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Analysis of TNFα promoter SNPs and the risk of cervical cancer in urban populations of Posadas (Misiones, Argentina)

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ABSTRACT

Background: Human papillomavirus (HPV) plays a central role in cervical cancer development. However, only a small fraction of infected women develop the disease. Additional risk factors, including SNPs in immune system and cytokine genes, are likely to be important determinants.
Objective: We investigated the potential role of cytokine TNF-α promoter SNPs (TNFα-375A, TNFα-307A, TNFα-243A, and TNFα-237A) in the development of high-grade cervical lesions and cancer in urban women from Posadas (Misiones, Argentina).
Study design: Fifty-six cases (CINIII and invasive carcinoma) and 113 age-matched controls were included in the study. HPV genotype detection was conducted by PCR. TNFα SNP genotyping was conducted through PCR amplification and direct sequencing of genomic DNA.
Results: We observed differences in the allelic distribution of TNFα-307A and TNFα-375A SNPs among cases and controls ($p < 0.05$). The TNFα-307A variant was associated with cervical cancer at an OR 2.4 (CI 95% 1.1–5.4), while the TNFα-375A SNP was identified in 8.8% of the controls and none of the cases. Moreover, the TNFα-375A always occurred in association with the TNFα-237A SNP, indicating linkage disequilibrium between them.
Conclusion: Our study suggests that the presence of the high producer allele TNFα-307A is associated with an increased risk for the development of cervical cancer in the Posadas population. We also speculate that the "protective effect" of the TNFα-375A/-237A haplotype, which was restricted to controls, may be related to HLA genes linked on chromosome 6. These findings contribute to our understanding of immune gene variation in an Argentinean population, and its role in disease susceptibility.
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1. Background

The relationship between genital infection by certain types of human papillomavirus (HPV) and the development of cervical cancer is firmly established.[1] These clinically important HPVs belong to the *Papillomaviridae* family, *Alpha-Papillomavirus* Genus, species *A6*, *A7* and *A9*, and are generally identify as High-Risk Types (HPV-HR).[2,3] However, epidemiological studies have shown that only a small fraction of women infected with HPV-HR will develop high-grade lesions and invasive cancer. Therefore, other factors acting in conjunction with HPV infection likely influence this transition to cancer. Along with environmental and lifestyle factors,[4] human genetic markers are likely to be involved in this process.[5]

The Tumor Necrosis Factor-alpha (TNFα) is a multifunctional cytokine that plays a central role in the human immune response against a wide range of pathogens, including HPV. Despite its beneficial functions, the systemic excessive production of TNFα can also contribute to the development of autoimmune and malignant diseases.[6,7] Moreover, increased levels of serum TNFα have been described in cervical cancer patients, and are associated with a poor disease outcome.[8] This effect has been explained by its ability to induce angiogenesis.[9,10] Overall, the dual role of TNFα, which acts

Abbreviations: HPV, human papillomavirus; TNFα, Tumor Necrosis Factor-alpha; SNPs, single nucleotide polymorphisms; CIN-3, cervical intraepithelial neoplasia grade 3; H-SIL, high-grade squamous intraepithelial lesion; ISCC, invasive squamous cell carcinoma; HLAs, Human Leukocyte Antigens.

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as an agent of both innate immunity and inflammatory and malignant pathology, poses a considerable challenge for understanding the genetic regulation of its production.

