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

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A B S T R A C T

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 -1082A/G and -819C/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 allele 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 -1082G allele (p = 0.0162) and -819GG genotype (p = 0.0332) among HPV-infected patients who used oral contraceptives. Our findings suggest that -1082A/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
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 IL10-1082A/G (rs1800870), -819C/T (rs1800871) and -592A/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 (-1082A/G and -819C/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. 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’-CGTCCMARRGGAWEACTGATC-3’) and MY11 (5’-GCMACGGGCWCAAAATGCGGG-3’) (Manos et al., 1989; Karlsen et al., 1996).

The MY09/11 PCR that was tested positive was purified with the Invitrogen® Fragment Cleanup (Invitrek) 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 -1082A/G and -819C/T polymorphisms

The genotyping of polymorphisms in the promoter of IL10 gene, -1082A/G (rs1800896) and -819C/T (rs1800871), was performed by means of the Rotor-Gene 6000 apparatus (Rotor-Gene, University-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 -819C/T and 75 to 85 °C for -1082A/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
matrices of pairwise linkage disequilibrium (LD) between the SNPs and haplotype associations (Barrett et al., 2005). All the tests were two-tailed and the level of significance for all the statistical results was set at \( p < 0.05 \).

3. Results

3.1. Characteristics of study populations

The features of the cases and controls employed in this study are summarized in Supplementary Table S2. The ages of the patients with HPV-positive cervical abnormality ranged from 18 to 63 (average \( 34.7 \pm 10.8 \)), and the age of the controls ranged from 18 to 65 years (average \( 34.7 \pm 11.2 \)). Significant differences were observed between the cases and controls with regard to the use of contraceptives \( (p = 0.04) \) and the number of sexual partners \( (p < 0.001) \). The age variable was also taken into account to determine its association with the development of cervical lesions and presence of HPV, but no significant association was found \( (p = 0.13) \).

3.2. HPV analysis

A total of 364 DNA cervical cell samples were examined for the presence of HPV, using L1 consensus primers (MY09/MY11): 171 (47\%) were positive for HPV and 193 (52\%) were HPV-negative.

The most common high-risk HPV type, HPV-16, was found in 49 (28.6\%) of the HPV-positive patients. The other HPV types which showed a notable prevalence were Types 31 (19.9\%), 58 (5.8\%) and 33 (4.7\%). Nineteen patients had co-infections. Details of specific HPV type frequencies are shown in Supplementary Table S3.

The most common high-risk HPV type, HPV-16, was found in 49 (28.6\%) of the HPV-positive patients. The other HPV types which showed a notable prevalence were Types 31 (19.9\%), 58 (5.8\%) and 33 (4.7\%). Nineteen patients had co-infections. Details of specific HPV type frequencies are shown in Supplementary Table S3.

3.3. Genotyping of \( IL10 \) -819 C/T and -1082 A/G polymorphisms

The results of the experiment with \( IL10 \) -819 C/T and -1082 A/G polymorphisms are displayed in Table 1. All the polymorphisms (in both cases and controls groups) were in conformity to Hardy–Weinberg equilibrium.

Regarding to the \( IL10 \) -819 C/T polymorphism for the case group, the frequencies of the alleles C and T were 0.59 and 0.41, respectively. For the control group, the frequencies of the alleles C and T frequencies were 0.63 and 0.37, respectively. The observed genotype frequency in the case group was 33\% (CC), 52\% (CT) and 15\% (TT). In the control group, the genotype frequency was 41\% (CC), 44\% (CT) and 15\% (TT). Thus, CC was the genotype used as a reference point, and the relative disease association of CT and TT genotypes was expressed by calculating the odds ratios (OR) and their 95\% CI (Table 1).

With regard to the allele frequencies, there was no statistically significant differences between patients with HPV/cervical lesion and the healthy controls (C vs. T allele: OR = 1.15, 95\% CI: 0.84–1.57 and \( p = 0.36 \)). In the case of the genotypic frequencies, as stated earlier, no differences were identified between the control and case groups (CC vs. CT genotype: OR = 1.48, CI: 0.92–2.39 and \( p = 0.11 \); CC vs. TT genotype: OR = 1.15, CI: 0.58–2.27 and \( p = 0.75 \) (Table 1).

