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Significant association between *IL10*-1082/-819 and *TNF*-308 haplotypes and the susceptibility to cervical carcinogenesis in women infected by Human papillomavirus

Bárbara Simas Chagas[a], Rita de Cássia Pereira de Lima[a], Sérgio de Sá Leitão Paiva Júnior[b], Ruany Cristyne de Oliveira Silva[a], Marcelo Nazário Cordeiro[a], Jacinto da Costa Silva Neto[c], Marcus Vinicius de Aragão Batista[d], Anna Jéssica Duarte Silva[a], Ana Pavla Almeida Diniz Gurgel[e], Antonio Carlos de Freitas[a,*]

[a] Laboratory of Molecular Studies and Experimental Therapy, Department of Genetics, Federal University of Pernambuco, Pernambuco, Brazil
[b] Academic Unit of Serra Talhada - Federal Rural University of Pernambuco, Pernambuco, Brazil
[c] Laboratory of Molecular and Cytological Research, Department of Histology, Federal University of Pernambuco, Pernambuco, Brazil
[d] Department of Biology, Federal University of Sergipe, Sergipe, Brazil
[e] Department of Engineering and Environment, Federal University of Paraiba, Paraiba, Brazil

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ABSTRACT

Human papillomavirus (HPV) is responsible for high-grade cervical lesions and cervical cancer. The inflammation plays a key role in cervical cancer progression. In this context, studies propose an association between *TNFα* and *IL10* SNPs and susceptibility to HPV infection. The present work aimed to investigate the possible association between *IL10* and *TNFα* promoter polymorphisms and HPV infection in the cervical carcinogenesis risk in women from Brazil. A total of 654 samples was evaluated in this study. HPV detection was performed by PCR and HPV genotyping was performed by PCR and sequencing of positive MY09/11 PCR product. Genotyping of *IL10* SNPs (rs1800871 and rs1800896) was performed by High Resolution Melt analysis. Genotyping of *TNFα SNP* (rs1800629) was performed by fluorogenic allele-specific probes. The distribution of TNF-308 (rs1800629) allelic ($p = 0.03$) and genotype ($p = 0.03$) frequencies and HPV-58 infection has showed a statistically significant difference between case and control groups for the assessed *TNFα* polymorphism. When it comes to TNFα (rs1800629) allelic and genotypic distribution and HPVs 18 and 31 infections, no statistically significant differences between case and control groups were observed for the studied TNFα polymorphism. The allelic and genotypic distribution of IL10-819 (rs1800871) and IL10-1082 (rs1800896) and HPV infection (HPVs 58, 18 and 31) has showed no statistically significant differences between case and control groups for the assessed *IL10* polymorphisms. Furthermore, it was observed that haplotypes were associated with an increased cervical cancer risk in HPVs 16, 18 and 58-positive women. It was observed that women carrying the GTA and ATG haplotypes had 3.85 and 17.99-fold, respectively, increased cervical cancer susceptibility when infected by HPV-58. In women infected with HPV-16 and HPV-18, statistically significant results in women carrying the GTA and ATA haplotypes was observed. They had a 2.32 and 3.67-fold, respectively, increased cervical cancer susceptibility when infected by these two HPV types. The analysis of the haplotypes distribution in women infected with HPV-31 has showed no statistically significant results. Our study indicates that the association of genetic polymorphism in inflammation-related genes represents a risk to the susceptibility in the development of cervical cancer in women infected by HPVs 16, 18 and 58.

1. Introduction

Papillomaviruses (PVs), as members of the *Papillomaviridae* family, are double-strand DNA viruses that infect squamous or mucosal epithelia and produce a range of epithelial neoplasms, both benign and malignant [1]. Human papillomavirus (HPV) is responsible for most viral infection of the reproductive tract in USA population [2], which can increase the risk of cervical cancer. More than 200 types of HPV

* Corresponding author at: Av. Prof. Moraes Rego, 1235, Cidade Universitária, 50670-901 Recife, PE, Brazil.
E-mail address: acf_ufpe@yahoo.com.br (A.C.d. Freitas).

