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ORIGINAL ARTICLE – CANCER RESEARCH

# *FOXP3* immunoregulatory gene variants are independent predictors of human papillomavirus infection and cervical cancer precursor lesions

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## Abstract

**Purpose** FOXP3 is a marker of the T regulatory (Treg) cell subset and drives its function and homeostasis. Its expression maintains the host immunosuppressive state that favors persistence of human papillomavirus (HPV) infection and squamous intraepithelial lesion (SIL) appearance. The present study evaluated the effects of the rs3761548 and rs2232365 intronic single-nucleotide variants (SNVs) and their haplotypes on HPV infection and SIL diagnosis in HPV-infected and -uninfected women.

**Methods** HPV DNA-based detection in cervical specimens was performed by PCR. *FOXP3* variants were genotyped by PCR-restriction fragment length polymorphism and haplotype recombination sites were inferred for 208 HPV-infected and 218 HPV-uninfected women diagnosed or not with low- or high-grade intraepithelial lesions of cervix. Case–control analyses were carried out by logistic regression adjusted for several socio-demographic, sexual lifestyle, and clinical data.

**Results** The homozygous genotype of the rs3761548 variants (A/A) (related to decreased *FOXP3* expression) may exert a protective role against HPV infection in women ($OR_{Aj}$: 0.60; 95% CI 0.36–0.99; $p=0.049$) and was an independent predictor of protection against HSIL development ($OR_{Adj}$: 0.28; 95% CI 0.11–0.68; $p=0.006$). In addition, the homozygous genotype (G/G) of the rs2232365 variants (related to increased *FOXP3* expression) was independently associated with the HPV infection ($OR_{Adj}$: 2.10; 95% CI 1.06–4.15; $p=0.033$). Haplotype analysis revealed no significant associations in our study.

**Conclusions** Our results reveal the significant and independent associations between *FOXP3* genetic variants and susceptibility to HPV infection and SIL diagnosis and their role as biomarkers of HPV infection and cervical lesion management.

**Keywords** Human papillomavirus · Squamous intraepithelial lesion · *FOXP3* · Genetic variants · Haplotypes

## Introduction

Infection by human papillomavirus (HPV) is the most common sexually transmitted disease among sexually active people. Persistent chronic infection with high oncogenic risk types (HR-HPV) is a well-understood etiology of oral and anogenital cancers, particularly cervical cancer (CC), the fourth most common cancer among women (Morshed et al. 2014; Ferlay et al. 2015). The overall global burden of HPV infection was widely assessed by polymerase chain reaction (PCR) and hybrid capture techniques, and was estimated at approximately 11–12% (Forman et al. 2012).

HR-HPVs have two transcriptional units, E6 and E7, which encode oncoproteins essential for viral replication, allowing for tumor suppression inactivation and unchecked

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cell-cycle progression through p53 and retinoblastoma protein inhibition, particularly in infected keratinocytes (Morshed et al. 2014). Integration of the HPV genome into the host genome is an essential step in tumorigenesis and often results in the loss of regulation and overexpression of oncogenes *E6* and *E7* (Jeon and Lambert 1995).

Approximately 80% of primary CC cases arise from preexisting squamous dysplasia, formally classified in the Bethesda System terminology as squamous intraepithelial lesion (SIL) and stratified as low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL) (Waggoner 2003; Solomon et al. 2002). LSIL encompasses mild dysplasia/cervical intraepithelial neoplasia (CIN1), while HSIL refers to moderate and severe dysplasia (CIN 2/CIN3) and carcinoma in situ (Solomon et al. 2002).

Persistent HPV infection does not act alone to cause SIL progression, and the immune system plays a key role. Although an effective immune response is responsible for eliminating viral infection in healthy women and inducing immunoprotection through type-1 helper T-cell response activation, HPV eventually begins modulating cellular immunity towards an immune-tolerant microenvironment, characterized by $CD4^+$ $CD25^+$ $FOXP3^+$ T regulatory (Treg) cell recruitment (Sasagawa et al. 2012).

*FOXP3* is a critical regulator that maintains a heritable genetic program for $CD4^+$ $CD25^+$ Treg cells to function as suppressor T cells. Given their importance in preventing the development of autoimmunity and their therapeutic potential, the molecular mechanisms governing $CD4^+$ $CD25^+$ Treg development and function are of great interest (Fontenot et al. 2003).

*FOXP3* is located at Xp11.23 and encodes a cluster of transcription factors that are members of the Forkhead/winged-helix family of transcriptional factors. Alternatively spliced transcript variants encoding different isoforms have been identified, which may influence the differentiation or functionality of Treg cells in vivo (Lu et al. 2017).

Progressive up-regulation of FOXP3 has been reported in the course of SIL pathogenesis. In addition, FOXP3 immunostaining is correlated with the expression of $p16^{INK4a}$, a key marker for the integration of HR-HPV into host cells (Zeng et al. 2013).

Genetic variants in *FOXP3* may influence protein expression and function, dampening Treg cell activity and leading to autoimmunity development (Shen et al. 2010). Intronic variants may affect the alternative splicing process by disrupting or creating binding sites for splicing, transcriptional, and other regulatory factors (Mudvari et al. 2015). *FOXP3* variants have been positively associated with several disorders such as endometriosis, idiopathic arthritis, atopy, Crohn's disease, unexplained recurrent spontaneous abortion, diabetes, and breast cancer (for review see Oda et al. 2013).

Moreover, *FOXP3* loci were associated with some infectious and parasitic diseases, including tuberculosis (Beiranvand et al. 2017), malaria (Koukouikila-Koussounda et al. 2013), and chronic hepatitis B (Chen et al. 2012). To date, no studies have investigated *FOXP3* variants in HPV infection and SIL development.

Therefore, the present study was conducted to evaluate the involvement of the *FOXP3* genetic variants (SNVs) rs3761548 and rs2232365 in HPV infection and SIL pathogenesis in a South Brazilian cohort, known to present a complex pattern of genetic and ethnic admixture.

## Materials and methods

### Study design and ethical approval

This study included 426 women who underwent outpatient cytology testing and were recruited from several health services in Londrina (Paraná, Southern Brazil), including the ambulatory colposcopy facility of the International Consortium of Health of the Middle Paranapanema (Cismepar), University Hospital and Clinic Center of the State University of Londrina, and two Basic Health-care Units, between March 2015 and December 2016. All study subjects received clear instructions regarding the purpose of the study and procedures to which they would be subjected prior to sample collection and signed formal consent. Next, each subject was interviewed for several socio-demographic, sexual lifestyle, gynecological and obstetric background. Patients who underwent the loop electrosurgical excision procedure for SIL treatment or presented an inconclusive molecular detection for HPV were excluded. The present study was approved by the Institutional Ethics Committee Involving Humans of the State University of Londrina (Londrina, PR, Brazil) (CEP/UEL 466/2012; CAAE 05505912.0.0000.5231).

### Sampling

Cervical epithelial scrapings were obtained from women undergoing clinical evaluation in outpatient appointments. After sample collection for cytology, the cytobrushes were stored in 2 mL of TE buffer (10 mM Tris–HCl and 1 mM EDTA pH 8.0) at − 20 °C until molecular analysis. Peripheral blood was collected by venipuncture into vacutainer tubes containing EDTA and stored at − 20 °C until analysis. Blood samples were used for *FOXP3* SNV analysis, while cervical samples were tested for HPV detection and genotyping.

### Cytological examination of cervical samples

Cervical smears were obtained at the time of enrollment. Experienced pathologists graded and reported *Pap* smears according to the Bethesda System (2001) diagnosis criteria at the Public Health System Laboratory. Patients were deemed to have LSIL, HSIL, or negative for intraepithelial lesion and malignancy (NILM) if cytology samples presented normal morphology (da Silva et al. 2012).

