Universidade Estadual de Londrina

KLEBER PAIVA TRUGILO

VARIAÇÕES GENÉTICAS, NÍVEIS CERVICAIS E PLASMÁTICOS DE TGFB1:

IMPLICAÇÕES NA INFECÇÃO PELO HPV E NO CÂNCER CERVICAL

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Orientadora: Profa. Dra. Karen Brajão de Oliveira

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Londrina, 03 de abril de 2020.

Dedico esta aos meus pais,

meus eternos mestres.

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"Seguir em frente e ter a certeza de que
apesar de às vezes estar no escuro,
o sol vai voltar a brilhar."
(Irmã Dulce)

TRUGILO, Kleber Paiva. **Variações genéticas, níveis cervicais e plasmáticos de TGFB1: implicações na infecção pelo HPV e no câncer cervical.** Tese de Doutorado – Programa de Pós-Graduação em Patologia Experimental, Departamento de Ciências Patológicas, Centro de Ciências Biológicas – Universidade Estadual de Londrina, 2020.

RESUMO

O papilomavírus humano (HPV) é o principal agente causador das lesões intraepiteliais cervicais e do câncer cervical. Entretanto, a resposta imune apresenta um impacto considerável no desfecho da infecção. Neste contexto, o fator de transformação do crescimento β 1 (TGFB1) parece desempenhar um papel dual, atuando como supressor ou favorecendo a progressão das lesões e do câncer. Como variações genéticas podem influenciar a produção de TGFB1, o presente estudo teve como objetivo verificar o papel das variações genéticas c.–1638G>A, c.–1347T>C, c.29C>T e c.74G>C de *TGFB1* e suas estruturas haplotípicas na suscetibilidade à infecção pelo HPV e no desenvolvimento das lesões cervicais e do câncer, além de analisar se os haplótipos influenciam os níveis cervicais e plasmáticos de TGFB1. Um total de 402 mulheres participaram do estudo. Amostras de muco cervical e sangue periférico foram coletadas de 351 mulheres submetidas a exame citológico e amostras de tecido tumoral embebidas em parafina de 51 pacientes foram fornecidas pelo Hospital do Câncer de Londrina. As participantes foram estratificadas com base na presença ou ausência de DNA do HPV, testada pela reação em cadeia da polimerase (PCR), e de acordo com o status cervical, conforme determinado por análise citológica ou biópsia cervical [sem lesão cervical, lesões de baixo e alto grau (LIEBG e LIEAG) e câncer]. Dados clínicos e histopatológicos de pacientes com câncer e dados sociodemográficos de todos as participantes também foram coletados. Os genótipos das variações de *TGFB1* foram determinados por PCR seguida de digestão enzimática e as estruturas haplotípicas foram inferidas pelo software PHASE 2.1.1. Os níveis cervicais e plasmáticos de TGFB1 foram medidos por ensaio imunofluorimétrico. As análises caso-controle foram realizadas por regressão logística ajustada para possíveis fatores de confusão e por testes de Mann-Whitney e Kruskal-Wallis. Para a primeira produção científica (artigo 1), foram incluídas 190 mulheres não infectadas por HPV (controles) e 161 infectadas. Entre as infectadas (com exclusão de cinco pacientes), 80 (51,3 %) não apresentavam lesão cervical, 23 (14,7 %) possuíam LIEBG e 53 (34,0 %) tinham LIEAG. A análise das variantes genéticas mostrou que os alelos –1347T (P = 0,028) e 29C (P = 0,006) e o genótipo 29CC (P = 0,023) foram proporcionalmente mais frequentes nas mulheres infectadas do que nas não infectadas (40.1%, 50.0%, 24.8% e 32.1%, 39.7%, 17.4%, respectivamente), enquanto não houve diferença entre os grupos de lesão. Considerando os haplótipos, oito estruturas foram inferidas. Dessas, GCTG, aqui denominado **4*, foi mais frequente no grupo infectado do que no não infectado (P = 0,003). A análise por regressão logística mostrou que mulheres –1347TT e –1347CT+TT foram mais suscetíveis à infecção pelo HPV do que –1347CC (OR = 2,16; $IC_{95\%}$ = 1,10 – 4,25 e OR = 1,62; $IC_{95\%}$ = 1,03 – 2,54). Da mesma forma, 29CT, 29CC e 29CT+CC aumentaram a suscetibilidade ao HPV quando comparados com 29TT (OR = 1,77; $IC_{95\%}$ = 1,06 – 2,97; OR = 2,31; $IC_{95\%}$ = 1,23 – 4,34 e OR = 1,92; $IC_{95\%}$ = 1,18 – 3,12, respectivamente). Por outro lado, portadoras do haplótipo **4* (que contém os alelos –1347C e 29T) em homozigose foram menos suscetíveis ao HPV do que as não portadoras de **4* (OR = 0,39; $IC_{95\%}$ = 0,21 – 0,72). Por fim, comparando

os dois haplótipos mais frequentes, *3* (GTCG) com *4*, mulheres *3/*3* e *3/*4* foram mais suscetíveis ao HPV do que mulheres *4/*4* (OR = 2,13; $IC_{95\%}$ = 1,13 – 4,00 e OR = 2,81; $IC_{95\%}$ = 1,29 – 6,10, respectivamente). As pacientes infectadas pelo HPV apresentaram níveis maiores de TGFB1 no plasma [4575,19 (IQR = 4392,34) pg/mL] e no muco cervical [53,17 (IQR = 56,46) pg/mg de proteínas] comparadas com as não infectadas [2964,80 (IQR = 3091,45) pg/mL e 32,57 (IQR = 54,49) pg/mg de proteínas, respectivamente] (*P* = 0,001 e *P* = 0,008, respectivamente). Maiores níveis também foram observados no plasma do grupo LIEBG [6653,45 (IQR = 5098,44) pg/mL] do que no grupo sem lesão [3689,4 (IQR = 3383,8) pg/mL] (*P* = 0,010). Com relação aos haplótipos, mulheres infectadas portando *3*/outros (heterozigotas) apresentaram níveis menores de TGFB1 plasmático do que mulheres infectadas não portadoras (outros/outros) [3067,13 (IQR 4200,20) pg/mL e 4836,23 (IQR 4313,38) pg/mL, respectivamente, *P* = 0,03]. Da mesma forma, mulheres infectadas *3/*3* + *3*/outros [3993,99 (IQR = 4173,05)] tiveram níveis mais baixos de TGFB1 plasmático (*P* = 0.04) do que as infectadas não portadoras [4836,23 (IQR = 4313,38)]. No segundo artigo, foram incluídas apenas pacientes infectadas (n = 130) e distribuídas nos grupos sem lesão (79/60,8 %) e câncer (51/39,2 %). Inicialmente, nenhuma das variações genéticas enquanto marcadores isolados foi associada com o câncer. No entanto, o haplótipo GTCG nos modelos dominante e recessivo aumentou a suscetibilidade ao câncer cervical (OR = 2,48; IC95% = 1,05 – 5,83 e OR = 4,73; IC95% = 1,18 – 19,02; respectivamente). Com base nos resultados dos artigos 1 e 2, esta é a primeira vez que haplótipos de *TGFB1* foram associados com a infecção pelo HPV e com o câncer cervical, sugerindo as estruturas haplotípicas de *TGFB1* (c.–1638G>A, c.–1347T>C, c.29C>T e c. 74G>C) como potenciais *loci* candidatos de suscetibilidade para estas doenças.

Palavras-chave: Polimorfismo; SNP; Câncer de colo do útero; rs1800468; rs1800469; rs1800470; rs1800471.

TRUGILO, Kleber Paiva. **Genetic variations, cervical and plasma levels of TGFB1: implications on HPV infection and cervical cancer.** Doctoral thesis – Postgraduate Program in Experimental Pathology, Department of Pathological Sciences, Biological Science Center – State University of Londrina, 2020.

ABSTRACT

Human papillomavirus (HPV) is the main agent for cervical lesions and cancer development. However, the immune response presents a considerable impact on the infection outcome. In this context, transforming growth factor beta 1 (TGFB1) seems to play a dual role, acting as suppressor or favoring lesions progression. Since genetic variations may influence TGFB1 production levels, the present study purpose was to verify the role of *TGFB1* variations (c.–1638G>A, c.–1347T>C, c.29C>T and c.74G>C) and their haplotype structures on HPV infection susceptibility and on cervical lesions and cancer development, and further, to analyze if the haplotype structures influence TGFB1 cervical and plasma levels. A total of 402 women participated in the study. Cervical mucus and peripheral blood samples were collected from 351 women who underwent outpatient cytology testing and tumor tissue samples embedded in paraffin from 51 patients were provided by the Londrina Cancer Hospital. Participants were stratified based on presence or absence of HPV DNA, as tested by polymerase chain reaction (PCR), and based on cervical status, as determined by cervical cytology or biopsy [no cervical lesion (NL), low- and high-grade lesions (LSIL and HSIL) and cancer]. Clinical and histopathological data from cancer patients and sociodemographic data from all participants were also collected. *TGFB1* genotypes were assessed by PCR followed by enzymatic digestion and haplotype structures were inferred by PHASE software 2.1.1. Cervical and plasma levels of TGFB1 were determined by immunofluorimetric assay. Case–control analyses were performed by logistic regression adjusted for possible confounding factors and by Mann-Whitney and Kruskal-Wallis tests. For the first scientific production (Paper 1), 190 HPV-uninfected women (controls) and 161 HPV-infected were included. Among the infected (excluding five patients), 80 (51.3 %) presented NL, 23 (14.7 %) had LSIL whereas 53 (34.0 %) had HSIL. Analysis of the genetic variants showed that the –1347T ($P = 0.028$) and 29C ($P = 0.006$) alleles and the 29CC genotype ($P = 0.023$) were proportionally more frequent in infected than in uninfected women (40.1%, 50.0%, 24.8% e 32.1%, 39.7%, 17.4%, respectively), while there was no difference between the lesion groups. Regarding the haplotype, eight structures were inferred. Of these, GCTG, here designated **4*, was more frequent in the infected than in the uninfected group ($P = 0.003$). Logistic regression analysis showed that –1347TT and –1347CT+TT women were more susceptible to HPV infection than –1347CC (OR = 2.16; $CI_{95\%}$ = 1.10 – 4.25 and OR = 1.62; $CI_{95\%}$ = 1.03 – 2.54). Likewise, 29CT, 29CC and 29CT+CC increased susceptibility to HPV when compared with 29TT (OR = 1.77; $CI_{95\%}$ = 1.06 – 2.97; OR = 2.31; $CI_{95\%}$ = 1.23 – 4.34 and OR = 1.92; $CI_{95\%}$ = 1.18 – 3.12, respectively). On the other hand, carriers of the **4* haplotype (which contains the –1347C and 29T alleles) in homozygosity were less susceptible to HPV than those without **4* (OR = 0.39; $CI_{95\%}$ = 0.21 – 0.72). Finally, comparing the two most frequent haplotypes, **3* (GTCG) with **4*, women **3/*3* and **3/*4* were more susceptible to HPV than those **4/*4* (OR = 2.13; $CI_{95\%}$ = 1.13 – 4.00 and OR = 2.81; $CI_{95\%}$ = 1.29 – 6.10, respectively). HPV-infected patients had higher levels of TGFB1 in plasma [4575.19 (IQR = 4392.34) pg/mL] and cervical mucus [53.17 (IQR = 56.46) pg/mg of protein] compared with uninfected ones [2964.80 (IQR = 3091.45) pg/mL and 32.57

(IQR = 54.49) pg/mg of protein, respectively] (*P* = 0.001 and *P* = 0.008, respectively). Higher plasma levels were also observed in the LSIL group [6653.45 (IQR = 5098.44) pg/mL] than in the NL group [3689.4 (IQR = 3383.8) pg/mL] (*P* = 0.010). Regarding the haplotypes, infected women carrying **3*/others (heterozygotes) had lower plasma levels of TGFB1 than infected non-carriers (others/others) [3067.13 (IQR 4200.20) pg/mL and 4836.23 (IQR 4313.38) pg/mL, respectively, *P* = 0.03]. Likewise, infected women **3*/**3* + **3*/others [3993.99 (IQR = 4173.05)] had lower plasma levels of TGFB1 (*P* = 0.04) than infected non-carriers [4836.23 (IQR = 4313.38)]. In the second paper, only HPV-infected patients (n = 130) were included and divided in NL (79/60.8 %) and cervical cancer (51/39.2 %). Initially, none of the genetic variations as single markers was associated with cancer. However, GTCG haplotype in both dominant and recessive models increased the susceptibility to cervical cancer (OR = 2.48, $CI_{95\%}$ = 1.05 – 5.83 and OR = 4.73, $CI_{95\%}$ = 1.18 – 19.02, respectively). Based on results in the paper 1 and 2, this is the first time that *TGFB1* haplotypes were associated with HPV infection and cervical cancer, suggesting c.–1638G>A, c.–1347T>C, c.29C>T and c.74G>C *TGFB1* haplotype structures as potential candidate of susceptibility *loci*.

Keywords: Polymorphism; SNP; Cancer of uterine cervix; rs1800468; rs1800469; rs1800470; rs1800471.