The human TNFα gene has been mapped to human chromosome 6 (6p21.3) in the Human Leukocyte Antigen (HLA) region between the Class I HLA-B and the Class II HLA-DR loci.[11] The analysis of genetic variation in the TNFα promoter resulted in the discovery of single nucleotide polymorphisms (SNPs) located at positions −1030, −862, −856, −574, −375, −307, −243, and −237 from the transcription start point.[12–16] Furthermore, studies indicated that certain SNPs were in linkage disequilibrium with neighboring HLA genes,[12–14,16,17] occurred in distinct patterns in different ethnic groups,[17] and influenced TNFα transcription.[18,19]

Although the last point is still controversial,[20,21] these SNPs have been classified based on their functional effect as "high" or "low" producers and studied in the context of cervical cancer development. For example, the TNFα-307 SNP is a transition (G → A) that reportedly doubled TNFα production *in vitro*,[19] and is considered a risk factor for cancer.[22] On the other hand, the TNFα-237 SNP is a transition (G → A) that reportedly decreases TNFα production.[23] It has further been associated with a protective genotype for invasive squamous cell carcinoma development.[24]

In Argentina, Misiones Province has one of the highest mortality rates for cervical carcinoma in the country, with values of 10 cases in 100,000 women compared to 4.5 in 100,000 in Buenos Aires.[25] In addition, epidemiological studies have showed an elevated HPV genital infection in asymptomatic white urban women (43%) and Guarani Indian women inhabiting the area (64%).[26,27] For these reasons, screening women for cervical cancer remains an important health and economic concern throughout the region. However, investigations designed to delineate the underlying immunological mechanisms involved in the natural control of HPV infection and cervical cancer are also needed. Thus, in this study, we evaluate the potential role of TNFα allelic diversity in cervical cancer in a population from Posadas.

2. Objective

To investigate the potential role of cytokine TNF-α promoter SNPs (TNFα-375A, TNFα-307A, TNFα-243A, and TNFα-237A) in the development of high-grade cervical lesions and cancer in urban women from Posadas (Misiones Province, Argentina).

3. Study design

3.1. Study design and bioethics

One hundred and sixty nine patients were selected for a case–control study. Case samples of cervical intraepithelial neoplasia grade 3 (CIN-3), which includes high-grade squamous intraepithelial lesion (H-SIL) and *in situ* carcinoma, and invasive squamous cell carcinoma (ISCC) were obtained without identifiers from the Tumor Registry database (years 2005–06) at the Department of Pathology, Hospital Escuela de Agudos "Dr Ramón Madariaga", Posadas, Misiones (Argentina). For each case, two control subjects were recruited from unrelated women visiting the Department of Gynecology at the same institution for routine gynecological care between the same periods. All controls gave their informed consent to participate in the study. The general criteria for inclusion were (a) normal cytology by Pap smears, (b) age-matched (5 years range), and (c) the same area of residence and time of sample collection.

The final study groups comprised 56 cases with histopathologically confirmed high-grade cervical lesions and cancer (27 CIN3 [13 H-SIL and 14 carcinoma *in situ*] and 29 ISCC), and 113 healthy women (normal cytology). Information on ethnicity was not collected. However, judging from the usual demographics for patients at the hospital, we estimated that the patients and controls were similar, and broadly representative of the population of Posadas (i.e., white-admixed of Amerindian-European descent).

The Research Ethics Committee of the Argentinian institutions (Comité de Bioética del Departamento de Docencia e Investigación del Hospital Escuela de Agudos Dr. Ramón Madariaga) and Institutional Review Board of the University of Pennsylvania approved the study design. All procedures were carried out in accordance with the Helsinki Declaration.

3.2. Samples

Genomic DNA was isolated from fresh cervical samples (endo–ecto-cervical) collected with cytobrush (controls) and 5–10 10-μm sections of archival paraffin-embedded, formaldehyde-fixed tissue samples (cases) using the QIAGEN Extraction Kit as recommended by the manufacturer.