### Genomic DNA extraction

For the *FOXP3* genotyping, genomic DNA from peripheral blood samples was extracted using the Biopur Mini Plus Kit (Biometrix, Curitiba, PR, Brazil). For the HPV detection and genotyping, the genomic DNA was obtained from cervical cytobrushes using DNAzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and stored at − 20 °C. The DNA concentration was measured with a NanoDrop 2000c™ Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 260 nm and purity was assessed by measuring the 260/280 ratio.

### HPV detection and genotyping

HPV polymerase chain reaction (PCR) was carried out using the MY09 (5′-CGTCCMAARGGAWACTGATC-3′) and MY11 (5′-GCMCAGGGWCATAAYAATGG-3′) primers according to the GenBank Accession number AJ236888. These primers were designed to amplify a conserved region of approximately 450 base pairs (bp) of *L1* in HPV (Bauer et al. 1991). Reaction conditions were 190 nM of dNTPs (Invitrogen, Carlsbad, CA, USA), 500 nM of each primer, 2 mM of $MgCl_2$, 1× of buffer (20 mM of Tris–HCl PH 8,5; 50 mM of KCl), approximately 80 ng of DNA, and 1.25 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), with an annealing temperature of 55 °C. Co-amplification of the human *β-globin* gene (approximately 268 bp) was performed as an amplification control using primers GH20 (5′-GAAGAGCCAAGGACAGGTAC-3′) and PC04 (5′-CAACTTCATCCACGTTCACC-3′) (da Silva et al. 2012) under the same conditions as used in HPV PCR. A negative control sample with no DNA was included in all reaction sets to exclude possible contamination. A positive control consisting of DNA from the HeLa cervical cancer cell lineage, which contains the HPV18 genome integrated into the cell genome, was used. In addition, to identify the HPV type, HPV-positive samples were subjected to an enzymatic restriction with *Hpy*CH4V (New England Biolabs, Ipswich, MA, USA) by restriction fragment length polymorphism analysis of PCR amplicons (Santiago et al. 2006). All PCR and digested products were analyzed by 10% polyacrylamide gel electrophoresis, stained with silver nitrate. According to this method, the HPV types detected in these samples were classified as high-risk (16, 18, 31, 33, 45, 52, 56, 58, 59, 68, and 73) and low-risk (06, 11, 70, 72, 74, 81, and 83).

### *FOXP3* genetic variant genotyping

PCR-restriction fragment length polymorphism analysis was carried out using peripheral blood genomic DNA to detect the rs2232365 and rs3761548 SNVs, according to GenBank Accession number NG_007392.1. In rs2232365 SNV, an adenine is replaced by a guanine. In rs3761548 SNV, a cytosine is replaced by an adenine. For rs2232365 genotyping, the following primers were used: 5′-AGGAGA AGGAGTGGGCATTT-3′ (forward) and 5′-TGTGAGTGG AGGAGCTGAGG-3′ (reverse) (Paradowska-Gorycka et al. 2015). The rs3761548 genotyping was performed with the following primers: 5′-GGCAGAGTTGAAATCCAAGC-3′ (forward) and 5′-CA ACGTGTGAGAAGGCAGAA-3′ (reverse) (He et al. 2013). The reaction contained 1× of PCR buffer (20 mM of Tris–HCl PH 8.5; 50 mM of KCl), 1 mM of $MgCl_2$, 0.1 mM of dNTP (Invitrogen, Carlsbad, CA, USA), 0.2 µM of each primer, 1 U/µL of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), and 2.5 ng/µL of genomic DNA diluted in ultra-pure H2O (Milli-Q—Merck KGaA, Darmstadt, Germany) to a final volume of 25 µL per reaction tube, with 5% glycerol added to the rs2232365 reaction. A negative control sample with no DNA was included in all reaction sets to exclude possible contamination. The cycling protocol, used for both *FOXP3* SNVs, was as follows: denaturation at 95 °C for 5 min, 35 cycles of 45 s at 95 °C, 45 s at 59 °C for rs2232365 or 30 s at 65 °C for rs3761548, 1 min at 72 °C, and 10 min of final elongation at 72 °C. PCR products (3 µL) of rs2232365, which were 249 bp, were digested overnight at 55 °C with 1.5 U/reaction of the *Bsm*BI restriction endonuclease (New England Biolabs, Ipswich, MA, USA), which generated two fragments of 132 and 117 bp corresponding to allele G. The PCR product (6 µL) of rs3761548, which was 155 bp, was digested overnight at 37 °C with 2.0 U/reaction of the *Pst*I restriction endonuclease (New England Biolabs, Ipswich, MA, USA), generating two fragments of 80 and 75 bp that correspond to allele C. All PCR and digested products were analyzed by 10% polyacrylamide gel electrophoresis and silver nitrate staining.

### Statistical analysis

Analyses of contingency tables by Pearson's Chi-square ($\chi^2$) test were used to evaluate differences in the frequency distributions of selected socio-demographic and clinical categorical variables and *FOXP3* variants inheritance models between controls and case groups (i.e., HPV status/SIL diagnosis). Bonferroni correction was used as a post hoc test

to avoid false-positive (type I error) findings arising from multiple comparisons. Departures from Hardy–Weinberg equilibrium and frequency differences between groups were evaluated by the Chi-square test. *FOXP3* haplotype frequencies were also compared with those of populations worldwide using the Chi-square test. The results are expressed as absolute values and percentages. The continuous variable distribution compared in our design (age) was tested for Gaussian distribution by the Kolmogorov–Smirnov test and normalized on a logarithmic scale; when normality was not observed, we used the non-parametric Mann–Whitney test and Kruskal–Wallis test with Dunn's post hoc test to identify age differences between groups. In this case, data are expressed as the median and interquartile range (IQR 25–75). Inference of recombination sites between *FOXP3* alleles of women studied was performed using PHASE software version 2.1.1 (Stephens et al. 2001; Stephens and Scheet 2005). The web-based application SNPstats (Catalan Institute of Oncology, Barcelona, Spain) (https://www.snpstats.net) was used to analyze linkage disequilibrium between *FOXP3* genetic variants (Solé et al. 2006; Machiela and Chanock 2015). Binary and multinomial logistic regression with a stepwise method was performed to identify among socio-demographic and clinical data possible confounding factors that may have biased the case–control association analysis. Confounding factors were defined as the variables that were associated with dependent variable analyzed (HPV infection or SIL diagnosis) with a significance level lower than 0.05 found in the bivariate analysis. Binary and multinomial logistic regression controlled by confounders in the forced entry method was employed to predict independent associations between SNV inheritance models and haplotypes as explanatory variables and case groups (i.e., HPV/SIL) as dependent variables. Correlations between haplotypes and SIL diagnosis were evaluated by Kendall's tau-b rank correlation coefficient. All tests were two-tailed, with a $p$ value $< 0.05$ considered statistically significant. Odds ratios (ORs) and 95% confidence intervals (CI) were estimated. Statistical analyses were carried out using SPSS Statistics 22.0 software (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 7.0 for Windows (GraphPad Software, Inc., La Jolla, CA, USA).

## Results

### Sample characterization according to HPV infection, socio-demographic, and clinical data

First, 426 women were included in the study and categorized as HPV-infected (208/48.8%) and HPV-uninfected or controls (218/51.2%). The median age of HPV-infected patients was 34 years (IQR 26–46), while that of HPV-uninfected women was 42 (IQR 32–51), showing a significant difference ($p < 0.001$). The socio-demographic, sexual lifestyle, gynecological, and obstetric data of these patients are summarized in Tables 1 and 2.