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LISTA DE ABREVIATURAS E SIGLAS

Aa	*Amino acids*
ACMG	*American College of Medical Genetics*
ACT	*Activin*
ACTR	*Activin receptor*
$AgNO_3$	*Silver nitrate*
AIS	*Adenocarcinoma in situ*
AKT	*"Ak" thymoma protein*
ALK	*Activin-like Kinase*
AMH	*Anti-Mullerian hormone*
AMHR	*Anti-Mullerian hormone receptor*
AP1	*Activator protein 1*
APC	*Antigen presenting cell*
ASC-H	*Atypical squamous cells, cannot exclude HSIL*
ASC-US	*Atypical squamous cells of undetermined significance*
ATF	*Activating transcription factor*
ATP	*Adenosine triphosphate*
B*a*P	*Benzo[a]pyrene*
BAX	*BCL2-associated X protein*
BCL-Xl	*B-cell lymphoma-extra large*
BCL2	*B-cell lymphoma 2*
BIM	*BCL-2-interacting mediator of cell death*
BMF	*BCL-2-modifying factor*
BMP	*Bone morphogenetic protein*
BMPR	*BMP receptor*
Bp	*Base pairs*
CAF	*Cancer-associated fibroblasts*
CaSki	*Cell line of cervical carcinoma metastasis containing integrated HPV16 and HPV18 genomes*
CD	*Cluster of differentiation*
CDC42	*Cell division cycle 42*
CDK	*Cyclin-dependent kinase*

CEP/UEL	*Institutional Ethics Committee Involving Humans of the State University of Londrina*
CIN1, 2 ou 3	*Cervical intraepithelial neoplasia grade 1, 2 or 3*
c-MYC	*v-MYC avian myelocytomatosis viral oncogene homolog*
Co-SMAD	*Common partner SMAD*
CREB	*cyclic-AMP response element binding protein*
CSCC	*Cervical squamous cell carcinoma*
CTL	*Cytolytic T lymphocytes*
CXCR	*CXC-chemokine receptor*
DNA	*Deoxyribonucleic acid*
dNTP	*Deoxynucleotide*
E2F	*E2 promoter-binding factor*
ECM	*Extracellular matrix*
EDTA	*Ethylenediaminetetraacetic acid*
EGFR	*Epidermal growth factor receptor*
EMT	*Epithelial-to-mesenchymal transition*
ERK1/2	*Extracellular signal-regulated MAP kinase1/2*
FOX	*Forkhead Box*
FOXP3	*Forkhead box P3*
G2	*Gap2 (cell cycle)*
GDF	*Growth and differentiation factors*
GDNF	*Glial-derived neurotrophic factors*
GIPC	*GAIP-interacting protein C-terminus*
GTPase	*Guanosine triphosphatase*
HeLa	*Cell line of cervical adenocarcinoma containing integrated HPV18 genome*
HGVS	*Human Genome Variation Society*
HLA-I	*Human leukocyte antigen class 1*
HOX	*Homeobox*
HPV	*Human Papillomavirus*
HR-HPV	*High-risk HPV*
HSIL	*High-grade squamous intraepithelial lesions*
hTERT	*Human telomerase reverse transcriptase*
IL	*Interleukin*
INH	*Inhibin*

I-SMAD	*Inhibitory SMAD*
JNK	*c-Jun N-terminal kinase*
LAP	*Latency-associated peptide*
LCR	*Long control region*
ROS	*Reactive oxygen species*
LEFTY	*Left-right determination factor*
LR-HPV	*Low-risk HPV*
LSIL	*Low-grade squamous intraepithelial lesions*
$MgCl_2$	*Magnesium chloride*
MIS	*Müllerian inhibiting substance*
MMP	*Matrix metalloproteinases*
mRNA	*Messenger ribonucleic acid*
MSC	*Mesenchymal stromal cells*
mTOR	*Mechanistic target of rapamycin complex*
NF1	*Nuclear factor 1*
NFκB	*Nuclear factor kappa-light-chain-enhancer of activated B cells*
NK	*Natural-killer cell*
NODAL	*Nodal growth differentiation factor*
OC	*Oral contraceptive*
ORF	*Open reading frames*
P/CAF	*p300/CREB-binding protein-associated factor*
p15; p21; p27	*CDK inhibitors*
p38 MAPK	*p38 mitogen-activated protein kinase*
p53	*Protein 53*
PCR	*Polymerase chain reaction*
PI3K	*Phosphoinositide 3-kinase*
PKB	*Protein Kinase B*
pRb	*Retinoblastoma protein*
pSMAD	*Phosphorylated SMAD*
RAC	*RAS-related C3 botulinum toxin substrate*
RAS	*Rat sarcoma viral oncoprotein homolog*
RFLR	*Restriction fragment length polymorphism*
RHO	*RAS homolog protein*

R-SMAD	*Receptor-bound SMAD*
RUNX	*Runt-related transcription factor*
SAPK	*Stress-activated protein kinases*
SiHa	*Cell line of cervical carcinoma containing integrated HPV16 genome*
Ski	*Sloan-Kettering Institute proto-oncoprotein*
SMAD	*Small mothers against decapentaplegic protein*
SMURF	*Smad ubiquitination-related factor*
SNP	*Single nucleotide polymorphisms*
SNV	*Single nucleotide variation*
Sp1	*Specificity protein 1 transcription factor*
TAK1	*TGFB-associated kinase 1*
TE	*Tris-EDTA*
TGFB	*Transforming growth factor β*
TGFBR	*TGFB receptor*
TRAF	*TNF receptor-associated factor*
Treg	*Regulatory T cell*
TSP-1	*Trombospondin-1*
UEL	*State University of Londrina*
UR-HPV	*Undetermined-risk HPV*
URR	*Upstream regulatory region*
VEGF	*Vascular endothelial growth factor*
WNT	*Wingless-type MMTV integration site family*

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Introdução

1 INTRODUÇÃO

O papilomavírus humano (HPV) é o agente etiológico da infecção viral mais frequente do trato anogenital. A maioria dos homens e mulheres sexualmente ativos é infectada pelo HPV em algum momento da vida, mas nem sempre a infecção apresenta lesões clínicas detectáveis. Nas mulheres, o HPV possui papel central no desenvolvimento de lesões intraepiteliais cervicais e do câncer cervical – neste último, está virtualmente presente em 100% dos casos (SCHIFFMAN et al., 2016).

Embora existam formas de prevenir a infecção e, consequentemente, a doença maligna – a vacina, o exame papanicolau de rotina, o uso de preservativos entre outros, em 2018, foram estimadas cerca de 570.000 mulheres diagnosticadas com câncer cervical invasivo e 311.000 mortes em todo o mundo. Com esses números, o câncer cervical ocupa a quarta posição entre os tipos de cânceres (excluindo os de pele não-melanoma) mais incidentes e de maior mortalidade feminina. Porém, nos países com baixo e médio índice de desenvolvimento humano, este câncer ocupa a segunda posição (BRAY et al., 2018). No Brasil, as estimativas para o ano de 2018 indicaram o câncer de colo de útero como a terceira causa de morte feminina por câncer, com aproximadamente 8.000 casos (BRUNI et al., 2019a). Já o número de casos novos estimados para cada ano do triênio 2020-2022 é de 16.590, com um risco estimado de 15,43 casos a cada 100 mil mulheres. A taxa de incidência varia entre as regiões brasileiras, chegando a ser o segundo tipo de câncer mais incidente entre as mulheres na região Norte (21,20/100 mil), Nordeste (17,62/100 mil) e Centro-Oeste (15,92/100 mil), quarto na região Sul (17,48/100 mil) e o quinto na região Sudeste (12,01/100 mil), sem considerar os tumores de pele não melanoma (INSTITUTO NACIONAL DE CÂNCER JOSÉ ALENCAR GOMES DA SILVA, 2019).

Entretanto, ao longo da vida sexual, a maioria das mulheres é infectada por algum dos tipos de HPV e apenas uma pequena porcentagem desenvolve a doença invasiva (WOODMAN; COLLINS; YOUNG, 2007). A regressão ou progressão das lesões depende em grande parte da ação do sistema imunológico (SCHIFFMAN et al., 2007) cujas células e moléculas neste microambiente desempenham um papel de fundamental importância na tumorigênese. Neste contexto, o fator de transformação do crescimento β (TGFB), uma citocina conhecida por sua ação anti-proliferativa, possui um papel paradoxal na patogênese das neoplasias malignas, atuando como supressor tumoral, em fases iniciais, ou

estimulador da progressão, invasão e metástase tumoral, em fases avançadas (CUI et al., 1996; ODA; GUEMBAROVSKI; WATANABE, 2012).

Ao longo das últimas décadas, estudos vêm mostrando a associação de variações genéticas do *TGFB1* com vários tipos de doenças, inclusive o câncer (CARNEIRO et al., 2013; CHANG et al., 2014; EWART-TOLAND et al., 2004; FAN et al., 2014; POOJA et al., 2013; ZHANG et al., 2011). Entretanto, a relação com o câncer cervical ou com as lesões induzidas pelo HPV ainda não está bem estabelecida. Portanto, este trabalho pretende abordar o papel do TGFB1 na carcinogênese cervical, avaliando a associação das variações de nucleotídeo único (SNVs) rs1800468 (c.-1638G>A), rs1800469 (-1347T>C), rs1800470 (c.29C>T) e rs1800471 (c.74G>C) do gene *TGFB1* com a infecção pelo HPV e com as lesões e o câncer cervical.

2 PAPILOMAVÍRUS HUMANO (HPV)

O HPV é um vírus pertencente à família *Papillomaviridae* e seus mais de 200 tipos conhecidos (BZHALAVA; EKLUND; DILLNER, 2015) são distribuídos em cinco grandes gêneros: Alphapapillomavirus, Betapapillomavirus, Gammapapillomavirus, Mu papillomavirus e Nu papillomavirus (BZHALAVA et al., 2013; DE VILLIERS et al., 2004).

Este vírus, não-envelopado, é composto por capsídeo proteico e ácido desoxirribonucleico (DNA) circular dupla-fita. O genoma viral possui cerca de 8000bp e é constituído de oito ou nove *open reading frames* (ORF), além de um segmento longo não-codificante chamado de *long control region* (LCR) ou *upstream regulatory region* (URR) que controla a transcrição e replicação do DNA viral. As ORFs podem ser divididas em regiões de expressão precoce (E) e tardia (L), de acordo com as etapas de replicação viral e os estágios de maturação das células epiteliais. A região de expressão precoce codifica as proteínas necessárias para a replicação viral (E1, E2, E4, E5, E6 e E7), enquanto a de expressão tardia codifica as proteínas do capsídeo (L1 e L2), indispensáveis para a montagem dos novos vírions (DOORBAR, 2006; INTERNATIONAL AGENCY FOR RESEARCH ON CANCER, 2007; KIRNBAUER et al., 1992).

2.1 Proteínas Virais

As proteínas E1 e E2 estão envolvidas com o início da replicação viral. E1 tem função de helicase dependente de *adenosine triphosphate* (ATP) e E2 atua principalmente no recrutamento de E1 ao sítio de origem de replicação e também como importante reguladora da transcrição da região de expressão precoce, ativando quando em baixos níveis ou reprimindo se presente em grandes quantidades (HEBNER; LAIMINS, 2006). Portanto, a alta produção de E2 regula negativamente a expressão de E6 e E7 e ativa a expressão dos genes tardios L1 e L2 (JOHANSSON; SCHWARTZ, 2013).

A proteína E4, no ciclo de vida viral, parece contribuir para uma maior eficiência da amplificação do genoma do vírus por sequestrar CiclinaB/*Cyclin-dependent kinase* 1 (CDK1) para o citoplasma, mantendo a célula por mais tempo na fase *gap* 2 (G2) do ciclo celular, colaborando com a síntese de novas partículas virais. Também, E4 parece facilitar a liberação/transmissão dos vírions recém formados através da desestruturação da rede de citoqueratina (DOORBAR, 2013).

A proteína E5 está localizada na membrana do complexo de Golgi, retículo endoplasmático e, em menor quantidade, na membrana plasmática (FEHRMANN; KLUMPP; LAIMINS, 2003; HEBNER; LAIMINS, 2006). É considerada uma oncoproteína com função ainda pouco esclarecida no contexto de uma infecção natural por HPV. Porém existe um acúmulo de evidências sugerindo que E5 possa contribuir para a carcinogênese através do aumento do potencial de imortalização celular de E6 e E7, incluindo regulação positiva da via de sinalização disparada pelo receptor do fator de crescimento epidermal (EGFR), angiogênese e efeito anti-apoptótico (KIM et al., 2010).

As oncoproteínas E6 e E7 têm como alvo uma série de reguladores negativos do ciclo celular, principalmente os supressores tumorais proteína 53 (p53) e proteína do retinoblastoma (pRB), respectivamente. Durante o ciclo replicativo viral, E6 e E7 facilitam a manutenção estável de epissomas virais e estimulam células diferenciadas a entrarem na fase S (FEHRMANN; KLUMPP; LAIMINS, 2003).

As proteínas estruturais L1 e L2 são responsáveis pela formação do invólucro do vírus. L1 é o elemento estrutural primário, contendo 360 cópias desta proteína organizadas em 72 capsômeros. L2 é o componente menor do capsídio e está presente no centro dos capsômeros pentavalentes nos vértices do vírion. Ambas

as proteínas são de suma importância para garantir uma eficiente infectividade viral (DOORBAR, 2006; MODIS; TRUS; HARRISON, 2002).

2.2 Transmissão do HPV, Infecção Produtiva e Lesões Cervicais

A transmissão do HPV se dá pelo contato pele-a-pele ou mucosa-a-mucosa (SCHIFFMAN et al., 2007). O vírus infecta células basais do epitélio, expostas geralmente por microlesões, e o seu material genético se instala no núcleo na forma epissomal. As células basais infectadas servem como um reservatório da infecção para sustentar a lesão. A célula basal infectada se divide e o genoma viral é replicado. O aumento da densidade celular na camada basal induz o comprometimento de células filhas infectadas com a diferenciação. Neste processo, em camadas mais superiores, o estímulo de fatores de crescimento locais diminui, os sinais extracelulares que conduzem a diferenciação aumentam e o padrão de expressão viral muda (aumento de E1, E2 e E4 e diminuição de E6 e E7). Assim, o genoma do vírus é amplificado e novas partículas são formadas para a propagação da infecção. A conclusão das etapas produtivas do ciclo de vida do HPV depende, então, do fluxo constante de células infectadas do reservatório da camada basal para as camadas epiteliais superiors (DOORBAR, 2018; FERNANDES et al., 2015).