3.3. HPV detection and typing

HPV detection was performed with L1 consensus primers MY09–MY11.[28] The typing of HPV DNA positive samples was performed by E6-Nested Multiplex PCR (E6-NMPX) with cocktails of primers C-1 (HPV-HR 16, 18, 31, 45, and 59) and C-2 (HPV-HR 33, 56, 52, 58 and HPV low Risk 6 and 11).[29] Samples which could not be typed by E6-NMPX PCR were subjected to RFLP analysis.[28]

3.4. TNFα promoter SNPs

We developed two pair of primers for this study (Table 1). The target is shorter in the PCR protocol for the case group, since the amplification of large genomic sections was difficult in archival tissue, probably due to DNA degradation. PCR reactions were carried out in 75 μl of solution containing 100 ng of template DNA, 50 mM KCl, 20 mM Tris–HCl (pH 8.4), 1.5 mM $MgCl_2$, 200 μM of each dNTPs, 1 μM of each primer and 2 U of Taq polymerase. Positive (human DNA) and negative (no template) controls were included in all amplifications. Amplification conditions for each protocol are detailed in Table 1. Ten μl of PCR products were run in 1.5% agarose gels and visualized through ethidium bromide staining.

The remaining sample was purified and subjected to sequencing using the amplification primers with Big Dye Terminator Pre-Mix v3.1 kits (Applied Biosystems) as described in 30, 31. All sequences were read on an ABI 3130xl Gene Analyzer, and the resulting sequence data aligned and analyzed using Codon Code aligner software v 3.0.1 (CodonCode Corporation). In order to test the accuracy of genotyping, we randomly repeated 40 sequences (24.1%), and obtained identical results.

3.5. Statistical analysis

Allele and genotype frequencies were calculated by direct counting. Genotype proportions for the controls were tested for Hardy–Weinberg equilibrium using Arlequín 3.1.[32] Haplotypes were computationally inferred from sequence data for linkage disequilibrium analysis (DNAsp version 5.00.04).[33] The distribution of genotypes among cases and controls were compared by χ^2 or two-tailed Fisher exact test. Logistic regression was used to estimate the odds ratio (OR) and 95% confidence intervals (CIs).

Table 1
Primers and PCR profiles.

Primers	Primer position[a]	Product	PCR profile[b]
Controls			
5′-CCAGCATTATGAGTCTCCG-3′	−678 to −660	722 bp	40 cycles of 95 °C, 1 min (denaturation); 61 °C, 1 min (annealing); 72 °C, 1 min (extension)
5′-TAGCTGGTCCTCTGCTGTC-3′	+31 to +49		
Cases			
5′-CACAGGCCTCAGGACTCAAC-3′	−490 to −471	363 bp	45 cycles of 94 °C, 1 min (denaturation); 62 °C, 1 min (annealing); 72 °C, 1 min (extension)
5′-ACCTTCTGTCTCGGTTTCTTC-3′	−128 to −148		

[a] Positions are indicated as minus (−) or plus (+) to indicate their position relative to the transcription start point in the TNF-α human gene.
[b] The PCR profile includes an initial denaturation step of 95 °C for 3 min and a final extension of 72 °C for 5 min.

4. Results

4.1. Study population profile

The mean age of the 56 patients included in this study was 44.4 years (SD = 11.2; range 23–71; median 43.0). This age distribution was not different (median test $p = 0.11$) from that of the 113 healthy controls, whose mean age was 41.9 years (SD = 10.4; range 28–70; median 40).

4.2. HPV detection and typing

HPV DNA was detected in 31.0% of control women and 80.4% of cases. Fourteen patients presented co-infections and twelve could not be typed. Details on HPV type specific frequencies are shown in Table 2. In agreement with worldwide data, infection with HPV-16 and -18 was higher in the case group compared to controls (57.1% vs. 5.3%). Based on these findings, the associated risk for HPV-16/18 infection and cervical cancer was estimated at an OR of 23.8 (8.9–63.2).

4.3. Genotyping of TNFα promoter SNPs

The allele frequencies of TNFα promoter SNPs are shown in Table 3. The frequency of the TNFα-237A allele was similar between cases and controls (0.053 and 0.079, respectively; $p > 0.05$). We found only two carriers for the TNFα-243A polymorphism and they occurred in the case group (0.018). In addition, the frequency of the TNFα-307A allele was 0.125 in the cases and 0.053 in the controls, a difference that was statistically significant ($p < 0.05$). The allele TNFα-375A was found only in controls (0.044), which also reached statistical significance ($p < 0.05$).

