the association of genotype frequencies with risk and clinicopathological characteristics were estimated using multiple logistic regression models after adjusting with covariates (age, sex, smoking, chewing and drinking). A two-sided p-value of less than 0.05 was considered for statistical significance.

## Results

### Demographic characteristics of the study population

The frequency distribution of demographic and clinical characteristics of the 312 HNSCC patients and 312 controls showed in Table 1. The power calculation analysis showed that the case control pair of 312 samples was sufficient to achieve a 80% power for effective sample size. The majority of the participants were male in both groups, patients (82.69%) and controls (79.17%). Most of the patients in the study cohort were with tobacco habit (73.08%) as compared to control group (29.49%). The sites affected in majority of the patients were oral cavity and oropharynx. The patients with advanced stage (III +IV) were presented in maximum number (84.94%).

### PCR-RFLP of NFKB1 and NFKB1A gene

Analysis of the NFKB1 and NFKB1A were performed by the PCR-RFLP method. Genotype of the NFKB1 -94 del/ins ATTG was determined as del/del genotype without PflMI restriction site, the PCR product of 281 bp remained undigested. On the other hand, the insertion variants were cleaved by PflMI (Van91I) restriction enzyme into two fragments of 240bp and 45 bp. Heterozygotes showed all three bands (Figure. 1 a). The genotypes of NFKB1A -826 C/T polymorphisms were determined as CC (200 bp), TT (180 and 20 bp), and heterozygote C/T(200 bp, 180 bp and 20 bp) (Figure. 1 b). The presence or absence of a polymorphism was monitored by UV transilluminator (Bio-Rad Gel Doc XR+) system. The genotype results were confirmed via direct DNA sequencing of the amplified fragments. Ten percent of the total samples were used for sequencing.

### Association of NFKB1 and NFKB1A polymorphisms with HNSCC

Genotype distribution of promoter polymorphisms

Table 1. Frequency Distribution of Selected Variables of the Controls and HNSCC Patients

Variable	Controls (n=312)	Patients (n=312)	p value
Age (y)			
20-40	114 (36.54%)	82 (26.28%)	
41-60	115 (36.86%)	158 (50.64%)	
>60	83 (26.60%)	72 (23.08%)	p=0.002
Mean age (year±SD)	45.00±15.94	49.39±11.90	
Sex			
Male	247 (79.17%)	258 (82.69%)	
Female	65 (20.83%)	54 (17.31%)	p=0.262
Smoking Status			
Yes	92 (29.49%)	228 (73.08%)	
No	220 (70.51%)	84 (26.92%)	p=0.0001
Chewing Status			
Yes	86(27.56%)	143 (45.83%)	
No	226 (72.44%)	169 (54.17%)	p=0.0001
Alcoholic Status			
Yes	110 (35.26%)	83(26.60%)	
No	202 (64.74%)	229 (73.40%)	p=0.019
Histopathology			
SCC	-	66 (21.15%)	
WDSCC	-	55 (17.63%)	
MDSCC	-	178 (57.05%)	
PDSCC	-	13 (4.17%)	
Stage			
I+II	-	47 (15.06%)	
III+IV	-	265 (84.94%)	
Tumor Size			
T1+T2	-	70 (22.44%)	
T3+T4	-	242 (77.56%)	
Node			
N0	-	127 (40.71%)	
N+	-	185 (59.29%)	
Site			
Nasopharynx	-	11 (3.53%)	
Larynx	-	45 (14.42%)	
Oropharynx	-	107 (34.29%)	
Oral Cavity	-	149 (47.76%)	

MDSCC, Moderately Differentiated Squamous Cell Carcinoma; WDSCC, Well Differentiated Squamous Cell Carcinoma; PDSCC, Poorly Differentiated Squamous Cell Carcinoma; SCC, Squamous Cell Carcinoma; Chi square test was performed to analyze differences between controls and patients.

in NFKB1-94 del/ins ATTG and NFKB1A -826 C/T between patients and controls, as well as their associations with HNSCC risk were presented in Table 2. Chi square test showed genotype frequencies of NFKB1-94 del/ins ATTG in controls were (p=0.0641) in accordance to Hardy–Weinberg Equilibrium (HWE). The frequencies of the del/del, del/ins, and ins/ins genotypes were 5.77%, 51.92% and 42.31% among the cases,

