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Characterization of Single-Nucleotide Polymorphisms in the Tumor Necrosis Factor α Promoter Region and in Lymphotoxin α in Squamous Intraepithelial Lesions, Precursors of Cervical Cancer[1]

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Abstract

Development of cervical cancer is a long process of abnormal cancerous cell growth in the cervix and is primarily the result of infection with specific high-risk types of human papillomavirus (HPV). The cytokines tumor necrosis factor α (TNFα) and lymphotoxin α (LTA) have an important role in all stages of cervical cancer and have the ability to induce the regression or promote the development of human tumors. Biologically important single-nucleotide polymorphisms (SNPs) occur within the TNFα and LTA genes. Therefore, the purpose of this study was to investigate the SNPs in the TNFα promoter region (−163, −238, −244, −308, −376, −857, −863, and −1031) and in the first intron of LTA (+252) in women with precursor lesions of cervical cancer. Overall, we studied 396 women from Mexico City. A total of 191 patients with HPV infection and precursor cervical lesions were subdivided in two groups: those with low-grade squamous intraepithelial lesions (n = 132) and those with high-grade squamous intraepithelial lesions (n = 59). Women (n = 205) negative for HPV and without cervical lesions were also included in the study. DNA was extracted from peripheral white blood cells and from cervical samples, and detection of biallelic polymorphisms of TNFα and LTA was performed using the polymerase chain reaction–sequence-specific oligonucleotide probe and restriction fragment length polymorphism techniques, respectively. We demonstrated that risk is associated with the genotype G/A (odds ratio = 2.48) and that protection is associated with the genotype G/G of SNP TNFα −376 (odds ratio = 0.37).

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Introduction

Cervical cancer is the second most common cancer in women worldwide, but it is the most common one in most developing countries, where 80% of the cases occur. Cervical cancer is the leading cancer-related cause of death in Mexico. The principal risk factors are poverty-related factors, including a lack of formal education, unemployment, a low socioeconomic level, rural residence, and insufficient access to healthcare [1]. The most extensively studied aspect of human papillomavirus (HPV) infection has been the molecular pathogenesis of cervical cancer and its precursor lesions, which are frequently referred to as low- and high-grade squamous intraepithelial lesions (LSIL and HSIL,

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respectively). Certain HPV genotypes, such as HPV-16 and -18, have been definitively associated with the development of cervical cancer [2–4]. Although the virus is usually cleared in 70% to 90% of HPV-infected individuals, in a small percentage of patients, the virus persists and may lead to the development of SILs [5].

The cellular immune response mediated by cytokines is the main defense against tumors related to cervical carcinogenesis. Previous studies have suggested that a T-helper 1 (T_H1) response is associated with the clearance of HPV infection and the regression of cervical cancer and that T_H2 responses are associated with cervical carcinogenesis owing to the stimulation of tumor growth [6,7]. Although each study was different in the observed profiles of increased cytokine, the results revealed that both T_H1 and T_H2 cytokines are increased in cervical cancer. Different studies of candidate genes have demonstrated that genetic polymorphisms in cytokine genes contribute to the variation in the levels of cytokines produced, and this variation may influence the severity of cervical cancer [7,8].

Tumor necrosis factor α (TNFα) and lymphotoxin α (LTA) are members of the tumor necrosis factor family of cytokines. The TNFα and LTA genes are located within the major histocompatibility complex, between the HLA class I and II regions on chromosome 6p21.3. Several studies of different diseases have investigated the effect of single-nucleotide polymorphisms (SNPs) in the TNFα promoter region (−163, −238, −244, −308, −376, −857, −863 and −1301) [9,10] and the *Nco*I restriction fragment length polymorphism (+252) in the first intron of LTA [11]. Most SNPs located within the TNFα promoter region have a change of G to A, as is found at positions −163, −238, −244, −308, and −376. The −857 and −1031 SNPs have a C-to-T and T-to-C transition, respectively, whereas the SNP at the −863 position is a C-to-A transition and LTA +252 has an A-to-G transition. All SNPs within the TNFα promoter and LTA seem to be biologically important in the transcription of these cytokines and are associated with various diseases [12,13].

We selected these cytokines for our study because they are involved in the immune response to diverse forms of cellular stress, inflammation, and exposure to carcinogens. The objective of this study was to examine the relationship of genetic polymorphisms in the TNFα promoter region and in the intronic position of LTA with the development of LSIL and HSIL in Mexican women.

Materials and Methods

Studied Population

We studied 396 Mexican women older than 25 years who attended the Mexican Institute of Social Security of Mexico City from December 2003 to July 2006. These women were divided in two study groups. One group was the controls and consisted of 205 healthy women with a mean age of 42 years with three Papanicolaou test samples negative for HPV infection, without SILs, and with normal cytology and colposcopy results. Each sample was analyzed at a 1-year interval. The second group consisted of 191 patients with HPV infection and SILs based on cytology, histology, and colposcopy examination. The patients in this group had a mean age of 37 years. This group was subdivided according to the Bethesda classification [14]: 132 women with LSIL (women diagnosed with HPV infection and cervical intraepithelial neoplasia 1 [CIN 1]) and 59 women with HSIL (women diagnosed with CIN 2 and CIN 3). Any patient who had received preventive treatment, who was pregnant or up to 2 months postpartum, with a history of hysterectomy, or with a severe chronic disease was excluded from the study. Study participants completed an interviewer-administered risk factor questionnaire that assessed reproductive, sexual, and medical histories and demographic data.