Na cérvix uterina, o HPV pode ser eliminado, permanecer num estado latente ou provocar lesões. Numa fase pré-maligna, as lesões são classificadas de acordo com o grau de severidade correspondentes às anormalidades histológicas ou citológicas encontradas. Obedecendo as regras brasileiras, utiliza-se para o exame histopatológico a classificação de Richart (1967): neoplasias intraepiteliais cervicais grau (CIN) 1 (displasia leve), CIN 2 (displasia moderada) ou CIN 3 (displasia severa/carcinoma *in situ*), baseando-se na proporção de espessura do epitélio escamoso constituído de células maduras e diferenciadas. Para o exame citológico, adota-se a classificação citológica brasileira, baseada no Sistema Bethesda (2001): lesões intraepiteliais escamosas de baixo ou alto grau (LSIL ou HSIL) e adenocarcinoma *in situ*. LSIL corresponde a CIN1 e HSIL a CIN2 e CIN3 (Tabela 1) (INSTITUTO NACIONAL DE CÂNCER JOSÉ ALENCAR GOMES DA SILVA, 2012, 2016).

Embora a presença do vírus somente seja confirmada por técnicas de biologia molecular, a pesquisa dessas lesões pré-malignas pelo exame citopatológico

e histopatológico é a ferramenta mais utilizada nos programas de rastreio para prevenção do câncer de colo do útero no mundo todo.

Apenas uma minoria das lesões pré-malignas, quando não tratadas, evolui para o câncer cervical. Nesta fase, o DNA viral geralmente se integra ao genoma do hospedeiro (SCHIFFMAN et al., 2007), provocando a perda de E2 e consequente quebra do *feedback* negativo que controla a expressão dos oncogenes (WOODMAN; COLLINS; YOUNG, 2007). Como consequência, E6 e E7 se tornam ativamente expressas, iniciando a transformação tumoral.

Tabela 1 Nomenclaturas citopatológica e histopatológica para lesões cervicais.

Classificação citológica de Papanicolaou (1941)	Classificação histológica da OMS (1952)	Classificação histológica de Richart (1967)	Classificação citológica brasileira (2006)*
Classe I	-	-	Dentro dos limites de normalidade
Classe II	-	-	Alterações benignas
	-	-	Atipias de significado indeterminado
Classe III	Displasia leve	CIN1[a]	LSIL[b]
	Displasia moderada	CIN2[a]	HSIL[c]
	Displasia severa	CIN3[a]	HSIL
Classe IV	Carcinoma *in situ*	CIN3	HSIL
			AIS[d]
Classe V	Câncer Invasor	Câncer Invasor	Câncer Invasor

Fonte: adaptado de INSTITUTO NACIONAL DE CÂNCER JOSÉ ALENCAR GOMES DA SILVA (2016, p. 26). *Baseado no Sistema Bethesda de classificação citológica (2001). [a]CIN1, 2 ou 3, neoplasia intraeptelial cervical grau 1, 2 ou 3; [b]LSIL, lesão intraepitelial escamosa de baixo grau; [c]HSIL, lesão intraepitelial escamosa de alto grau; [d]AIS, adenocarcinoma *in situ*.

2.3 Tipos de HPV e a Relação com as Lesões e o Câncer Cervical

A associação do HPV com as lesões intraepiteliais e com o câncer levaram a classificação dos tipos de HPV em: alto risco carcinogênico (HR-HPV), risco indeterminado (UR-HPV) e baixo risco carcinogênico (LR-HPV). Dentre os 47 tipos de HPVs que infectam a mucosa genital, todos do gênero Alphapapillomavirus (BZHALAVA et al., 2013), até o momento 25 tipos são considerados HR-HPVs, mas apenas 12 tipos são confirmados pela epidemiologia como “carcinógenos humanos”

(HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 e 59) (DOORBAR, 2018; SCHIFFMAN et al., 2016). Destes, o HPV16 e o HPV18 são os mais prevalentes nas lesões cervicais e juntos são responsáveis por cerca de 70% dos casos de câncer de colo do útero invasivo. Os LR-HPVs tipos 6 e 11 são os mais encontrados nas lesões genitais benignas, causando 90% dos condilomas (BRUNI et al., 2019b; SCHIFFMAN et al., 2007; SMITH et al., 2007). Além do câncer de colo do útero, o HPV tem sido associado ao câncer de vagina (ALEMANY et al., 2014), vulva (SIRIAUNKGUL et al., 2014), pênis (LEBELO et al., 2014), ânus (ALEMANY et al., 2015), cabeça e pescoço (CORRENTI; RIVERA; CAVAZZA, 2004; GUDLEVICIENE et al., 2009) e mama (FU et al., 2015).

No entanto, a infecção por HPV é necessária, porém insuficiente para causar o câncer cervical (KOWLI et al., 2013; ZUR HAUSEN, 2000). A maioria das mulheres é infectada por algum dos tipos de HPV ao longo da vida sexual e apenas uma pequena parcela desenvolve a doença invasiva (WOODMAN; COLLINS; YOUNG, 2007), uma vez que a maioria das infecções por HPV é resolvida pela resposta imunológica (SCHIFFMAN et al., 2007). Todavia, as infecções causadas por HR-HPVs tendem a progredir para HSIL ou câncer invasivo como resultado da interação de células e moléculas do sistema imunológico com o microambiente da lesão (PATEL; CHIPLUNKAR, 2009).

Neste cenário, a inflamação crônica resultante possui papel fundamental na tumorigênese, contribuindo com a iniciação do tumor através da produção de estresse genotóxico, com a promoção, pela indução de proliferação celular, e com a progressão, por aumentar a angiogênese e invasão tecidual (CHOW; MÖLLER; SMYTH, 2012). As citocinas e quimiocinas do ambiente inflamatório influenciam direta ou indiretamente as propriedades proliferativas e invasivas das células tumorais (AMEDEI; PRISCO; M. D'ELIOS, 2013; DE OLIVEIRA et al., 2014), inibindo o desenvolvimento e progressão do tumor ou, paradoxalmente, promovendo crescimento, diminuição da apoptose e facilitando a invasão e metástase (DRANOFF, 2004).

Entre essas moléculas, o fator de transformação do crescimento β (TGFB), que é uma citocina multifacetada, atrai atenção no âmbito da relação entre o câncer e a inflamação. Esta citocina é reconhecida não somente por regular o comportamento celular e possuir um papel essencial na modulação do crescimento,

maturação e diferenciação das células, mas também pelo fato de estar envolvida na progressão do tumor (ZARZYNSKA, 2014).

3 FATOR DE TRANSFORMAÇÃO DO CRESCIMENTO β (TGFB)

O TGFB pertence a uma família de proteínas diméricas, estruturalmente conservadas (DELA CRUZ; REIS, 2015) e com peso molecular de aproximadamente 25kDa (ROBERTS; SPORN, 1993). Em mamíferos, três isoformas do TGFB são conhecidas: TGFB1, TGFB2, e TGFB3 (PAPAGEORGIS, 2015; VAIDYA; KALE, 2015), sendo cada uma codificada por um gene diferente. O gene *TGFB1* está localizado no cromossomo 19q13, o *TGFB2* no 1q41 e o *TGFB3* no 14q24 (KRSTIC; SANTIBANEZ, 2014; NEEL; HUMBERT; LEBRUN, 2012). No ser humano, o TGFB1 é a isoforma predominante e praticamente quase todas as células podem sintetizá-lo, com destaque para plaquetas, células T regulatórias (Treg) e outros linfócitos, macrófagos/monócitos, fibroblastos, células epiteliais e células dendríticas (KAJDANIUK et al., 2013).

Inicialmente, o TGFB é sintetizado como pré-pró-TGFB, um monômero de 55kDa, contendo 390 resíduos de aminoácidos (aa) que compõem uma estrutura formada por um peptídeo sinal N-terminal de 29 aa, uma pró-região de 249 aa chamada de *Latency-associated peptide* (LAP) e uma sequência C-terminal de 112 aa que corresponde ao TGFB ativo. Após uma série de etapas de proteólises e alterações na conformação e ligação à proteína, o TGFB é secretado na forma latente e interage covalentemente com componentes da matriz extracelular (ECM), como fibrilina-1 e fibronectina (PONIATOWSKI et al., 2015). Posteriormente, ele é convertido por enzimas na sua forma de dímero ativo (PIEK; HELDIN; TEN DIJKE, 1999). A ativação do TGFB também ocorre em níveis baixos de pH no microambiente (LYONS; KESKI-OJA; MOSES, 1988) ou pela produção de espécies reativas de oxigênio induzidas por irradiação (BARCELLOS-HOFF et al., 1994; PAPAGEORGIS, 2015).

Os efeitos do TGFB ativo são variados e complexos e dependem da sua ligação aos seus receptores na membrana de diversos tipos celulares. Em humanos, são conhecidos os receptores: tipo 1, tipo 2 (MASSAGUÉ; GOMIS, 2006) e tipo 3 ou betaglicana, o qual apresenta função reguladora (MEYER et al., 2014). A sinalização começa quando o TGFB ativo se liga a um complexo localizado na

membrana celular, formado por dois receptores tipo 2 (TGFBR2) e dois receptores tipo 1 (TGFBR1) (DERYNCK; AKHURST, 2007; MASSAGUÉ; GOMIS, 2006). O TGFBR2, com atividade serina/treonina quinase, fosforila o TGFBR1 (MASSAGUÉ, 2012; WRANA et al., 1994) e este promove a sinalização intracelular por via dependente (canônica) e independente (não canônica) de *Small mothers against decapentaplegic proteins* (SMADs). Na via canônica, o TGFBR1 ativo fosforila e ativa as *receptor-bound SMADs (R-SMADs)*, SMAD 2/3, permitindo a translocação para o núcleo com auxílio da SMAD4, uma *common partner SMAD (Co-SMAD)*. Uma vez no núcleo, as SMADs ativas formam um complexo que regula a transcrição de genes alvos, podendo promover ativação ou repressão gênica (MASSAGUÉ; GOMIS, 2006). Diferentes genes em diferentes tipos celulares são regulados pelas SMADs associadas a diferentes proteínas (MASSAGUÉ, 2000). A disponibilidade de tais proteínas depende do tipo de célula e isso, em parte, determina as respostas gênicas de cada célula ao TGFB (FENG; DERYNCK, 2005; MASSAGUE, 2005; MASSAGUÉ; GOMIS, 2006).

A via não canônica de sinalização do TGFB, independente de SMADs, emprega outras moléculas efetoras que induzem vias como a da *extracellular signal-regulated MAP kinase* (ERK) 1/2, p38 *mitogen-activated protein kinase* (MAPK), *c-Jun N-terminal kinase* (JNK) (MULDER, 2000; VAIDYA; KALE, 2015), *protein kinase B* (PKB)/*"Ak" thymoma protein* (AKT), a via *phosphoinositide 3-kinase* (PI3K)-AKT-*mechanistic target of rapamycin complex* (mTOR) (LAMOUILLE; DERYNCK, 2007), *nuclear factor kappa-light-chain-enhancer of activated B cells* (NFκB) e a via *wingless-type MMTV integration site family* (WNT)/β-catenin (BAKIN et al., 2002; GINGERY et al., 2008), *tumor necrosis factor (TNF)-receptor-associated factor* (TRAF) 6 e *TGFB-associated kinase* (TAK) 1 e as *guanosine triphosphatases* (GTPases) *rat sarcoma viral oncoprotein homolog (RAS)-homolog protein* (RHO), *RAS-related C3 botulinum toxin substrate* (RAC), e *cell division cycle* (CDC) 42, e a via RAS-ERK-MAPK (MU; GUDEY; LANDSTRÖM, 2012).

A sinalização do TGFB é amplamente regulada. Primariamente, três tipos de proteínas: *inhibitory SMADs (I-SMADs)*, ubiquitina-ligases da família dos *Smad ubiquitination-related factors* (SMURFs) e fosfatases cumprem o papel de controlar a via do TGFB por mecanismos de competição, inativação e degradação do complexo ligante-receptor (MASSAGUE, 2005; PARDALI; MOUSTAKAS, 2007). Desbalanços gerados por alterações na produção e atividade do TGFB, TGFBR1/2/3

e SMADs contribuem para uma ampla variedade de doenças, entre elas, o câncer (DRABSCH; TEN DIJKE, 2012; FABREGAT et al., 2014; GATZA; OH; BLOBE, 2010).

3.1 TGFB E HPV NAS LESÕES CERVICAIS

A manutenção de uma infecção persistente por HPV e o desenvolvimento das lesões cervicais com progressão para o câncer requer a evasão da resposta imunológica. O HPV, por si só, consegue evadir da resposta imune através de uma íntima interação com o TGFB1 (GUAN et al., 2010). Esta citocina pode desempenhar um importante papel na diminuição das moléculas de *human leukocyte antigen class 1* (HLA-I) nas células tumorais cervicais e protegê-las contra a lise por linfócitos T citolíticos (CTL) (GARCÍA-ROCHA et al., 2015), diminuir a sinalização via receptor de IL-2 em células T e a expressão de IL-12 pelas células apresentadoras de antígenos (APC) e induzir a expressão de IL-10 por macrófagos, contribuindo para a imunossupressão durante a carcinogênese cervical (PERALTA-ZARAGOZA et al., 2006; TORRES-POVEDA, 2014).

Colaborando com o microambiente imunossuprimido, células T reguladoras produzindo TGFB foram encontradas em quantidade abundante em HSIL, no carcinoma cervical e também no linfonodo de drenagem (KOBAYASHI et al., 2005). Além disso, tem sido observado que os níveis de TGFB1 e do seu *messenger ribonucleic acid* (mRNA) aumentam gradativamente conforme a evolução do tecido normal para o carcinoma invasivo (GARCÍA-ROCHA et al., 2015; KI et al., 2009; PEGHINI et al., 2012; XU et al., 2006). Um estudo prévio demonstrou que a expressão e produção de TGFB1 por linhagens de células de metástase de carcinoma cervical contendo os genomas do HPV16 e HPV18 integrados (CaSki), de adenocarcinoma cervical contendo o genoma do HPV18 integrado (HeLa) e de carcinoma cervical contendo o genoma do HPV16 integrado (SiHa) foram maiores do que em células sem o genoma do vírus e isso foi positivamente relacionado com a presença de E7 (XU et al., 2006). As oncoproteínas E6 e E7 induzem a expressão de TGFB1 através do fator de transcrição *specificity protein 1* (Sp1). Os complexos E6-Sp1 e E7-Sp1 migram para o núcleo e estimulam a expressão do gene desta citocina (PERALTA-ZARAGOZA et al., 2006; TORRES-POVEDA, 2014).

