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Genetic polymorphisms in tumor necrosis factor alpha and interleukin-10 are associated with an increased risk of cervical cancer

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ABSTRACT

Most cases of cervical cancer are the result of infection with specific high-risk types of human papillomavirus (HPV). Investigating the genetic basis of the host immune response, particularly cytokine function, could help further characterize the progression of cervical HPV infection into neoplasia. Prior studies have demonstrated a correlation between genetic variants of tumor necrosis factor alpha (TNF-α, *TNF* gene) and/or interleukin-10 (IL-10, *IL10* gene) and cervical cancer susceptibility. However, some of the results have been contradictory. We sought to resolve these discrepancies by carrying out our study in a large cohort of Chinese women. In order to assess the association of *TNF* and *IL10* genotypes with cervical cancer susceptibility, the polymorphisms in *TNF* (-238 G/A, -308 G/A) and *IL10* (-592 C/A, -819 C/T, -1082 A/G) were genotyped and odds ratios for the genotype and allele frequencies between cervical cancer patients and healthy controls were calculated. Also, the functional relevance of these polymorphisms was evaluated using enzyme-linked immunosorbent assays (ELISAs) and in vitro lymphocyte proliferation assays. The *TNF*-238 AA genotype frequency was lower in patients than in controls ($p < 0.05$). *TNF*-308 AA, *IL10*-592 CA/AA, and *IL10*-819 CC/CT genotype frequencies were higher in cervical cancer patients than in controls ($p < 0.05$). The frequency of the *TNF*-238 A allele was significantly lower in patients, while the frequency of the -308 A allele was significantly higher ($p < 0.05$). No significant differences between patients and controls were found in the genotype or allele frequencies of *IL10*-1082 A/G ($p > 0.05$). Furthermore, the combinations of *TNF*-238 GA or GG and *IL10*-592 CC; *TNF*-238 GA or GG and *IL10*-592 CA or AA; *TNF*-308 AA and *IL10*-592 CC; and *TNF*-308 AA and *IL10*-592 CA or AA in cervical cancer patients were statistically significant ($p < 0.0167$). Upon stimulation with PHA, peripheral blood mononuclear cells (PBMCs) with the *TNF*-308AA genotype exhibited significantly higher proliferation rates, elevated IL-4, TGF-β levels, and lower IL-2 levels ($p < 0.05$). For *IL10*-592C/A, the AA and CA genotypes were significantly associated with higher proliferation rates, elevated IL-4 and IL-10 levels ($p < 0.05$). We also found that for *TNF*-308 G/A or *IL10*-592 C/A variants, the combination of *TNF*-308 GG or GA with *IL10* CA or AA had an association with the severity of cervical cancer. Taken together, these results suggest that *TNF*-308 AA and *IL10*-592 CA/AA genotypes may increase susceptibility to cervical cancer by altering the immune response of an individual.

1. Introduction

Persistent infection with oncogenic human papillomaviruses (HPVs) is the most common cause of cervical cancer, the second most prevalent cancer in women throughout the world [1]. Only a fraction of women infected with HPV develops cancer of the cervix, and there is a period of latency between the primary infection and detectable neoplasia. This pattern of disease progression suggests that the host immune response, including cell-mediated immunity (CMI) regulated by cytokines produced by T helper (Th) cells, monocytes and macrophages, is involved in cervical carcinogenesis [2]. Indeed, differences in genetic susceptibility and immune responses have been found to increase the incidence of HPV-associated lesions and thus, cervical cancer [3].

Cytokines play an important role in immune defense against HPV infections by modulating viral replication and altering the Th1/Th2 polarization [4]. A predominance of Th1 polarization, with production

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of interleukin (IL)-2 and interferon-gamma (IFN-γ), has been associated with clearance of HPV and regression of squamous intraepithelial lesions (SIL). In addition, tumor necrosis factor alpha (TNF-α) is also known to have antiviral properties [5,6]. By contrast, immuno-inhibitory cytokines such as IL-10 and TGF-β are associated with a Th2/Th3 response and can lead to the persistence of HPV and progression to SIL by suppressing CMI [7]. Thus, investigating genetic factors that affect the immune response of the host could help explain why some infections persist and cause malignancy. Several studies have demonstrated that polymorphisms in cytokine genes can affect the immune response to HPV infection, thereby possibly modifying susceptibility to cervical cancer [8]. Among them, TNF-α and IL-10 are of particular interest, as the genes for these cytokines are located in the central major histocompatibility complex (MHC).

TNF-α, secreted mainly by activated macrophages, plays a critical role in immune homeostasis, inflammation, and host defense [9]. Multiple polymorphisms in the *TNF* gene have been found to be associated with susceptibility to many cancers, including those of the stomach, breast, prostate, and lung. [10–13]. One of the most common polymorphisms occurs in a putative repressor site located in a 25 bp stretch that includes position -238. A G to A substitution here can affect TNF-α levels [14]. The other common polymorphism is a G to A substitution at position -308 in the promoter of *TNF* and this SNP might also have an effect on TNF-α levels [15]. Accordingly, it is reasonable to assume that the *TNF*-238G/A *and* -308G/A SNPs may have an impact on the progression of cervical cancer. Although the association of *TNF*-238G/A and -308G/A polymorphisms with cervical cancer has been widely studied, the results are still inconclusive or contradictory [16,17].