Genomic and Cervical DNA Extraction

Genomic DNA was extracted from a 5-ml total blood sample drawn from each woman. DNA was isolated from these cells using a salting-out microtechnique [15] and was frozen at −20°C until tested.

Cervical cell DNA was extracted from cytologic samples taken from the cervical transformation zone after colposcopic examination of the cervix and before the application of acetic acid. The sample was placed in 1 ml of cold phosphate-buffered saline solution for subsequent DNA extraction by the standard sodium dodecyl sulfate–proteinase K lysis technique [16] and was frozen at −20°C until tested.

Polymerase Chain Reaction Detection of HPV

We used two sets of general primers (MY09/MY11 and GP5/GP6) and the polymerase chain reaction and cycling conditions described previously for the detection of HPV DNA in cervical cells [17,18]. As a positive control, HPV DNA obtained from the HeLa cell line, which contains 10 to 50 copies of the HPV-18 L1 ORF, was used [19].

HPV DNA Type Analysis

HPV-positive samples were subsequently assayed for HPV typing with the HPVFast 2.0 kit (Pharma Gen S.A., Madrid, Spain) according to the manufacturer's instructions.

TNFα Promoter Region Typing

A 1355-base pair (bp) fragment of the TNFα promoter region sequence was amplified with previously published primers [20,21]. Polymerase chain reaction was performed in a reaction mixture containing 0.16 ng of DNA, 0.25 pmol of each primer, 0.03 U of Taq polymerase (Invitrogen, Carlsbad, CA), 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 16 mM Tris-HCl (pH 8.4), and 50 mM KCl. The amplification conditions were 94°C for 3 minutes, 64°C for 1 minute, and 72°C for 1 minute followed by 35 cycles of 94°C for 1 minute, 64°C for 1 minute, and 72°C for 1 minute, and a final cycle of 94°C for 1 minute, 64°C for 1 minute, and 72°C for 5 minutes.

The samples were genotyped for SNPs within the TNFα promoter region gene sequence (GenBank accession number Z15026) using the sequence-specific oligonucleotide probes (SSOP) technique as follows: −163 (G→A) (without rs number), −238 (G→A) (rs361525), −244 (G→A) (rs673), −308 (G→A) (rs1800629), −376 (G→A) (rs1800750), −857 (C→T) (rs1799724), −863 (C→A) (rs1800630), and −1301 (T→C) (rs1799964).

The −163 (CTTCTCCATCG***C***GGGGGC), −244 (GATTC***C***-GAGGGGGGTCTT), −308 (AACCCCGTCC***C***CATGCCC), and −376 (CCTTCTAA***C***TTCGAGACA) probes and their counterparts were designed by our laboratory, and the other probes used (−238, −857, −863, and −1031) have been published elsewhere [22].

Eight SSOP and their counterparts were used to identify the SNPs in the TNFα promoter region. The experimental conditions for the SSOP method were as described previously [23] with the following modification for the SNPs in TNFα promoter region. The hybridization temperatures were as follows: 38°C for probes SNP −376[A] and −1031[T]; 40°C for probe −376[G]; 43°C for probes −857[T], −863[C], and −1031[C]; 47°C for probes −244[A], −857[C], and −863[A]; 50°C for probes −238[A] and −244[G]; 53°C for probes −163[A], −238[G], and −308[A]; 55°C for probe −163[G]; and 56°C for probe −308[G].

The stringent wash temperatures were as follows: 50°C for probes −376[G], −376[A], and −863[C]; 55°C for probe −1031[T]; 57°C for probe −1031[C]; 58°C for probes −244[A], −857[C], −857[T], and −863[A]; 62°C for probes −163[G], −163[A], −238[G], −244[G], −308[G], and −308[A]; and 66°C for probe −238[A]. As a positive control, we used DNA obtained from a B-lymphoblastoid cell line (Boleth) included in the International Histocompatibility Workshop cell lines panel [24].

LTA +252 Typing

The DNA samples were genotyped for the polymorphism within the intronic region gene sequence of LTA +252 (A→G) (rs909253) (GenBank accession number Z15026) using the restriction fragment length polymorphism technique. We used previously published 5′ and 3′ primers and cycling conditions [25].

Statistical Analysis

The allele and genotype frequencies were compared between the control and patient groups (LSIL and HSIL). Significant associations were evaluated by either the χ^2 test or the Fisher exact test with 2 × 2 contingency tables using the Epi Info 6.04D 2001 USA program. The level of significance was established at $P < .05$. The magnitude of the associations was assessed using the odds ratio (OR), and 95% of confidence intervals (CI) were calculated for the OR [26]. The gene-dose effect was determined with a linear trend analysis using the dominant homozygote genotype for each SNP studied, and the χ^2 test was performed using the Epidat 3.1 Software [27], with a level of significance at $P < .05$. The attributable proportion (AP) value was calculated using the formula: AP = PRF × (1 − 1 / OR), in which PRF represents percentage of risk factor [28]. A χ^2 analysis was used to test for any deviation of the genotype distribution from Hardy-Weinberg equilibrium. Haplotypes of the TNFα and LTA SNPs were assessed using HaploView 4.1 Software [29].