When HPV-infected women and HPV-uninfected women were compared, a higher frequency of HPV was observed in women younger than 24 years old ($p < 0.001$), receiving < 1 minimum wage ($p = 0.022$), single ($p = 0.001$), smokers ($p = 0.017$), and nulliparous ($p = 0.034$). Moreover, HPV infection was more common among women with an age at first intercourse of less than 17 years ($p = 0.018$) and had at least four sexual partners during their lifetime ($p < 0.001$).

For convenience, HPV-infected women were divided into two groups according to the carcinogenic potential of the HPV type screened: HR-HPV infection and LR-HPV infection. We found no association between socio-demographic, sexual lifestyle, gynecological, and obstetric data and low/high-risk infection (data not shown).

In addition, women in this study were divided into three groups based on cytological abnormalities detected and classified according to the Bethesda System classification as follows: NILM (control group) (304/74%), LSIL (30/7.3%), and HSIL (77/18.7%) groups. The median age of control subjects was 40 years (IQR 30–51), LSIL was 34 years (IQR 22–42), and HSIL was 34 years (IQR 28–46). HSIL showed a significant difference from the controls ($p = 0.033$). Socio-demographic, sexual lifestyle, gynecological, and obstetric data of these patients are presented in Tables S1 and S2 (Supplemental information). A positive significant association was found between LSIL and HSIL diagnosis and age lower than 24 years ($p = 0.023$), LSIL and hormonal contraceptive method use ($p = 0.009$), and LSIL and abortion ($p = 0.011$). HSIL diagnosis was associated with women receiving < 1 minimum wage ($p < 0.001$), smoking status ($p = 0.006$), at least four sexual partners during their lifetime ($p = 0.007$), and sexual partners within the past 6 months ($p = 0.010$).

### *FOXP3* genetic variants and HPV infection/SIL susceptibility

The minor allele frequency (MAF) of rs3761548 was consistent with that reported by the 1000 Genome Project (http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/). The MAF of rs2232365 differed from the global frequency found by the 1000 Genome Project. However, when analyzing different populations separately, the MAF of rs2232365 corresponded to Ad Mixed American and East Asian populations.

Case–control analysis was performed to assess the influence of *FOXP3* intronic variants on HPV infection and SIL susceptibility. Hardy–Weinberg equilibrium (HWE) of rs3761548 and rs2232365 in HPV-infected and control

**Table 1** Socio-demographic data of HPV-uninfected and HPV-infected women

| Variable | HPV-uninfected | | HPV-infected | | *p* value* |
|---|---|---|---|---|---|
| | *n* = 218[a] | (%) | *n* = 208[a] | (%) | |
| Ethnicity | | | | | 0.223 |
| Caucasian | 115 | (52.8) | 94 | (45.2) | |
| Non-Caucasian | 97 | (44.5) | 101 | (48.6) | |
| Missing data | 6 | (2.7) | 13 | (6.2) | |
| Age range (years) | | | | | **< 0.001** |
| ≤ 24 | 12 | (5.5) | 44[b] | (21.2) | |
| 25–34 | 53 | (24.3) | 63 | (30.3) | |
| 35–44 | 54 | (24.8) | 44 | (21.2) | |
| 45–54 | 60 | (27.6) | 28 | (13.4) | |
| ≥ 55 | 36 | (16.5) | 27 | (13.0) | |
| Missing data | 3 | (1.3) | 2 | (0.9) | |
| Educational level[c] | | | | | 0.836 |
| Incomplete elementary | 64 | (29.4) | 62 | (29.8) | |
| Complete elementary | 26 | (11.9) | 24 | (11.5) | |
| Incomplete secondary | 29 | (13.3) | 29 | (13.9) | |
| Complete secondary | 69 | (31.7) | 66 | (31.7) | |
| Incomplete higher education | 7 | (3.2) | 6 | (2.9) | |
| Complete higher education | 17 | (7.8) | 9 | (4.3) | |
| Missing data | 6 | (2.7) | 12 | (5.9) | |
| Monthly income[d] | | | | | **0.022** |
| < 1 minimum wage | 55 | (25.2) | 73[b] | (35.1) | |
| 1 to < 3 minimum wages | 140 | (64.2) | 101 | (48.6) | |
| > 3 minimum wages | 17 | (7.8) | 15 | (7.2) | |
| Missing data | 6 | (2.8) | 19 | (9.1) | |
| Marital status | | | | | **< 0.001** |
| Single | 22 | (10.1) | 51[b] | (24.6) | |
| Married/civil partner | 157 | (72.0) | 119 | (57.2) | |
| Divorced | 27 | (12.4) | 24 | (11.5) | |
| Widowed | 11 | (5.0) | 14 | (6.7) | |
| Missing data | 1 | (0.5) | 0 | (0.0) | |
| Smoking status | | | | | **0.017** |
| No | 180 | (82.6) | 150 | (72.1) | |
| Yes | 35 | (16.0) | 52[b] | (25.0) | |
| Missing data | 3 | (1.4) | 6 | (2.9) | |
| Knowledge about HPV | | | | | 0.502 |
| No | 42 | (19.3) | 48 | (23.0) | |
| Have ever heard | 117 | (53.7) | 100 | (48.1) | |
| Yes | 55 | (25.2) | 49 | (23.6) | |
| Missing data | 4 | (1.8) | 11 | (5.3) | |

Bold values represent statistical significant values

*HPV* human papillomavirus

*Analysis by two-sided Pearson's Chi-square ($\chi^2$) test, with $p < 0.05$ considered significant

[a]For association analysis between HPV-uninfected and HPV-infected women, not all 426 patients were included, with variations depending on the characteristic analyzed

[b]$p < 0.05$, tested by Bonferroni post hoc test for multiple comparisons

[c]Based on Brazilian educational system

[d]Based on Brazilian minimum wage (approximately US$ 250.00)

**Table 2** Sexual lifestyle, gynecological, and obstetric data of HPV-uninfected and HPV-infected women

| Variable | HPV-uninfected | | HPV-infected | | *p* value* |
|---|---|---|---|---|---|
| | *n* = 218[a] | (%) | *n* = 208[a] | (%) | |
| Contraceptive method | | | | | 0.433 |
| No | 120 | (55.0) | 96 | (46.2) | |
| Yes, hormonal | 66 | (30.3) | 77 | (37.0) | |
| Yes, condom | 24 | (11.0) | 27 | (13.0) | |
| Yes, both | 8 | (3.7) | 8 | (3.8) | |
| Missing data | 0 | – | 0 | – | |
| Parity | | | | | **0.034** |
| 0 | 19 | (8.7) | 35[b] | (16.8) | |
| 1 | 37 | (17.0) | 45 | (21.6) | |
| 2 | 71 | (32.6) | 47 | (22.6) | |
| 3 | 45 | (20.6) | 47 | (22.6) | |
| 4 | 24 | (11.0) | 18 | (8.7) | |
| > 5 | 22 | (10.1) | 16 | (7.7) | |
| Missing data | | | | | |
| Abortion | | | | | 0.973 |
| No | 155 | (71.1) | 135 | (64.9) | |
| Yes | 44 | (20.2) | 38 | (18.3) | |
| Missing data | 19 | (8.7) | 35 | (16.8) | |
| Age at first sexual intercourse (years) | | | | | **0.018** |
| ≤ 17 years | 108 | (49.5) | 125[b] | (60.1) | |
| ≥ 18 years | 109 | (50.0) | 79 | (38.0) | |
| Missing data | 1 | (0.5) | 4 | (1.9) | |
| Age at menarche (years) | | | | | 0.694 |
| ≤ 11 | 47 | (21.6) | 51 | (24.5) | |
| 12 | 51 | (23.4) | 54 | (26.0) | |
| 13 | 53 | (24.3) | 45 | (21.6) | |
| ≥ 14 | 65 | (29.8) | 55 | (26.5) | |
| Missing data | 2 | (0.9) | 3 | (1.4) | |
| Sexual partners during lifetime | | | | | **< 0.001** |
| 1 | 87 | (39.9) | 45 | (21.6) | |
| 2–3 | 69 | (31.7) | 68 | (32.7) | |
| ≥ 4 | 58 | (26.6) | 82[b] | (39.4) | |
| Missing data | 4 | (1.8) | 13 | (6.3) | |
| Sexual partners within the past 6 months | | | | | 0.098 |
| 0 | 30 | (13.8) | 28 | (13.5) | |
| 1 | 176 | (80.7) | 148 | (71.2) | |
| ≥ 2 | 2 | (0.9) | 8 | (3.8) | |
| Missing data | 10 | (4.6) | 24 | (11.5) | |