IL-10 is a Th2-type cytokine with anti-inflammatory properties that dampens the immune response in multiple ways [18]. IL-10 is produced by activated lymphocytes, monocytes, and fibroblasts [18]. IL-10 potently inhibits T cell proliferation and production of Th1 cytokines. As a strong immunosuppressive cytokine, the biological significance of IL-10 production by tumor cells remains unclear. Studies have shown that the capacity for IL-10 production is influenced by genetic makeup [19] and have proposed IL-10 as a risk gene for cervical cancer [20]. The *IL10* gene is located on chromosome 1, and several SNPs have been identified in prior work [21]. The production of IL-10 is regulated at the transcriptional level and may vary with differences in the genetic composition of the *IL10* locus [22,23]. Numerous recent studies have demonstrated an association between cervical cancer and several *IL10* SNPs (-592 C/G, -819 C/T and -1082 G/A) [24]. However, the results have been somewhat contradictory, possibly due to limited sample sizes or differences between ethnic groups.

Therefore, our objective was to investigate the distribution of *TNF* (-238 G/A, -308 G/A) and *IL10* (-592 C/G, -819 C/T and -1082 G/A) SNPs and their relationship to cervical cancer in a large cohort of Chinese women. In the present study, we functionally characterized the effects of these SNPs by measuring serum cytokine levels of the patients and controls. Additionally, we performed analyses of T-cell proliferation and cytokine production using PBMCs from healthy controls with diverse *TNF* and *IL10* genotypes.

2. Materials and methods

2.1. Study subjects

A total of 522 Chinese women with cervical cancer were enrolled from March 2014 to July 2017 at the Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital (Table 1). Diagnosis of cervical cancer was determined by histopathological examinations according to the International Federation of Gynecology and Obstetrics (FIGO). Meanwhile, 550 healthy women were selected as controls from the medical examination center of the same hospital within the same period. The selection criteria for the control group were as follows: no history of cancer or cervical intraepithelial neoplasia, negative HPV test, and normal examination (within the past two years) and cytology of the cervix. Information about tobacco smoking and menopausal status was obtained as part of the patient history. All participants indicated informed consent in writing for study participation and blood collection.

Table 1
Clinical and demographic characteristics of patients and controls.

Characteristics	Frequency, no. (%) Patients (n = 522)	Frequency, no. (%) Controls (n = 550)	p value
Age (yr)			
≤35	123 (23.6)	126 (22.9)	0.836
36–50	238 (45.6)	245 (44.5)	
> 50	161 (30.8)	179 (32.5)	
Tobacco smoking			
Nonsmokers	461 (88.3)	470 (85.5)	0.166
Smokers	61 (11.7)	80 (14.5)	
HPV status			
HPV+	425 (81.4)	0 (0)	
HPV-	97 (18.6)	550 (100)	
Pregnancy			
Never	56 (10.7)	49(8.9)	0.317
Ever	466 (89.3)	501(91.1)	
Menopausal status			
Premenopausal	214 (41.0)	229 (41.6)	0.832
Postmenopausal	308 (59.0)	321 (58.4)	
Tumor stage			
Stage 0	184 (35.2)		
Stage I	138 (26.4)		
Stage II	97 (18.6)		
Stage III	69 (13.2)		
Stage IV	34 (6.5)		
Histological grade			
G1	176 (33.7)		
G2	183 (35.1)		
G3	120 (30.0)		
Gx	43 (8.2)		
Histological type			
Squamous cell carcinoma	451(86.4)		
Adenocarcinoma	46(8.8)		
Other[a]	25(4.8)		

a, Other type includes 20 cases of adenosquamous carcinoma, 2 cases of neuroendocrine tumours, and 3 cases of undifferentiated carcinoma.

2.2. DNA extraction and genotyping

Genomic DNA from peripheral leukocytes of all subjects was extracted using a genomic DNA Extraction Kit (Qiagen, Germany). The polymorphic regions of *TNF*-238 G/A (rs361525), -308 G/A (rs1800629), *IL10*-592 C/A (rs1800872), -819 C/T (rs1800871), and -1082 A/G (rs1800896) were amplified by PCR, with a 25 μl reaction volume containing 0.3 μg template DNA, 1 × PCR buffer, 0.3 mM $MgCl_2$, 0.2 mM each dNTP, 2 U PrimeStar HS DNA polymerase (Takara, Japan), and 0.1 μmol of each primer (Invitrogen, China). The primer pairs employed were as follows: *TNF*-238 G/A (Sense: AGACCCCCCT CGGAATC; antisense: ATCTGGAGGAAGCGG TAGTG), from 4735 to 4883 (149 bp); *TNF*-308 G/A (Sense: GCAATAGGTT TTGAGGGGCAT; antisense: TCCCTGCTCCGATTCCG), from 4660 to 4761(102 bp); *IL10*-592 A/C (Sense: GGTGAGCACTACCTGACTAGC; antisense: CCTAGG TCACAGTGACGTGG), from 4257 to 4668 (412 bp); *IL10*-819 T/C (Sense: CCAGATATCTGAAGAAGTCCTG; antisense: TGGGGGAAGTGG GTAAG AGT), from 3768 to 4326 (559 bp); and *IL10-1082* A/G (Sense: ACTAAGGCT TCTTTGGGA; antisense: GTGCCAACTGAGAATTT), from 3925 to 4178 (254 bp). SNP genotyping was performed using Sanger sequencing carried out by a 3730 DNA analyzer (Applied biosystems, Foster City, CA, USA).