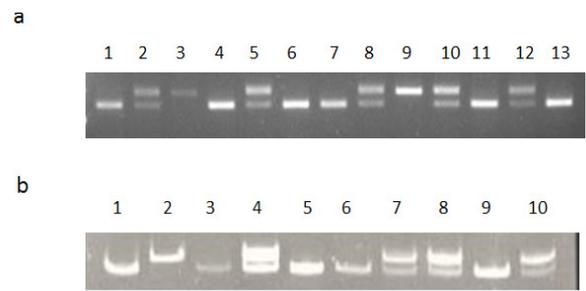


Figure 1. a) The Pattern of PfIMI Enzyme Digestion. Lanes 2, 5, 8, 10 and 12 are heterozygous del/ins ATTG; lanes 1, 4, 6, 7, 11 and 13 are homozygous ins/ins ATTG; and Lane 3 and 9 is homozygous del/del ATTG genotype. b) The BfaI Digestion Profile. Lanes 4, 7, 8 and 10 are heterozygous CT; lanes 1, 3, 5, 6 and 9 are homozygous GG; and Lane 2 is homozygous CC genotype.

respectively, and 7.05%, 46.79%, and 46.15% among the controls, respectively. Using the del/del genotypes of NFKB1-94 as reference, 1.12 fold increased risk of HNSCC was observed with ins/ins genotype (OR=1.12, 95% CI =0.57-2.18). We also found that the del/ins

Variable	Distribution Frequencies for NFKB1 Promoter -94 del/ins ATTG and NFKB1A -826 C/T Genotypes in Controls and Patients.		OR (95% CI)	p value	AOR (95% CI)	p value
	Controls (n=312)	Patients (n=312)				
<b>NFKB1 Genotype</b>						
del/del	22 (7.05%)	18 (5.77%)	1 (ref)		1 (ref)	
del/ins	146 (46.79%)	162 (51.92%)	1.35 (0.69-2.62)	0.367	2.36 (1.06-5.23)	0.034
ins/ins	144 (46.15%)	132 (42.31%)	1.12 (0.57-2.18)	0.738	1.75 (0.79-3.89)	0.164
del/ins+ins/ins	290 (92.95%)	294 (94.23%)	1.23 (0.65-2.35)	0.514	2.04 (0.94-4.41)	0.068
<b>NFKB1A Genotype</b>						
CC	16 (5.13%)	32 (10.26%)	1 (ref)		1 (ref)	
CT	208 (66.67%)	199 (63.78%)	0.47 (0.25-0.89)	0.022	0.47 (0.22-0.98)	0.046
TT	88 (28.21%)	81 (25.96%)	0.46 (0.23-0.90)	0.024	0.42 (0.19-0.92)	0.031
CT+TT	296 (94.87%)	280 (89.94%)	0.47 (0.25-0.88)	0.018	0.46 (0.22-0.94)	0.034

Table 3. Interactive Effect of Smoking, Chewing and Alcohol Consumption on NFKB1 –94 del/ins ATTG Polymorphism

Variable	Controls	Patients	OR (95% C.I.)	p value	AOR (95% C.I.)	p value
<b>Smoking</b>						
del/del without NS	11 (3.65%)	5 (1.67%)	1 (ref)		1 (ref)	
del/ins or ins/ins without S	209 (69.44%)	79 (26.42%)	0.83 (0.28-2.46)	0.74	1.03 (0.32-3.29)	0.06
del/ins or ins/ins with S	81 (26.91%)	215 (71.91%)	5.83 (1.96-17.32)	0.001	13.96 (4.22-46.11)	0.0001
<b>Chewing</b>						
del/del without NC	14 (66.67%)	7 (2.33%)	1(ref)		1(ref)	
del/ins or ins/ins without C	212 (69.74%)	162 (53.82%)	1.52 (0.60-3.87)	0.371	2.15 (0.77-6.03)	0.143
del/ins or ins/ins with C	78 (25.66%)	132 (43.85%)	3.38 (1.30-8.74)	0.012	10.92 (3.68-32.40)	0.0001
<b>Alcoholic</b>						
del/del without NA	14 (4.61%)	14 (4.55%)	1 (ref)		1 (ref)	
del/ins or ins/ins without A	188 (61.84%)	215 (69.81%)	1.14 (0.53-2.46)	0.731	2.06 (0.81-5.26)	0.129
del/ins or ins/ins with A	102 (33.55%)	79 (25.65%)	0.77 (0.34-1.71)	0.53	0.81 (0.31-2.13)	0.679

OR, Odd Ratios; AOR, Adjusted Odd Ratios; NS, Non smoking; S, Smoking; NC, Non Chewing; NA, Non alcoholic; A, alcoholic; ORs with 95% CIs were estimated by logistic regression models; AORs with 95% CIs were estimated by multiple logistic regression models after controlling for sex, age, smoketobacco chewing and alcohol consumption status.

genotype was significantly ( $p=0.034$ ) associated with a increased risk of HNSCC (AOR=2.36, 95% CI=1.06-5.23) after adjusting co-variates sex, age, smoking, chewing and alcoholic status. While no significant association of NFKB1A -826 C/T polymorphism was detected (OR=0.46, 95% CI= 0.23-0.90) with TT genotype as compared to wild type CC genotype.