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have been identified through DNA sequencing [3] and more than 30 HPV types infect the cervix, vagina, vulva, penis and anus [4,5]. Those classified as oncogenic HPV or high-risk HPV are the most frequent found in high grade cervical lesions and cervical cancer. Among them, HPV-16 is commonly identified as the most prevalent in most countries, including Brazil. However, the second and third most prevalent types may be different according to the assessed population. Our previous results demonstrated the prevalence of different HPV types including HPV-16, HPV-18, HPV-31 and HPV-58 in women from northeastern Brazil [6].

This HPV prevalence linked to population features may be a consequence of particular interactions between the virus and the host immune response. Recent evidence suggests that inflammation plays a key role in cancer progression, including cervical cancer. Cancer-related inflammation can influence tumor cell proliferation, invasion, cell survival and angiogenesis [7]. The unique cytokines profile of each ethnic group is related to the respective functional polymorphisms or haplotypes in cytokine genes or other closely linked genes, and its activity might be crucial to induce protective immune responses [8]. Interleukin-10 (IL10) is an anti-inflammatory cytokine that is produced mainly by monocytes during inflammation process, with pleiotropic effects on immunological regulation, including pro-inflammatory cytokines downregulation, as well as tumor necrosis factor-alpha (TNFα), IL-1, IL-6, IL-8, IL-12, and IFN-γ [9]. IL10 activity, whose serum levels are influenced by at least three polymorphic sites within its promoter [10], is so critical to HPV infection that high-risk HPVs modulate immune cells to create an immunosuppressive microenvironment and induce these immune cells to produce IL10 [11]. IL10 GTC haplotype has been associated to viral load and cervical cancer development [12]. On the other hand, TNFα is one of the critical innate cytokines that plays an important role as inflammation state mediators. The tumor microenvironment is largely affected by inflammation and many cancers arise from the infection and chronic inflammation sites, where TNFα polymorphisms may interfere on neoplasm progression [13]. Recent studies suggest an association between TNFα SNPs and the susceptibility to HPV infection [14], but the results may depend on different ethnic backgrounds [15]. Li et al. [16] suggest that TNFα SNPs is associated with the degree of malignancy of cervical cancer.

Human papillomatosis seems to be a polygenic disease, which means that frequent genetic variants with slight effects may have consequences on disease susceptibility as well as disease phenotype and outcome. Therefore, the present study aimed to investigate the possible association between *IL10* and *TNFα* promoter polymorphisms and HPV-related cervical carcinogenesis risk.

2. Materials and methods

2.1. Study group

In this study, 654 samples were randomly collected by cervical scraping from voluntary patients, aged from 18 to 82 years (average 34.6 ± 10.8), during their cervical cancer screening at the gynecological unit of the Clinical Hospital (HC) and Oswaldo Cruz University Hospital (HUOC) in Recife, Pernambuco State, Northeastern Brazil.

Table 1
Clinical characteristics of the patients.

	N	%
Cytological results		
LSIL[a]	126	19.3
HSIL[b]	121	18.5
Normal	407	62.2
Total number of patients	654	100

[a] LSIL: Low-grade squamous intraepithelial lesions.
[b] HSIL: High-grade squamous intraepithelial lesions.

Table 1 shows the clinical characteristics of the patients involved in the present study.

This study was approved by the Ethics Committee of the Federal University of Pernambuco (CEP/CCS/UFPE N° 491/11) and Ethical Committee of Oswaldo Cruz University Hospital (HUOC/PROCAPE 64/2010). All the women signed the informed consent. The inclusion criteria were to agree in participating in the study. On the other hand, the exclusion criteria were current pregnancy, having undergone hysterectomy or to be HIV-positive.

Cervical cells were collected with cytobrush, added in pH 7.4 phosphate-buffered saline (PBS) and subsequently used to DNA extraction by DNeasy Blood Tissue Kit (Qiagen), in accordance with the manufacturer's manual.