Entretanto, a resposta de células infectadas ao TGFB1 leva a uma redução da atividade transcricional da LCR do HPV com consequente queda na

transcrição dos genes de expressão precoce – entre eles E6 e E7, como demonstrado por um modelo *in vitro* que utiliza células do epitélio genital humano imortalizadas por HPV16 (WOODWORTH; NOTARIO; DIPAOLO, 1990). Também foi observado que o TGFB1 diminui a expressão de E6 e E7 em células CaSki, resultando no resgate da expressão de p53 e da via de resposta a pRb e na indução da senescência (DONALISIO et al., 2008). A atividade da LCR na célula hospedeira é regulada pelo complexo NF1/Ski, formado pelo Fator Nuclear 1 (NF1), um fator de transcrição, e pela oncoproteína *Sloan-Kettering Institute proto-oncoprotein* (Ski) (BALDWIN; PIRISI; CREEK, 2004; TARAPORE et al., 1997). Este complexo ativa a transcrição dos oncogenes virais E6 e E7(BALDWIN; PIRISI; CREEK, 2004; KOWLI et al., 2013). O TGFB1 diminui a formação do complexo NF1/Ski, resultando na inibição da atividade transcricional da LCR e, consequentemente, supressão dos níveis de E6 e E7 (BALDWIN; PIRISI; CREEK, 2004; KOWLI et al., 2013).

A regulação autócrina da expressão gênica do HPV16 pelo TGFB1 poderia representar um dos mecanismos de vigilância intracelular direcionado contra a transcrição do genoma do HPV. Na verdade, o efeito repressor do TGFB1 sobre a expressão do genoma viral varia significativamente em diferentes linhagens celulares, nas quais, frequentemente, ocorre uma resistência parcial a esse efeito inibitório do TGFB1, tanto sobre a expressão dos genes precoces do HPV quanto sobre o crescimento celular (WOODWORTH; NOTARIO; DIPAOLO, 1990). *In* vitro, o efeito de inibição do crescimento celular do TGFB1 sobre células epiteliais contendo o DNA do HPV16 diminuiu progressivamente durante o processo de malignização. A resistência à ação anti-crescimento do TGFB pode ser explicada pela redução de 50% na sinalização de SMAD, associada a uma perda completa de expressão de genes inibidores de crescimento (KOWLI et al., 2013) e à perda parcial do TGFBR1 (MI et al., 2000). A diminuição na expressão de SMAD4 e de TGFBR2 também foi observada em células de linhagem de tumores cervicais (FRENCH et al., 2013; XU et al., 2006), assim como a atenuação da fosforilação de SMAD2 e da translocação nuclear de SMAD4 na presença de E5/HPV16 (FRENCH et al., 2013).

Portanto, o efeito anti-proliferativo do TGFB parece predominar nas fases iniciais da transformação, enquanto ao final, prevalece o efeito pró-tumorigênico sobre a célula maligna, com indução de *epithelial-to-mesenchymal transition* (EMT) e motilidade celular, favorecendo a metástase.

A EMT é um processo vital para a morfogênese durante o desenvolvimento embrionário e que pode ser reativada anormalmente em tecidos adultos em condições patológicas, como a fibrose e o câncer. A EMT envolve a indução de um programa transcricional reversível em que células epiteliais fortemente conectadas e organizadas se transdiferenciam em células mesenquimais móveis e desorganizadas. Neste processo, as junções entre células epiteliais são desfeitas – entre as proteínas perdidas está a E-caderina, um marcador epitelial; e o citoesqueleto sofre remodelamento levando a perda celular da polaridade apical-basal e para assumir uma morfologia semelhante à de um fibroblasto. Além disso, a célula adquire outras características do fenótipo mesenquimal, como a expressão de N-caderina, vimentina, fibronectina e actina de músculo liso (marcadores mesenquimais) (PAPAGEORGIS, 2015). O TGFB tem um papel fundamental na regência destas alterações, induzindo ou diminuindo a expressão de genes cujo resultado é a EMT (KOWLI et al., 2013; PAPAGEORGIS, 2015).

Sabe-se que a infecção por HPV não é determinante isolado para o desenvolvimento de HSIL e câncer cervical, visto que, na grande maioria das vezes, a infecção e a LSIL sofrem regressão espontânea. Como visto, o TGFB tem importante participação no controle da transcrição dos genes precoces virais além de exercer efeito anti-proliferativo sobre as células epiteliais nas fases iniciais da doença, assim como estimular a EMT, tardiamente, e provocar imunossupressão e angiogênese. Portanto, variações da quantidade de TGFB neste microambiente poderiam influenciar no desfecho da doença.

Variações genéticas no *TGFB1* e no gene dos seus receptores podem alterar a expressão e produção dessas proteínas (DUNNING et al., 2003; GRAINGER, 1999; HEALY et al., 2009; SHAH et al., 2006; WOOD et al., 2000a), fato que contribuiria para a variabilidade interindividual de resposta no eixo ligante-receptor (GRAINGER, 1999; SHAH et al., 2006), sugerindo uma explicação para a resistência ou suscetibilidade a desenvolver doenças relacionadas a esta citocina.

3.2 POLIMORFISMOS GENÉTICOS DO *TGFB1*

Polimorfismos genéticos são alterações na sequência de DNA que dão origem a dois ou mais alelos para um determinado locus, quando comparadas com uma sequência de referência. Eles são herdados de pai para filho e o alelo menos

frequente (alelo raro) está presente na população numa frequência igual ou superior a 1% (KARKI et al., 2015). No entanto, segundo Den Dunnen e colaboradores (2016), a *Human Genome Variation Society* (HGVS) e a *American College of Medical Genetics* (ACMG) recomendam que o termo "polimorfismo" deixe de ser utilizado e termos neutros como "variante", "alteração" ou "mudança" sejam preferidos.

As variantes de sequência de DNA podem acontecer em todo o genoma, incluindo regiões intergênicas, codificantes (codificam proteínas), regulatórias (controlam a expressão gênica) e intrônicas (separam regiões codificantes ou éxons dentro de um gene). Dependendo da localização, elas podem modificar a expressão gênica e a produção, estrutura e função das proteínas (BALASUBRAMANIAN et al., 2004).

Dentre as variantes de sequência, as variações de nucleotídeo único (SNVs) ou polimorfismos de nucleotídeo único (SNPs, como foram chamados até o momento) são os mais comuns e são caracterizados pela substituição (>) de um nucleotídeo por outro. As SNVs são utilizadas como "assinaturas genéticas" na população que permitem estudar a suscetibilidade para certas características que os indivíduos apresentam, incluindo as doenças (BALASUBRAMANIAN et al., 2004; KARKI et al., 2015).

Várias variações genéticas na sequência do gene *TGFB1* foram descritas (CEBINELLI et al., 2016). Duas SNVs em região promotora (c.-1638G>A e c.-1347T>C) e duas no peptídeo sinal (c.29C>T e c.74G>C) têm sido associadas com a suscetibilidade a certos tipos de cânceres (FAN et al., 2014; JIN et al., 2008; POOJA et al., 2013; WEI et al., 2007b).

A SNV c.-1638G>A (rs1800468 ou G-800A) é caracterizada pela troca de uma guanina (G) por uma adenina (A) na posição -1638 em relação à primeira base (+1) da região codificante (c.). O alelo variante (-1638A) está associado com a diminuição da expressão de *TGFB1* (GRAINGER, 1999; JIN et al., 2004). Na variação c.-1347T>C (rs1800469 ou T-509C), a substituição de uma timina (T) por uma citosina (C) está relacionada com a diminuição dos níveis plasmáticos de TGFB1 (GRAINGER, 1999; GUO et al., 2011; SHAH et al., 2006).

A SNV c.29C>T (rs1800470 ou Pro10Leu) ocorre em região codificante do DNA. Está localizada a 29pb posteriores à primeira base (+1) da região codificante, no códon 10 do peptídeo sinal (SHAH et al., 2006). A troca de C por T resulta na substituição de uma prolina (Pro) por uma leucina (Leu) na proteína. Da

mesma forma, a troca de G por C na variação c.74G>C (rs1800471 ou Arg25Pro) acarreta a substituição de uma arginina (Arg) por uma prolina (Pro) na posição 25 da proteína. Os alelos c.29C e c.74G produziram maior secreção do TGFB1 *in vitro* (AWAD et al., 1998; DUNNING et al., 2003; GU et al., 2012) e têm mostrado forte relação com o aumento da concentração sérica desta citocina (GUO et al., 2011; POOJA et al., 2013; TAUBENSCHUSS et al., 2013; YOKOTA et al., 2000). O peptídeo sinal é uma sequência de 29aa que sinaliza a exportação da proteína em síntese para as membranas do retículo endoplasmático. Estruturalmente possui três regiões: uma região N-terminal carregada positivamente, um núcleo hidrofóbico central e uma região polar C-terminal. Existem especulações de que modificações na composição de aminoácidos do peptídeo sinal poderiam afetar a sua polaridade e provocar diferenças nas taxas de exportação da proteína para o retículo endoplasmático (WOOD et al., 2000a), explicando a maior produção de TGFB1 na presença de 29C e 74G.

Estas variações no gene *TGFB1* têm sido associadas com vários tipos de cânceres, como os de: mama (DUNNING et al., 2003; POOJA et al., 2013; VITIELLO et al., 2018; XU et al., 2011; ZHANG et al., 2011; ZIV et al., 2001), próstata (EWART-TOLAND et al., 2004; LI et al., 2004), pulmão (FAN et al., 2014), cabeça e pescoço (CARNEIRO et al., 2013; GUAN et al., 2010; WEI et al., 2007a), esôfago (JIN et al., 2008; WEI et al., 2007b), estômago (CHANG et al., 2014; GUO et al., 2011; JIN et al., 2007; XU et al., 2011) e colorretal (LIU et al., 2012).

Por outro lado, a relação destas SNVs com a infecção pelo HPV ou com o câncer de colo do útero ainda não está bem estabelecida. Apenas dois estudos observaram diferenças de suscetibilidade ao câncer associada com a variação c.-1638G>A (RAMOS-FLORES et al., 2013) e com a c.-1347T>C (SINGH; JAIN; MITTAL, 2009), enquanto um estudo verificou a associação das SNVs c.29C>T e c.74G>C com a infecção e com o desenvolvimento de lesões (TRUGILO et al., 2019). Portanto, estudos adicionais são necessários para melhor compreensão do papel destas variações genéticas, isoladas ou combinadas em haplótipos, na patogênese do câncer cervical e na produção do TGFB1 no plasma e no microambiente cervical.

Objetivos

4 OBJETIVOS

4.1 OBJETIVO GERAL

Este trabalho teve como objetivo geral avaliar o papel das variações genéticas c.-1638G>A, c.-1347T>C, c.29C>T e c.74G>C do *TGFB1* na infecção pelo HPV e no desenvolvimento das lesões intraepiteliais cervicais e do câncer invasivo e níveis plasmáticos e cerivais de TGFB1.

4.2 OBJETIVOS ESPECÍFICOS

- Realizar a detecção do HPV em amostras de mulheres atendidas pelos programas de prevenção contra o cânder de colo do útero do Sistema Único de Saúde (SUS) na região norte do Paraná;

- Analisar a relação da infecção pelo HPV e das lesões cervicais com dados sociodemográficos destas mulheres, como idade, etnia e hábito tabagista, e com variáveis sexuais como menarca e idade da primeira relação sexual;

- Avaliar a frequência dos alelos, genótipos e haplótipos das variações genéticas c.-1638G>A, c.-1347T>C, c.29C>T e c.74G>C do *TGFB1* nestas mulheres;

- Avaliar a suscetibilidade destas mulheres à infecção pelo HPV e ao desenvolvimento de lesões de baixo e alto grau e do câncer cervical associada aos genótipos e haplótipos das variações genéticas c.-1638G>A, c.-1347T>C, c.29C>T e c.74G>C do *TGFB1*;

- Verificar os níveis de TGFB1 no sangue periférico e no microambiente cervical destas mulheres, comparando os grupos de mulheres não infectadas e infectadas pelo HPV e de infectadas sem lesão, com lesão de baixo grau e com lesão de alto grau;

- Investigar o impacto das estruturas haplotípicas das variações genéticas c.-1638G>A, c.-1347T>C, c.29C>T e c.74G>C do *TGFB1* sobre os níveis plasmáticos e cervicais destas mulheres (não infectadas e infectadas pelo HPV e infectadas sem lesão, com lesão de baixo grau e com lesão de alto grau).

Produção Científica

5 PRODUÇÃO CIENTÍFICA

5.1 ARTIGO 1

<u>Haplotype structures and protein levels of *TGFB1*: candidate susceptibility marker for HPV infection</u>

ABSTRACT

Background: human papillomavirus (HPV) is the main agent for cervical lesions development. However, the immune response presents a considerable impact on the infection outcome. In this context, transforming growth factor-beta 1 (TGFB1) plays a dual role, acting as a suppressor or favoring lesions' progression. Since genetic variants may influence TGFB1 production levels, the present study objective was to verify the role of *TGFB1* variants (c.–1638G>A, c.–1347C>T, c.29C>T and c.74G>C) and their haplotype structures on HPV infection susceptibility and cervical lesions development, and to analyze if the haplotype influence TGFB1 cervical and plasma levels.
Methods: *TGFB1* genotypes were assessed by PCR followed by enzymatic digestion and haplotype were inferred for 190 HPV-uninfected and 161 HPV-infected women. TGFB1 plasma and cervical levels were determined by immunofluorimetric assay. Case-control analyses were performed by logistic regression adjusted for possible confounders.
Results: the –1347T and 29C alleles and the 29CC genotype were more frequent in HPV-infected than in uninfected women (40.1%, 50.0%, 24.8% and 32.1%, 39.7%, 17.4%, respectively). Logistic regression showed that women carrying –1347TT or –1347CT+TT were more likely to have HPV than –1347CC ones and women carrying 29CT, 29CC, or 29CT+CC, were more susceptible to HPV infection when compared to 29TT. Combining the variants, 8 haplotype structures were inferred, with the most probable ancestral being **1 PAN* and the most frequent haplotypes (over 5%) being **4* (48.3%), **3* (34.2%), **5B* (6.0%), and **2* (5.7%). Among them, women **4*/**4* were less likely to have HPV than those with no copy of **4*. Comparing **3* and **4*, women carrying **3*/**4* or **3*/**3* were more susceptible to HPV than **4*/**4*. The TGFB1 plasma and cervical levels were higher in the infected patients. Plasma levels were also higher in the infected women with low-grade lesions compared to the no lesion group. HPV-infected patients carrying **3*/*Other* and **3*/*Other* +**3*/**3* presented lower TGFB1 plasma levels than those with **3* no copy.
Conclusions: the current study suggests c.–1638G>A, c.–1347C>T, c.29T>C, and c.74G>C haplotype structures in the *TGFB1* gene as a possible candidate susceptibility *loci*.