Bold values represent statistical significant values

*HPV* human papillomavirus

*Analysis by two-sided Pearson's Chi-square ($\chi^2$) test, with $p < 0.05$ considered significant

[a]For association analysis between HPV-uninfected and HPV-infected women, not all 426 patients were included, with variations depending on the characteristic analyzed

[b]$p < 0.05$, tested by Bonferroni post hoc test for multiple comparisons

groups was assessed and genotype frequencies presented deviation from HWE, excepted rs3761548 in HPV group. Allelic and genotype distributions and *p* values for the Chi-square test are shown in Table 3. Associations were tested considering the codominant model (heterozygotes or variant homozygotes versus major allele homozygotes), dominant model (heterozygotes and variant homozygotes versus major allele homozygotes), recessive model (variant homozygotes

**Table 3** *FOXP3* genotype and allele distribution considering HPV infection status/SIL diagnosis and inheritance models testing

| *FOXP3* genotypes | HPV-uninfected | | HPV-infected | | *p* value* | NILM | | LSIL | | HSIL | | *p* value* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *n* = 218[a,1] | (%) | *n* = 208[a,2] | (%) | | *n* = 304[a] | (%) | *n* = 30[a] | (%) | *n* = 77[a] | (%) | |
| rs3761548 | | | | | | | | | | | | |
| Codominant model | | | | | **0.018** | | | | | | | **0.036** |
| C/C | 63 | (31.7) | 66 | (32.2) | | 90 | (31.0) | 11 | (36.7) | 27 | (35.5) | |
| C/A | 81 | (40.7) | 105[b] | (51.2) | | 126 | (43.4) | 12 | (40.0) | 42 | (55.3) | |
| A/A | 55 | (27.6) | 34 | (16.6) | | 74[b] | (25.5) | 7 | (23.3) | 7 | (9.2) | |
| Dominant model | | | | | 0.908 | | | | | | | 0.552 |
| CC | 63 | (31.7) | 66 | (32.2) | | 90 | (31.0) | 11 | (39.3) | 27 | (35.5) | |
| C/A + A/A | 136 | (68.3) | 139 | (67.8) | | 200 | (69.0) | 17 | (60.7) | 49 | (64.5) | |
| Recessive model | | | | | **0.007** | | | | | | | **0.008** |
| AA | 55 | (27.6) | 34 | (16.6) | | 74[b] | (25.5) | 5 | (17.9) | 7 | (9.2) | |
| C/C + C/A | 144 | (72.4) | 171[b] | (83.4) | | 216 | (74.5) | 23 | (82.1) | 69 | (90.8) | |
| Overdominant model | | | | | **0.034** | | | | | | | 0.175 |
| C/C + A/A | 118 | (59.3) | 100 | (48.8) | | 164 | (56.6) | 16 | (57.1) | 34 | (44.7) | |
| C/A | 81 | (40.7) | 105[b] | (51.2) | | 126 | (43.4) | 12 | (42.9) | 42 | (55.3) | |
| Alelle | | | | | | | | | | | | |
| C | 207 | (52.0) | 237 | (57.8) | 0.097 | 306 | (52.7) | 34 | (60.7) | 96 | (63.2) | 0.050 |
| A | 191 | (48.0) | 173 | (42.2) | | 274 | (47.3) | 22 | (39.3) | 56 | (36.8) | |
| rs2232365 | | | | | | | | | | | | |
| Codominant model | | | | | 0.426 | | | | | | | 0.582 |
| A/A | 46 | (22.0) | 34 | (17.3) | | 56 | (19.2) | 6 | (21.4) | 14 | (19.4) | |
| A/G | 129 | (61.7) | 124 | (63.3) | | 188 | (64.6) | 15 | (53.6) | 42 | (58.3) | |
| G/G | 34 | (16.3) | 38 | (19.4) | | 47 | (16.2) | 7 | (25.0) | 16 | (22.2) | |
| Dominant model | | | | | 0.239 | | | | | | | 0.962 |
| A/A | 46 | (22.0) | 34 | (17.3) | | 56 | (19.2) | 6 | (21.4) | 14 | (19.4) | |
| A/G + G/G | 163 | (78.0) | 162 | (82.7) | | 235 | (80.8) | 22 | (78.6) | 58 | (80.6) | |
| Recessive model | | | | | 0.412 | | | | | | | 0.289 |
| GG | 34 | (16.3) | 38 | (19.4) | | 47 | (16.2) | 7 | (25.0) | 16 | (22.2) | |
| A/A + A/G | 175 | (83.7) | 158 | (80.6) | | 244 | (83.8) | 21 | (75.0) | 56 | (77.8) | |
| Overdominant model | | | | | 0.749 | | | | | | | 0.361 |
| A/A + G/G | 80 | (38.3) | 72 | (36.7) | | 103 | (35.4) | 13 | (46.4) | 30 | (41.7) | |
| A/G | 129 | (61.7) | 124 | (63.3) | | 188 | (64.6) | 15 | (53.6) | 42 | (58.3) | |
| Alelle | | | | | 0.268 | | | | | | | 0.757 |
| A | 221 | 52.9 | 192 | 49.0 | | 300 | (51.5) | 27 | (48.2) | 70 | (48.6) | |
| G | 197 | 47.1 | 200 | 51.0 | | 282 | (48.5) | 29 | (51.8) | 74 | (51.4) | |

Bold values represent statistical significant values

*HPV* human papillomavirus, *NILM* negative for intraepithelial lesion and malignancy, *LSIL* low-grade squamous intraepithelial lesion, *HSIL* high-grade squamous intraepithelial lesion

*Analysis by two-sided Pearson's Chi-square ($\chi^2$) test, with $p < 0.05$ considered significant

[a]For association analysis not all 426 patients were included because of technical issues in *FOXP3* genotyping

[b]$p < 0.05$, tested by Bonferroni post hoc test for multiple comparisons

[1]Hardy–Weinberg equilibrium $\chi^2$: rs3761548 = 0.51, $p > 0.05$; rs2232365 = 13.85, $p < 0.05$

[2]Hardy–Weinberg equilibrium $\chi^2$: rs3761548 = 6.78, $p < 0.05$; rs2232365 = 11.89, $p < 0.05$

versus major allele homozygotes and heterozygotes), and overdominant model (variant heterozygotes versus variant and major allele homozygotes).

The rs3761548 codominant, recessive, and overdominant models were associated with the HPV infection. In agreement with these results, when the models were adjusted by age and partners during lifetime in the binary logistic regression, only the recessive model (i.e., assessing the A/A genotype effect) was independently associated with virus infection, displaying a protective role ($OR_{Adj} = 0.60$; 95%

CI 0.36–0.99; $p = 0.049$) (Table 4). rs2232365 did not reach significance in the Chi-square test, while multivariate analyses detected an association of the codominant model with HPV infection ($OR_{Adj} = 2.10$; 95% CI 1.06–4.15; $p = 0.033$). Association studies between low/high-risk HPV infection and *FOXP3* genetic variants showed no significant association (data not shown).

