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An interleukin-10 gene polymorphism associated with the development of cervical lesions in women infected with Human Papillomavirus and using oral contraceptives

Bárbara Simas Chagas [a,*], Ana Pavla Almeida Diniz Gurgel [a], Heidi Lacerda Alves da Cruz [b], Carolina Maria Medeiros Amaral [a], Marcus Vinicius Cardoso [c], Jacinto da Costa Silva Neto [d], Luiz Antônio Ferreira da Silva [e], Eugênia Maria Bezerra de Albuquerque [f], Maria Tereza Cartaxo Muniz [g], Antonio Carlos de Freitas [a,*]

[a] Laboratory of Molecular Studies and Experimental Therapy, Department of Genetics, Federal University of Pernambuco, Pernambuco, Brazil
[b] Laboratory of Genomics and Gene Expression, Central Laboratory/Centre of Biological Sciences, Federal University of Pernambuco, Pernambuco, Brazil
[c] Laboratory of Human Molecular Genetics, Department of Genetics, Federal University of Pernambuco, Pernambuco, Brazil
[d] Molecular and Cytological Research Laboratory, Department of Histology, Federal University of Pernambuco, Pernambuco, Brazil
[e] Institute of Biological Sciences and Health, Federal University of Alagoas, Maceió, Brazil
[f] Gynaecological Unit, University Hospital Oswaldo Cruz, Pernambuco, Brazil
[g] Laboratory of Molecular Biology of Center of Pediatric Oncohematologia, University of Pernambuco, Pernambuco, Brazil

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ABSTRACT

Human Papillomavirus (HPV) infection plays a crucial role in the development of cervical lesions and tumors, however most lesions containing high-risk HPVs do not progress to cervical tumors. Some studies suggest that the use of oral contraceptives may increase the risk of cervical carcinogenesis, but this has not been confirmed by all the studies. Cytokines are important molecules that act in the defense of an organism against viral infections. Several genetic studies have attempted to correlate cytokine polymorphisms with human diseases, including cancer. The significance of *IL10* polymorphisms for cancer is that they have both immunosuppressive and antiangiogenic properties. We aimed to investigate the role of promoter polymorphisms in the *IL10* gene in women with cervical lesions associated with HPV infection, in the presence of the use of oral contraceptives. Using High Resolution Melt analysis (HRM), we analyzed an SNP $_{-1082}$A/G and $_{-819}$C/T in interleukin-10 promoter region in 364 Brazilian women: 171 with cervical lesions and HPV infection, and 193 with normal cytological results and HPV-negative. We observed no significant differences in genotype and allele frequencies in the two loci between patients and healthy controls. Furthermore, in the haplotype analysis of *IL10*, we found that CA haplotype was significantly more frequent in patients infected with HPV than in the control group ($p = 0.0188$). We did not find any genotype and allelic association of the *IL10* gene polymorphisms between cases and controls. However, in this study, when the HPV-positive patients were stratified according to their use of contraceptives, we found a significant association between the $_{-1082}$G allele ($p = 0.0162$) and $_{-1082}$GG genotype ($p = 0.0332$) among HPV-infected patients who used oral contraceptives. Our findings suggest that $_{-1082}$A/G gene polymorphism represents a greater susceptibility to progressive cervical lesions in HPV- infected women who use oral contraceptives.

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1. Introduction

At present, cervical cancer represents 9% of cases of female cancer and is the third leading cause of cancer death in women worldwide, with more than 529.800 new cases and 275.100 deaths per year (Jemal et al., 2011; Freitas et al., 2012).

It is generally accepted that Human Papillomavirus (HPV) infection plays a crucial role in the development of cervical lesions, and it is estimated that about 98% of cervical tumors are associated with HPV (zur Hausen, 1996; Baseman and Koutsky, 2005). The contribution of HPV infection in cervical disease depends on the HPV type and period of viral replication in the epithelial cells of the cervical transformation zone (Harmsel et al., 1999; Hopman et al., 2000). However, most lesions containing high-risk HPVs do

* Corresponding authors. Address: Av. Prof. Moraes Rego, 1235, Cidade Universitária, 50670-901 Recife-PE, Brazil. Fax: +55 81 21268512.
E-mail addresses: babisimas@gmail.com (B.S. Chagas), antonio.freitas@pq.cnpq.br, acf_ufpe@yahoo.com.br (Antonio Carlos de Freitas).

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not progress to cervical tumors. Thus, genetic and environmental cofactors may also be involved in predisposition to disease (Freitas et al., 2012). Some studies suggest that the use of oral contraceptives may increase the risk of cervical carcinogenesis (Ylitalo et al., 1999), but this has not been confirmed in all the studies (Thomas et al., 2001).

The host immune response is also important for the control of neoplastic growth and viral infection. The prevalence of HPV infection and progression of cervical lesions is more frequent in immunodeficient women than immunocompetent women (Parkin et al., 2002).

Cytokines are molecules that are important in the defense of organisms against viral infections. They are produced by macrophages, monocytes, and lymphocytes, and act in an indirect way by determining a pattern of immune response or directly by inhibiting viral replication (de Waal Malefyt et al., 1991; Fernandes et al., 2005). IL-10 is of particular interest with regard to cancer because it has both immunosuppressive (potentially cancer-promoting) and antiangiogenic (potentially cancer-inhibiting) properties (Howell and Rose-Zerilli, 2007).