Table 2
Genotype and allele frequencies of the TNFα gene (*TNF*) in cervical cancer patients and controls.

Genotype	Frequency, No. (%) Patients (n = 1044)	Frequency, No. (%) Controls (n = 1100)	OR (95% CI)	p value
-238, G/A				
Codominant model				
GG	866 (83.0)	888 (80.7)	Reference	
GA	146 (14.0)	128 (11.6)	0.855 (0.662,1.103)	0.228
AA	32 (3.0)	84 (7.7)	0.391 (0.257,0.593)	< 0.01
Dominant model				
GG	866 (83.0)	888 (80.7)	Reference	
GA + AA	178 (17.0)	212 (19.3)	0.861 (0.691,1.073)	0.182
Recessive model				
GG + GA	1012 (96.9)	1016 (92.4)	Reference	
AA	32 (3.1)	84 (7.6)	0.382 (0.252,0.580)	< 0.01
Alleles				
G	939 (89.9)	952 (86.5)	Reference	
A	105 (10.1)	148 (13.5)	0.719 (0.551,0.938)	0.015
-308, G/A				
Codominant model				
GG	780 (74.7)	862 (78.4)	Reference	
GA	168 (16.1)	182 (16.5)	1.020 (0.810,1.285)	0.866
AA	96 (9.2)	56 (5.1)	1.895 (1.344,2.671)	< 0.01
Dominant model				
GG	780 (74.7)	862 (78.4)	Reference	
GA + AA	264 (25.3)	238 (21.6)	1.226 (1.003,1.498)	0.046
Recessive model				
GG + GA	948 (90.8)	1044 (94.9)	Reference	
AA	96 (9.2)	56 (5.1)	1.888 (1.342,2.655)	< 0.01
Alleles				
G	864 (82.8)	953 (86.6)	Reference	
A	180 (17.2)	147 (13.4)	1.351 (1.066,1.711)	0.013

2.3. Isolation and culturing of PBMCs

Peripheral blood mononuclear cells (PBMCs) of healthy controls were isolated from heparinized venous blood by centrifugation using the Ficoll-Hypaque gradient method. The PBMCs were washed with PBS, resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), and incubated with or without 25 μg/ml phytohemagglutinin (PHA; Sigma) for 6 h.

2.4. Cell proliferation assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to quantify cell proliferation. 5×10^3 PBMCs were plated in each well of a 96-well plate. Lymphocytes were or were not stimulated with PHA for 6 h, and then 20 μl of the MTT reagent (5 mg/ml) was added to each well. After another 4 h of incubation at 37 °C, the medium was discarded, and 150 μl DMSO was added. Absorbance values were measured at 570 nm using a microplate reader from BIO-RAD (Hercules, CA). Proliferation assays were done in triplicate.

2.5. ELISAs

Cytokine levels were assessed in serum samples and PBMC culture supernatants using enzyme-linked immunosorbent assays (R&D, Minneapolis, MN, USA). Each cytokine was measured according to the manufacturer's instructions. In brief, 200 μl of standard, control or samples were added and incubated at room temperature for 2 h. After four washes with washing buffer, 200 μl of cytokine conjugate per well was then added. After 2 h of incubation at room temperature, the plate was washed, and 200 μl of substrate solution was added for color development. Optical densities (OD) were measured, and the results were recorded in pg/ml.

2.6. Statistical analysis

All data are expressed as means ± standard deviation. The χ^2 test was used to determine whether the distribution of genotypes in patients and controls was in Hardy-Weinberg equilibrium. The χ^2 test was also used to evaluate differences in age and smoking status between patients and controls. The reference variant was the homozygous form of the most common allele. Student's *t*-test or one-way analysis of variance was used to determine differences in lymphocyte proliferation and cytokine concentrations between the different genotypes. SPSS software (version 14.0; SPSS, Chicago, IL, USA) was used to calculate odds ratios (OR) and 95% confidence intervals (95% CI). For statistical significance, $\alpha = 0.05$ unless multiple comparisons were made, in which case Bonferroni's correction was applied.

3. Results

3.1. Participant demographics

Characteristics of the two groups are shown in Table 1. Among the patients with cervical cancer, 81.4% were HPV positive, and all of these were confirmed to have a single infection. No statistical differences were found between patients and controls in terms of age, smoking status, pregnancy and menopausal status ($p = 0.836$, $p = 0.166$, $p = 0.317$, and $p = 0.832$). HPV genotype was determined for all those who tested positive: 60.1% were positive for HPV type 16, 11.4% for HPV type 18, and 9.9% for other HPV types (2.5% positive for HPV type 31, 2.6% for HPV type 58, 1.7% for HPV type 45, 1.7% for HPV type 52, and 1.4% for HPV type 69). The FIGO (1988) cervical cancer staging system was used to categorize the severity of the disease found in each patient (Table 1).