Results

Study Population

A total of 396 women were typed for SNPs in the promoter region of TNF and LTA. From this group, 205 women who were negative for HPV and who had no SILs were selected as the control group. From the 191 HPV-positive patients, 132 had LSIL and 59 had HSIL. Thirteen types of HPV were detected (6, 11, 16, 18, 31, 45, 53, 55, 56, 58, 82, 83, and 90), and 18 samples were negative for HPV infection in the patient group (3 in LSIL and 15 in HSIL). Infection with HPV type 16 was the most common at 50.74% and 48.83% in LSIL and HSIL, respectively. This result was followed by type 58 at 12.68% and 17.74% and type 18 at 8.95% and 14.52% in LSIL and HSIL, respectively. In one sample, we found a coinfection of two HPV types (6 and 82). All of the samples studied were in Hardy-Weinberg equilibrium for all SNPs analyzed, and there were no statistically significant differences when the frequencies of the observed and expected genotypes were compared.

Polymorphisms in the Promoter Region of TNFα

The distribution of alleles and genotypes of the polymorphisms in the promoter region of TNFα in patients and controls is shown in Table 1. A comparison between the allele frequencies of the controls and patients was performed, and the results showed that, in patients with LSIL, the TNFα −376 "A" allele is associated with the development of LSILs ($P < .05$, OR = 2.81, 95% CI = 1.09-7.44). In both patients and controls, the genotype G/G was the most frequent at positions −163, −238, −244, −308, and −376; the C/C genotype was present at −857 and −863, whereas genotypes T/T and A/A were defined at positions −1031 and +252, respectively. There were no differences in the distribution of the genotype frequencies at positions −163, −238, −244, −308, −857, −863, and −1031 of the promoter region of TNFα. However, we observed a risk trend for the TNFα −376 position ($P < .05$, OR = 2.48, 95% CI = 0.98-6.25) in the genotype G/A by comparing the LSIL group with the control group (Table 1). The calculated AP value was 5.45%, indicating that approximately 5% of patients with LSIL may be predisposed to the development of cervical lesions under the influence of allele "A." When the frequencies of the heterozygous and homozygous genotypes of the SNPs in the promoter region of TNFα and LTA were grouped, the genotype G/G at the TNFα −376 position was significantly different ($P < .05$, OR = 0.37, 95% CI = 0.14-0.99) in patients with LSIL. By analyzing the genotypes of the TNFα promoter region for HPV infection, we found no association between HPV-positive patients and HPV-negative patients as well as between HPV-negative patients and controls (data not shown).

The LTA +252 Polymorphism

The frequency distributions of the alleles and genotypes at position +252 of the LTA gene in the control group and patients are shown in Table 1. In the control group and LSIL, the most common genotype was A/A at 44.87% (92/205) and 45.45% (60/132), respectively, whereas the frequency of genotype A/G was more frequent (54.23%) in patients with HSIL. There was no statistically significant association between any of the polymorphisms of the alleles and genotypes of the LTA +252 position and the risk of developing SILs when the three groups were compared. When the frequencies of the recessive homozygous (G/G) and heterozygous (A/G) genotypes at position +252 of LTA were grouped, no statistically significant differences were observed between patients with LSIL and HSIL and the control group (Table 1). By comparing the LTA +252 position polymorphism and the HPV infection status, we found that the allele "G" was not statistically significant in patients with SILs regardless of a positive or negative HPV status (data not shown).

Combined TNFα Promoter Region and LTA Genotypes

We tested whether combinations of the genotypes of the promoter region of TNFα and LTA were related to susceptibility or protection in the patient groups (LSIL and HSIL) compared with the control group. Although 56 different genotype combinations are possible, only 20 were significant (data not shown). In contrast, the combination genotype G-G/G-G/G-G/G-G/G-G/C-T/C-C/T-T/G-A showed a trend of risk for HSIL, although the result was not significant ($P = .052$; Table 2).

Haplotypes

A total of eight SNPs across the TNFα/LTA loci were analyzed. Figure 1 shows the linkage disequilibrium (LD plot) of the SNPs in the TNFα/LTA genes of patients with LSIL (Figure 1*A*) and HSIL (Figure 1*B*). Table 3 shows the association analysis of the eight SNPs across the TNFα/LTA loci established in healthy women (controls) and in patients with LSIL and HSIL. Three haplotype blocks were identified, with 10 haplotypes for the control group, 8 haplotypes in patients with LSIL, and 7 haplotypes for patients with HSIL. The ATCCGGGG haplotype was the most frequent in all of the groups.

Table 1. TNFα Promoter Region and LTA SNP Polymorphisms Frequencies in the Control Group and in Patients with LSIL and HSIL.