The *IL10* gene is located on human chromosome 1, between 1q31 and 1q32 (Eskdale et al., 1997). Many single nucleotide polymorphisms (SNPs) have been detected within the cytokine gene sequence, especially within the promoter regions, including *IL10* $_{-1082}$A/G (rs1800870), $_{-819}$C/T (rs1800871) and $_{-592}$A/C (rs1800872). These polymorphisms may be associated with differential levels of gene transcription, since some alleles can produce low, medium and high amounts of IL-10 (Eskdale et al., 1998). The ability to secrete different cytokines seems to be important in the immune response (Hutchinson et al., 1999). Genetic studies have been conducted in an attempt to correlate these cytokine polymorphisms with some types of cancer, although with mixed results (Stanczuk et al., 2001; Roh et al., 2002; Szoke et al., 2004; Matsumoto et al., 2010).

Thus, in this work, a case-control study was carried out to investigate the role of *IL10* gene promoter polymorphisms ($_{-1082}$A/G and $_{-819}$C/T) in women with cervical lesions associated with HPV infection in the presence of environmental cofactors.

2. Materials and methods

2.1. Study group

The samples evaluated in this study were obtained by cervical scraping from a total of 364 voluntary patients who underwent cervical cancer screening at the Gynecological Clinic at the "Oswaldo Cruz University Hospital (HUOC)" in Pernambuco State, Northeastern Brazil. 171 women (median age 34.7 ± 10.8) with cervical abnormalities (low-grade squamous intraepithelial lesions and high-grade squamous intraepithelial lesions) and HPV infection were classified as cases. In addition, 193 women (median age 34.7 ± 11.2) with normal cytological results and HPV-negative, were classified as controls. All the patients and control subjects were from the same geographical area (Northeastern, Brazil), and belonged to the same ethnic group; they were HIV-negative and not being treated with immunosuppressive medication.

A short questionnaire about social and demographic features such as age, sexual behavior and the use of oral contraceptives was carried out to investigate the increased risk of cervical neoplasia. Approval of the Ethical Committee (HUOC/PROCAPE 64/2010) and informed consent from all women in the study were obtained.

The cervical cells collected with cytobrush were placed in phosphate-buffered saline (PBS) pH 7.4 and stored at −80 °C prior to DNA extraction.

2.2. DNA isolation

Genomic DNA was extracted from cervical cells using the DNeasy Blood and Tissue Kit (Qiagen), in accordance with the following stages: resuspension of the cell pellet in PBS (pH 7.4), cell lysis, purification, and washing and drying the material to obtain the DNA elution.

2.3. HPV analysis

Human Papillomavirus DNA was detected by employing the PCR method based on the amplification of the viral L1 gene fragment using degenerate primers MY09 (5′-CGTCCMARRGGAWACTGATC-3′) and MY11 (5′-GCMCAGGGWCATAAYAATGG-3′) (Manos et al., 1989; Karlsen et al., 1996).

The MY09/11 PCR that was tested positive was purified with the Invisorb® Fragment Cleanup (Invitek) kit and sequenced by using ABI PRISM BigDyeTM Terminator Cycle Sequencing v3.1 Ready reaction (Applied Biosystems). The HPV genotype was identified by comparing the sequence with that reported in GenBank using Basic Local Alignment Search Tool (BLAST), available at <http://www.ncbi.nlm.nih.gov/blast>.

2.4. Genotyping of IL10 $_{-1082}$A/G and $_{-819}$C/T polymorphisms

The genotyping of polymorphisms in the promoter of *IL10* gene, $_{-1082}$A/G (rs1800896) and $_{-819}$C/T (rs1800871), was performed by means of the Rotor-Gene 6000 apparatus (Rotor-Gene, Uniscience-Cobert Research). This apparatus used High Resolution Melt analysis (HRM), a fluorescence-based method for rapid mutation screening after standard PCR amplification in the presence of dsDNA intercalating EvaGreen fluorescent dye.

HRM was carried out for the detection of DNA sequence variants and was first applied for genotyping (Wittwer et al., 2003). This simple approach allowed us to discriminate between the three possible *IL10* promoter alleles immediately. For each allele, a specific melting curve is created at the end of the precise warming of the amplicon. The decreasing fluorescence signal is converted to a graphic representation and each genotype gives a melt curve that is slightly different from the others. With this simple assay it is possible to distinguish between all three alleles (Vossen et al., 2009).

The PCR amplification reactions were performed in a final volume of 25 μl, using HRM PCR Master Mix 2X (Qiagen), 10 μM of each primer (Supplementary Table S1) and roughly 50 ng of genomic DNA as a template. After amplification, all the samples were analyzed by heating to 95 °C for 1 min, cooling to 40 °C for 1 min and then melting at 0.1 °C/s with continuous acquisition of fluorescence from 78 to 88 °C for $_{-819}$C/T and 75 to 85 °C for $_{-1082}$A/G.

Some homozygous and heterozygous samples were analyzed by direct sequencing to validate the data obtained by the HRM method. The same results were observed in both techniques.

2.5. Statistical analysis

A chi-square test was used to verify the Hardy–Weinberg equilibrium using the Genotype Transposer program (Version 1.0) and Fisher's exact test was used for pair-wise comparison of alleles and genotypes using contingency tables as appropriate, through the open-source R package (available at the <http://www.r-project.orgsite>). The genetic frequency testing and association analysis between the comparison groups and risk factors were performed with UNPHASED v.3.121 (Dudbridge, 2008). This software carries out a retrospective likelihood test (the probability of observing genotypes for given phenotypes) using a multinomial logistic regression model. HAPLOVIEW v.4.2 was used to evaluate the

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