Keywords: polymorphism, rs1800468, rs1800469, rs1800470, rs1800471

BACKGROUND

The cervical epithelium infection by high-risk human papillomaviruses (HR-HPVs) is necessary for cervical cancer development, as well as the local immune response, is an important determinant of progression and disease outcome (BOSCH; DE SANJOSÉ, 2007). Cytokines play a crucial role in mounting and maintaining immune responses against a host of pathogens, including viral infections and tumors (HARDIKAR *et al.*, 2015).

In this context, we highlight the transforming growth factor-beta 1 (TGFB1), a pleiotropic cytokine that plays an important role in several biological processes, including cell replication, differentiation, apoptosis, angiogenesis, and immune system regulation (CHIN *et al.*, 2004; KUBICZKOVA *et al.*, 2012). Its signaling pathway has also been established as essential for cancer progression, because of its prominent role in the regulation of cell growth, differentiation, and migration (PICKUP; NOVITSKIY; MOSES, 2013).

The *TGFB1* gene is located in the 19q13.2 chromosomal region, comprises 7 exons separated by 6 very large introns (DERYNCK *et al.*, 1987), and presents various sequence variations that can be classified as functional, non-functional, or with undetermined function. Until now, 8 single nucleotide variations (SNVs) and one deletion/insertion variant have been reported to be associated with a functional impact on TGFB1 production (CEBINELLI *et al.*, 2016). Among them we highlight rs1800468 (c.–1638G>A, G–800A, g.4245G>A) and rs1800469 (c.–1347C>T, C–509T, g.4536C>T), both located on the *TGFB1* promoter region, and rs1800470, on codon 10 (c.29C>T, Pro10Leu, g.5911C>T), and rs1800471, on the codon 25 (c.74G>C, Arg25Pro, g.5956G>C), both on the signal peptide sequence.

Genetic variations may alter gene expression, messenger RNA (mRNA) stability, alternative splicing, microRNA target sequence, protein exportation to endoplasmic reticulum via signal peptides, or alter protein function when an amino acid is changed (SHASTRY, 2009).

Much has been discovered about the role of TGFB1 in HPV infection, such as the immunosuppression caused by TGFB1 favoring infection or the cytokine increasing by the action of viral oncoproteins. However, many pieces are lacking for complete elucidation of the mechanisms involving TGFB1 participation in the infection, intraepithelial lesion development, and cervical cancer establishment. Therefore, looking for one of these pieces, this work analyzes four genetic variations of *TGFB1*

(c.–1638G>A, c.–1347C>T, c.29C>T c.74G>C) and their haplotype structures in HPV-uninfected and infected patients, and in patients who developed or did not suffer premalignant lesions caused by HPV, as well as to verify the haplotype structures impact on plasma and cervical TGFB1 levels.

MATERIALS AND METHODS

PATIENTS AND SAMPLES

The present study enrolled 351 women who underwent outpatient cytology testing between 2013 and 2015 at an ambulatory colposcopy facility of the Intermunicipal Consortium of Health of the Middle Paranapanema, at the University Hospital and Clinic Center of the State University of Londrina, and Basic Healthcare Units in Londrina-PR, Brazil. Initially, participants signed an informed consent form and were interviewed using a structured questionnaire. After cervical sample collection, cytobrushes were stored in 2 mL of TE buffer [10 mM Tris–HCl, 1 mM ethylenediamine tetra-acetic acid (EDTA) pH 8.0]. Peripheral blood was drawn into sterile syringes containing EDTA as an anticoagulant. Cervical and blood samples were kept at 4 ºC for a maximum of 4 hours, enough time to transport them to the laboratory and start the analysis. As soon as possible, 200 µL of peripheral blood were intended for DNA extraction and the remaining had the plasma separated and stored at –20 °C until TGFB1 dosage. The suspension of cervical cells in TE buffer was centrifuged and cervical cells were used for DNA extraction while the supernatant was stored at –20 °C until TGFB1 dosage.

Participants were stratified based on the presence or absence of HPV DNA, as tested by PCR, and based on lesion grade, as determined by cervical cytology.

DNA EXTRACTION

Genomic DNA was obtained from cervical cytobrushes using DNAzol (Invitrogen^{TM}, Carlsbad, CA, USA) according to the manufacturer’s instructions, and stored at − 20 °C until use. Genomic DNA was also extracted from peripheral blood using a Biopur Mini Spin Plus Kit (Biometrix, Curitiba, PR, Brazil). DNA concentration

was measured at 260 nm on a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and purity was assessed by the A260/A280 ratio.

HPV DETECTION BY PCR

HPV was detected by Polymerase Chain Reaction (PCR) using the primers MY09 (5′-CGTCCMAARGGAWACTGATC-3′) and MY11 (5′-GCMCAGGGWCATAA-YAATGG-3′), which are designed to amplify a conserved region of approximately 450 base-pairs (bp) in the HPV L1 gene (GenBank Accession number: AJ236888) (BAUER, 1991). A fragment of human b-globin with a length of 268 bp was co-amplified as an amplification control using primers GH20 (5′-GAAGAGCCAAGGACAGGTAC-3′) and PC04 (5′-CAACTTCATCCACGTTCACC-3′) (DA SILVA *et al.*, 2012). Reactions without template DNA were used as a negative control to test for contamination, and DNA from HeLa cells, which are stably integrated with HPV18, was used as a positive control. PCR products were electrophoresed on 10% polyacrylamide and stained with silver nitrate.

CERVICAL CYTOLOGY

According to the Bethesda System (2001), Cytology samples were graded at the Public Health System Laboratory. Patients were deemed to have low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL), or no lesions (NL) if cytology samples were normal, i.e., were not indicated as having low- or high-grade squamous intraepithelial lesions, cervical carcinomas, atypical squamous cells of undetermined significance, or other atypical squamous cells that cannot be excluded as high-grade squamous intraepithelial lesions (DA SILVA *et al.*, 2012).

TGFB1 GENETIC VARIANTS GENOTYPING

Genetic polymorphisms were analyzed by PCR followed by Restriction Fragment Length Polymorphism (RFLP) analysis, according to that described by Jin *et al.* (2004) with modifications. All PCR reagents were purchased from Invitrogen™ (Carlsbad, CA, USA), and all restriction enzymes were from New England Biolabs® (Ipswich, MA, USA). Briefly, two primers pairs were designed based on the *TGFB1* gene reference sequence (NCBI gene bank accession number NG_013364.1): one encompassing the two promoter region variants (c.–1638G>A and c.–1347C>T) and

the other encompassing the signal peptide variants (c.29C>T and c.74G>C) (JIN *et al.*, 2004). The PCR conditions for the two reactions were the same. Both reactions were performed in a final volume of 25 µL containing PCR buffer (1x), dNTP (0.1 mM), primers (0.2 µM), $MgCl_2$ (1.0 mM), Taq DNA polymerase (1 U/reaction) and genomic DNA (approximately 3 ng/µL). PCR reactions were performed along with a negative control with no DNA addition to check for exogenous DNA contamination. The sequences of the primers flanking the promoter region polymorphisms were: 5′-GCAGTTGGCGAGAACAGTTG-3′ and 5′-CCAGAACGGA-AGGAGAGTCAG-3′, generating a 597 (bp) amplicon (annealing temperature: 59 °C). The restriction enzyme *HpyCH4IV* was used for enzymatic digestion of c.–1638G>A polymorphisms generating 402 and 195 bp fragments for G allele and *Bsu36I* restriction enzyme was used for c.–1347C>T genotyping, generating 488 and 109 bp fragments for C allele. Restriction conditions followed the manufacturer's protocol. Primer sequences for the signal peptide region were: 5′-TTCCCTCGAGGCCCTCCTA-3′ and 5′-GCCGCAGCTTGGACAGGATC-3′. The annealing temperature was set at 62 °C. *MspA1I* restriction enzyme was used to determine c.29C>T genotypes, cleaving the 294 bp amplicon in 161, 67, 40, and 26 bp fragments for T allele, and in 149, 67, 40, 26, and 12 bp fragments for C allele. *BglI* restriction enzyme cleaved this same amplicon in 131, 103, and 60 bp fragments for G allele from c.74G>C polymorphism and in 163 and 131 bp fragments for C allele. Restriction conditions followed the manufacturer's instruction. Amplicons and restriction fragments were analyzed by electrophoresis on polyacrylamide gel (10%) visualized after silver staining.

To confirm the genotyping method accuracy, one individual for each genotype from all genetic variants analyzed was sequenced in a 3500 Genetic Analyzer® (Applied Biosystems) using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Austin, TX, USA) and at least 5% of the total sample were repeated for PCR–RFLP analysis, obtaining 100% of concordance between results.

HAPLOTYPE ANALYSIS

Inference of recombination sites between *TGFB1* SNV alleles of women studied was performed using PHASE software version 2.1.1 (STEPHENS; SCHEET, 2005; STEPHENS; SMITH; DONNELLY, 2001). The web-based application SNPstats (Catalan Institute of Oncology, Barcelona, Spain) (https://www.snpstat.net) was used to analyze linkage disequilibrium between *TGFB1* genetic variants (MACHIELA;

CHANOCK, 2015; SOLÉ *et al.*, 2006). The haplotype tree was constructed using the Maximum Parsimony method in MEGA7 (https://www.megasoftware.net/).

TGFB1 LEVELS

Venous blood samples obtained with anticoagulant EDTA were centrifuged at 3000 rpm for 15 min for plasma obtaining and stored at −20 °C until use. Cervical mucus collected by cytobrush was suspended in TE buffer, centrifuged at 3000 rpm for 15 min, and recovered supernatants were stored at –20 ºC. TGFB1 levels were determined using microspheres immunofluorimetric assay (Novex™, Life Technologies, Frederick, MD, USA) for Luminex platform (MAGPIX™, Luminex Corp., Austin, TX, USA), that was performed according to the manufacturer's instructions and its reference value. Total protein in the supernatant normalized cervical TGFB1 levels and results were expressed as pg/mg of total protein. Plasma levels were reported as pg/mL.

STATISTICAL ANALYSIS

Analyses of contingency tables by Pearson's Chi-square (χ2) test were used to evaluate differences in the frequency distributions of selected socio-demographic and clinical categorical variables and *TGFB1* 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. We assessed the differences in continuous variables between groups using the Mann–Whitney test or Kruskal-Wallis test with Dunn-Bonferroni's post hoc test to identify differences between groups. Categorical variables were expressed as absolute number (n) and percentage (%) and continuous variables were expressed as the median and interquartile range (IQR). Binary and multinomial logistic regression controlled by possible 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 and SIL) as dependent variables. Adjusted odds ratios (ORs) and 95% confidence intervals (CI) were estimated. All tests were two-tailed, with a p-value (*P*) < 0.05 considered statistically significant. Statistical analyses were carried out using SPSS Statistics 25.0 software (SPSS, Inc., Chicago, IL, USA).

RESULTS

SAMPLE CHARACTERIZATION ACCORDING TO HPV INFECTION, SOCIO-DEMOGRAPHIC, AND CLINICAL DATA

First, 351 women were included in the study and categorized as HPV-infected (161/45.9%) and HPV-uninfected (HPV control group - 190/54.1%). The HPV-infected women were divided into three groups based on cytological abnormalities detected and classified according to the Bethesda System classification as follows: no cervical lesion (NL, lesion control group - 80/51.3%), LSIL (23/14.7%), and HSIL (53/34.0%) groups. For lesion analyses, 5 patients were excluded, 1 woman without cervical cytology result and 4 women diagnosed with cervical cancer (Figure 1).

Patients' features such as age, age at menarche, age at first sexual intercourse, pregnancies, oral contraceptive usage, marital status, sexual partners during the lifetime, and smoking status are summarized in Table 1. In this, a relevant higher proportion of infected women compared to uninfected were observed under the age of 35 years, with 2.29 times more likely to have HPV than those over 35 years of age (OR = 2.29; $CI_{95\%}$ = 1.35 – 3.90). About lesion grades groups (infected women), differences in age range distribution were not observed. However, these women with 3 or more pregnancies were more likely to have LSIL (OR = 5.64; $CI_{95\%}$ = 1.56 – 20.39) and HSIL (OR = 3.30; $CI_{95\%}$ = 1.31 – 8.28) than those with up to 2 pregnancies, as well as having less than 3 lifelong sexual partners decreased the chances of HSIL (OR = 0.41; $CI_{95\%}$ = 0.17 – 0.99) compared to those who had 3 or more partners.

DISTRIBUTION OF ALLELES, GENOTYPES, AND HAPLOTYPES OF *TGFB1* GENETIC VARIATIONS AND SUSCEPTIBILITY TO HPV INFECTION AND CERVICAL LESIONS

Alleles and genotype distribution are in Table 2. Women were genotyped for *TGFB1* SNVs c.–1638G>A, c.–1347C>T, c.29C>T, and c.74G>C. Only one sample (HPV-infected) could not be genotyped for c.–1347C>T SNV, and was excluded from further analyses involving this genetic variation. For each SNV, all groups were tested for Hardy-Weinberg Equilibrium and no deviation from expected genotype frequencies

was found (P > 0.05). Additionally, higher linkage disequilibrium was observed between c.–1347C>T and c.29C>T (D' = 0.95, r^2 = 0.63) (Figure 2).