2.2. HPV genotyping

HPV DNA was detected by PCR using degenerate primers MY09/11 [17]. HPV typing was performed by PCR using specific primers for HPV-6, HPV-11, HPV-16, HPV-18, HPV-31, HPV-33 and HPV-58 (Supplementary Table 1), as well as sequencing of positive MY09/11 PCR product, which were purified with Wizard SV Gel and PCR Clean-Up System Kit (Promega) prior to sequencing using ABI PRISM Big Dye TM Terminator Cycle Sequencing v3.1 ready reaction (Applied Biosystems). After DNA sequencing, the identification of HPV isolates was performed by using Basic Local Alignment Search Tool (BLAST).

2.3. IL10 and TNFα genotyping of polymorphisms

Genotyping of −819 C/T (rs1800871) and −1082 A/G (rs1800896) polymorphisms in the promoter of *IL10* gene was performed as described by Chagas et al. [18]. Genotyping of -308 G/A (rs1800629) polymorphism in the *TNFα* promoter was performed with fluorogenic allele-specific probes (TaqMan® Probes, Applied Biosystems) using 50 ng of DNA and the ABI7500 Real-Time PCR platform (Applied Biosystems). SDS software 2.3 (Applied Biosystems) was used for allelic discrimination.

2.4. Statistical analysis

The statistical analysis was performed using open-source R software [19]. The Hardy-Weinberg equilibrium was assessed using chi-square test with Yates' continuity correction. Fisher's exact test was used for pairwise comparison of alleles and genotypes contingency tables. In order to verify the association between cases and controls, the following analyses were performed: the genetic frequency testing, the association analysis between groups, and the risk factors with multinomial logistic regression model.

When it comes to haplotype analysis, this study has investigated whether polymorphisms of the *IL10* and *TNFα* genes play a role in cervical cancer development. So, three loci haplotypes were constructed, and their distribution was compared as follows:

- HPV-58 → Case: HPV-58-positive patients (with and without coinfection with other HPV). Control: All HPV-58-negative patients.
- HPV-16 → Case: HPV-16-positive patients (with and without coinfection with other HPV). Control: All HPV-16-negative patients.
- HPV-18 → Case: HPV-18-positive patients (with and without coinfection with other HPV). Control: All HPV-18-negative patients.
- HPV-31 → Case: HPV-31-positive patients (with and without coinfection with other HPV). Control: All HPV-31-negative patients.

Haplotypes were formed by joining the alleles of rs1800629, rs1800871 and rs1800896 SNPs. ORs and the 95% confidence interval were calculated considering the frequencies of the haplotypes between the case and control groups.

Table 2
Frequency distribution of HPV types.

HPV types	Cases	
	N	%
High risk		
HPV-16	34	8.5
HPV-18	6	1.5
HPV-31	49	12.3
HPV-33	4	1.0
HPV-35	8	2.0
HPV-39	2	0.5
HPV-45	1	0.2
HPV-51	9	2.3
HPV-52	7	1.8
HPV-53	5	1.3
HPV-56	9	2.3
HPV-58	5	1.3
HPV-59	3	0.8
Low risk		
HPV-6	7	1.8
HPV-11	2	0.5
HPV-66	7	1.8
HPV-68	2	0.5
HPV-70	7	1.8
HPV-73	4	1.0
HPV-81	1	0.2
HPV-82	3	0.8
Co-infections	215	54
Positive undetermined	7	1.8
Total	397	100

3. Results

A total of 654 samples were examined for the presence of HPV, using *L1* consensus primers (MY09/MY11): 397(60.7%) were positive for HPV and 257 (39.3%) were HPV-negative. The most common high-risk HPV type was HPV-31 (12.3%) followed by HPV-16 (8.5%). Also, it was observed HPV coinfection. The distribution of HPV is shown in Table 2.

Table 3 presents the genomic localization and the results of Hardy-Weinberg equilibrium tests for *TNFα* (rs1800629) and *IL10* (rs1800871 and rs1800896) polymorphisms in both case and control groups. Genotypic and allelic frequencies of *TNFα* (rs1800629) and *IL10* (rs1800871 and rs1800896) polymorphisms in relation to the infection by HPVs 58, 18 and 31 are displayed in Table 4.