SNP Site/Allele Genotype	Control, *n* (%)	LSIL, *n* (%)	OR* (95% CI)	HSIL, *n* (%)	OR† (95% CI)
TNFα					
−163					
G	403 (98.59)	264 (100.0)		118 (100.0)	
A	7 (1.70)	0 (0.0)		0 (0.0)	
GG	198 (96.58)	132 (100.0)	Reference	59 (100.0)	Reference
GA	7 (3.14)	0 (0.0)	—	0 (0.0)	—
Carrier A	7 (3.14)	0 (0.0)	—	0 (0.0)	—
−238					
G	403 (98.30)	264 (100.0)		118 (100.0)	
A	7 (1.70)	0 (0.0)		0 (0.0)	
GG	199 (97.07)	132 (100.0)	Reference	59 (100.0)	Reference
GA	5 (2.93)	0 (0.0)	—	0 (0.0)	—
AA	1 (0.48)	0 (0.0)	—	0 (0.0)	—
Carrier A	6 (2.92)	0 (0.0)	—	0 (0.0)	—
−244					
G	404 (98.53)	261 (98.86)		118 (100.0)	
A	6 (1.46)	3 (1.13)		0 (0.0)	
GG	199 (97.07)	129 (97.72)	Reference	59 (100.0)	Reference
GA	6 (2.93)	3 (2.27)	—	0 (0.0)	—
Carrier A	6 (2.92)	3 (2.27)	NS	0 (0.0)	—
−308					
G	387 (94.40)	250 (94.69)		115 (97.45)	
A	23 (5.60)	14 (5.30)		3 (2.54)	
GG	183 (89.26)	122 (92.42)	Reference	56 (94.91)	Reference
GA	21 (10.24)	10 (7.57)	NS	3 (5.08)	NS
AA	1 (0.48)	0 (0.0)	—	0 (0.0)	—
Carrier A	22 (10.73)	10 (7.57)	NS	3 (5.08)	NS
−376					
G	402 (98.04)	250 (94.69)		117 (99.15)	
A	8 (1.95)	14 (5.30)		1 (0.84)	
GG	197 (96.09)	119 (90.15)	Reference	58 (98.30)	Reference
GA	8 (3.90)	12 (9.09)	**2.48 (0.98-6.25)**‡	1 (1.69)	—
AA	0 (0.0)	1 (0.75)	—	0 (0.0)	—
Carrier A	8 (3.90)	13 (9.85)	NS	1 (1.69)	NS
−857					
C	304 (74.14)	186 (70.45)		83 (70.33)	
T	106 (25.85)	78 (29.54)		35 (29.66)	
CC	114 (55.60)	63 (47.72)	Reference	30 (50.84)	Reference
CT	76 (37.07)	59 (44.69)	NS	23 (38.98)	NS
TT	15 (7.31)	10 (7.57)	NS	6 (10.16)	NS
Carrier T	91 (44.40)	69 (52.28)	NS	29 (49.15)	NS
−863					
C	360 (87.80)	237 (89.77)		110 (93.22)	
A	50 (12.19)	27 (10.22)		8 (6.77)	
CC	160 (78.04)	106 (80.30)	Reference	52 (88.13)	Reference
AC	40 (19.51)	25 (18.93)	NS	6 (10.16)	NS
AA	5 (2.43)	1 (0.75)	NS	1 (1.69)	NS
Carrier A	45 (21.95)	26 (19.70)	NS	7 (11.87)	NS
−1031					
T	345 (84.14)	223 (84.46)		102 (86.44)	
C	65 (15.85)	41 (15.53)		16 (13.55)	
TT	147 (71.70)	93 (70.45)	Reference	45 (76.27)	Reference
CT	51 (24.87)	37 (28.03)	NS	12 (20.33)	NS
CC	7 (3.41)	2 (1.51)	NS	2 (3.38)	NS
Carrier C	58 (28.30)	29 (29.55)	NS	14 (23.73)	NS
LTA					
+252					
A	273 (66.58)	176 (66.66)		74 (62.71)	
G	137 (33.41)	88 (33.33)		44 (37.28)	
AA	92 (44.87)	60 (45.45)	Reference	21 (35.59)	Reference
AG	89 (43.41)	56 (42.42)	NS	32 (54.23)	NS
GG	24 (11.70)	16 (12.12)	NS	6 (10.16)	NS
Carrier G	113 (55.13)	72 (54.55)	NS	38 (64.40)	NS

NS indicates that the *P* value is not significant.
*Odds ratio calculated by comparing LSIL *versus* control.
†Odds ratio calculated by comparing HSIL *versus* control.
‡$P < .05$.

It was observed in 30.8% of control subjects, 45.6% of patients with LSIL, and 34.0% of patients with HSIL. We found that the haplotypes GTCTGGGG and ATCCGGGG were more frequent in the patient groups (45.6% and 25.4% in LSIL and 34.0% and 21.1% in HSIL, respectively), but only the GTCTGGGG genotype was associated with an eight-fold increase in risk of LSIL development ($P < .05$, OR = 8.33, 95% CI = 5.12-13.61). For patients with HSIL, the risk increased by almost four-fold ($P < .05$, OR = 3.85, 95% CI = 2.05-7.23). In

Table 2. Genotypes Relevant to Risk.