#### *Interactive effects of environmental risk factors on genetic polymorphism*

Interactive effects were also analysed between environmental risk factors (smoking, chewing and alcohol status) and polymorphism of NFKB1–94 del/ins ATTG. The ins/ins genotypes was found to be 13.96 fold increased risk of HNSCC ( $p=0.0001$ ) with smoking habit (OR= 13.96, 95% CI 4.22-46.11) as compared to del/del genotype with non smoking (NS) after adjusting sex, age, chewing and alcohol consumption. Similarly, after adjusting variables, ins/ins genotype showed 10.92 fold higher risk of HNSCC (OR=10.92, 95% CI=3.68-32.40,  $p=0.0001$ ) with chewing (C) as compared to del/del with non chewing. No significant association of HNSCC risk was observed with alcohol consumption (Table 3).

#### *Association of NFKB1 –94 del/ins ATTG and NFKB1A -826 C/T with clinicopathologic variables*

No significant association was observed between NFKB1 promoter –94 del/ins ATTG and NFKB1A -826 C/T genotypes and the clinicopathological variables such as stage, tumor size and lymph node metastasis of to the overall risk of HNSCC (Supplementary Table 1).

## **Discussion**

The primary risk factors for the HNSCC are tobacco chewing, cigarette smoking, alcohol consumption and HPV infection (Vigneswaran and Williams, 2014). Among all, smoking and chewing are the most common etiologic factors in Indian subcontinent. We have observed a higher

number of patients affected at oral cavity and oropharynx sites in the study group due to high prevalence of tobacco chewing and smoking habits. Middle age group (40-60 years) is the most susceptible to the disease, because of the accumulation of toxic compounds into the cells due to long term tobacco consumption. The NF- $\kappa$ B signaling pathway plays an important role in cell regulation, expression of genes, influencing a broad range of biological processes which include apoptosis, inflammation, stress responses, infection, etc. and dysregulation of this pathway leads to develop tumor and cause cancer (Karin et al., 2002). Various polymorphic sites of NFKB1 and NFKB1A have been reported in various journals, among them NFKB1-94 del/ins ATTG and NFKB1A -826 C/T are the most significant and have biologic importance, too. To our knowledge this study is the first to identify the association between NFKB1 -94 del/ins ATTG and NFKB1A -826 C/T polymorphism in the promoter region with HNSCC risk in an Indian population. We have observed different frequency of NFKB1 -94 del/del genotype in our population as compared to the Taiwanese population due to the ethnicity differences. The risk of HNSCC was analysed higher in NFKB1 -94 ins/ins as compared to del/del genotype. There is an evidence that the NFKB1 -94 polymorphism containing ins/ins genotype has the higher binding ability to the promotor than del/del genotype (Karban et al., 2004) and consequently enhanced the expression of NF- $\kappa$ B. The frequencies of the smoking and tobacco chewing were observed higher in patients as compared to controls. Carcinogenic compounds of tobacco are accumulated in cells over time and may interfere with DNA replication and damage the DNA of the cell, leads to development of tumor (Hoeijmakers, 2009). Present study analysed the interactive effect of smoking, tobacco chewing and alcohol consumption with NFKB1 -94 ins/ins as higher risk factor of HNSCC. Over time predisposition of toxic compounds into the cell make the individual susceptible for the disease. Hence, ins/ins genotype is known risk factor of HNSCC due to

up-regulation of NF- $\kappa$ B protein expression and tobacco smoking further increases the risk of HNSCC in presence of ins/ins genotype in Indian population.

The functional activity of NF- $\kappa$ B complex is regulated by inhibiting through I $\kappa$ B in the cytoplasm and differential expression of I $\kappa$ B leads to dysregulation of NF- $\kappa$ B signaling pathways. Therefore, we also studied polymorphism of NFKB1A -826 C/T which may influence the expression of NF- $\kappa$ B. In this study, NFKB1A -826 C/T polymorphism showed the protective role in population and no association of NFKB1A -826 C/T with risk of HNSCC in Indian population while in other population (Lin et al., 2012), NFKB1A -826 C/T polymorphism was found to be associated with risk of cancer.

Hence, from our research result, it can be summarized that in Indian population, NFKB1-94 ATTG ins/ins genotype along with smoking or chewing habit is more susceptible for developing HNSCC as compared to del/del genotype with non-smoking or non-chewing. NFKB1-94 ATTG ins/ins genotyping may help for prediction of HNSCC risk, while NFKB1A -826 C/T indicates TT genotype plays a protective role in the general population of India.

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