Differences in allele and genotype distributions between groups were assessed by the $\chi 2$ test. Differences were noted regarding the c.–1347C>T (*P* = 0.028) and c.29C>T (*P* = 0.006) allele distribution, and c.29C>T genotype distribution (*P* = 0.023) between the infection groups but not the lesion grades groups. The –1347T and 29C alleles and the 29CC genotype were more frequent in HPV-infected than in uninfected women (40.1%, 50.0%, 24.8% and 32.1%, 39.7%, 17.4%, respectively).

Combinations of investigated variants resulted in 8 inferred haplotype structures, two of them possibly recombinant (Figure 3). According to the degree of sequence identity with the *Pan troglodytes TGFB1* gene sequence, the most probable ancestral haplotype (named as **1 PAN*) is formed by c.–1638G, c.–1347C, c.29C and c.74G alleles (for short, GCCG*)*. Differences between groups were found in the frequencies of **4* (GCTG) when infected and uninfected women were compared (*P* = 0.003) (Table 3). For further analysis, only haplotypes over 5 % frequency across the study population were tested; they are **4* (GCTG, 48.3 %), **3* (GTCG, 34.2 %), **5B* (ACTG, 6.0 %), and *2 (GCCC, 5.7 %). SNV alleles were represented in haplotype structures according to their position in the *TGFB1* gene, following the order: c.–1638G>A, c.–1347C>T, c.29C>T and c.74G>C.

Binary and multinomial logistic regression adjusted for “age range, age at first sexual intercourse, marital status and sexual partners during lifetime” or “pregnancies, oral contraceptive usage, marital status, sexual partners during the lifetime and smoking status” was conducted to assess the influence of SNVs on the susceptibility to HPV infection and development of low and high-grade lesions, respectively (Table 4). Relevant influences were only observed for c.–1347C>T, c.29C>T, and **4* haplotype regarding the HPV infection. Both women carrying c.–1347TT and women with –1347CT or TT were more likely to have HPV than –1347CC ones, with respective odds ratios and confidence intervals (95%) of 2.16 (1.10 – 4.25) and 1.62 (1.03 – 2.54). Susceptibility to infection was also greater among women carrying 29CT, 29CC, or 29CT+CC when compared to 29TT, with odds ratios and confidence intervals (95%) of 1.77 (1.06 – 2.97), 2.31 (1.23 – 4.34), and 1.92 (1.18 – 3.12), respectively. Regarding haplotypes, women with 2 copies (homozygotes) of **4* (GCTG) were less likely to have HPV compared to women with no copy of **4* (OR = 0.39, $CI_{95\,\%}$ = 0.21 – 0.72). Furthermore, **3* (GTCG) in comparison to the **4* (the two more

frequent haplotypes) evidenced higher susceptibility to HPV infection to women carrying *3/*4 or *3/*3 than *4/*4 (OR = 2.13, $CI_{95\%}$ = 1.13 – 4.00, and OR = 2.81, $CI_{95\%}$ = 1.29 – 6.10, respectively).

IMPACT OF THE *TGFB1* HAPLOTYPES ON PLASMA AND CERVICAL LEVELS OF PROTEIN

After having observed the influence of *TGFB1* genetic variations on susceptibility to HPV infection, their impact on plasma and cervical TGFB1 levels was evaluated.

Initially, plasma and cervical levels were found to be higher in infected patients [4575.19 (IQR 4392.34) pg/mL and 53.17 (IQR 56.46) pg/mg of total protein, respectively] than in uninfected [2964.80 (IQR 3091.45) pg/mL and 32.57 (IQR 54.49) pg/mg of total protein, respectively] ($P < 0.001$ and $P = 0.008$, respectively). However, among the lesion groups, there was a difference only in plasma levels ($P = 0.007$), whose group of women with LSIL presented higher TGFB1 levels than the NL group [6653.45 (IQR 5098.44) and 3689.42 (IQR 3383.84) pg/mL, respectively, $P = 0.010$] (Table 5). Thus, TGFB1 plasma levels were investigated in the uninfected and HPV-infected women groups according to the haplotype structure inheritance (Table 6). The difference was observed in the HPV-infected group regarding the *3 (GTCG) haplotype. Comparing patients *3 no carriers, homozygotes (*3/*3) and heterozygotes (*3/Other), heterozygotes presented a lower amount of TGFB1 than those with *3 no copy [3067.13 (IQR 4200.20) pg/mL and 4836.23 (IQR 4313.38) pg/mL, respectively, $P = 0.03$]. There was also a lower TGFB1 level in *3 carriers (homozygotes + heterozygotes) when compared with *3 no carriers [3993.99 (4173.05) and 4836.23 (4313.38), respectively, $P = 0.04$]. For TGFB1 cervical levels, there was no difference neither in uninfected nor in HPV-infected women concerning the haplotype inheritance (Table 7). The results above were very similar when analyses were done after outliers exclusion.

DISCUSSION

As far as we are aware, this was the first study to address the haplotypes structures of these *TGFB1* genetic variations in the HPV infection and the development of low- and high-grade cervical lesions. It is also the first time to assess the impact of

TGFB1 haplotypes on cervical and plasma TGFB1 levels according to the disease context.

This case-control study comprised 351 women, of whom 161 were HPV-infected and 190 were not. The presence of HPV in the uterine cervix may lead to the development of the intraepithelial lesions and, therefore, only the infected group was included in the cervical lesions analyses.

Among the extrinsic factors to HPV-infection, as younger age, or to cervical intraepithelial lesions, as the high number of pregnancies and sexual partners were in agreement with other studies (COSER et al., 2016; DE SANJOSÉ et al., 2007). Genetic factors have also been associated with HPV infection in the cervical microenvironment, especially variations in the genes of immune system components. This research group has found an association between HPV infection and genetic variation in *FOXP3* (CEZAR-DOS-SANTOS *et al.*, 2019), *CXCL12* (OKUYAMA *et al.*, 2018), *IL-10* (BERTI *et al.*, 2017), and *TGFB1* (TRUGILO *et al.*, 2018).

TGFB1 gene regulation and expression levels are affected by the presence of SNVs in the gene locus (SHAH; HURLEY; POSCH, 2006). The c.–1638G>A SNV is located in the enhancer region 1. Reduced affinity for cAMP response element-binding protein (CREB) family in the presence of allele A is associated with lower TGFB1 levels (GRAINGER *et al.*, 1999).

The c.–1347C>T variation is located in the first negative regulatory region and T allele carriers have almost double plasma levels in comparison to C allele carriers. Furthermore, as reviewed by CEBINELLI and colleagues (2016), several *in vitro* studies using *TGFB1* promoter-luciferase reporter plasmids demonstrated that the T allele increases relative luciferase activity, compared to the C allele. One hypothesis is the loss of negative regulation by the T allele increasing *TGFB1* transcription. It was also reported that the presence of thymine instead of cytosine at this locus increases the bind of transcription factor Yin-Yang 1 (YY1) and hence transcriptional activity.

The c.29C>T and c.74G>C SNVs are located in the signal peptide sequence and cause amino acid substitutions, proline to leucine, and arginine to proline exchanges at positions 10 and 25, respectively. Modifications in the signal peptide amino acid composition could affect its polarity and result in different rates of protein export (WOOD *et al.*, 2000). Alleles 29C and 74G have been shown to increase TGFB1 serum concentration (AWAD *et al.*, 1998; TAUBENSCHUSS *et al.*, 2013; YOKOTA *et al.*, 2000).

Concerning the frequency of the investigated variants, the HPV-uninfected group (which were also cervical lesion-free) presented the minor allele frequencies very similar to the Southern European population as reported in the Genome Aggregation Database (gnomAD), using the dataset gnomAD v2.1.1 (Controls) (https://gnomad.broadinstitute.org/; KARCZEWSKI *et al.*, 2020). Although the high miscegenation observed in the Brazilian population, these data evidence the Southern European ancestry in the current Southern Brazilian cohort, a region which was mainly colonized by the European population (LINS *et al.*, 2009; PARRA *et al.*, 2003).

Previously in HPV infection, Trugilo and colleagues (2018) evaluated only two genetic variations in *TGFB1*, c.29C>T and c.74G>C. They found a higher frequency of 29CC and 74GC genotypes in the infected patients than in the uninfected, with the 29CC /74GC combined genotypes increasing the infection susceptibility. In the current study, 29CC, CT, and CT+CC were independently associated with greater susceptibility to HPV infection than 29TT carriers were. Similarly, Guan and colleagues (2010) reported that American male and female patients with 29CC genotype were more likely to have HPV16-positive squamous cell carcinoma of the oropharynx than 29TT carriers. They also observed that even statistically non-significant, 74GG genotype distribution was slightly more frequent in HPV16-positive tumor patients than HPV-negative ones. Diversely, other studies in different Brazilian regions with smaller sample sizes evaluated these two genetic variants in HPV-infected and uninfected patients but found no association (FERNANDES *et al.*, 2008; LIMA JÚNIOR *et al.*, 2016; MARANGON *et al.*, 2013).

The –1347T allele was associated with infection, as well as the –1347TT and CT+TT were in the adjusted analysis. Differently, Guan and colleagues (2010), in the same study previously mentioned, observed that c.–1347C>T genotypes had a similar distribution between the HPV16-positive tumor patients and HPV16-negative control group. Further, Singh, Jain, and Mittal (2009) found that in Indian cervical cancer patients and no cervical lesion controls, the c.–1347C>T allele frequencies were quite similar.

However, phenotype could be better explained in a natural context by a set of genetic variations, such as the haplotypes, rather than variations studied in isolation. Here, four *TGFB1* SNVs were analyzed and eight haplotype structures were inferred to the current study population. The frequency of haplotypes in the HPV-uninfected group (control) was similar to that found by Vitiello and colleagues (2018) in their

cancer-free control group that was part of a study carried out in the same Brazilian region. This agreement may represent with confidence the distribution of *TGFB1* haplotypes in this population.

Of all the eight haplotype structures, **4* (GCTG) was more frequent among uninfected women, being independently associated with protection against HPV infection, as were the single alleles –1347C and 29T harbored by it. The protective effect remained when **4* was compared with **3* (GTCG). Thus, could any haplotype influence protein levels in such a population?

Firstly, plasma and cervical levels of TGFB1 were measured (Table 5). Increased levels were observed in HPV-infected patients (plasma and cervical secretion) and the LSIL group (plasma). HPV and TGFB1 have been closely related: 1) in epithelial cells containing HPV DNA, E6, and E7 oncoproteins can interact with the specificity protein 1 transcription factor (Sp1) and form the E6-Sp1 and E7-Sp1 complexes which can migrate into the nucleus and induce the *TGFB1* gene expression (PERALTA-ZARAGOZA *et al.*, 2006; TORRES-POVEDA *et al.*, 2014); 2) on the other hand, TGFB1 suppresses the LCR-driven transcriptional activity and downregulates at the transcriptional level the early HPV16 expression genes (KOWLI *et al.*, 2013; WOODWORTH; NOTARIO; DIPAOLO, 1990). In addition to the infected cell, $TCD4^+$ cells of cervical tissue also produce TGFB1. Bonin and colleagues (2019) observed that double labeling ($CD4^+$/TGFB) by immunohistochemistry was higher in cervical tissue with a high viral load than in uninfected tissue. At the same time, they saw that the double labeling $CD25^+$/$FOXP3^+$ was also higher in the infected cervix. Evidence suggests that $CD4^+$ $CD25^+$ $FOXP3^+$ T regulatory (Treg) cells may play an important role in an immune-tolerant microenvironment and the failure of HPV elimination. Not only were $CD25^+$/$FOXP3^+$ Tregs producing TGFB, but also TGFB itself induces the conversion of $FOXP3^-$ T cells into $FOXP3^+$ T cells (ANSA-ADDO *et al.*, 2017). It is well known that TGFB1 has a growth-inhibitory effect both on normal epithelial cells and on cells of the immune system, such as pro-inflammatory T-cells (PAPAGEORGIS, 2015). Thus, TGFB seems to be compromised with an immunosuppressed microenvironment, which could favor the persistence of HPV infection.

Looking for an answer to the question above, the association between *TGFB1* haplotypes and protein levels was assessed in uninfected and infected women. No differences in the TGFB1 plasma and cervical levels were found in uninfected ones according to the haplotype inheritance. Otherwise, **3*/Other patients and **3* carriers

(*3/*3 + *3/Other) presented lower TGFB1 plasma levels than patients *3 no carriers (Other/Other) in the infected group. Surprisingly, *3 haplotype harbors the –1638G, –1347T, 29C and 74G alleles that were associated with higher TGFB1 production compared to their respective alternative alleles (AWAD *et al.*, 1998; GRAINGER *et al.*, 1999; TAUBENSCHUSS *et al.*, 2013; YOKOTA *et al.*, 2000). However, a possible explanation is that TGFB1 production could be more strongly affected by another unevaluated variation in linkage disequilibrium, inside or outside the gene; or another possibility is that the presence of the –1347T and/or 29C alleles cause a change in affinity for the E6-Sp1 / E7-Sp1 complexes. As discussed earlier, these complexes increase *TGFB1* expression, and thus, if the affinity for them is weakened, TGFB1 production could be reduced.

Although the small number of patients with LSIL and the exclusion of patients with cervical cancer may have limited the sensitivity of the cervical lesion grade analyses in the current study, the strengths lie in the analysis of haplotypes and the adjustment for potential confounding factors. To our knowledge, this is the first time that *TGFB1* haplotype was associated with HPV infection: *4/*4 conferring protection against HPV infection as well as *3/*4 and *3/*3 increasing susceptibility to HPV compared to *4/*4 patients. Furthermore, in the HPV-infected group, *3/Other patients and *3 carriers (*3/*3 + *3/Other) were associated with lower TGFB1 plasma levels in comparison to patients with no *3 copy (Other/Other). Although further studies are warranted to confirm the results, the current study suggests c.–1638G>A, c.–1347C>T, c.29T>C, and c.74G>C haplotype structures in the *TGFB1* gene as a possible candidate susceptibility marker.