The distribution of TNF-308 (rs1800629) allelic frequencies and HPV-58 infection has showed a statistically significant difference between case and control groups for the assessed *TNFα* polymorphism (G vs. A allele: OR = 2.21, CI: 1.07–4.54 and $p = 0.03$) (Table 4). With respect to the distribution of genotypic frequencies, we noted more than twofold increased cervical cancer risk in HPV-58-positive patients (single infection or coinfection), which were GA/AA genotype-positive as compared to controls (GG vs. GA/AA genotype: OR = 2.67, CI: 1.13–6.34 and $p = 0.03$) (Table 4).

When it comes to TNFα (rs1800629) allelic and genotypic distribution and HPVs 18 and 31 infections, no statistically significant differences between case and control groups were observed for the studied TNFα polymorphism (Table 4).

The allelic and genotypic distribution of IL10-819 (rs1800871) CC and CT/TT and IL10-1082 (rs1800896) AA and AG/GG and HPV infection (HPVs 58, 18 and 31) has showed no statistically significant differences between case and control groups for the studied *IL10* polymorphisms (Table 4).

Haplotype analysis were conducted, and the six most frequent haplotypes are shown in Table 5. It was observed that women carrying the GTA and ATG haplotypes had 3.85 and 17.99-fold, respectively, increased cervical cancer susceptibility when infected by HPV-58 in comparison to the haplotype GCA (Table 5). In women infected with HPV-16 and HPV-18, statistically significant results in women carrying the GTA and ATA haplotypes was observed. They had a 2.32 and 3.67-fold, respectively, increased cervical cancer susceptibility when infected by these two HPV types (Table 5). The analysis of the haplotypes distribution in women infected with HPV-31 has showed no statistically significant results (Table 5).

Table 3
Identity, genomic localization and results of Hardy-Weinberg equilibrium (HWE) tests for *TNFα* and *IL-10* polymorphisms.

	SNP	Base change	SNP region	Chromosome	HWE p-value
TNFα (rs1800629)	TNF-308	G > A	−308 (Promoter)	6p21.3	0.55
IL-10 (rs1800871)	IL10-819	C > T	−819 (Promoter)	1q31-1q32	0.88
IL-10 (rs1800896)	IL10-1082	A > G	−1082 (Promoter)	1q31-1q32	0.61

4. Discussion

Studies indicate that several genetic polymorphisms in cytokine genes have been related to the increased risk of cervical carcinogenesis [16,20,21], which is considered as an important public health problem worldwide [22]. In Brazil, cervical cancer was responsible for 265.000 deaths in women in 2012 [23]. Here we found that the *TNFα*-308 polymorphism was significantly associated with HPV-58 infected patients. In addition, haplotypes were associated with increased risk of developing cervical cancer in women infected by HPVs 16, 18 and 58.

It is known that cervical epithelial infection by high-risk oncogenic HPV is important for the establishment of cervical carcinogenesis. However, it is believed that the immune response may be a determinant factor for the development of cervical disease [24]. Cytokines are signaling molecules produced by many cells linked to the immune response, playing a key role in the formulation of the response against viral infection and tumors [20,25]. Therefore, genetic variations in genes related to cytokine coding may be determinant to the carcinogenesis, once they may cause changes in the function or amount of cytokine produced [26–29].

TNFα is a pro-inflammatory cytokine related to cell proliferation and differentiation [30]. Single base polymorphisms that may indicate a particular biological significance occur within the *TNFα* gene and several studies suggest that these polymorphisms may somehow serve as a tumor promoter in cancers such as prostate, breast, ovary and cervical cancer [31–35]. Our data showed that the variation G-308A in the *TNFα* promoter region has been found associated with the risk of cervical cancer, in which women with GA/AA genotypes were estimated to present a significantly higher risk of development of cervical cancer than women with GG genotype when infected by high-risk HPV-58. Therefore, this result may suggest the contribution of this polymorphism to cervical cancer development. According to Mehta et al. [36], in several populations, this polymorphism has been found to be associated with the development of cervical neoplasia in HPV-16-infected women. However, in the present study, it was observed that the same polymorphism is associated with cervical neoplasia development in HPV-58-infected women. In the study performed by Li et al. [16], that investigated the relationship between *TNFα*-308 polymorphism and cervical cancer susceptibility, it was observed that patients with A allele have higher susceptibility for cervical cancer development.