Genotypes	Control, *n* = 205 (%)	LSIL, *n* = 132 (%)	OR* (95% CI)	HSIL, *n* = 59 (%)	OR† (95% CI)
G-G/G-G/G-G/G-G/C-C/C-C/T-T/G-A	22 (10.73)	13 (9.84)	—	9 (15.25)	1.50 (0.60-3.69)
G-G/G-G/G-G/G-G/C-T/C-C/T-T/A-A	22 (10.73)	21 (15.9)	1.57 (0.79-3.14)	7 (11.86)	1.12 (0.41-2.96)
G-G/G-G/G-G/G-G/C-T/C-C/T-T/G-A	22 (10.73)	18 (13.63)	1.31 (0.64-2.68)	12 (20.33)	2.12 (0.91-4.89)‡
G-G/G-G/G-G/G-G/C-C/C-C/T-T/G-G	17 (8.29)	11 (8.33)	1.01 (0.42-2.36)	6 (10.16)	1.25 (0.42-3.60)
G-G/G-G/G-G/G-G/T-T/C-C/T-T/A-A	15 (7.31)	8 (6.06)	—	5 (8.47)	1.17 (0.35-3.66)
G-G/G-G/G-G/G-G/C-C/A-C/T-C/A-A	7 (3.41)	6 (4.54)	1.35 (0.39-4.58)	2 (3.38)	—
G-G/G-G/G-G/G-G/C-T/A-C/T-C/A-A	7 (3.41)	9 (6.81)	2.07 (0.68-6.35)	1 (1.69)	—
G-G/G-G/G-G/G-G/C-C/A-C/T-C/G-A	6 (2.92)	8 (6.06)	2.14 (0.66-7.14)	3 (5.08)	1.78 (0.34-8.34)
G-G/G-G/G-G/A-G/C-C/C-C/T-T/G-A	5 (2.43)	3 (2.27)	—	3 (5.08)	2.14 (0.39-10.71)
G-G/G-G/G-G/G-G/C-C/C-C/T-C/G-A	4 (1.95)	1 (0.75)	—	3 (5.08)	2.69 (0.46-14.78)
G-G/G-G/G-G/G-G/C-T/C-C/T-C/A-A	2 (0.97)	2 (1.51)	1.56 (0.16-15.70)	2 (3.38)	3.63 (0.36-36.98)
G-G/G-G/G-G/G-G/A/C-C/C-C/T-T/G-A	1 (0.48)	1 (0.75)	1.56 (0.0-57.45)	1 (1.69)	3.52 (0.0-130.89)

*Comparisons between LSIL and the control group.
†Comparisons between HSIL and the control group.
‡P = .052.

contrast, the haplotypes ACACGGGG and ATCCGGGG presented a low-grade risk of only four-fold (P < .05, OR = 4.36, 95% CI = 3.09-6.15) and almost three-fold (P < .05, OR = 2.68, 95% CI = 1.09-6.75), respectively, for developing cervical lesions. The ATCTGGGG haplotype was associated with a protective effect against lesion development (P < .05, OR = 0.39, 95% CI = 0.21-0.70). The ATACGGGG haplotype was related to protective effects in patients with LSIL (P < .05, OR = 0.19, 95% CI = 0.06-0.59), whereas in patients with HSIL, this haplotype yielded a two-fold increase in susceptibility to developing high-grade lesions (P < .05, OR = 2.43, 95% CI = 1.26-4.69; Table 3).

Discussion

Human Papillomavirus

The frequency of HPV-16 in our study (50.0%) was similar to the frequency found in women with HSIL (reported as CIN grades 2 and 3) in South Africa (56.6%) [30]. We confirmed that infection with HPV-16 remains one of the main factors predisposing to the development of SILs and cervical cancer. Interestingly, the HPV type 58 was the second most common virus in the samples (14.28%), similar to what has been observed in other countries, such as Africa (12.8%) [31] and Jamaica (18.8%) [32]. González-Losa [33] found that, in women diagnosed with LSIL and HSIL in the state of Yucatan (in the southeast of Mexico), HPV type 58 was the most frequent (23.3% and 30.6% in LSIL and HSIL cases, respectively), whereas in women treated for dysplasia in clinics of the Mexican Social Security Institute in the state of Nayarit (Western Mexico), the frequency of this type was 12.12% [34]. These data demonstrate that, although there are different distribution patterns of the types of HPV in different countries and even within the same country, HPV type 58 is one of the most common causes of cervical and vaginal infections and is a precursor for cervical lesions and cancer. Our results provide further evidence of the distribution of the most frequent HPV types in an area of Mexico City, and this information could be