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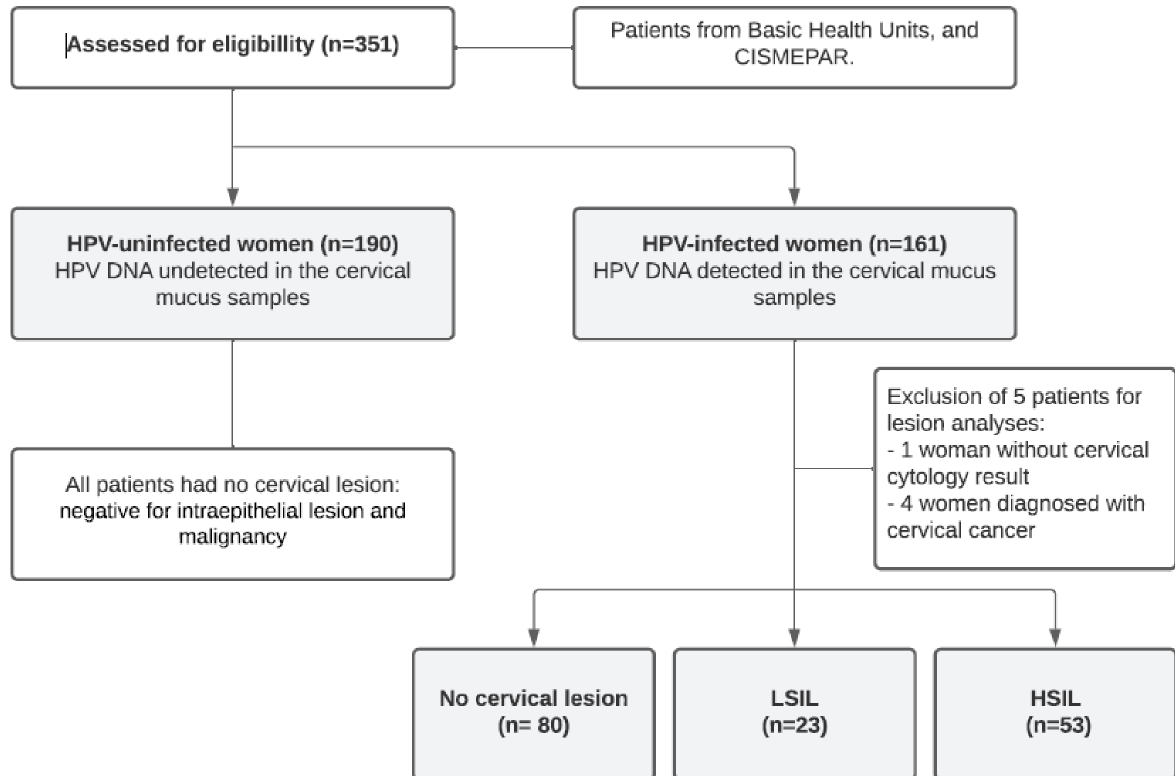

Figure 1. Study flow chart.

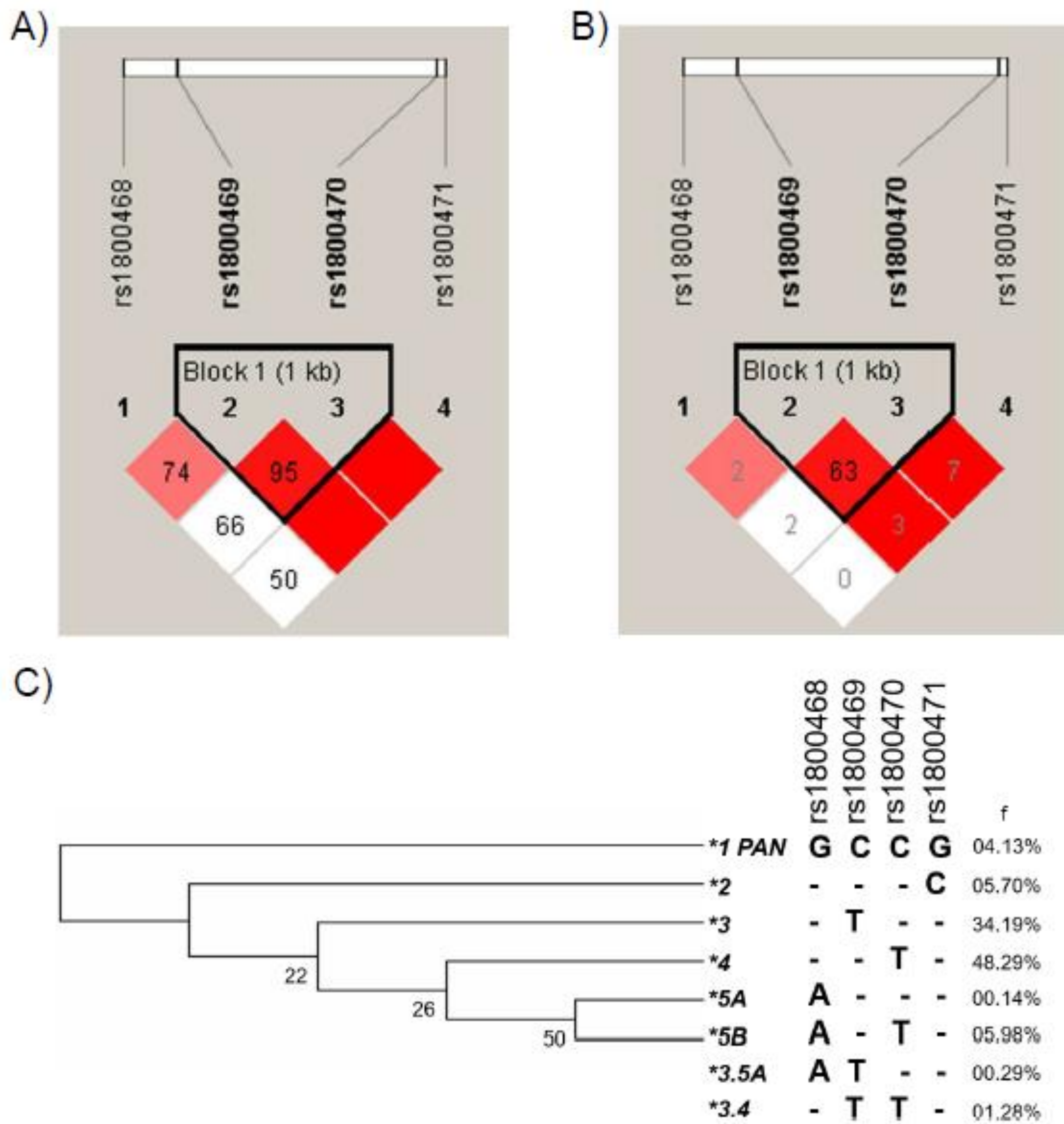

Fig. 2 Heatmap linkage disequilibrium and the maximum parsimony analysis of taxa – Values for D’ (A) and r^2 (B). The evolutionary history was inferred using the maximum parsimony method (C). *TGFB1* SNVs: rs1800468 (c.–1638 G>A), rs1800469 (c.–1347 C>T), rs1800470 (c.29 T>C) and rs1800471 (c.74 G>C).

Table 1 Age, smoking status and ethnicity regarding HPV infection and lesion grade status.

Characteristics		HPV			Lesion grade (Infected patients)				
		Uninfected (n=190)	Infected (n=161)	OR ($CI_{95\%}$)	NL (n=80)	LSIL (n=23)	HSIL (n=53)	OR_{LSIL} ($CI_{95\%}$)	OR_{HSIL} ($CI_{95\%}$)
Age range (years)	< 35	57 (30.0)	80 (49.7)	**2.29 (1.35-3.90)**	40 (50.0)	12 (52.2)	26 (49.1)	1.79 (0.51-6.35)	1.16 (0.45-2.95)
	≥ 35	133 (70.0)	81 (50.3)	Reference	40 (50.0)	11 47.8)	27 (50.9)	Reference	Reference
Age at menarche (years)	< 13	86 (45.5)	84 (52.2)	1.16 (0.74-1.83)	42 (52.5)	11 (47.8)	29 (54.7)	Reference	Reference
	≥ 13	103 (54.5)	77 (47.8)	Reference	38 (47.5)	12 (52.2)	24 (45.3)	1.26 (0.45-3.54)	0.79 (0.37-1.71)
Age at first sexual intercourse (years)	< 18	92 (48.4)	99 (61.5)	Reference	47 (58.8)	16 (69.6)	32 (60.4)	Reference	Reference
	≥ 18	98 (51.6)	62 (38.5)	0.89 (0.54-1.46)	33 (41.3)	7 (30.4)	21 (39.6)	0.87 (0.27-2.74)	1.37 (0.60-3.16)
Pregnancies	< 3	108 (56.8)	95 (59.0)	Reference	56 (70.0)	10 (43.5)	28 (52.8)	Reference	Reference
	≥ 3	82 (43.2)	66 (41.0)	1.27 (0.76-2.12)	24 (30.0)	13 (56.5)	25 (47.2)	**5.64 (1.56-20.39)**	**3.30 (1.31-8.28)**
Oral contraceptive usage	No	132 (69.5)	105 (65.2)	Reference	56 (70.0)	13 (56.5)	32 (60.4)	Reference	Reference
	Yes	58 (30.5)	56 (34.8)	0.96 (0.57-1.62)	24 (30.0)	10 (43.5)	21 (39.6)	3.11 (0.97-9.90)	1.83 (0.78-4.29)
Marital status	Married[a]	139 (73.2)	97 (60.2)	Reference	48 (60.0)	10 (43.5)	36 (67.9)	Reference	Reference
	Single[b]	51 (26.8)	64 (39.8)	1.58 (0.98-2.56)	32 (40.0)	13 (56.5)	17 (32.1)	2.47 (0.85-7.15)	0.59 (0.26-1.37)
Sexual partners during the lifetime	< 3	111 (58.4)	65 (40.4)	0.63 (0.39-1.00)	37 (46.3)	10 (43.5)	17 (32.1)	1.29 (0.41-4.05)	**0.41 (0.17-0.99)**
	≥ 3	79 (41.6)	96 (59.6)	Reference	43 (53.8)	13 (56.5)	36 (67.9)	Reference	Reference
Smoking status	No	142 (74.7)	114 (70.8)	Reference	62 (77.5)	13 (56.5)	37 (69.8)	Reference	Reference
	Yes	48 (25.3)	47 (29.2)	1.06 (0.64-1.76)	18 (22.5)	10 (43.5)	16 (30.2)	2.33 (0.74-7.31)	1.12 (0.46-2.76)

Data were analyzed by logistic regression, with $P<0.05$ considered significant (bold). “Uninfected” and “no cervical lesion” groups were reference for HPV infection and Lesion grade analysis, respectively. NL, no cervical lesion; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; OR ($CI_{95\%}$), odds ratio with 95% of confidence interval. [a]Married and civil partner. [b]Single, divorced and Widowed.

Table 2 *TGFB1* genetic variations in HPV infection and cervical lesion status.

***TGFB1* SNVs**	**HPV**			**Lesion grade (Infected patients)**			
	Uninfected (n=190)	**Infected (n=161)**	***P***	**NL (n=80)**	**LSIL (n=23)**	**HSIL (n=53)**	***P***
c.–1638G>A							
GG	169 (88.9)	140 (87.0)	0.708	68 (85.0)	23 (100.0)	45 (84.9)	0.213
Ga	19 (10.0)	20 (12.4)		12 (15.0)	0	7 (13.2)	
aa	2 (1.1)	1(0.6)		0	0	1 (1.9)	
Allele G	357 (94.0)	300 (93.2)	0.674	148 (92.5)	46 (100.0)	97 (91,5)	0.136
Allele a	23 (6.0)	22 (6.8)		12 (7.5)	0	9 (8.5)	
c.–1347C>T							
tt	22 (11.6)	28 (17.4)	0.104	10 (12.7)	6 (26.1)	11 (20.8)	0.398
tC	78 (41.1)	73 (45.3)		38 (48.1)	7 (30.4)	24 (45.3)	
CC	90 (47.4)	60 (37.3)		31 (39.2)	10 (43.5)	18 (34.0)	
Allele t	122 (32.1)	129 (40.1)	**0.028**	58 (36.7)	19 (41.3)	46 (43.4)	0.537
Allele C	258 (67.9)	193 (59.9)		100 (63.3)	27 (58.7)	60 (56.6)	
c.29C>T							
cc	33 (17.4)	40 (24.8)	**0.023**	17 (21.3)	5 (21.7)	16 (30.2)	0.768
cT	85 (44.7)	81 (50.4)		41 (51.2)	13 (56.5)	25 (47.2)	
TT	72 (37.9)	40 (24.8)		22 (27.5)	5 (21.7)	12 (22.6)	
Allele c	151 (39.7)	161 (50.0)	**0.006**	75 (46.9)	23 (50.0)	57 (53.8)	0.544
Allele T	229 (60.3)	161 (50.0)		85 (53.1)	23 (50.0)	49 (46.2)	
c.74G>C							
GG	173 (91.1)	138 (85.7)	0.117	71 (88.8)	19 (82.6)	45 (84.9)	0.683
Gc	17 (8.9)	23 (14.3)		9 (11.3)	4 (17.4)	8 (15.1)	
Allele G	363 (95.5)	299 (92.9)	0.128	151 (94.4)	42 (91.3)	98 (92.4)	0.702
Allele c	17 (4.5)	23 (7.1)		9 (5.6)	4 (8.7)	8 (7.6)	

Data presented as absolute number and percentage. Two-sided χ^2 test, with $P<0.05$ considered significant. [#] Yates correction. SNV, single nucleotide variant; NL, no cervical lesion; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion.

Table 3 Comparison of the frequency of *TGFB1* haplotypes in HPV and cervical lesion groups.

