

# TNF $\alpha$ –308G/A polymorphism as a risk factor for HPV associated Cervical Cancer in Indian population

Indu Kohaar<sup>a</sup>, Nisha Thakur<sup>a</sup>, Sudha Salhan<sup>b</sup>, Swaraj Batra<sup>c</sup>, Veena Singh<sup>d</sup>, Anita Sharma<sup>a</sup>, Pushpa Sodhani<sup>e</sup>, B.C. Das<sup>f</sup>, Debi P. Sarkar<sup>g</sup> and Mausumi Bharadwaj<sup>a,\*</sup>

<sup>a</sup> Division of Molecular Genetics & Biochemistry, Institute of Cytology & Preventive Oncology (ICMR), I-7, Sector 39, Noida, India

<sup>b</sup> Department of Obstetrics and Gynaecology, Safdarjung Hospital, New Delhi, India

<sup>c</sup> Department of Obstetrics & Gynaecology, LNJP Hospital, New Delhi, India

<sup>d</sup> Division of Clinical Oncology, Institute of Cytology & Preventive Oncology (ICMR), I-7, Sector 39, Noida, India

<sup>e</sup> Division of Cytopathology, Institute of Cytology & Preventive Oncology (ICMR), I-7, Sector 39, Noida, India

<sup>f</sup> Division of Molecular Oncology, Institute of Cytology & Preventive Oncology (ICMR), I-7, Sector 39, Noida, India

<sup>g</sup> Department of Biochemistry, University of Delhi (South Campus), New Delhi, India

**Abstract.** *Background:* Investigation of the potential association of single nucleotide polymorphisms (SNPs) at –308 G/A and –238 G/A of Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) with susceptibility to HPV-16 associated cervical cancer in Indian women. *Methods:* The study included 165 histologically confirmed cases with 45 precancer and 120 cancer patients and an equal number (165) of healthy controls with normal cervical cytology. PCR-RFLP was employed to analyze TNF $\alpha$  promoter polymorphisms, which were confirmed by direct sequencing. Both patients and controls were screened for Human Papillomavirus (HPV) infection. *Results:* The frequency of –308 A allele in TNF $\alpha$  was significantly higher in cases compared with control subjects (21% in cases vs. 9% in controls;  $p < 0.01$ ), with an odds ratio of 2.7 (95% CI = 1.41–5.15). Also, women carrying A allele for this locus presented 3 times increased susceptibility to HPV 16 infection as evident from carrier genotype distribution between HPV positive cases and control subjects (24% in HPV positive cases vs. 9% in controls;  $p < 0.01$ ; OR = 3.1; 95% CI = 1.60–6.03). No such association was found for TNF $\alpha$ –238 (G/A) polymorphism with the risk of development of cervical cancer. *Conclusion:* It suggests that SNP at –308 (G/A) of TNF $\alpha$  promoter may represent an increased risk for HPV infection and development of cervical cancer in Indian women.

**Keywords:** Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), single nucleotide polymorphism, –308 promoter polymorphism, –238 promoter polymorphism, cervical precancer, cervical cancer, human papillomavirus, genetic susceptibility

## 1. Introduction

Cancer of the uterine cervix is the second most common cancer among women worldwide but it is the most common cancer in Indian women [6]. A wealth of epidemiologic and molecular biologic data established an etiologic link between high-risk human papillomavirus

(HR-HPV) infection and cervical cancer. In 70–90% of HPV-infected individuals the virus is naturally cleared while in small percentage of patients persistent infection with HR-HPV such as HPV type 16 and 18 lead to the development of cervical intraepithelial neoplastic lesion (CIN), a precursor of cervical cancer [26]. Thus, the variations in effective host immune response may be an important determinant of persistence of HPV infection and susceptibility to cervical cancer. Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), being a potent proinflammatory cytokine, has been implicated in the control of HPV infection in several *in vivo* and *in vitro* studies [2,17,24]. It is known to induce apoptosis in cervical

\*Corresponding author: Mausumi Bharadwaj, Division of Molecular Genetics & Biochemistry, Institute of Cytology & Preventive Oncology (ICMR), I-7, Sector 39, Noida 201301, India. Tel.: +91 95120 2579471; Fax: +91 95120 2579473; E-mail: mausumi02@yahoo.com, bharadwajm@icmr.org.in.