Interleukin-10 is an anti-inflammatory cytokine and polymorphisms found in the *IL10* gene has been shown to influence the production levels of this cytokine [37]. The present study did not show significant association of the *IL10* promoter polymorphisms (C-819T and A-1082G) with cervical cancer risk. Our findings were similar to those obtained by Roh et al. [38] in a study carried out with Korean women.

Table 4
TNFα and *IL-10* polymorphisms in HPV infected patients.

	HPV-18			HPV-31			HPV-58		
	Case[a]	Control[b]	*p-value*; OR (95% CI)	Case[c]	Control[d]	*p-value*; OR (95% CI)	Case[e]	Control[f]	*p-value*; OR (95% CI)
TNFα rs1800629									
Alelles									
G	52 (90%)	703 (88%)	Reference	214 (90%)	541 (88%)	Reference	38 (76%)	308 (88%)	Reference
A	6 (10%)	93 (12%)	0.75; 0.87 (0.36–2.08)	24 (10%)	75 (12%)	0.39; 0.80 (0.49–1.31)	12 (24%)	44 (12%)	**0.03; 2.21 (1.07–4.54)**
Genotypes									
GG	23 (79.3%)	311 (78.1%)	Reference	95 (79.8%)	239 (77.6%)	Reference	14 (56%)	136 (77.3%)	Reference
GA / AA	6 (20.7%)	87 (21.9%)	0.88; 0.93 (0.37–2.36)	24 (20.2%)	69 (22.4%)	0.61; 0.88 (0.52–1.47)	11 (44%)	40 (22.7%)	**0.03; 2.67 (1.13–6.34)**
IL-10 rs1800871									
Alelles									
C	28 (58%)	479 (61%)	Reference	115 (62%)	392 (60%)	Reference	14 (47%)	240 (62%)	Reference
T	20 (42%)	307 (38%)	0.71; 1.11 (0.61–2.01)	69 (38%)	258 (40%)	0.59; 0.91 (0.65–1.27)	16 (53%)	144 (38%)	0.09; 1.90 (0.90–4.01)
Genotypes									
CC	7 (29.2%)	144 (36.6%)	Reference	35 (38%)	116 (35.7%)	Reference	4 (26.7%)	73 (38%)	Reference
CT / TT	17 (70.8%)	249 (63.4%)	0.45; 1.40 (0.57–3.47)	57 (62%)	209 (64.3%)	0.09; 1.76 (0.88–3.49)	11 (73.3%)	119 (62%)	0.37; 1.69 (0.52–5.49)
IL-10 rs1800896									
Alleles									
A	23 (48%)	455 (58%)	Reference	104 (57%)	374 (58%)	Reference	15 (50%)	464 (58%)	Reference
G	25 (52%)	331 (42%)	0.17; 1.49 (0.83–2.67)	80 (43%)	276 (42%)	0.80; 1.04 (0.74–1.45)	15 (50%)	342 (42%)	0.41; 1.35 (0.65–2.81)
Genotypes									
AA	7 (29.2%)	133 (33.8%)	Reference	30 (32.6%)	110 (33.9%)	Reference	78 (34.7%)	62 (32.3%)	Reference
AG/GG	17 (70.8%)	260 (66.2%)	0.63; 1.24 (0.50–3.07)	62 (67.4%)	215 (66.2%)	0.47; 0.79 (0.42–1.48)	147 (65.3%)	130 (67.7%)	0.61; 0.90 (0.60–1.35)

$p < 0.05$ – statistically significant. Dominant model.
[a] Case: Patients HPV-18 infected (with and without coinfection with other HPV).
[b] Control: All patients not infected with HPV-18.
[c] Case: Patients HPV-31 infected (with and without coinfection with other HPV).
[d] Control: All patients not infected with HPV-31.
[e] Case: Patients HPV-58 infected (with and without coinfection with other HPV).
[f] Control: All patients not infected with HPV-58.

Table 5
Haplotype frequencies and the association between case and control groups.