3.2. Prevalence of TNF polymorphisms in cervical cancer patients

The genotype and allele distributions of *TNF* polymorphisms in patients with cervical cancer and controls are shown in Table 2. The

Table 3
Genotype and allele frequencies of the IL-10 gene (*IL10*) in cervical cancer patients and controls.

Genotype	Frequency, No. (%) Patients (n = 1044)	Frequency, No. (%) Controls (n = 1100)	OR (95% CI)	p value
-592, C/A				
Codominant model				
CC	380 (36.4)	458 (41.6)	Reference	
CA	522 (50.0)	520 (47.3)	1.210 (1.008,1.452)	0.040
AA	142 (13.6)	122 (11.1)	1.403 (1.063,1.852)	0.017
Dominant model				
CC	380 (36.4)	458 (41.6)	Reference	
CA + AA	664 (63.6)	642 (58.4)	1.247 (1.048,1.483)	0.013
Recessive model				
CC + CA	902 (86.4)	978 (88.9)	Reference	
AA	142 (13.6)	122 (11.1)	1.262 (0.975,1.634)	0.077
Alleles				
C	641 (61.4)	718 (65.3)	Reference	
A	403 (38.6)	382 (34.7)	1.182 (0.991,1.409)	0.063
-819, T/C				
Codominant model				
TT	258 (24.7)	318 (28.9)	Reference	
CT	584 (55.9)	558 (50.7)	1.290 (1.055,1.577)	0.013
CC	202 (19.4)	224 (20.4)	1.112 (0.865,1.429)	0.410
Dominant model				
TT	258 (24.7)	318 (28.9)	Reference	
CC + CT	786 (75.3)	782 (71.1)	1.239 (1.023,1.501)	0.028
Recessive model				
CT + TT	842 (80.6)	876 (79.6)	Reference	
CC	202 (19.4)	224 (20.4)	0.938 (0.759,1.160)	0.556
Alleles				
T	550 (52.7)	597 (54.3)	Reference	
C	494 (47.3)	503 (45.7)	1.066 (0.900,1.263)	0.460
-1082, A/G				
Codominant model				
AA	546 (52.3)	564 (51.3)	Reference	
AG	310 (29.7)	340 (30.9)	0.942 (0.776,1.143)	0.544
GG	188 (18.0)	196 (17.8)	0.991 (0.786,1.250)	0.938
Dominant model				
AA	546 (52.3)	564 (51.3)	Reference	
AG + GG	498 (47.7)	536 (48.7)	0.960 (0.810,1.137)	0.635
-1082, A/G				
Recessive model				
AA+AG	856 (82.0)	904 (82.2)	Reference	
GG	188 (18.0)	196 (17.8)	1.013 (0.812,1.263)	0.909
Alleles				
A	701 (67.1)	734 (66.7)	Reference	
G	343 (32.9)	366 (33.3)	0.981 (0.820,1.175)	0.837

Table 4
Frequency of *TNF* and *IL10* genotype combinations in cervical cancer patients and controls.

Genotype	Frequency, No. (%) Patients (n = 1044)	Frequency, No. (%) Controls (n = 1100)	OR (95% CI)	p value
TNF-238/*IL10*-592				
AA/CC	24	62	Reference	
GA or GG/CC	356	364	2.527 (1.543,4.138)	< 0.01
AA/CA or AA	26	54	1.244 (0.640,2.416)	0.519
GA or GG/CA or AA	638	620	2.658 (1.638,4.313)	< 0.01
TNF-308/*IL10*-592				
GG or GA/CC	316	398	Reference	
AA/CC	62	46	1.698 (1.128,2.555)	0.011
GG or GA/CA or AA	602	610	1.243 (1.032,1.497)	0.022
AA/CA or AA	64	46	1.752 (1.167,2.632)	0.006

two SNPs genotyped in case and control cohorts were in Hardy-Weinberg equilibrium ($p > 0.05$). Table 2 shows the association analysis of *TNF*-238 G/A, -308 G/A and the risk of cervical cancer as odds ratios (ORs). For -238 G/A, the AA genotype was protective, as it was negatively associated with cervical cancer, with an OR of 0.391 (95% CI: 0.257–0.593) for the codominant model. This result was confirmed in the recessive model, in which the AA genotype was negatively associated with cervical cancer, with an OR of 0.382 (95% CI: 0.252–0.580), $p < 0.01$. For alleles, a strong negative association with cervical cancer was found for the A allele, with an OR of 0.719 (95% CI: 0.551–0.938), $p = 0.015$.

For -308 G/A, the AA genotype was positively associated with cervical cancer, with an OR of 1.895 (95% CI: 1.344–2.671), $p < 0.01$ in the codominant model. This result was confirmed in the dominant and recessive models. For the alleles, a significant positive association with cervical cancer was found for the A allele, with an OR of 1.351 (95%

Table 5
Associations of *TNF* and *IL10* polymorphisms with cytokine levels in cervical cancer patients and controls.