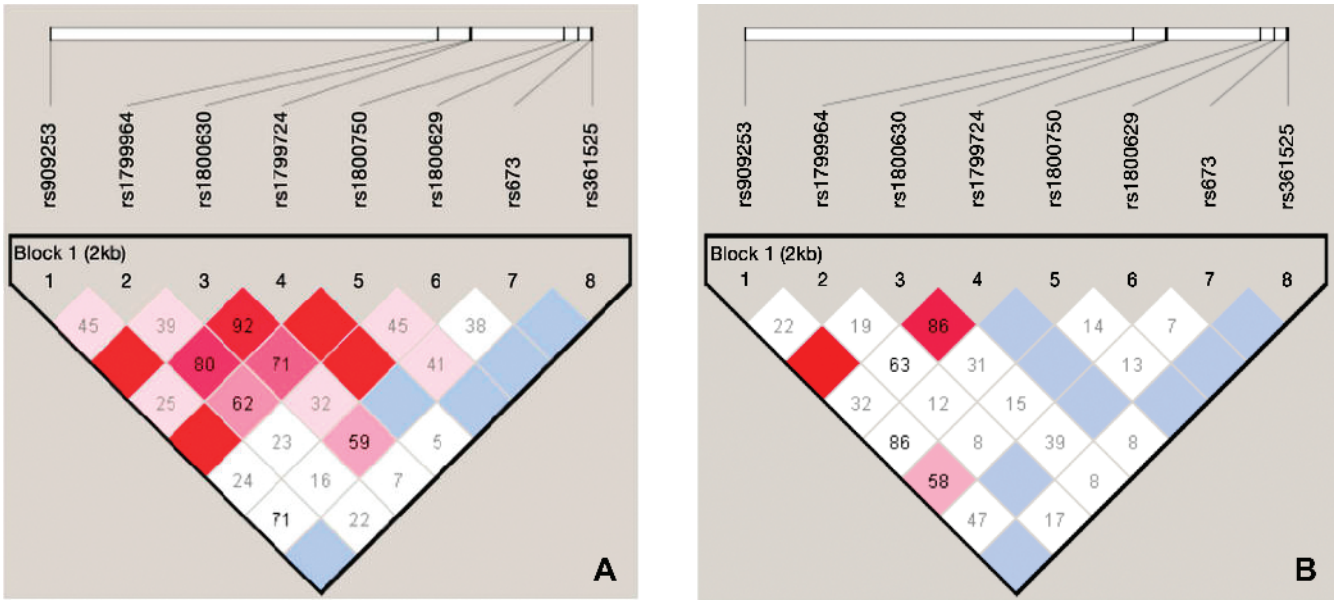

Figure 1. Linkage disequilibrium plot of SNP estimated as r^2 using Haploview 4.1. (A) LD plot for patients with LSIL. (B) LD plot for patients with HSIL.

Table 3. Common Haplotypes and Association Analysis for the Control Group and the LSIL and HSIL Groups.

Haplotypes	Control* (%)	LSIL* (%)	χ^2	*P*	OR† (95% CI)	HSIL* (%)	χ^2	*P*	OR‡ (95% CI)
ATCCGGGG	30.8	45.6	15.97	6.43*e* – 05	4.36 (3.09-6.15)	34.0	2.93	0.0867	—
GTCCGGGG	15.3	7.9	8.74	0.0031	—	13.5	2.33	0.1263	—
ATCTGGGG	15.1	4.5	20.27	6.70*e* – 06	0.39 (0.21-0.70)	8.5	1.02	0.3122	—
ATACGGGG	7.3	1.0	13.50	2.00*e* – 04	0.19 (0.06-0.59)	16.2	30.10	4.09*e* – 08	2.43 (1.26-4.69)
GTCTGGGG	6.7	25.4	50.99	9.26*e* – 13	8.33 (5.12-13.61)	21.1	0.19	0.6626	3.85 (2.05-7.23)
ACCCGGGG	5.6	6.4	0.22	0.6363	—	1.7	5.37	0.0204	—
GCCCGGGG	4.4	0.0	12.55	4.00*e* – 04	—	0.0	4.77	0.0289	—
GTCCGAGG	3.9	0.0	10.83	0.001	—	1.7	0.05	0.8133	—
ACACGGGG	2.2	3.9	1.61	0.2041	2.68 (1.09-6.75)	0.0	5.80	0.016	—
ACCTGGGG	2.0	0.0	5.34	0.0208	—	0.0	2.16	0.1415	—
ACACAAGG§	0.0	2.7	10.98	9.00*e* – 04	—	0.0	—	—	—

*Frequency of haplotype.
†Comparison between the LSIL and control groups; all significant data are shown ($P < .05$).
‡Comparison between the HSIL and control groups; all significant data are shown ($P < .05$).
§Haplotype only found in patients with LSIL.

used for the formulation of appropriate vaccines to combat specific HPV infections among Mexican women.

SNPs in the Promoter Region of TNFα

Most genetic association studies of TNFα with diseases in different patient populations have been conducted with different SNPs in the promoter regions of TNFα and LTA [35,36]. In our study, most SNPs examined were in the promoter region of TNFα (−163, −238, −244, −308, −376, −857, −863 and −1031) and at position +252 of the first intron of LTA to acquire a more complete perspective of the associations of SNPs in TNFα and LTA with the development of precursor lesions of cervical cancer in Mexican women.

Some studies have suggested that polymorphisms of SNPs in the promoter region of TNFα may be a contributing factor for the development of cervical cancer [37,38]. However, in our study, the SNPs at TNFα −163, −238, −244, −308, −857, −863, and −1031 showed no association with SILs.

When comparing the TNFα −163 and −244 SNP genotype frequencies from our study with those of two other studies, one with Hispanic women with invasive cervical cancer and HPV-16–positive tumors (TNFα −161, 100%-G/G, 0.0%-G/A, and 0.0%-A/A; and TNFα −243, 99.2%-G/G, 0.8%-G/A, and 0.0%-A/A) [38] and one from patients with cervical cancer treated at the Mayo Clinic (US population) (TNFα −244, 100%-G/G, 0.0%-G/A and 0.0%-A/A) [37], only minimal differences are observed. This information might indicate that the frequencies observed in the three populations are very similar. When we compared our results against TNFα −244 sarcoidosis patients in Hokkaido, Japan [25], and patients of the Irani National Reference Laboratory for Tuberculosis [39], we noted that both of the other populations presented only the genotype G/G, whereas our population also had the genotype G/A, with a 2.93% frequency. Another possible explanation may be the fact that the TNFα −244 SNP has been reported to be the first SNP of TNFα that is correlated to extended HLA haplotypes of African origin, which may indicate that this SNP of TNFα is highly conserved even after several years of migration [40].