The genotype distribution and potential association in the healthy controls and cancer patients is shown in Table 4. Genotype frequencies for TNFα-307, −237 and −375 were found to be in Hardy–Weinberg equilibrium. TNFα-307A was associated with cervical cancer development with an OR of 2.4 (1.1–5.4).

4.4. Haplotype analysis

Haplotypes inferred from genotype data are shown in Table 5. Haplotype analysis also indicated linkage disequilibrium between

Table 2
HPV types in the control and case groups.

HPV types	Controls		Cases	
	n	%	*n*	%
High risk				
HPV-16	2	1.8	25	44.5
HPV-18	–	–	2	3.6
HPV-33	8	7.1	1	1.8
HPV-45	–	–	1	1.8
HPV-52	3	2.6	–	–
HPV-56	5	4.4	–	–
HPV-31	1	0.9	–	–
HPV-58	1	0.9	–	–
HPV-61	2	1.8	–	–
Low risk[a]				
HPV-6/11	1	0.9	2	3.6
Mixed infections				
HPV-16 + HPV-18 + HPV-6/11	1	0.9	–	–
HPV-16 + HPV-6/11	3	2.6	1	1.8
HPV-33 + HPV-56	1	0.9	–	–
HPV-33 + HPV-52	1	0.9	–	–
HPV-31 + HPV-45	1	0.9	–	–
HPV-56 + HPV-6/11	1	0.9	1	1.8
HPV-16 + HPV-52	–	–	1	1.8
HPV-16 + HPV-33	–	–	2	3.6
HPV-16 + HPV-18	–	–	1	1.8
Positive undetermined	4	3.5	8	14.3
Negative	78	69.0	11	19.6
Total	113	100.0	56	100.0

[a] HPV-6 and HPV-11 are indicated as a combined prevalence (HPV-6/11), according to Sotlar et al.[29]

Table 3
Allele frequencies of TNFα promoter SNPs in cases and controls.

SNPs	Allele	Control (n = 226)	Cases (n = 112)	p-Value
−375	G	0.956 (216)	1.000 (112)	**0.03**
	A	0.044 (10)	0.000 (0)	
−307	G	0.947 (214)	0.875 (98)	**0.03**
	A	0.053 (12)	0.125 (14)	
−243	G	1.000 (226)	0.982 (110)	0.1
	A	0.000 (0)	0.018 (2)	
−237	G	0.921 (208)	0.947 (106)	0.5
	A	0.079 (18)	0.053 (6)	

The number of allele counted is noted in parentheses.
The p-values that are statistically significant are indicated in bold (p < 0.05).

Table 4
Genotype frequencies (%) of TNFα promoter SNPs and potential associations between case and control subjects.

SNPs	Genotype	Control (n = 113)	Cases (n = 56)	OR (CI 95%) or p-value[a]
−375	G/G	91.2 (103)	100 (56)	0.03
	G/A	8.8 (10)	0 (0)	
−307	G/G	89.4 (101)	78.6 (44)	1 (Ref)
	G/A	10.6 (12)	17.8 (10)	2.4 (1.1–5.4)
	A/A	0 (0)	3.6 (2)	
−243	G/G	100 (113)	96.4 (54)	0.6
	G/A	0 (0)	3.6 (2)	
−237	G/G	84.9 (96)	89.3 (50)	0.11
	G/A	14.2 (16)	10.7 (6)	
	A/A	0.9 (1)	0 (0)	

[a] χ^2 or two-tailed Fisher exact test.
The frequency of each genotype is followed by the total number of individuals counted which is noted in parentheses.

the TNFα-237A and TNFα-375A SNPs (coefficient of linkage disequilibrium, D' = 1; p = 0.000). The overall distribution of haplotypes was significantly different between case subjects and control subjects. Haplotype #2, defined by the TNFα-237A and TNFα-375A SNPs, was present in only control subjects (p = 0.03), whereas haplotype #3, defined by the TNFα-307A SNP, was present at a significantly higher frequency in case subjects compared to control subjects (p = 0.03).