Furthermore, we investigated whether *FOXP3* genetic variants are involved in LSIL and HSIL pathogenesis. The rs3761548 codominant and recessive models showed a protective role in SIL development. Indeed, A/A genotype carriers were more likely to present with normal cytology. This finding was confirmed by multinomial logistic regression adjusting by age and abortion, with the A/A genotype found to be an independent protective factor in HSIL development ($OR_{Adj} = 0.28$; 95% CI 0.11–0.68; $p = 0.006$). The rs3761548 overdominant model (i.e., assessing the C/A genotype effect) was found to be independently associated with the HSIL development ($OR_{Adj} = 1.77$; 95% CI 1.01–3.11; $p = 0.046$) (Table 4).

**Table 4** Case–control multivariate analysis considering HPV infection status/SIL diagnosis and inheritance models

| Models | Case groups [OR (CI 95%)] | | |
|---|---|---|---|
| | HPV infection[a] | LSIL[b] | HSIL[b] |
| rs3761548 | | | |
| Codominant model | | | |
| C/C | Reference | Reference | Reference |
| C/A | 1.21 (0.75–1.95) | 1.11 (0.42–2.93) | 1.22 (0.66–2.27) |
| A/A | 0.67 (0.38–1.20) | 0.68 (0.19–2.40) | **0.31** (0.12–0.83) |
| Dominant model | | | |
| CC | Reference | Reference | Reference |
| C/A + A/A | 1.00 (0.64–1.56) | 0.94 (0.38–2.32) | 0.87 (0.48–1.55) |
| Recessive model | | | |
| C/C + C/A | Reference | Reference | Reference |
| A/A | **0.60** (0.36–0.99) | 0.64 (0.20–2.00) | **0.28** (0.11–0.68) |
| Overdominant model | | | |
| C/C + A/A | Reference | Reference | Reference |
| C/A | 1.42 (0.94–2.15) | 1.30 (0.54–3.11) | **1.77** (1.01–3.11) |
| rs2232365 | | | |
| Codominant model | | | |
| A/A | Reference | Reference | Reference |
| A/G | 1.43 (0.83–2.45) | 1.11 (0.35–3.50) | 0.98 (0.46–2.10) |
| G/G | **2.10** (1.06–4.15) | 1.85 (0.50–6.88) | 1.55 (0.63–3.77) |
| Dominant model | | | |
| A/A | Reference | Reference | Reference |
| A/G + G/G | 1.55 (0.91–2.63) | 1.29 (0.43–3.84) | 1.11 (0.53–2.30) |
| Recessive model | | | |
| A/A + A/G | Reference | Reference | Reference |
| G/G | 1.59 (0.92–2.73) | 1.71 (0.62–4.72) | 1.57 (0.79–3.09) |
| Overdominant model | | | |
| A/A + G/G | Reference | Reference | Reference |
| A/G | 1.00 (0.65–1.53) | 0.81 (0.33–1.98) | 0.78 (0.43–1.39) |

Bold values represent $p < 0.05$

*HPV* human papillomavirus, *NILM* negative for intraepithelial lesion and malignancy, *LSIL* low-grade squamous intraepithelial lesion, *HSIL* high-grade squamous intraepithelial lesion

[a]OR (odds ratio) and CI (confidence interval) 95% estimated by binary logistic regression with "HPV-uninfected group" as reference and controlling by age and partners during lifetime

[b]OR (odds ratio) and CI (confidence interval) 95% estimated by multinomial logistic regression with "NILM group" as reference and controlling by age and abortion

## *FOXP3* haplotype structures and HPV infection/SIL susceptibility

Four possible haplotype combinations from rs2232365 and rs3761548 were investigated in our experimental design: AC, AA, GC, and GA.

Analysis of linkage disequilibrium among rs2232365 and rs3761548 showed that these SNVs are not good surrogate markers of each other ($D' = 0.16$; $r^2 = 0.15$). Once they are inherited in the same chromosome with a high independency level, it is important to assess their combined effects.

In the association study of *FOXP3* haplotypes, the following models were analyzed: AC dominant (AA, GC, and GA carriers versus AC carriers), AC recessive (AA, GC, and GA carriers versus ACAC), AA dominant (AC, GC, and GA carriers versus AA carriers), AA recessive (AC, GC, and GA carriers versus AAAA carriers) GC dominant (AC, AA, and GA carriers versus GC carriers), GC recessive (AC, AA, and GA carriers versus GCGC), GA dominant (AC, AA, and GC carriers versus GA carriers), and GA recessive (AC, AA, and GC carriers versus GAGA). The predominant haplotype was AC in our patient cohort, while the less frequent haplotype was AA.

In the present study, no significant association between haplotypes and HPV infection (adjusted for age and partners during lifetime) or degrees of SIL diagnosis (adjusted for age and abortion) were detected when the binary or multinomial logistic regression was employed, respectively (Table 5). At this point, for statistical purposes, we grouped our SIL samples into two groups: NILM and SIL diagnosis. Using binary logistic regression adjusting by monthly income and partners during lifetime, we found a trend of association between the AA haplotype in the dominant model ($p = 0.077$) and strong trend between the AA haplotype in the recessive model ($p = 0.058$) with SIL protection. In addition, correlation analysis revealed a strong trend for a negative correlation between the AA haplotype in the dominant model and SIL diagnosis ($r = -0.094$; $p = 0.056$) (Table 6).

**Table 5** Haplotype distribution considering HPV infection status/grade of SIL diagnosis

| Haplotypes (rs2232365/ rs3761548) | HPV-infected *n* (%) | OR (CI 95%)[a] | *p* value | LSIL *n* (%) | OR (CI 95%)[b] | *p* value | HSIL *n* (%) | OR (CI 95%)[b] | *p* value |
|---|---|---|---|---|---|---|---|---|---|
| A/C dominant | 144 (69.2) | 0.96 (0.62–1.47) | 0.854 | 18 (60.0) | 0.62 (0.24–1.61) | 0.333 | 58 (75.3) | 1.61 (0.85–3.02) | 0.137 |
| A/C recessive | 17 (8.2) | 0.73 (0.36–1.51) | 0.409 | 5 (16.7) | 1.61 (0.42–6.12) | 0.424 | 7 (9.1) | 0.79 (0.29–2.16) | 0.657 |
| A/A dominant | 37 (17.8) | 0.71 (0.43–0.18) | 0.193 | 5 (16.7) | 1.29 (0.40–4.17) | 0.666 | 10 (13.0) | 0.70 (0.30–1.60) | 0.400 |
| A/A recessive | 7 (3.4) | 0.40 (0.14–1.11) | 0.079 | 0 (0.0) | NA | | 2 (2.6) | 0.28 (0.03–2.54) | 0.261 |
| G/A dominant | 125 (60.1) | 1.28 (0.85–1.93) | 0.231 | 19 (63.3) | 1.65 (0.67–4.06) | 0.729 | 42 (54.5) | 0.90 (0.51–1.60) | 0.790 |
| G/A recessive | 7 (3.4) | 0.70 (0.26–1.85) | 0.477 | 0 (0.0) | NA | | 3 (3.9) | 0.83 (0.22–3.11) | 0.783 |
| G/C dominant | 69 (33.2) | 1.35 (0.87–2.10) | 0.174 | 9 (30.0) | 0.99 (0.36–2.71) | 0.984 | 29 (37.7) | 1.48 (0.81–2.69) | 0.199 |
| G/C recessive | 9 (4.3) | 1.46 (0.51–4.14) | 0.473 | 3 (10.0) | 2.46 (0.41–14.77) | 0.324 | 3 (3.9) | 0.90 (0.22–3.61) | 0.891 |

Reference genetic model employed in this analysis was the corresponding genetic model (Ex: A/C dominant vs. A/C recessive and vice versa)