The SNPs TNFα −238 and −308 have been intensively studied in patients with precursor lesions of cervical cancer and invasive cervical cancer, and the genotype G/G is the most frequent in different populations, such as the United Kingdom (89.0% and 52.0%) [41], New Delhi (96.0% and 91.0%) [42], and Switzerland (95.3% and 83.3%) [43]. With other diseases, the frequency of the genotype G/G for the same SNPs was more frequent, as evidenced by a report in a Mexican population of patients with systemic lupus erythematosus (92.7% and 94.5%) [44], another report on UK women with breast cancer (87.7% and 62.9%) [45] and, finally, a study of patients with recurrent spontaneous absorption in the Chinese population (88.8% and 90.8%) [35]. Although we confirmed that the genotype G/G was the most frequent in both SNPs, we found no statistically significant association between either SNP and the development of precursor lesions. In our study, the TNFα −238 SNP genotype was present only as the homozygous G/G (dominant) genotype in all patients (LSIL and HSIL). In other populations, it has been shown that the genotype G/G was prevalent, whereas genotypes that carry the recessive allele (G/A and A/A) have lower frequencies (1%-14%), depending on the study population [37,38,41–43]. The genotype G/A for the TNFα −238 SNP has been associated with a protective role in cervical cancer patients in the United States (OR = 0.33, 95% CI = 0.11-0.96) [37], whereas for patients in North Korea, the TNFα −238 allele "A" is associated with a decreased risk of developing different types of cancer (cervical, gastric, and renal carcinoma) (OR = 0.25, 95% CI = 0.10-0.64) [46]. When considering the genotypes that carry the allele "A" in the TNFα −308 (G/A and A/A) recessive position, we found that the genotype frequencies were lower among patients with LSIL and HSIL when compared with the frequencies of the control group. This finding resembles the decrease in the TNFα −308 genotype frequencies observed in UK patients with cervical precursor lesions; in that study, Kirkpatrick et al. [41] found that this SNP indicates a susceptibility for developing low dysplasia ($P < .05$). Other studies have shown that patients with the allele "A" and the genotype G/A + A/A have an up to two times higher risk to develop cervical cancer, as demonstrated for a population in New Delhi (OR = 2.7, 95% CI = 1.41-5.15) [42] and in India (OR = 2.24 for genotype G/A and OR = 2.05 for genotype A/A) [47] and in northern Portugal (OR = 1.88, 95% CI = 1.20-2.94) [28]. The differences in the allele and genotype frequencies of these SNPs in our results and earlier data are probably explained by the statistical power of the samples studied or by ethnic differences between the populations.

With respect to the TNFα −376 position, our data showed that 9.09% (12/132) of patients with LSIL had the genotype G/A compared to 3.90% (8/205) in the control group. This difference was statistically significant ($P < .05$; Table 1). To date, there is uncertainty about the biologic effect of the TNFα −376 SNP. However, this SNP is located in the promoter region of TNFα gene. Knight et al. [48] suggested that this SNP induces multiple DNA-protein interactions

and that it is the less common allele (TNFα −376 "A") and recruits the transcription factor Oct-1 to that area. Oct-1 is a transcription factor whose interactions with other proteins control diverse effects on gene regulation. In most cases, it enhances transcription, but in some other contexts, it functions as a transcription repressor [49]. The importance of the potential activity of the transcription factor Oct-1 in association with the allele "A" of the TNFα −376 SNP was indicated in a study in which this interaction resulted in an increase of four times the risk of cerebral malaria in two genetically distinct populations in Africa (Gambia and Kenya) [48]. It is known that the SNPs located in the promoter region of different genes can alter the affinity of association of transcription factors. Therefore, based on our results, the genotype G/A at the TNFα −376 position likely confers a susceptibility to develop low-grade precursor lesions by a modification of the transcription product from the allele "A." This idea is supported by the value obtained from AP, which indicates that approximately 5% of patients with LSIL may develop this injury under the influence of allele "A." Moreover, when the genotypes were grouped (G/A and A/A), our results show that the genotype G/G yields protection for the development of LSIL ($P < .05$, OR = 0.37, 95% CI = 0.14-0.99). Calhoun et al. [37] studied four SNPs of the TNFα promoter region in patients with cervical cancer, one of which was the TNFα −376 SNP, which is a risk factor for cervical cancer development. Our findings lead us to propose that the TNFα −376 SNP may be a susceptibility factor from the early stages of precursor lesions to cervical cancer.

We noted that genotypes with the recessive allele "T" (C/T and T/T) at the position TNFα −857 were associated with a protective trend, as Deshpande et al. [38] described in Hispanic women with cervical cancer. However, in the same study, the genotypes A/C and A/A at position TNFα −863 opposed our results (there is a risk trend; Table 1). In both positions, the data were not statistically significant, possibly because the number of samples analyzed in our group of patients. However, Ivansson et al. [43] reported that position −857 of TNFα and other SNPs had no association with cervical cancer, although it was a study with sufficient power to detect these associations.