Cytokine		*TNF*-238 G/A				*TNF*-308 G/A				*IL10*-592 C/A			
		GG	GA	AA	*p*	GG	GA	AA	*p*	CC	CA	AA	*p*
TNF-α	Patient	6.71 ± 0.26	6.05 ± 0.21	6.24 ± 0.12	0.141	5.21 ± 0.22	5.84 ± 0.16	6.06 ± 0.20	0.046	3.01 ± 0.28	3.32 ± 0.32	3.41 ± 0.31	0.188
	Control	4.92 ± 0.29	5.23 ± 0.30	5.44 ± 0.22	0.293	5.09 ± 0.27	5.11 ± 0.37	5.20 ± 0.36	0.778	2.98 ± 0.18	3.03 ± 0.31	2.71 ± 0.25	0.352
IL-10	Patient	5.25 ± 0.22	4.77 ± 0.18	4.27 ± 0.19	< 0.01	5.04 ± 0.16	5.02 ± 0.18	5.77 ± 0.16	0.006	3.55 ± 0.19	4.28 ± 0.22	4.37 ± 0.26	< 0.01
	Control	5.22 ± 0.29	5.08 ± 0.29	4.94 ± 0.31	0.275	4.80 ± 0.25	5.04 ± 0.23	4.75 ± 0.22	0.190	3.11 ± 0.27	2.98 ± 0.27	3.02 ± 0.35	0.875
IL-2	Patient	5.01 ± 0.31	5.22 ± 0.26	5.69 ± 0.15	0.026	5.77 ± 0.31	5.18 ± 0.28	5.02 ± 0.36	0.021	5.11 ± 0.30	5.73 ± 0.31	4.89 ± 0.26	0.064
	Control	4.98 ± 0.27	4.98 ± 0.31	4.41 ± 0.26	0.692	5.0 ± 0.37	5.11 ± 0.19	5.05 ± 0.25	0.757	3.00 ± 0.27	2.88 ± 0.30	2.98 ± 0.32	0.671
IL-4	Patient	5.35 ± 0.11	4.27 ± 0.29	4.12 ± 0.33	0.040	4.87 ± 0.33	5.09 ± 0.27	5.74 ± 0.36	0.053	5.41 ± 0.43	5.66 ± 0.41	6.12 ± 0.37	0.019
	Control	5.36 ± 0.21	5.11 ± 0.31	4.77 ± 0.29	0.311	5.17 ± 0.31	5.22 ± 0.43	5.44 ± 0.32	0.516	4.44 ± 0.36	4.55 ± 0.28	4.28 ± 0.33	0.865
TGF-β	Patient	5.46 ± 0.21	5.09 ± 0.27	4.01 ± 0.45	0.033	4.84 ± 0.18	5.36 ± 0.21	5.91 ± 0.19	0.048	5.12 ± 0.25	5.01 ± 0.21	5.29 ± 0.23	0.724
	Control	4.70 ± 0.26	4.54 ± 0.19	4.41 ± 0.11	0.310	4.77 ± 0.26	5.06 ± 0.31	5.37 ± 0.22	0.231	3.56 ± 0.31	3.55 ± 0.33	3.76 ± 0.29	0.621

All values are given in pg/ml. For −238 G/A, the difference between AA and GA + GG was calculated. For −308 G/A, the difference between AA and GA + GG was calculated. For −592 C/A, the difference between CC and CA + AA was calculated.

CI:1.066–1.711), $p = 0.013$.

3.3. Prevalence of IL10 polymorphisms in cervical cancer patients

IL10-592 C/A, -819 C/T, -1082 A/G genotypes were in Hardy-Weinberg equilibrium in both groups ($p > 0.05$). Compared with controls, the genotypes CA and AA in -592 C/A were positively associated with the case group (Table 3); respective ORs were 1.210 (95% CI: 1.008–1.452), $p = 0.04$ (marginally significant after Bonferroni correction), and 1.403 (95% CI: 1.063–1.852), $p = 0.017$ in the codominant model. In the dominant model, the combination of CA and AA remained positively associated with cervical cancer, with an OR of 1.247 (95% CI, 1.048–1.483), $p = 0.013$. For alleles, a marginally positive association was found for the A allele, with an OR of 1.182 (95% CI, 0.991–1.409), $p = 0.063$. For −819C/T, we found that the CT SNP was associated with cervical cancer in the codominant model, with an OR of 1.290 (95% CI: 1.055–1.577), $p = 0.013$. The combination of CC and CT was significant in the dominant model, with an OR of 1.239 (95% CI: 1.023–1.501). No significant differences between patients and healthy controls were found in the genotype or allele frequencies for −1082 A/G ($p > 0.05$).