*HPV* human papillomavirus, *NILM* negative for intraepithelial lesion and malignancy, *LSIL* low-grade squamous intraepithelial lesion, *HSIL* high-grade squamous intraepithelial lesion, *NA* not applicable

[a]OR (odds ratio) and CI (confidence interval) 95% estimated by binary logistic regression with "HPV-uninfected group" as reference and controlling by age and partners during lifetime

[b]OR (odds ratio) and CI (confidence interval) 95% estimated by multinomial logistic regression with "NILM group" as reference and controlling by age and abortion

**Table 6** Haplotype distribution and correlation analysis considering SIL diagnosis

| Haplotypes (rs2232365/ rs3761548) | NILM *n* (%) | SIL *n* (%) | OR (CI 95%)[a] | *p* value | *r*[b] | *p* value |
|---|---|---|---|---|---|---|
| A/C dominant | 200 (65.8) | 76 (71.0) | 1.13 (0.66–1.92) | 0.063 | 0.049 | 0.322 |
| A/C recessive | 26 (8.6) | 12 (11.2) | 1.40 (0.65–3.16) | 0.411 | 0.040 | 0.414 |
| A/A dominant | 69 (22.7) | 15 (14.0) | 0.54 (0.27–1.07) | 0.077 | −0.094 | 0.056 |
| A/A recessive | 18 (5.9) | 2 (1.9) | 0.13 (0.01–1.07) | 0.058 | −0.083 | 0.094 |
| G/A dominant | 176 (57.9) | 61 (57.0) | 0.96 (0.58–1.58) | 0.889 | −0.008 | 0.873 |
| G/A recessive | 17 (5.6) | 3 (2.8) | 0.73 (0.20–2.65) | 0.639 | −0.057 | 0.250 |
| G/C dominant | 91 (29.9) | 38 (35.5) | 1.5 (0.90–2.53) | 0.117 | 0.053 | 0.285 |
| G/C recessive | 11 (3.6) | 6 (5.6) | 1.36 (0.44–4.16) | 0.582 | 0.044 | 0.375 |

*NILM* negative for intraepithelial lesion and malignancy, *SIL* squamous intraepithelial lesion, *r* correlation coefficient

[a]OR (odds ratio) and CI (confidence interval) 95% estimated by binary logistic regression controlling by monthly income and partners during lifetime. Reference genetic model employed in this analysis was the corresponding genetic model (Ex: A/C dominant vs. A/C recessive and vice versa)

[b]Correlation analysis was performed by Kendall's tau-b rank correlation coefficient considering the haplotype genetic model and SIL diagnosis

## Discussion

We analyzed the *FOXP3* rs3761548 and rs2232365 genetic variants in outpatients infected with HPV carrying CC premalignant lesions and HPV-negative and lesion-free controls. The main findings of our study were that the A/A homozygous genotype of the rs3761548 variant is a good predictor of protection from HPV infection in women; this genotype is also an independent protection predictor for HSIL development. Furthermore, women carrying the G/G homozygous genotype of the rs2232365 variant are more prone to be infected with the virus.

Although the effects of *FOXP3* intronic variants have been reported in other gynecological diseases, our results reveal that the rs3761548 and rs2232365 SNVs are biomarkers of HPV infection and SIL diagnosis. As far as we are aware, this is the first genetic association study to investigate the influence of *FOXP3* variants under such pathological conditions, despite more than 1 decade of consistent evidence that $FOXP3^+$ cells infiltration plays a marked

deleterious role in the course of HPV-associated carcinogenesis (Kobayashi et al. 2008).

We found a positive association between the variables age lower than 24 years, low monthly income, single marital status, smoking status, nulliparity, early age at first sexual intercourse, and high number of sexual partners during the lifetime with HPV infection. Although nulliparity was associated with infection in this study, this result may be biased by the younger age of the patients. SIL diagnosis was associated with age lower than 24 years, low monthly income, smoking status, hormonal contraceptive method, abortion, early age at first sexual intercourse, and high number of sexual partners.

Because infection by oncogenic HPV is a necessary but not sufficient cause of CC (Walboomers et al. 1999), the variables described above are all well-known risk factors associated with HPV infection and HPV-related cervical oncogenesis, and our findings confirm the accumulated epidemiological data reported by our research group and extensive medical literature (Trugilo et al. 2018; Okuyama et al. 2018; Chelimo et al. 2013; Castellsagué and Muñoz 2003). Social factors may not be directly involved in lesions and cancer pathology but may predispose individuals to HPV infection and the transition to cervical malignancy because of restricted access to educational, prevention, and sexual and reproductive health tools (Krishnan et al. 2008).

*FOXP3* is an X-linked gene that encodes a transcription factor, the most specific and reliable biomarker of Treg cells. Indeed, canonical *FOXP3* expression is essential for driving $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cell function (Müller et al. 2010; Passerini et al. 2014). The previous studies demonstrated that mutations in *FOXP3* may cause immune response impairment and contribute to autoimmune disease development (Katoh et al. 2013), infectious processes (Beiranvand et al. 2017), and cancer (Wang et al. 2009).

Evidence from psoriatic patients showed that $CD4^+$ $CD25^+$ $FOXP3^+$ T cells carrying the rs3761548 A/A genotype have lower FOXP3 levels caused by slowed transcription/expression and reduced luciferase reporter activity when allele A was present (Shen et al. 2010). C>A substitution causes binding loss to E47 and c-Myb transcription factors, impairing *FOXP3* gene transcription.

We found a significant association between allele C of rs3761548 as confirmed in the codominant, recessive, and overdominant models and HPV infection. In agreement with this, the A/A genotype was found to have independent protective effects against infection. The A/A genotype was more frequent among NILM patients and was found to be independently associated with protection against HSIL development. The C/A genotype was associated with the HSIL development. A recent meta-analysis found that the rs3761548 genetic variants, particularly the recessive model (A/A vs C/A + C/C: OR = 1.45, 95% CI 1.03–2.02, $p = 0.03$), is associated with cancer risk in the overall population (Cheng, Guo and Ming 2018). In a case–control study that evaluated the association between *FOXP3* genetic variants (rs3761548 and rs5902434) and endometrial cancer (EC) in Chinese Han women, the authors verified that the frequency of allele A in rs3761548 was significantly lower than that in healthy controls [20.3% vs 26.4%, odds ratio (OR) 0.71, 95% confidence interval (CI) 0.54–0.93, $p = 0.012$], while heterozygous AC genotype showed a significant protective effect on EC in codominant, dominant, and overdominant models (You et al. 2018).

We inferred that the C allele increases HPV susceptibility by up-regulating *FOXP3* expression, but the opposite may be true: the A allele may protect against HPV infection and high-grade dysplasia by decreasing *FOXP3* expression, inducing down-regulation of Treg-mediated immunoregulatory mechanisms and establishing effective anti-viral cellular immunity. This assumption is in agreement with a study, showing that high frequencies of HPV 16-specific Treg cells were strongly correlated with HPV persistent infection and HSIL progression (Molling et al. 2007).

Studies of the roles of *FOXP3* in neoplastic cells have shown conflicting results. *FOXP3* expression in Siha HPV-16-positive cervical cancer cells following lentivirus-mediated RNA interference showed that *FOXP3* silencing inhibited cell proliferation and invasiveness, induced apoptosis, reduced cells in S phase and G2 phase, and blocked cells in G1 phase (Wang et al. 2010). Some studies indicated that *FOXP3* acts as a tumor suppressor gene and is a favorable prognostic factor in several tumors, including HPV-positive tonsillar squamous cell carcinoma (Wang et al. 2010; Park et al. 2013). In this context, decreased *FOXP3* expression would impair FOXP3-mediated oncogene repression and tumor suppressor activation, explaining why the A/A genotype increases cancer susceptibility (Cheng et al. 2018; Zuo et al. 2007).