A multivariate logistic regression analysis based on environmental risk factors, showed that the use of contraceptives and number of sexual partners did not indicate any significant differences in either genotypic or allelic frequencies of \( IL10 \) -819 C/T polymorphisms (data not shown).

When the \( IL10 \) -1082 A/G polymorphisms were analyzed for the case groups, the frequencies of the alleles A and G were 0.56 and 0.44, respectively. With respect to the control group, the A and G frequencies were 0.60 and 0.40, respectively. The genotype frequency in the case group was 33\% (AA), 45\% (AG) and 22\% (GG). In the control group, the genotype frequency was 36\% (AA), 48\% (AG) and 16\% (GG). For this reason, AA was regarded as the reference-point of the genotype, and the relative association of the AG and GG genotype with disease was expressed by calculating the odds ratios (OR) and their 95\% CI (Table 1).

With regard to the allele frequencies, there were no statistically significant differences between patients with HPV/cervical lesion and the healthy control groups (A vs. G allele: OR = 1.18, 95\% CI: 0.86–1.60 and \( p = 0.29 \)). As observed earlier, no differences were identified for the genotypic frequencies, between the control and case groups (AA vs. AG genotype: OR = 1.02, CI: 0.62–1.65 and \( p = 1 \); AA vs. GG genotype: OR = 1.44, CI: 0.76–2.73 and \( p = 0.23 \) (Table 1).

A multivariate logistic regression analysis using environmental risk factors showed that the use of contraceptives revealed a

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Healthy Control</th>
<th>( p )-value; OR [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>( IL10 ) -819 C/T</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>202 (0.59)</td>
<td>105 (0.59)</td>
</tr>
<tr>
<td>T</td>
<td>140 (0.41)</td>
<td>73 (0.41)</td>
</tr>
<tr>
<td>Genotypes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>56 (0.33)</td>
<td>29 (0.33)</td>
</tr>
<tr>
<td>CT</td>
<td>90 (0.53)</td>
<td>47 (0.53)</td>
</tr>
<tr>
<td>TT</td>
<td>25 (0.14)</td>
<td>13 (0.19)</td>
</tr>
<tr>
<td><strong>( IL10 ) -1082 A/G</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>190 (0.56)</td>
<td>102 (0.57)</td>
</tr>
<tr>
<td>G</td>
<td>152 (0.44)</td>
<td>76 (0.43)</td>
</tr>
<tr>
<td>Genotypes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>56 (0.33)</td>
<td>30 (0.34)</td>
</tr>
<tr>
<td>AG</td>
<td>78 (0.46)</td>
<td>42 (0.47)</td>
</tr>
<tr>
<td>GG</td>
<td>37 (0.21)</td>
<td>20 (0.24)</td>
</tr>
</tbody>
</table>

\( p < 0.05 \) – Statistically significant.

HC: Healthy Control.

\( \text{a HPV/LSIL: HPV + low-grade squamous intraepithelial lesions.} \)

\( \text{b HPV/HSIL: HPV + high-grade squamous intraepithelial lesions.} \)
Haplotype frequencies and association between case and control groups.

Table 2

<table>
<thead>
<tr>
<th>Alleles</th>
<th>HPV and no contraceptive use</th>
<th>HPV and contraceptive use</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>114 (0.51)</td>
<td>76 (0.64)</td>
<td>1</td>
</tr>
<tr>
<td>G</td>
<td>110 (0.49)</td>
<td>42 (0.36)</td>
<td>1.75 (1.10–2.76)</td>
</tr>
<tr>
<td>Association</td>
<td></td>
<td></td>
<td>(\chi^2 = 5.78 / p-value = 0.0162)</td>
</tr>
<tr>
<td>Genotypes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>29 (0.46)</td>
<td>27 (0.46)</td>
<td>1</td>
</tr>
<tr>
<td>AG</td>
<td>56 (0.37)</td>
<td>22 (0.37)</td>
<td>1.06 (0.44–2.55)</td>
</tr>
<tr>
<td>GG</td>
<td>27 (0.17)</td>
<td>10 (0.17)</td>
<td>2.51 (1.02–6.15)</td>
</tr>
<tr>
<td>Association</td>
<td></td>
<td></td>
<td>(\chi^2 = 6.81 / p-value = 0.0332)</td>
</tr>
</tbody>
</table>