3.4. Prevalence of TNF and IL10 polymorphism combinations in cervical cancer patients

Combinations of *TNF* and *IL10* genotypes were analyzed in order to determine whether their interactions had an additive effect on their association with cervical cancer (Table 4). Statistically significant models with the strongest positive odds ratios from Tables 2 and 3 were used in the calculations. For the combination of *TNF*-238 G/A with *IL10*-592 C/A, the *TNF*-238 GA or GG and *IL10*-592 CC; and *TNF*-238 GA or GG and *IL10*-592 CA or AA were positively associated with cervical cancer. Respective ORs were 2.527 (95% CI: 1.543–4.138), $p < 0.01$, and 2.658 (95% CI: 1.638–4.313), $p < 0.01$. For *TNF*-308 G/A with *IL10*-592 C/A, compared with controls, the frequency of combinations of *TNF*-308 AA and *IL10*-592 CC and *TNF*-308 AA and *IL10*-592 CA or AA in cervical cancer patients were of statistical significance ($p < 0.0167$).

3.5. Association of TNF and IL10 polymorphisms with serum cytokine levels

Serum cytokine values of patients and controls were analyzed and compared using the same models from above. The *TNF*-238 G/A, -308 G/A and *IL10*-592 C/A genotypes had no associations with serum TNF-α, IL-10, IL-2, IL-4, or TGF-β levels in the controls (all $p > 0.05$; Table 5). However, for the case group, the AA genotype in *TNF*-238 was significantly associated with an increase in the serum IL-2 level ($p = 0.026$) and a decrease in serum IL-10 ($p < 0.01$), IL-4 ($p = 0.040$), and TGF-β ($p = 0.033$) levels compared to GG and GA genotypes. The AA genotype of −308 was associated with increases in serum TNF-α ($p = 0.046$), IL-10 ($p = 0.006$), and TGF-β ($p = 0.048$) and a decrease in IL-2 ($p = 0.021$) levels compared to GA and GG genotypes. Also, the CA and AA genotypes of -592C/A of *IL10* were significantly associated with increases in serum IL-10 ($p < 0.01$) and IL-4 ($p = 0.019$) levels compared to the CC genotype. Together, these results demonstrate that TNF-α-238 GG/GA and -308 AA genotypes are associated with cervical cancer. A possible mechanism is a decrease in circulating Th1-type cytokines and a concomitant increase in Th2/Th3-type cytokines. Similarly, *IL10*-592 CA/AA genotypes are associated with cervical cancer and with the same pattern of elevated Th2-type cytokine levels.

3.6. Effects of different TNF and IL10 genotypes on T-cell proliferation and cytokine levels

PHA was used to stimulate PBMCs with different *TNF* (-238 G/A,

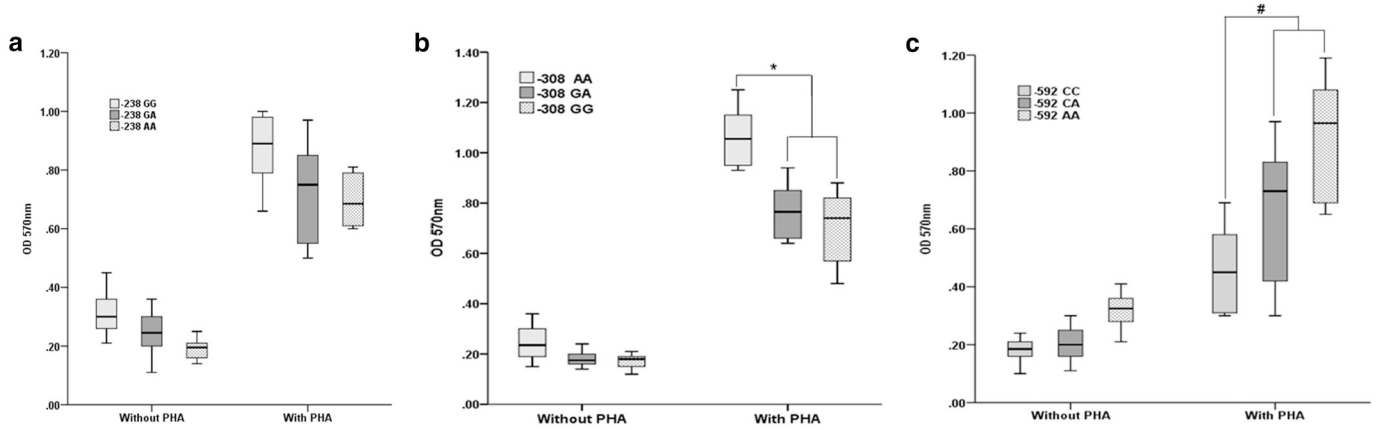

Fig. 1. T lymphocyte activation and proliferation in PBMCs from healthy individuals carrying different *TNF*-238 G/A (A), -308 G/A (B) and *IL10*-592 C/A (C) genotypes. Cell proliferation was assessed using the MTT assay, which showed that, when stimulated with PHA, T lymphocytes carrying the *TNF*-308 AA genotype had significantly higher rates of proliferation than those carrying-308 GA and GG. Likewise, T lymphocytes carrying either *IL-10*-592 CA or AA genotypes had significantly higher rates of proliferation than those carrying -592 CC. The line inside each box is the median, while the upper and lower limits of the box are the 75th and 25th percentiles, respectively. The vertical bars above and below the box indicate the maximum and minimum values, respectively. *AA vs. GA + GG, $p < 0.05$. #CC vs. CA + AA, $p < 0.05$.