5. Discussion

The role of host genetic variation in determining susceptibility to cervical cancer development is the focus of much research around the world.[5,34,35] In Argentina, human genetic factors analyzed during the last few years include polymorphisms in the oncogene ras and the tumor suppressor gene p53.[36–38] However, information about variation in immune system genes (HLA, cytokines) is still scarce.[39]

In this work, our association study shows that individuals carrying TNFα-307A allele were more frequently members of the case group (21.5%) than the control group (10.6%), at an OR value of 2.4 (CI 95% 1.1–5.4). This is an important finding, as the TNFα-307A polymorphism has been associated with invasive cervical cancer in European (Portuguese) and Indian women,[40,41] but not those from Africa.[42,43] TNFα SNPs have also been reported to occur at different frequencies within different ethnic groups.[17] These differences may be due to endogamy, population stratification, selection or symptoms of disease association. The Misiones province from which the study populations were drawn is ethnically diverse, with the white population (Amerindian-European admixed) living in rural and urban areas and Amerindians of the Guarani tribe inhabiting the rainforest of the region. In this regard, it is interesting to note that, within Misiones, the TNFα-307A is absent in Guarani Indians.[44]

As a final point, we detected a haplotype defined by the TNFα-375A and TNFα-237A SNPs that was restricted to controls, and hypothesize that this haplotype may confer a "protective effect" to individuals having it. However, the TNFα gene is particularly difficult to study in isolation because, in being part of the MHC class III cluster, it is an integral component of this HLA gene complex. Thus, previous studies have shown an association between the −375, −307, −237 and −243 variants and extended HLA

Table 5
TNFα promoter haplotypes among cases and controls.

Haplotype/SNPs	−375	−307	−243	−237	Controls (n = 226)	Cases (n = 112)	p-Value
H1	G	G	G	G	196	91	0.2
H2[a]	**A**	**G**	**G**	**A**	10	0	**0.03**
H3	**G**	**A**	**G**	**G**	12	14	**0.03**
H4	G	G	A	G	0	1	0.3
H5	G	G	G	A	8	5	0.6
H6	G	G	A	A	0	1	0.3

Haplotypes statistically inferred by DNAsp for the TNFα promoter region.
[a] Haplotype analysis also indicated linkage disequilibrium between TNF-237A and TNF-375A (coefficient of linkage disequilibrium, D' = 1; p = 0.000).

haplotypes.[16,17] For example, the polymorphism at TNFα-307 is linked with the HLA A1-B8-DR3-DQ2,[16] and within this haplotype HLA-DR3 has been specifically linked with cervical cancer.[45] Studies of HLA distributions in Argentinean women show that HLA-DRB1*04 and HLA-DQB1*0302 are risk factors for malignant progression, whereas HLADRB1*13 and HLA-DQB1*02 have a protective effect.[39] Based on these observations, it will be necessary to extend our study to the evaluation of HLA alleles in linkage disequilibrium with the described TNF-α SNPs.

This paper is the first report of TNFα SNP variation in Misiones, and will contribute to the characterization of immunological genetic markers in Argentina. In this respect, we are aware that small population studies have low power to detect statistically significant associations. Therefore, future pooling efforts are needed to confirm the patterns of genetic diversity and disease association observed in this project.

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Competing interests

The Funding Agencies have not been involved in the study design, data collection, analysis, and paper writing and submission. The authors have no conflicts of interest to declare.

Ethical approval

This study has been approved by the Comité de Bioética del Departamento de Docencia e Investigación del Hospital Escuela de Agudos Dr. Ramón Madariaga, Posadas, Misiones, Argentina, and the Institutional Review Board at the University of Pennsylvania.

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