Bioinformatic analysis revealed that rs2232365 is located in a putative-binding site for the transcription factor GATA-3 (Wu et al. 2012). In addition, this transcription factor binds the promoter region of *FOXP3* only when the allele A is present. Binding of GATA-3 inhibits *FOXP3* expression, and this transcription factor must be removed from the promoter for *FOXP3* expression to occur (Maruyama et al. 2011). Next, G/G carriers lose their GATA-3-binding site, enabling *FOXP3* gene transcription. Accordingly, women infected with *Mycobacterium tuberculosis* carrying the G/G genotype of rs2232365 SNV show 2.28-fold higher *FOXP3* expression than A/A carriers (Beiranvand et al. 2017). In the present study, the G/G genotype of the rs2232365 gene variant was an independent predictor of HPV infection. An A>G substitution may contribute to Treg $FOXP3^+$ generation and exert a detrimental role in the anti-HPV immune response. *FOXP3* plays a dual role in cell proliferation, with the G/G

genotype showing a protective role in severe recurrent respiratory papillomatosis (i.e., LR-HPV infection-related) (Kwon et al. 2017).

The haplotype frequencies of controls were compared to those of African, European, American, and Asian populations using publicly available data from the 1000 Genome Project obtained through the web-based application LDlink (Machiela and Chanock 2015). *FOXP3* haplotype frequencies differed significantly ($p < 0.0001$ by Chi-square test) from those of the overall population. However, the frequencies from our cohort matched with the following populations: Puerto Ricans from Puerto Rico ($p = 0.707$); Colombians from Medellin, Colombia ($p = 0.338$); Utah residents of Northern and Western European Ancestry ($p = 0.826$); British in England and Scotland ($p = 0.154$), and Punjabi from Lahore, Pakistan ($p = 0.136$). In our cohort, the *FOXP3* haplotype distribution of the control group differed significantly from that of the global population reported in the 1000 Genomes Project data, which did not verify these values in the Brazilian population.

Our results confirm those of Banin-Hirata et al. (2017), who found a significant difference between *FOXP3* haplotype frequencies of neoplasia-free patients among a Southern Brazilian population and different countries and continents. Examining the populations separately, the *FOXP3* haplotype distribution matched that of European, Latin American, and Asian populations. The Southern Brazil region displays the highest proportion of European ancestry among geopolitical regions in the country (Lins et al. 2010). Moreover, urban centers present with a high degree of admixture because of migration between inland areas and other regions and intermarriage between individuals of different ethnic origins (Lins et al. 2010).

To the best of our knowledge, no previous studies have investigated the effects of the rs3761548 and rs2232365 haplotypes on HPV and SIL susceptibility. Although the lack of association between these conditions was found in our analysis of haplotypes, the present study revealed that the A/A genotype of the rs3761548 SNV is a good predictor of prognosis in CC premalignant lesions. In addition, the G/G genotype of the rs2232365 SNV is independently associated with HPV infection. Further studies of the associations between immune gene variations and infection and SIL susceptibility may reveal the molecular immunopathogenesis of CC and lead to the establishment of new health prevention practices based on molecular screening.

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## Compliance with ethical standards

**Conflict of interest** The authors have no conflict of interest.

**Ethical approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Institutional Ethics Committee Involving Humans of the State University of Londrina, Londrina, PR, Brazil, CEP/UEL 133/2012, CAAE 05505912.0.0000.5231) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

## References

Banin-Hirata B, Guembarovski RL, Vitielo GAF et al (2017) FOXP3 Allelic variants and haplotype structures are associated with aggressive breast cancer subtypes. Dis Mark. https://doi.org/10.1155/2017/6359603

Bauer HM, Ting Y, Greer CE, Chambers JC, Tashiro CJ, Chimera J et al (1991) Genital human papillomavirus infection in female university students as determined by a PCR-based method. JAMA 265:472–477. https://doi.org/10.1001/jama.1991.03460040048027

Beiranvand E, Abediankenari S, Khani S, Hosseini HM, Zeinali S, Beiranvand B et al (2017) G allele at -924 A>G position of FoxP3 gene promoter as a risk factor for tuberculosis. BMC Infect Dis 17:4–9. https://doi.org/10.1186/s12879-017-2762-5

Castellsagué X, Muñoz N (2003) Chapter 3: cofactors in human papillomavirus carcinogenesis–role of parity, oral contraceptives, and tobacco smoking. J Natl Cancer Inst Monogr 2003:20–28

Chelimo C, Wouldes TA, Cameron LD, Elwood JM (2013) Risk factors for and prevention of human papillomaviruses (HPV), genital warts and cervical cancer. J Infect 66:207–217. https://doi.org/10.1016/j.jinf.2012.10.024

Chen Y, Zhang H, Wang Y, Xie H, Fei R, Wang X et al (2012) Association of FOXP3 gene polymorphism with chronic hepatitis B in Chinese population. Infect Int 1:191–195. https://doi.org/10.1186/1756-9966-32-39

Cheng Z, Guo Y, Ming L (2018) Functional Foxp3 polymorphisms and the susceptibility to cancer: an update meta-analysis. Medicine (Baltimore) 97:1–8. https://doi.org/10.1097/MD.0000000000011927

da Silva MC, Martins HPR, de Souza JL, Tognim MCB, Svidzinski TIE, Teixeira JJV et al (2012) Prevalence of HPV infection and genotypes in women with normal cervical cytology in the state of Paraná, Brazil. Arch Gynecol Obstet 286:1015–1022. https://doi.org/10.1007/s00404-012-2399-y

Ferlay J, Soerjomataram II, Dikshit R, Eser S, Mathers C, Rebelo M et al (2015) Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136:E356–E359. https://doi.org/10.1002/ijc.29210

Fontenot JD, Gavin MA, Rudensky AY (2003) Foxp3 programs the development and function of CD4+ CD25+ regulatory T cells. Nat Immunol 4:330–336. https://doi.org/10.1038/ni904

Forman D, De Martel C, Lacey CJ, Soerjomataram I, Lortet-Tieulent J, Bruni L et al (2012) Global burden of human papillomavirus and related diseases. Vaccine 30:F12–F23. https://doi.org/10.1016/j.vaccine.2012.07.055

He Y, Bo Q, Yong W, Qiu Z, Li Y, Li W (2013) FoxP3 genetic variants and risk of non-small cell lung cancer in the Chinese Han population. Gene 531:422–425. https://doi.org/10.1016/j.gene.2013.08.066

Jeon S, Lambert PF (1995) Integration of human papillomavirus type 16 DNA into the human genome leads to increased stability of E6 and E7 mRNAs: implications for cervical carcinogenesis. Proc Natl Acad Sci USA 92:1654–1658

Katoh H, Zheng P, Liu Y (2013) FOXP3: genetic and epigenetic implications for autoimmunity. J Autoimmun 41:72–78. https://doi.org/10.1016/j.jaut.2012.12.004

Kobayashi A, Weinberg V, Darragh T, Smith-McCune K (2008) Evolving immunosuppressive microenvironment during human cervical carcinogenesis. Mucosal Immunol 1:412–420. https://doi.org/10.1038/mi.2008.33

Koukouikila-Koussounda F, Ntoumi F, Ndounga M, Tong HV, Abena AA, Velavan TP (2013) Genetic evidence of regulatory gene variants of the STAT6, IL10R and FOXP3 locus as a susceptibility factor in uncomplicated malaria and parasitaemia in Congolese children. Malar J 12:1. https://doi.org/10.1186/1475-2875-12-9

Krishnan S, Dunbar MS, Minnis AM, Medlin CA, Gerdts CE, Padian NS (2008) Poverty, gender inequities, and women's risk of human immunodeficiency virus/AIDS. Ann N Y Acad Sci 1136:101–110. https://doi.org/10.1196/annals.1425.013