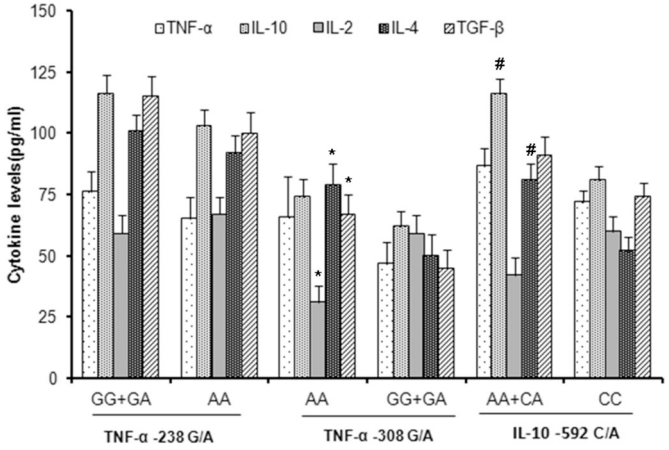

Fig. 2. Differential levels of cytokine production in PBMCs from healthy individuals carrying different *TNF*-238 G/A, -308 G/A and *IL10*-592 C/A genotypes. PBMCs extracted from healthy individuals were stimulated with PHA. Levels of TNF-α, IL-10, IL-2, IL-4, and TGF-β were detected in the cell culture supernatant. *AA vs. GG + GA, $p < 0.05$. #CC vs AA + CA, $p < 0.05$.

-308 G/A) or *IL10* (-592 C/A) genotypes. Then, the MTT assay was used to assess T cell proliferation (Fig. 1), while cytokine levels (TNF-α, IL-10, IL-2, IL-4, and TGF-β) in the cell culture supernatants were measured using ELISAs (Fig. 2). We observed that T cells were functionally activated after stimulation with PHA. MTT assays revealed that PBMCs with the *TNF*-308 AA or *IL10*-592 CA/AA genotypes had significantly higher rates of cell proliferation after PHA stimulation ($p < 0.05$). Analysis of TNF-α, IL-10, IL-2, IL-4, and TGF-β levels in cell culture supernatants showed that PBMCs with *TNF*-308 AA produced significantly less IL-2 but more IL-4 and TGF-β ($p < 0.05$). Similarly, for *IL10*-592 C/A, the presence of the AA/CA genotypes was associated with significantly higher IL-10 and IL-4 levels ($p < 0.05$). These findings mirror what is seen in the serum and clearly indicate that *TNF*-308 AA and *IL10*-592CA/AA genotypes have significant effects on T-cell proliferation and cytokine production.

Table 7
Frequency of *TNF* and *IL10* genotype combinations in cervical cancer patients with different clinicopathologic stages of disease.

Genotype	0	I	II	III	IV	χ^2	*p* value
TNF-238/*IL10*-592							
AA/CC	4	3	3	1	1	Reference	
GA or GG/CC	70	63	24	13	8	1.869	0.760
AA/CA or AA	1	2	4	3	3	4.109	0.391
GA or GG/CA or AA	109	70	66	52	22	0.647	0.958
TNF-308/*IL10*-592							
GG or GA/CC	65	57	16	10	10	Reference	
AA/CC	10	8	4	5	4	6.147	0.188
GG or GA/CA or AA	90	67	75	54	15	32.993	< 0.01
AA/CA or AA	19	6	2	0	5	9.771	0.044

3.7. Association of TNF and IL10 genotypes with clinicopathologic stages of cervical cancer

The stages of our 522 patients in the study were: Stage 0: 184; Stage I: 138; Stage II: 97; Stage III: 69; Stage IV: 34. The genotype frequencies for *TNF* (-238 G/A, -308 G/A) and *IL10* (-592 C/A) in these patients are shown in Tables 6 and 7. There was a strong association of the *TNF*-308 G/A and *IL10*-592 C/A variants with the clinicopathologic stages of cervical cancer. For the genotype combinations of *TNF*-308 G/A and

Table 6
Genotype frequencies of *TNF* and *IL10* in cervical cancer patients with different clinicopathologic stages of disease.

Stage	Cases (*n*)	-238G/A			*p*	-308G/A			*p*	-592C/A			*p*
		GG	GA	AA		GG	GA	AA		CC	CA	AA	
0	184	161	17	6	0.071	145	31	8	0.033	97	76	11	0.000
I	138	120	16	2		115	20	3		55	70	13	
II	97	77	16	4		92	4	1		14	68	15	
III	69	50	16	3		61	7	1		18	32	19	
IV	34	25	8	1		27	7	0		6	15	13	

IL10-592 C/A, *TNF*-308 GG or GA with *IL10* CA or AA was associated with the clinicopathologic stages of cervical cancer. However, for the combinations of *TNF*-238 G/A with *IL10*-592 C/A, no significant associations were found.