cancer cells and up-regulates vascular adhesion molecules as well as different chemokines leading to stimulation of inflammatory responses in the host. It also arrests growth of HPV infected keratinocytes, and down regulates HPV gene transcription [8]. Several studies have revealed a close association between -308 and -238 TNF $\alpha$  promoter polymorphisms with a number of inflammatory, infectious and autoimmune diseases [9]. These SNPs are also found to be associated with numerous cancers including cervical cancer in different populations [4,8,9,11,12,14,15,22]. Recently, several lines of studies have demonstrated that the transcription of this cytokine is highly affected by G/A polymorphism at -308 [16,25] and the A allele is associated with an increased risk of invasive cervical cancer in Portuguese population [9]. Another common regulatory polymorphism, G to A substitution at -238 locus in the TNF $\alpha$  promoter is shown to be associated with decreased susceptibility to various types of cancer in Korean women [14].

However, to the best of our knowledge, no report is available addressing the association of SNPs in the TNF $\alpha$  promoter with the susceptibility to HPV associated cervical cancer in Indian women. The present study has therefore been designed to investigate whether there is any association between the TNF $\alpha$  promoter variants and prevalence of cervical cancer and HPV infection.

## 2. Materials and methods

### 2.1. Subjects

We investigated the association of SNPs at -308 (G/A) and -238 (G/A) of TNF $\alpha$  promoter with the susceptibility to HPV associated cervical precancer and cancer cases in a hospital based case-control study. A total of 165 cases of Indo-Aryan ethnicity comprising cervical precancer (45) and invasive carcinoma (120) were employed for the study. The patients were recruited from Lok Nayak Jai Prakash and Safdarjung Hospitals, New Delhi, with histopathologically confirmed precancer/invasive carcinoma of uterine cervix. The patients had a mean age of  $49.4 \pm 12.4$  yrs.

The age and ethnicity matched control group consisting of 165 healthy women with no self or family history of any neoplastic disease and with normal cervical cytology were from outdoor patients of Department of Gynaecology, Safdarjung hospital, New Delhi, who came for routine checkup. Written consent

was obtained from all the participants and the study was carried out in accordance with the principles of Helsinki Declaration and was approved by the Ethics Committee of the Institute.

### 2.2. DNA extraction and HPV detection

Genomic DNA was extracted from fresh cervical tissue biopsy samples (patients) and cervical scrapes (control) by standard method using proteinase K followed by phenol/chloroform/isopropanol treatment [20].

HPV diagnosis was performed by PCR amplification using consensus primers MY09 and MY11 [18] and further typing was done by PCR using type specific primers for HPV 16 and HPV18 [19].

### 2.3. Analysis of TNF $\alpha$ G-308A and G-238A polymorphism by PCR-RFLP

We used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach to genotype these two loci of the TNF $\alpha$  promoter region with modification as employed by Jang et al. [14]. PCR products were digested overnight at 37°C in 10  $\mu$ l reaction volume containing 0.025 U of restriction enzyme (*Nco*I was used for -308 G/A polymorphism and *Bgl* II was used for -238 G/A polymorphism). The RFLP analysis was performed on 15% native polyacrylamide gel (Fig. 1A). The attributable proportion (AP) value was also calculated using formula:  $AP = PRF \times (1 - 1/OR)$  where PRF represents percentage of risk factor and OR is odds ratio [9].

### 2.4. DNA sequencing

We sequenced 20% of the patient samples randomly to validate the data generated by PCR-RFLP method. Sequencing reactions were performed according to the conventional dideoxy chain termination method using ABI Prism™ 310 Automated DNA Sequencer (Applied Biosystem, USA) (Fig. 1B).

### 2.5. Statistical analysis

The data analysis was performed using the computer software Statistical Package for the Social Sciences (SPSS) for Windows (version - 12.0). Chi-square test / Fisher's Exact Test (for smaller numbers on subgroup analysis) was used to compare the distributions of TNF $\alpha$  promoter polymorphisms between cancer patients and healthy controls.