Haplotype	rs1800629	rs1800871	rs1800896	Frequency	*p-value*; OR (95% CI)
HPV-58[a]					
1	G	C	A	0.31	Reference
2	G	C	G	0.21	0.66; 1.42 (0.29–6.85)
3	G	T	A	0.20	**0.04; 3.85 (1.01–14.63)**
4	G	T	G	0.12	0.76; 1.56 (0.09–26.63)
5	A	C	G	0.07	0.08; 5.97 (0.78–45.82)
6	A	T	G	0.02	**0.04; 17.99 (1.10–295.25)**
HPV-16[b]					
1	G	C	A	0.29	Reference
2	G	C	G	0.24	0.20; 1.55 (0.79–3.04)
3	G	T	A	0.23	**0.01; 2.32 (1.19–4.51)**
4	A	C	G	0.07	0.50; 1.53 (0.45–5.23)
5	A	T	A	0.04	0.18; 2.40 (0.67–8.54)
HPV-18[c]					
1	G	C	A	0.30	Reference
2	G	C	G	0.24	0.19; 1.82 (0.75–4.39)
3	G	T	A	0.22	0.67; 0.78 (0.25–2.48)
4	G	T	G	0.11	0.17; 1.92 (0.76–4.81)
5	A	T	A	0.04	**0.03; 3.67 (1.08–12.45)**
HPV-31[d]					
1	G	C	A	0.30	Reference
2	G	C	G	0.24	0.41; 1.24 (0.74–2.07)
3	G	T	A	0.22	0.75; 1.10 (0.63–1.90)
4	G	T	G	0.11	0.80; 0.92 (0.50–1.72)
5	A	C	G	0.06	0.88; 0.94 (0.42–2.12)
6	A	T	A	0.04	0.90; 0.94 (0.35–2.53)

$p < 0.05$ – statistically significant.
[a] Case: Patients HPV-58 infected (with and without coinfection with other HPV). Control: All patients not infected with HPV-58.
[b] Case: Patients HPV-16 infected (with and without coinfection with other HPV). Control: All patients not infected with HPV-16.
[c] Case: Patients HPV-18 infected (with and without coinfection with other HPV). Control: All patients not infected with HPV-18.
[d] Case: Patients HPV-31 infected (with and without coinfection with other HPV). Control: All patients not infected with HPV-31.

Studies involving analysis of haplotypes are related to the investigation of the association of SNPs arrangement with a given disease, being considered as an important tool for carrying out association studies [39]. Since it can be hypothesized that individual SNPs can weakly contribute to the disease development, but the arrangement of different alleles may have a greater influence on the disease. In the performed analysis, it was observed that women carrying the haplotypes GTA (OR = 3.85) and ATG (OR = 17.99) had increased risk of cervical carcinogenesis when infected by HPV-58 when compared to women infected by other HPV types. It was also observed that women carrying the haplotypes GTA (OR = 2.32) and ATA (OR = 3.67) had increased risk of cervical carcinogenesis when infected by HPV-16 and HPV-18, respectively. Our hypothesis is that these three polymorphisms together might influence susceptibility to cervical carcinogenesis, since it is known that *IL10* and *TNFα* may interfere in the immune response against HPV [12,35,40,41]. To the best of our knowledge, this is the first study that has investigated the role of IL10-1082/-819 and TNFα-308 haplotypes in the susceptibility of cervical carcinogenesis.

In summary, in this case-control study, our results show a statistically significant difference in the allele and genotype frequencies between case and control groups for the assessed *TNFα* polymorphism. The studied *IL10* polymorphisms have presented no statistically significant differences for allelic and genotypic frequencies between case and control groups. In addition, haplotypes were associated with an increased susceptibility of developing of cervical cancer in women infected by HPVs 16, 18 and 58. These results may reveal a public health problem for countries where there is circulation of HPV-58 and the 9-valent vaccine is not in use. Our study provides a contribution to the investigation of genetic host factors involved with the susceptibility to HPV infection, focusing on important genes involved in the immune response.

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6. Ethical approval

This study was approved by the Ethics Committee of the Federal University of Pernambuco (CEP/CCS/UFPE N° 491/11) and Ethical Committee of Oswaldo Cruz University Hospital (HUOC/PROCAPE 64/2010). All the women signed the informed consent.

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Conflict of Interest Statement

Authors certify no potential conflicts of interest.

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