Different studies have indicated a relationship between the expression of the cytokine TNFα and the SNPs −857 (C/T) and −863 (C/A) of the TNFα gene promoter region, suggesting that the recessive alleles −857 "C" and −863 "A" lead to higher production of TNFα, whereas the other alleles, −857 "T" and −863 "C," produce lower levels of this cytokine [9,50]. The allele "T" of TNFα−857, contains an anchor site for the transcription factor Oct-1 (ATGAAG**A**C) from position −858 to position −851 (the −857 SNP is underlined and in bold). The anchor sequence is present only with the allele "T" but not with the allele "C," thereby altering the transcriptional activity of the TNFα gene [51,52]. We observe that the frequencies of the C/T and T/T genotypes at position TNFα −857 in patients with LSIL and HSIL were increased, and we propose that the cells transformed after HPV infection avoid the host immune system in the early stages of carcinogenesis and become resistant to TNFα cytotoxicity. Alternatively, some reports suggest that C changes to an A at position −863 of the promoter region of TNFα, resulting in an increase in the production of the cytokine owing to the higher affinity of the transcription factor NF-κB for the allele "A." The transcription factor NF-κB is produced in two forms: one form is the canonical p65-p50 heterodimer, and the other form is a p50-p50 homodimer. The latter competitively inhibits the transcription factor NF-κB when anchored to the allele "A" position TNFα −863 [53]. The transcription factor NF-κB with the DNA anchor site is a transcriptional repressor in the promoter region of the TNFα gene [52,54]. We observed that the frequencies of the genotypes C/A and A/A in the TNFα −863 position in patients with LSIL and HSIL were reduced, and there was a trend of risk. These data lead us to conclude that TNFα production is insufficient and, therefore, is unable to destroy malignant cells.

The polymorphism at position TNFα −1031 has been associated with increased transcriptional activity and cytokine production of TNFα in different studies [22,55]. We observed no significant differences in the allele and genotype frequencies at this position between controls and patients with LSIL and HSIL, although this is the first report that examined the allele and genotype frequencies of TNFα −1031 position for this population. Our data are similar to the results of Negoro et al. [56], who studied this SNP in a Japanese population with Crohn disease. The TNFα −1031 SNP has been associated with other diseases, such as advanced stage endometriosis [57] and spontaneous deep intracerebral hemorrhage [58]. However, we propose that this position is not associated with the development of precursor lesions of cervical cancer. Further research is needed to determine the functional significance of this polymorphism for other diseases and different populations (ethnic groups).

Lymphotoxin α

When the LTA +252 polymorphism was studied, no association was established with precursor lesions of cervical cancer. This SNP has also been studied in patients with sarcoidosis, testicular germ cell tumors, chronic obstructive pulmonary disease, and tuberculosis in different populations [25,59–61]. We recently reported that the allele "A" predisposes patients to gastric cancer development [36]. However, the reported frequencies were different in this study, possibly because of the selection criteria used for the control subjects, which were the absence of gastroduodenal symptoms and sex (only females), leading to a bias in the frequencies.

Our frequencies of the genotypes G/G, G/A, and A/A for LTA position +252 (44.87%, 43.41%, and 11.70%) were similar to those of populations in northern Sweden (46.0%, 44.0%, and 11.0%) [59] and in the United States (43.1%, 44.4%, and 12.5%) [60], and we found no significant association of this SNP with the development of testicular germ cell tumors and chronic obstructive pulmonary disease. In contrast, Niwa et al. [62] studied positions +252 (G→A) and +804 (C→A) of LTA and found that there is a linkage disequilibrium between these two positions; the LTA +804 SNP was associated with a decreased cervical cancer risk, especially for squamous cell cervical cancer. These data suggest that position +252 is probably not associated with the development of precursor lesions of cervical cancer. Another study with a larger number of samples that was performed in Swiss women found no association between SNP +252 of LTA and women diagnosed with cervical tumors, severe dysplasia, and *in situ* and invasive cervical cancer [43]. Because of the above data, we suggest that other SNPs of the LTA gene should be considered in future studies to find possible associations with the development of SILs.

Genotype Combinations

The differences in the genotype combination frequencies between groups in our study showed no statistical significance, indicating that the genotype frequencies are not related to the development of cervical cancer precursor lesions. However, we noted that most genotypes showed a trend toward susceptibility, and the genotype G-G/G-G/G-G/G-G/G-G/C-T/C-C/T-T/G-A had an increased frequency as the

degree of injury advanced. This finding suggests that this genotype combination could be used as factor for risk surveillance.

Haplotypes

We studied the association of haplotypes including the SNPs of TNFα and LTA with the susceptibility for the development of cervical cancer precursor lesions. Ivansson et al. [43] reported that the SNPs −857, −572, −308, and −238 in the TNFα promoter region and LTA +252 in a Swiss population were not associated with the development of cervical cancer. We selected SNPs of the TNFα gene promoter region and the LTA gene based on their involvement in modifying the transcription of cytokines TNFα and LTA. Our results indicate that the haplotype GTCTGGGG is a potential genetic marker of a poor prognosis for the development of precursor lesions of cervical cancer. Most of the composition of this haplotype consists of alleles that produce fewer transcripts for the cytokines TNFα and LTA, possibly suggesting that the immune response is inefficient in cervical cells infected by HPV. In contrast, the statistical values observed for the haplotypes ATACGGGG and ATCTGGGG suggest that they could be considered protective genetic markers that differentiate progression in patients with low-grade lesions (LSIL). In this case, the immune response is still able to eliminate a certain number or volume of altered cells infected by HPV. Because the ATCCGGGG and ACACGGGG haplotypes are risk factors for patients with low injury, whereas the haplotype ATACGGGG is for patients with high-grade lesions, it is unlikely that the immune response of women is so efficient as to eliminate the altered cells infected by HPV.

In conclusion, our data provide evidence for the association of the TNFα −376 SNP and the GTCTGGGG and ATACGGGG haplotypes with the development of precursor lesions, and thus, these markers might serve as genetic markers of susceptibility. This study also illustrates the diversity in the effects of the TNFα and LTA SNPs in a Mexican population sample and the need to conduct further studies.

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