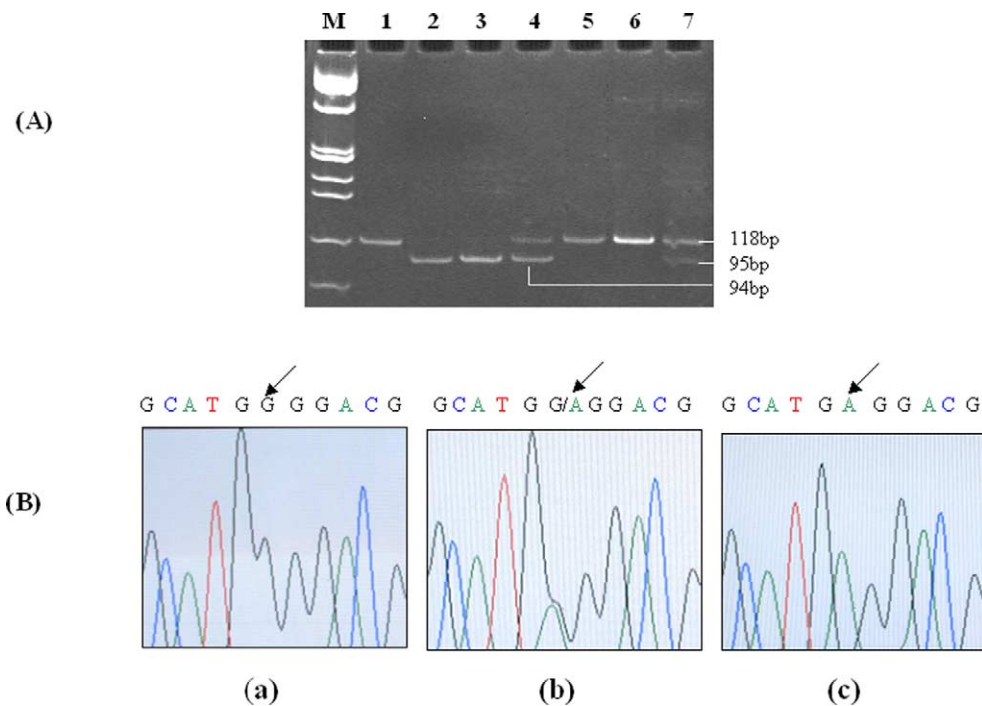


Fig. 1. (A) Analysis of the TNF $\alpha$  promoter genotypes at  $-308$  G/A &  $-238$  G/A by RFLP of the PCR products on 15% native PAGE gel. Lane M,  $\phi$ X1/Hae III digested Mol.Wt.Marker; lanes 1–4, *Nco* I digested products for  $-308$  locus (lane 1 – homozygous AA, lane 2 and 3 – homozygous GG, lane 4 – heterozygous GA); lanes 5–7, *Bgl* II digested products for  $-238$  locus (lane 5 and 6 – homozygous GG, lane 7 – heterozygous GA). (B). Partial electropherogram of TNF $\alpha$  promoter region depicting  $-308$  (G/A) polymorphism (polymorphic site depicted with the arrow) (a) homozygous G, (b) heterozygous G/A and (c) homozygous A.

The odds ratio (OR) and its 95% confidence Intervals (CI) were also calculated as a measure of the association between TNF $\alpha$  genotypes and cervical cancer risk. The significance of statistical test Chi-square / Fisher's Exact was considered as 2-tailed.

Genotypes were further checked for the conformance of Hardy Weinberg Equilibrium.

### 3. Results

#### 3.1. HPV prevalence

In the studied cohort about 82% (135/165) of cases and 0.61% (1/165) of normal healthy controls showed positivity for HPV DNA sequence. Out of the HPV positive cases, 98.5% (133/135) were infected with HPV type16 and rest 1.5% (2/135) were found to be positive for HPV type 18, while the only one HPV positive healthy control was found to be infected with HPV type 16.

#### 3.2. TNF $\alpha$ -308 G/A polymorphism

The genotype distribution and allelic frequencies for  $-308/-238$  loci of TNF $\alpha$  promoter in healthy controls and in patients with cervical precancer and cancer lesion is shown in Table 1. PCR-RFLP was performed to analyze these two loci, which was further confirmed by DNA sequencing for 20% of the samples. Both the techniques revealed similar results (Fig. 1). We observed that there was a significant difference ( $p < 0.01$ ) in TNF $\alpha$ -308 carrier A (GA/AA) genotype distribution between cases and controls with 21% (35/165) in cases and 9% (15/165) in controls. The estimated odds ratio of carrier genotype (GA/AA) to GG genotype was 2.7 (95% CI = 1.41–5.15), which indicated an increased risk for the development of the cervical cancer in women carrying A allele. When we stratified the cases according to the disease severity, both the precancerous and cancerous cases exhibited association for TNF $\alpha$ -308 A allele. The association was found to be highly significant ( $p < 0.01$ ) in carcinoma cases in comparison to the control group with an odds ratio of 2.8 (95% CI = 1.39–5.49) but in pre-