\(p < 0.05 – \text{Statistically significant.}\)
\(\chi^2: \text{Chi-square tests.}\)

significant difference in the genotype and allele frequencies (Table 2) in HPV-infected patients. However, with regard to the number of sexual partners, there was no significant difference in either genotypic or allelic frequencies for IL10 -1082A/G polymorphisms (data not shown).

3.4. Haplotype analysis of the IL10 gene

Haplotype analyses were conducted and the four possible haplotype frequencies are shown in Table 3. Despite the proximity of these two polymorphic sites of the IL10 gene, the linkage disequilibrium coefficient of 0.471 was not high \((p = 0.001)\). Haplotype analysis suggests that there may be an interaction between the two sites, with a haplotype distribution between the HPV-infected patients and control group. Global haplotype association was significant with a \(p\)-value of 0.0456 (Table 3). Among four possible haplotypes, only haplotype “1” (CA) was significantly more frequent in HPV-infected patients than the control group \((\chi^2 = 5.52, p = 0.0188; \text{OR} = 1.91 [1.21–3.04])\).

4. Discussion

The ability to produce different amounts of cytokines varies among individuals, and these differences may be genetically determined. Several studies have evaluated the possible influence of genetic factors and cytokine production on cervical lesions (Fernandes et al., 2005, 2008; Sharma et al., 2007).

This study has focused on whether the IL10 promoter polymorphisms -1082A/G and -819C/T can influence the development of cervical cancer associated with HPV infection, in the presence of environmental cofactors. Our data did not show significant differences in the allele and genotype frequencies between the IL10 promoter polymorphisms -819C/T and -1082A/G or any HPV-related cervical abnormalities. Roh et al. (2002), corroborated our findings that there was no apparent relationship between the IL10 gene promoter polymorphisms and the risk of cervical cancer. We also found that the linkage disequilibrium between position -819 and -1082 polymorphisms is not high. This may be surprising given that they are only 263 nucleotides apart and there are no intervening introns. However, this is congruent with other studies indicating that recombination frequency is not strictly proportional to chromosomal distance, and it is sensitive to ancestral effects; for example, Drysdale et al. (2000) found that “some pairs of close sites have reduced levels of linkage disequilibrium relative to more spaced pairs of sites”. Four possible haplotypes were demonstrated in the sample population and a significant genetic predisposition of haplotype CA (“1”) was found for cases, rather than the controls \((\text{OR} = 1.91, \text{CI} 1.21–3.04, p = 0.0188)\).

Although we did not find genotypic and allelic association of the IL10 gene polymorphisms between the cases and controls in this study, when HPV-positive patients were stratified according to their use of oral contraceptives, we observed a significant association between the -1082G allele/-1082G genotype and HPV infection in these patients. According to Gravitt et al. (2003), the IL-10 levels at the uterine cervix are influenced by cofactors like contraceptives. Our data showed that the -1082G allele and -1082G genotype were associated with a significantly increased risk of cervical cancer \((\text{OR} = 1.71, \text{CI} 1.10–2.76, p = 0.0162)\) and \((\text{OR} = 2.51, \text{CI} 1.02–6.15, p = 0.0332)\), respectively. To the best of our knowledge, this is the first study that has investigated this type of association between the IL10 gene polymorphisms and use of oral contraceptives, when there are HPV-related cervical abnormalities. There are controversial studies regarding the association of IL10 gene polymorphism and cervical cancer (Stanczuk et al., 2001; Roh et al., 2002; Szoke et al., 2004; Matsumoto et al., 2010), and perhaps one of the probable reasons for these divergent is the fact external factors are involved such as the use of oral contraceptives, which this study found led to an association between the polymorphism in the -1082 region and the risk of developing cervical cancer in the presence of viral infection. This means that this cofactor may cause an increased risk of cervical cancer in HPV-infected patients that carry the polymorphism -1082A/G, since these steroid hormones can accelerate the viral oncogene expression that is present in the HPV cellular genome (Salazar et al., 2005).