4. Discussion

Cervical cancer is regarded as a preventable disease because there is usually a long period during which lesions are precancerous [2]. Identifying additional risk factors that influence disease progression may help in prevention of this cancer [25]. It is widely accepted that HPV and smoking are the primary causes of virtually all cases of cervical intraepithelial neoplasia (CIN), although genetics play a crucial role as well [26]. This study aims to explore whether TNF-α and/or IL-10 polymorphisms can influence susceptibility to cervical cancer. If the association between *TNF* and/or *IL 10* gene SNPs and cervical cancer susceptibility can be better understood, it may contribute to our understanding of host control of HPV infection.

TNF-α is a well-established driver of inflammation that may promote the growth and progression of cancer [27]. The two most commonly studied SNPs are in the promoter region of *TNF*, two G versus A transitions at positions −238 and −308; these have been shown to influence the expression of TNF-α [28,29]. A large number of studies have investigated the associations of *TNF*-238 G/A and/or -308G/A variants with cervical cancer risk, but results are contradictory [30,31]. Our data showed that -238 GG/GA and -308 AA genotypes in *TNF* were associated with increased cervical cancer risk. These findings are consistent with some previous reports but not with others [32,33]. Relatively small sample sizes (and therefore, low statistical power), racial and ethnic differences, and publication bias may partly explain these inconsistencies.

Polymorphisms in *IL10*, another immunomodulatory gene, have also been implicated in cervical cancer [34]. In our study, three variants (-592 C/A, -819 C/T, -1082 A/G) in the promoter of *IL10* were investigated to find potential associations with cervical cancer. The results showed that −592 AA and CA genotypes were significantly associated with cervical cancer; this is consistent with previous studies [35]. We also found that the −819 CC and CT genotypes were associated with a modest increase in the odds of developing cervical cancer. No significant differences were found in the genotype or allele frequencies between the −1082 A/G variants and risk of cervical cancer.

Furthermore, in order to determine whether the presence of these SNPs had an additive effect on their association with cervical cancer, association of combinations of *TNF* and *IL10* genotypes with cervical cancer were analyzed. The results showed that combinations of *TNF*-238 GA or GG and *IL10*-592 CC; *TNF*-238 GA or GG and *IL10*-592 CA or AA; *TNF*-308 AA and *IL10*-592 CC; and *TNF*-308 AA and *IL10*-592 CA or AA were strongly associated with risk of cervical cancer ($p < 0.0167$). The increase in the odds ratios compared to the single SNPs alone suggests that there may be an additive effect of having high-risk genotypes at multiple loci.

We next wanted to characterize the functional consequences of these polymorphisms. Since cytokines play a significant role in the polarization of T cells [36], we investigated the association of *TNF* (-238 G/A, -308 G/A) and *IL10*-592C/A polymorphisms with proliferation and cytokine secretion in T cells. To the best of our knowledge, there have been no other studies done on the association of these SNPs with T-cell activity. For the proliferation assay, PBMCs carrying the *TNF*-308 AA or *IL10*-592 CA/AA genotypes showed significantly higher rates of T-cell proliferation. Because T lymphocytes play a major role in the surveillance of cancer cells, it is possible that the *TNF*-308 GG/GA or *IL10*-592 CC genotypes negatively regulate T-cell activation, which might confer increased susceptibility to cervical cancer. However, this finding contradicts the results above, warranting further investigation of the role of T cell proliferation in cervical cancer progression. In addition, as a major source of TNFα, the effect of monocytes on cervical cancer cannot be neglected and should be investigated in future work.

Several recent studies have reported a predominance of Th2-type cytokines in association with a diminished Th1 cytokine profile in cervical cancer patients. An impaired cell-mediated immune response due to the effect of immune suppressor cytokines such as IL-10 and TGF-β might be involved in the progression of cervical cancer. Our analyses showed that cytokine profiles in vitro were consistent with serum cytokine profiles in cervical cancer patients. Furthermore, the *TNF*-308 AA and *IL10*-592 AA/CA genotypes had strong associations with lower IL-2 (Th1-type cytokine), as well as higher IL-4, IL-10 (Th2-type cytokines) and TGF-β (Th3-type cytokine) levels. This Th2/Th3 pattern of cytokine expression suggests that HPV infection may induce immunosuppressive cytokines such as IL-10 and TGF-β as a means to escape the host immune system. This shift from Th1 to Th2/Th3 might facilitate cancer progression by allowing tumor cells to evade normal immune surveillance [37]. Additionally, the current study provides evidence that *TNF*-308 G/A and *IL10*-592C/A variants have associations with the severity of cervical cancer.

In conclusion, identifying host genetic factors that may facilitate the development of cervical cancer is very important, as it may aid in the development of effective preventive and treatment measures against HPV-related diseases. Our study demonstrated that *TNF*-308 G/A and *IL10*-592 C/A SNPs can affect cervical cancer susceptibility. Investigation of HPV type-specific and histological type-specific associations with *TNF*-308 G/A *and IL10*-592 C/A SNPs may further elucidate the relationship between the host immune response and HPV infection in cervical cancer.

Disclosure of conflict of interest

None.

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