Table 1

Distribution of -238 and -308 TNF $\alpha$  promoter genotypes among cervical precancerous and cancerous patients and healthy control subjects

		Genotypes (%)		<i>p</i> -value	OR (95% CI)	Allele G	Frequency A
		GG	GA/AA				
Control ( <i>n</i> = 165)	-238 G/A	159 (96)	6 (4)	Reference <sup>a</sup>		0.982	0.018
	-308 G/A	150 (91)	15 (9)	Reference <sup>b</sup>		0.955	0.045
Cases ( <i>n</i> = 165)	-238 G/A	162 (98)	3 (2)	0.50 <sup>a</sup>		0.991	0.009
	-308 G/A	130 (79)	35 (21)	<0.01 <sup>b</sup>	2.7 (1.41–5.15)	0.894	0.106
Precancer ( <i>n</i> = 45)	-238 G/A	43 (96)	2 (4)	0.80 <sup>a</sup>		0.978	0.022
	-308 G/A	37 (82)	8 (18)	0.04 <sup>b</sup>	2.5 (1.01–6.17)	0.911	0.089
Cancer ( <i>n</i> = 120)	-238 G/A	119 (99)	1 (1)	0.13 <sup>a</sup>		0.996	0.004
	-308 G/A	94 (78)	26 (22)	<0.01 <sup>b</sup>	2.8 (1.39–5.49)	0.892	0.108

OR, odds ratio; CI, confidence interval; *p*-value, probability from the  $\chi^2$  test comparing the genotype distribution for controls and cases. *p*-value for the genotype distribution for cancer vs. precancer = 0.12 (-238G/A), 0.82 (-308G/A).

<sup>a</sup>*p*-value for the carrier genotype distribution for -238 locus, <sup>b</sup>*p*-value for the carrier genotype distribution for -308 locus.

Table 2

Distribution of -238 and -308 TNF $\alpha$  promoter genotypes among HPV positive and negative patients (cervical precancer and cancer cases) and healthy control subjects

TNF $\alpha$ (locus) genotype	HPV + Cases <i>n</i> = 135 (%)	HPV - Cases <i>n</i> = 30 (%)	Control Subjects <i>n</i> = 165 (%)	<i>p</i> -value	OR (95% CI)
TNF $\alpha$ (-238) GG	132 (98)	30 (100)	159 (96)		
Carrier A (GA/AA)	3 (2)	0	6 (4)	0.52 <sup>a</sup> , 0.59 <sup>b</sup>	
TNF $\alpha$ (-308) Carrier A (GA/AA)	103 (76) 32 (24)	27 (90) 3 (10)	150 (91) 15 (9)	<0.01 <sup>a</sup> , 0.74 <sup>b</sup>	3.1 <sup>a</sup> (1.60–6.03)

OR, odds ratio; CI, confidence interval; *p*-value, probability from the  $\chi^2$  test comparing the genotype distribution for controls and cases.

<sup>a</sup> *p*-value/OR for distribution of carrier A genotype between HPV (+) cases vs. control.

<sup>b</sup> *p*-value for distribution of carrier A genotype between HPV (-) cases vs. control.

*p*-value for distribution of carrier A genotype between HPV (+) cases vs. HPV (-) cases = 0.4.

cancerous cases, it just reached statistical significance ( $p = 0.04$ ; OR = 2.50; 95% CI = 1.01–6.17). Also, the variant homozygous genotype -308 AA was found only in cases, 2.2% in precancer and 3.3% in cancer, but absent in control (data not shown). However, the statistical significance could not be calculated here due to very low frequency of AA carriers in the population. In the present scenario, AP value was found to be 13%, which showed that 13% of cases including cervical precancer and cancer could be attributed to the influence of the 'A' allele of the -308 TNF $\alpha$  promoter. But no significant association ( $p = 0.82$ ) was found between precancer and cancer cases with respect to carrier genotype (GA/AA) for this particu-