In HPV, the transcription of E6 and E7 oncoproteins is controlled by the regulation of the long control region (LCR). Furthermore, the LCR has been shown to contain response elements for progesterone and glucocorticoid (Park et al., 2003). In theory, progesterone and estrogens stimulate HPV-16 gene expression due to activation by nuclear receptors, followed by the interaction of the activated receptors with hormone-responsive elements within the LCR of HPV-16 (Chen et al., 1996). In addition, studies indicate that the use of oral contraceptives is a possible risk factor in progressive cervical cancers (Brinton et al., 1986; Hakama et al., 1993). Some reports have shown a correlation between the duration of the use of oral contraceptives and the presence of cervical intraepithelial neoplasia and HPV-associated cervical cancer (Hildesheim et al., 1990; Bosch et al., 1995), and that the risk of cervical cancer induced by HPV is doubled by prolonged use (Brissin et al., 1994). In studies conducted by Chen et al. (1996) it was demonstrated that the differential enhancement of HPV-16 gene expression was induced by some of the progesterones and estrogens.

With regard to the HPV vaccine Nardelli-Haefliger et al. (2003) reported that women that used contraceptives and who received the HPV vaccine, showed a considerable variation of serum and cervical concentrations of anti-HPV type 16 antibodies after vaccination, compared with those who did not use them. It can be assumed that hormonal modulation of immune cell function may cause a variation in the immune response to the HPV vaccine.
which can result in differences in the immunogenicity and efficacy of the vaccines (Marks et al., 2010).

The ability to secrete different cytokines appears to be important in the immune response against HPV infection and the development of cervical lesions. However, few studies have evaluated the cytokine polymorphisms in women who have cervical HPV-related lesions. Abnormal IL-10 production has already been reported in women with cervical precancerous lesions and invasive cancer (Giannini et al., 1998). Mota et al. (1999) demonstrated that the levels of the immunosuppressive cytokine IL-10 increased in cervical intraepithelial lesions, compared with normal cervixes where the IL10 is rarely detected (El-Sherif et al., 2001). In evaluating the -1082 polymorphism in the IL10 gene, Stanczuk et al. (2001) demonstrated that women with cervical cancer that carries the allele G appear to be immunogenetically predisposed to produce high levels of IL-10. Increased levels of IL-10 in HPV-infected women who have cervical lesions, compared with the women with normal uterine cervixes, suggests the occurrence of local immunosuppression. In contrast, IL-10 inhibits antigen presentation, the functioning of the cytotoxic T cells, the proliferation of T cells and secretion of proinflammatory cytokines, which leads to the development of cervical lesions after HPV infection (Giannini et al., 2002).

In summary, in this case-control study, it was found that the IL10 polymorphisms -1082A/G and -819C/T had no effect on cervical carcinogenesis in the studied population. However, we discovered that IL10 -1082A/G gene polymorphism was significantly associated with HPV-infected patients that use oral contraceptives. In addition, the CA haplotype was associated with the risk of cervical cancer to a significant degree. The main finding of our study suggests that genetic polymorphism of IL10 -1082A/G represents a risk to the susceptibility, development and progression of cervical lesions in HPV-infected women who use oral contraceptives. If the association between cytokine gene polymorphisms and the development of cervical cancer can be fully understood, this can lead to a comprehensive view of the immunological control of HPV infection.

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Competing interest

Ethical approval
This work has been approved by Ethics Committee on Human Research – Hospital Complex HUOC/PROCAPE (HUOC/PROCAPE 64/2010).

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Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2013.06.016.

References