Kwon T-K, Chung EJ, Lee N, Roh EY, Song EY (2017) Associations of FoxP3 gene polymorphisms with severe recurrent respiratory papillomatosis in Korean patients. J Otolaryngol Head Neck Surg 46:10–13. https://doi.org/10.1186/s40463-017-0197-z

Lins TC, Vieira RG, Abreu BS, Grattapaglia D, Pereira RW (2010) Genetic composition of Brazilian population samples based on a set of twenty eight ancestry informative SNPs. Am J Hum Biol 22:187–192. https://doi.org/10.1002/ajhb.20976

Lu L, Barbi J, Pan F (2017) The regulation of immune tolerance by FOXP3. Nat Rev Immunol 17:703–717. https://doi.org/10.1038/nri.2017.75

Machiela MJ, Chanock SJ (2015) LDlink: A web-based application for exploring population-specific haplotype structure and linking correlated alleles of possible functional variants. Bioinformatics 31:3555–3557. https://doi.org/10.1093/bioinformatics/btv402

Maruyama T, Li J, Vaque JP, Konkel JE, Wang W, Zhang B et al (2011) Control of regulatory T cell and Th17 cell differentiation by inhibitory helix-loop-helix protein Id3. Nat Immunol 12:86–95. https://doi.org/10.1038/ni.1965

Molling JW, De Gruijl TD, Glim J, Moreno M, Rozendaal L, Meijer CJLM et al (2007) CD4+ CD25hi regulatory T-cell frequency correlates with persistence of human papillomavirus type 16 and T helper cell responses in patients with cervical intraepithelial neoplasia. Int J Cancer 121:1749–1755. https://doi.org/10.1002/ijc.22894

Morshed K, Polz-Gruszka D, Szymañski M, Polz-Dacewicz M (2014) Human papillomavirus (HPV)—structure, epidemiology and pathogenesis. Otolaryngol Pol 68:213–219. https://doi.org/10.1016/j.otpol.2014.06.001

Mudvari P, Movassagh M, Kowsari K, Seyfi A, Kokkinaki M, Edwards NJ et al (2015) SNPlice: variants that modulate Intron retention from RNA-sequencing data. Bioinformatics 31:1191–1198. https://doi.org/10.1093/bioinformatics/btu804

Müller S, Poehnert D, Müller JA, Scheumann GWF, Koch M, Lück R (2010) Regulatory T cells in peripheral blood, lymph node, and thyroid tissue in patients with medullary thyroid carcinoma. World J Surg 34:1481–1487. https://doi.org/10.1007/s00268-010-0484-6

Oda JMM, Hirata BKB, Guembarovski RL, Watanabe MAE (2013) Genetic polymorphism in FOXP3 gene: imbalance in regulatory T-cell role and development of human diseases. J Genet 92:163–171

Okuyama NCM, Cezar-dos-Santos F, Pereira ÉR, Trugilo KP, Cebinelli GCM, Sena MM et al (2018) Genetic variant in CXCL12 gene raises susceptibility to HPV infection and squamous intraepithelial lesions development: a case-control study. J Biomed Sci 25:1–10. https://doi.org/10.1186/s12929-018-0472-y

Paradowska-Gorycka A, Jurkowska M, Felis-Giemza A, Romanowska-Prochnicka K, Manczak M, Maslinski S et al (2015) Genetic polymorphisms of Foxp3 in patients with rheumatoid arthritis. J Rheumatol 42:170–180. https://doi.org/10.3899/jrheum.131381

Park K, Cho KJ, Lee M, Yoon DH, Kim S-B (2013) Importance of FOXP3 in prognosis and its relationship with p16 in tonsillar squamous cell carcinoma. Anticancer Res 33:5667–5674

Passerini L, Santoni De Sio FR, Roncarolo MG, Bacchetta R (2014) Forkhead box P3: the peacekeeper of the immune system. Int Rev Immunol 33:129–145. https://doi.org/10.3109/08830185.2013.863303

Santiago E, Camacho L, Junquera ML, Vázquez F (2006) Full HPV typing by a single restriction enzyme. J Clin Virol 37:38–46. https://doi.org/10.1016/j.jcv.2006.06.001

Sasagawa T, Takagi H, Makinoda S (2012) Immune responses against human papillomavirus (HPV) infection and evasion of host defense in cervical cancer. J Infect Chemother 18:807–815. https://doi.org/10.1007/s10156-012-0485-5

Shen Z, Chen L, Hao F, Wang G, Fan P, Liu Y (2010) Intron-1 rs3761548 is related to the defective transcription of Foxp3 in psoriasis through abrogating E47/c-Myb binding. J Cell Mol Med 14:226–241. https://doi.org/10.1111/j.1582-4934.2008.00370.x

Solé X, Guinó E, Valls J, Iniesta R, Moreno V (2006) SNPStats: a web tool for the analysis of association studies. Bioinformatics 22:1928–1929. https://doi.org/10.1093/bioinformatics/btl268

Solomon D, Davey D, Kurman R, Moriarty A, Connor DO, Raab S et al (2002) The 2001 Bethesda System: terminology for reporting results of cervical cytology. JAMA 287:2114–2119. https://doi.org/10.1001/jama.287.16.2114

Stephens M, Scheet P (2005) Accounting for decay of linkage disequilibrium in haplotype inference and missing-data imputation. Am J Hum Genet 76:449–462. https://doi.org/10.1086/428594

Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. Am J Hum Genet 68:978–989. https://doi.org/10.1086/319501

Trugilo KP, Cebinelli GCM, Berti FCB, Okuyama NCM, Cezar-dos-Santos F, Sena MM et al (2018) Polymorphisms in the TGFB1 signal peptide influence human papillomavirus infection and development of cervical lesions. Med Microbiol Immunol 208:49–58. https://doi.org/10.1007/s00430-018-0557-y

Waggoner SE (2003) Cervical cancer. Lancet 361:2217–2225. https://doi.org/10.1016/S0140-6736(03)13778-6

Walboomers JMM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV et al (1999) Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 189:12–19. https://doi.org/10.1002/(SICI)1096-9896(199909)189:1%3c12:AID-PATH431%3e3.0.CO;2-F

Wang L, Liu R, Li W, Chen C, Katoh H, Chen GY et al (2009) Somatic single hits inactivate the X-linked tumor suppressor FOXP3 in the prostate. Cancer Cell 16:336–346. https://doi.org/10.1016/j.ccr.2009.08.016

Wang L, Liu R, Ribick M, Zheng P, Liu Y (2010) FOXP3 as X-linked tumor suppressor. Discov Med 10:322. https://doi.org/10.1002/ijc.22894

Wu Z, You Z, Zhang C, Li Z, Su X, Zhang X et al (2012) Association between functional polymorphisms of foxp3 gene and the occurrence of unexplained recurrent spontaneous abortion in a Chinese Han population. Clin Dev Immunol 2012:7. https://doi.org/10.1155/2012/896458

You D, Wang Y, Zhang Y, Li Q, Yu X, Yuan M et al (2018) Association of Foxp3 promoter polymorphisms with susceptibility to endometrial cancer in the Chinese Han women. Medicine (Baltimore). 97(18):e0582. https://doi.org/10.1097/MD.0000000000010582

Zeng C, Yao Y, Jie W, Zhang M, Hu X, Zhao Y et al (2013) Up-regulation of Foxp3 participates in progression of cervical cancer. Cancer Immunol Immunother 62:481–487. https://doi.org/10.1007/s00262-012-1348-8

Zuo T, Wang L, Morrison C, Chang X, Zhang H, Li W et al (2007) FOXP3 is an X-Linked breast cancer suppressor gene and an important repressor of the HER-2/ErbB2 oncogene. Cell 129:1275–1286. https://doi.org/10.1016/j.cell.2007.04.034