lar locus. The frequency of the -308 A allele in the cases was found to be 0.106 (precancer - 0.089 and cancer - 0.108) which is 2.4 times more than that of control (0.045). The -308 (G/A) TNF $\alpha$  promoter genotype with respect to HPV status revealed that "A" allele was significantly associated ( $p < 0.01$ ) with HPV positive cases of cervical precancer and cancer with a prevalence of 24% (32/135) when compared to controls with 9% (15/165). These results showed a three-fold increased risk for HPV infection in women carrying this allele (OR = 3.1; 95% CI = 1.60–6.03). AP value was found to be 16%, that indicated that about 16% of HPV positive cases could be attributed to the influence of the 'A' allele. But no significant associa-

tion was found between HPV negative cases and controls ( $p = 0.74$ ) as well as between HPV positive and negative cases. ( $p = 0.41$ ) (Table 2).

### 3.3. *TNF $\alpha$ -238 G/A polymorphism*

As shown in Table 1, the carrier genotype (GA/AA) distribution of the -238 TNF $\alpha$  promoter polymorphism in both precancer and cancer patient groups showed no significant difference when compared to the control group ( $p = 0.50$ ). The number of individuals possessing the -238 A allele were comparatively lower in cancer group than the control group, but it did not reach the limit of statistical significance. The polymorphic homozygous genotype AA was not found either in case or in control. The frequency of the -238 A allele in cases was found to be 0.009 (precancer - 0.022 and cancer - 0.004) which is 2 times lesser than that of control (0.018). After stratifying the cases by HPV status, no significant relation was found for -238 G/A TNF $\alpha$  polymorphism between cases and control subjects (Table 2).

## 4. Discussion

The availability of cervical cytology screening programs have significantly reduced the incidence of cervical cancer in developed countries in contrast to developing countries like India, where screening is either not available or too expensive. In India, more than 120,000 women develop this cancer every year, constituting about 16% of the world's annual incidence [5]. Infection of HPV type 16 has been found to be highest all over the world including India [7]. Recently, prevalence of other HR-HPV types have also been reported in cervical cancer cases from southern part of India but in the present study HPV type 16 alone was found to be as high as 98.5% while the rest was HPV 18 and no other high risk types were detected [10]. This reconfirmed the earlier report and suggested that there exists a regional variation in the prevalence of high-risk type HPV infection in India [7]. In spite of the fact that several other risk factors are involved including early age of marriage, promiscuity, smoking, use of contraceptives but persistent HR-HPV infection has been considered to be the principal etiologic factor. Although infection of HPV is essential but it is not sufficient for the development and progression of cervical cancer implying role of host genetic factors as evident from the 10% distribution of HPV positivity in healthy Indian women

[1]. Therefore, it is worthwhile to investigate the various host or viral markers in different stages of cervical cancer to understand the etiology of the disease, which may provide a very effective tool for the better management of this dreaded disease.

Our study indicated that carrier genotype (GA/AA) was more frequent among patients with precancer and cancer than in controls for -308 locus of TNF $\alpha$  promoter. Women who are A allele carriers presented a 2.5 fold increased risk of developing this carcinoma (Table 1) and 13% of these cases could be attributed to the influence of the studied polymorphism. Similar to our findings, it was shown in Portuguese population that -308 A allele increased two times the risk of development of cervical cancer [9]. In Korean population, it was shown that though there was a tendency for more individuals to carry the TNF $\alpha$ -308 A allele in the cervical cancer group but it could not attain statistical significance [14]. Several other studies also found similar results in different populations including South African population (Black and Mixed race ethnic groups), U.S. population, Zimbabwean population and Swedish population [4,11,12,22]. But Kirkpatrick et al. reported that in cervical intra-epithelial neoplasia (CIN) patients in UK population, TNF $\alpha$ -308 GG genotype was found to be significantly higher in comparison to control subjects [15], suggesting that ethnicity is playing a prominent role in TNF $\alpha$  promoter polymorphism and disease susceptibility. The present study also revealed that -308 carrier genotype (GA/AA) represented a 3-fold increased susceptibility to HPV 16 infection than control subjects (Table 2) with an AP value of 16%. But this was not in concordance with the findings of Deshpande et al. who found no association of this particular SNP with HPV infection in both Hispanic and Non-Hispanic races of US population [8] (Table 3).

Interestingly, the distribution for -308 A allele in Indian population is reported to be variable among different ethnic groups [13,21]. Present study showed that the frequency of this allele in the controls (0.045) is close to that of Korean population while it is higher than that of Japanese population and much lower than rest of the world populations [12,14].

However, this study, could not find any significant association for SNP at -238 of TNF $\alpha$  promoter with cervical precancer and cancer. But the frequency of heterozygotes (GA) was found to be more in controls than in cases, which is similar to the study reported by Deshpande et al. on Hispanic race of US population [8]. On the other hand, both in Korean and Caucasian

Table 3

Distribution (%) of TNF $\alpha$ -238 G/A and -308 G/A genotypes in HPV + women with cervical precancer/cancer among different world population

Population	Locus	Genotype n (%)		Association	Reference
		GG	GA/AA		
USA (Hispanic)	-238 G/A <i>n</i> = 126	119 (94)	7 (6)	No association	Deshpande et al. 2005 [8]
	-308 G/A <i>n</i> = 115	86 (75)	29 (25)		
USA (Non-Hispanic)	-238 G/A <i>n</i> = 182	167 (92)	15 (8)	No association	Deshpande et al. 2005 [8]
	-308 G/A <i>n</i> = 143	102 (71)	41 (29)		
UK (Bristol)	-238 G/A <i>n</i> = 84	79 (94)	5 (6)	No association of HR-HPV + low grade dysplasia w.r.t HR-HPV – low grade dysplasia	Kirkpatrick et al. 2004 [15]
	-308 G/A <i>n</i> = 84	63 (75)	21 (25)		
India (North)	-238 G/A <i>n</i> = 135	132 (98)	3 (2)	Positive association for -308 G/A locus but no association for -238 G/A locus	Present study
	-308 G/A <i>n</i> = 135	103 (76)	32 (24)		

population it was shown that, frequency of TNF $\alpha$ -238 A allele was more in cervical cancer group than in control group without any statistical significance [4, 14]. No association was found for -238 locus between cases and control subjects with respect to HR-HPV infection, similar to the study on UK population [15] (Table 3). The frequency of the -238 A allele in Indian population (0.018) is found to be similar to Japanese and Swedish populations but differ from that of other world populations [12,14].

Several functional studies revealed that A allele of -308 induces higher level of TNF $\alpha$  secretion [25]. This SNP is located within a consensus sequence of transcription factor AP-2 and -308 A allele has been found to be associated with higher constitutive and inducible levels of transcription of TNF $\alpha$ . The elevated level in serum may modulate the various immune responses towards the susceptibility to cancer of uterine cervix. Moreover, *in vivo* studies have also showed that TNF $\alpha$  promotes HPV immortalization and malignant changes in cervical epithelial cells, thus, mediating the escape of HPV infected cell from host immune surveillance mechanism. The increased level of the cytokine may cause aberrant pattern of interactions of cervix carcinoma cells with Extracellular Matrix Components (ECM) leading to tissue invasion. This ultimately pro-

motes angiogenesis at the site of inflammation through enhanced induction of VEGF [8,16,25]. Thus, it can be concluded that increased TNF $\alpha$  level exhibits an immunosuppressive and metastatic effect leading towards cervical carcinogenesis. Therefore, this polymorphism could be an important predisposing factor for cervical cancer development. But few studies showed no association of this allele with TNF $\alpha$  expression [3,23]. On the other hand, no transcriptional effect has been ascribed to TNF $\alpha$ -238 SNP. The functional significance of the rare TNF $\alpha$ -238 A allele is yet not clear, but a putative repressor site is supposed to be located in a 25-bp stretch including the position -238 [14].

In Indian women we showed for the first time a significant association between the carrier genotype (GA/AA) at -308 locus of TNF $\alpha$  and the development of cervical cancer. So in summary, it is suggested that TNF $\alpha$ -308 G/A polymorphism could serve as an important biomarker in Indian population for their susceptibility to cervical cancer as it may play a role in alteration of TNF $\alpha$  production and the inflammatory responses during the course of the disease. The findings could be useful as prediagnostic marker, which may help to detect women at risk of development of cervical cancer. Replication studies and functional analysis are required for further validation of our findings. Fu-

ture studies on linkage analysis and discovery of extended haplotypes with respect to other SNPs in TNF $\alpha$  promoter region as well as in the other genes in HLA complex may facilitate better insight and understanding of the role of TNF $\alpha$  promoter polymorphisms in the development of cervical cancer.

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