***TGFB1* Haplotypes**	**All**	**HPV**			**Cervical lesion grade (Infected patients)**			
	(n=702)	**Uninfected (n=380)**	**Infected (n=322)**	***P***	**NL (n=160)**	**LSIL (n=46)**	**HSIL (n=106)**	***P***
**1 PAN*	0.0413	0.0395	0.0435	0.790	0.0500	0.0435	0.0283	0.685
**2*	0.0570	0.0448	0.0714	0.128	0.0688	0.0870	0.0755	0.914
**3*	0.3419	0.3105	0.3789	0.057	0.3562	0.3695	0.4151	0.620
**4*	0.4829	0.5342	0.4224	**0.003**	0.4375	0.4565	0.3868	0.634
**5A*	0.0014	0.0026	0	1.000	0	0	0	-
**5B*	0.0598	0.0579	0.0621	0.814	0.0750	0	0.0660	0.166
**3.4*	0.0128	0.0105	0.0155	0.739	0.0125	0.0435	0.0094	0.270
**3.5A*	0.0029	0	0.0062	0.210	0	0	0.0189	0.141

Between groups comparison of a haplotype frequency with the sum of the other haplotypes frequency. Two-sided χ^2 test or Fisher Exact test when appropriated, with $P<0.05$ considered significant (bold).

Table 4 Susceptibility for HPV infection and LSIL or HSIL according *TGFB1* genetic variations.

***TGFB1* SNVs**	**Adjusted odds ratio [OR ($CI_{95\%}$)]**		
	HPV Infected	**Lesion grade (Infected patients)**	
		LSIL	**HSIL**
c.–1638G>A			
Ga vs GG	1.13 (0.56-2.26)	-	-
aa vs GG	1.03 (0.09-11.91)	-	-
Ga + aa vs GG	1.12 (0.57-2.19)	-	-
c.–1347C>T			
tC vs CC	1.47 (0.91-2.37)	0.44 (0.14-1.45)	0.95 (0.42-2.17)
tt vs CC	**2.16 (1.10-4.25)***	1.50 (0.39-5.78)	1.76 (0.57-5.38)
tC + tt vs CC	**1.62 (1.03-2.54)***	0.66 (0.23-1.88)	1.11 (0.51-2.43)
c.29C>T			
cT vs TT	**1.77 (1.06-2.97)***	1.52 (0.42-5.44)	1.01 (0.41-2.52)
cc vs TT	**2.31 (1.23-4.34)****	1.33 (0.29-6.07)	1.48 (0.52-4.19)
cT + cc vs TT	**1.92 (1.18-3.12)****	1.46 (0.43-4.96)	1.15 (0.49-2.71)
c.74G>C			
Gc *vs* GG	1.60 (0.80-3.20)	1.67 (0.44-6.38)	1.02 (0.37-2.85)
**4* (GCTG)			
Ht *vs* no copy	0.93 (0.55-1.56)	0.95 (0.30-3.04)	0.84 (0.37-1.90)
Hm *vs* no copy	**0.39 (0.21-0.72)****	1.15 (0.27-4.87)	0.66 (0.21-2.06)
Ht + Hm vs no copy	0.69 (0.42-1.11)	1.00 (0.33-3.00)	0.79 (0.36-1.74)
**3* (GTCG)			
Ht *vs* no copy	1.48 (0.92-2.38)	0.73 (0.24-2.21)	1.02 (0.46-2.28)
Hm *vs* no copy	1.81 (0.91-3.58)§	1.18 (0.28-5.09)	1.62 (0.53-4.95)
Ht + Hm vs no copy	1.56 (1.00-2.43)§	0.83 (0.30-2.30)	1.14 (0.53-2.43)
**5B* (ACTG)			
Ht + Hm vs no copy	1.12 (0.56-2.23)	-	1.16 (0.40-3.37)
**2* (GCCC)			
Ht + Hm vs no copy	1.60 (0.80-3.20)	1.67 (0.44-6.38)	1.02 (0.37-2.85)
**3/*4*			
Ht *vs* *4Hm	**2.13 (1.13-4.00)***	0.83 (0.16-4.19)	1.77 (0.53-5.88)
*3Hm *vs* *4Hm	**2.81 (1.29-6.10)****	1.34 (0.20-8.66)	2.39 (0.61-9.45)

Logistic regression adjusted for “age range, age at first sexual intercourse, marital status and sexual partners during lifetime” (HPV infection analysis) or “pregnancies, oral contraceptive usage, marital status, sexual partners during lifetime and smoking status” (lesion grade analysis), with “uninfected group” or “no cervical lesion group” as reference, respectively. Ht, heterozygote; Hm, homozygote; $CI_{95\%}$, 95% confidence interval. SNVs alleles in haplotype structures follow the order: c.–1638G>A, c.-1347Tt>C, c.29C>T and c.74G>C. Bolded values are significant, with *P<0.05 or **P<0.01. §0.05<P<0.1

Table 5 Plasma and cervical levels of TGFB1 in uninfected and infected women and in cervical lesions.

TGFB1 levels	HPV			Cervical lesion grade (Infected patients)			
	Uninfected	Infected	*P*	NL	LSIL	HSIL	*P*
Plasma (pg/mL)							
Median	2964.8	4575.8	0.001	3689.4[A]	6653.4[A]	4831.5	0.007
(IQR)	(3091.4)	(4405.8)		(3383.8)	(5098.4)	(5668.3)	
Cervical Secretion (pg/mg of proteins)							
Median	32.6	53.2	0.030	52.8	66.7	53.2	0.527
(IQR)	(54.5)	(56.5)		(59.9)	(57.7)	(51.5)	

Mann-Whitney test or Kruskal-Wallis test with Dunn-Bonferroni post-hoc test, with $P<0.05$ considered significant. [A]Significant pairwise comparison ($P<0.05$) in lesion grade categories. NL, no cervical lesion; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; IQR, interquartile range.

Table 6 Plasma of TGFB1 according to *TGFB1* haplotype models in uninfected and infected women.

TGFB1 Haplotypes	TGFB1 plasma level (pg/mL)					
	n	HPV uninfected	*P*	n	HPV infected	*P*
**4* (GCTG)						
Hm	60	3369.30 (3167.17)		28	4762.51 (4959.49)	
Ht	77	2888.33 (2858.02)	0.55	73	4565.70 (4093.63)	0.72
No copy	48	2891.69 (3338.91)		46	4352.38 (4697.96)	
Ht + Hm	137	3004.68 (3076.96)	0.75	101	4576.46 (4289.30)	0.73
No copy	48	2891.69 (3338.91)		46	4352.38 (4697.96)	
**3* (GTCG)						
Hm	22	2344.52 (3189.62)		22	4766.67 (4046.06)	
Ht	72	3084.49 (3667.38)	0.27	63	3067.13 (4200.20)[A]	0.03
No copy	91	3114.03 (3151.74)		62	4836.23 (4313.38)[A]	
Hm + Ht	94	2891.69 (3124.40)	0.45	85	3993.99 (4173.05)	0.04
No copy	91	3114.03 (3151.74)		62	4836.23 (4313.38)	
**5B* (ACTG)						
Hm + Ht	20	3528.98 (3796.48)	0.17	18	2474.66 (4087.90)	0.27
No copy	165	2908.18 (2983.04)		129	4706.97 (4414.91)	
**2* (GCCC)						
Hm + Ht	16	2298.79 (2713.65)	0.45	21	4831.49 (4719.33)	0.45
No copy	169	2974.81 (3205.66)		126	4548.31 (4189.74)	
**3 vs *4*						
**3* Hm	22	2344.52 (3189.62)		22	4766.67 (4046.06)	
Ht	54	2984.74 (3091.14)	0.22	44	3403.58 (3793.59)	0.23
**4* Hm	60	3369.30 (3167.17)		28	4762.51 (4959.49)	
**5B vs *4*						
**5B* Hm	2	4838.50 (---)		-	-	
Ht	7	3023.64 (3337.03)		9	3452.63 (3521.60)	0.39
**4* Hm	60	3369.30 (3167.17)		28	4762.51 (4959.49)	

Data presented as median and interquartile range (IQR). Mann-Whitney test or Kruskal-Wallis test with Dunn-Bonferroni's post hoc, with $P<0.05$ considered significant. Other, any other haplotype.

Table 7 Cervical levels of TGFB1 according to *TGFB1* haplotype models in uninfected and infected women.

TGFB1 Haplotypes	TGFB1 cervical level (pg/mL)					
	n	HPV uninfected	*P*	n	HPV infected	*P*
*4 (GCTG)						
Hm	38	35.28 (50.71)		11	66.67 (112.24)	
Ht	46	29.02 (75.07)	0.87	20	53.17 (49.93)	0.28
No copy	27	36.37 (38.99)		17	47.45 (64.76)	
Ht + Hm	84	31.29 (68.09)	0.88	31	60.33 (49.99)	0.24
No copy	27	36.37 (38.99)		17	47.45 (64.76)	
*3 (GTCG)						
Hm	13	29.42 (31.88)		7	47.45 (64.83)	
Ht	39	29.06 (77.90)	0.68	23	52.85 (42.88)	0.68
No copy	59	35.34 (51.42)		18	63.50 (69.55)	
Hm + Ht	52	29.24 (53.16)	0.43	30	52.82 (55.31)	0.38
No copy	59	35.34 (51.42)		18	63.50 (69.55)	
*5B (ACTG)						
Hm + Ht	9	30.00 (56.74)	0.83	5	68.05 (111.38)	0.87
No copy	102	33.90 (55.08)		43	53.15 (51.66)	
*2 (GCCC)						
Hm + Ht	12	65.05 (86.80)	0.10	5	52.84 (67.79)	0.95
No copy	99	29.42 (53.92)		43	53.19 (58.06)	
*3 vs *4						
*3 Hm	13	29.42 (31.88)		7	47.45 (64.83)	
Ht	30	25.03 (98.54)	0.69	14	53.17 (55.35)	0.45
*4 Hm	38	35.28 (50.71)		11	66.67 (112.24)	
*5B vs *4						
*5B Hm	1	-				
Ht	3	30.00 (---)		3	68.05 (---)	---
*4 Hm	38	35.28 (50.71)		11	66.67 (112.24)	

Data presented as median and interquartile range (IQR). Mann-Whitney test or Kruskal-Wallis test with Dunn-Bonferroni's post hoc, with P<0.05 considered significant. SNVs alleles in haplotype structures follow the order: c.–1638G>A, c.–1347C>T, c.29C>T and c.74G>C. Other, any other haplotype.

5.2 ARTIGO 2

TGFB1 haplotype structures is associated to Cervical Cancer

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ABSTRACT

Cervical cancer development is related to the persistent high-risk HPV infection. However, HPV is not sufficient to malignization and other factors such as genetic and/or epigenetic alterations are needed for the carcinogenesis. For this reason, the study purpose was to assess the influence of four transforming growth factor beta 1 (*TGFB1*) variants c.-1638 G>A, c.-1347 T>C, c.29 C>T, and c.74 G>C and their haplotype structures on cervical cancer. Cervical mucus and blood samples or formalin-fixed-parafin-embedded tumor tissues were collected from 130 HPV-infected women that were classified based on cytology as no cervical lesion (n = 79, controls) and cervical cancer (n = 51). Genotyping for *TGFB1* variations were performed by polymerase chain reaction followed by enzymatic restriction. Initially, none of the genetic variations as single markers was associated with cervical cancer. However, GTCG haplotype in both dominant and recessive models increased the susceptibility to this malignancy (OR= 2.48, $CI_{95\%}$= 1.05-5.83 and OR= 4.73, $CI_{95\%}$= 1.18-19.02, respectively). In conclusion, this study suggests *TGFB1* haplotype structures as potential susceptibility markers to cervical cancer in HPV-infected women.

Keywords: polymorphism, SNP, SNV, HPV, rs1800468, rs1800469, rs1800470, rs1800471

INTRODUCTION

Cervical cancer is the fourth most frequent cancer in women with an estimated 570,000 new cases in 2018 representing 7.5% of all female cancer deaths. Of the

estimated more than 311,000 deaths from cervical cancer every year, more than 85% of these occur in less developed regions (BRAY et al., 2018).

Cervical Cancer is caused by Human papillomavirus (HPV), which is the most common sexually transmitted viral infection worldwide (SATTERWHITE et al., 2013). Infection with high-risk HPV types has been established (biologically and epidemiologically) as the main cause for cervical cancer development (MUÑOZ et al., 2006). However, a persistent high-risk HPV infection is not sufficient to immortalize and transform the epithelial cells, and the presence of genetic and/or epigenetic alterations are needed for the carcinogenesis (FANG; ZHANG; JIN, 2014; SAAVEDRA; BREBI; ROA, 2012). Genetic variation in immune mediators has been shown to be an important determinant in susceptibility to neoplasms, as well as in progression and disease outcome, including the HPV-related epithelial transformation (HARDIKAR et al., 2015).

Since cytokines play a crucial role in mounting and maintaining immune responses against a host of pathogens, including viral infections and tumors, we emphasize the transforming growth factor beta 1 (TGFB1), whose role in cancer has been extensively documented, presenting a dual role. TGFB1 may present either a suppressive or a promoting role in tumor development, depending on tumor stage (PICKUP; NOVITSKIY; MOSES, 2013; ZARZYNSKA, 2014). Furthermore, sequence variations in *TGFB1* may alter its expression and activity (DUNNING et al., 2003; GRAINGER, 1999; HEALY et al., 2009; SHAH et al., 2006; WOOD et al., 2000b).

The *TGFB1* gene presents various polymorphisms that can be classified as functional, non-functional, or with undetermined function. Eight SNVs and one deletion/insertion polymorphism have been reported to be associated with a functional impact on TGFB1 production. Among them rs1800468 (c.-1638G>A), rs1800469 (c.-1347T>C), rs1800470 (c.29C>T), rs1800471 (c.74G>C) (CEBINELLI et al., 2016).

Investigating the genetic basis of the host immune response, particularly cytokine function, could help further characterize the progression of cervical HPV infection into neoplasia (Du et al., 2019). Therefore, the present study aimed to assess the influence of four *TGFB1* variants c.-1638 G>A, c.-1347 T>C, c.29 C>T, and c.74 G>C and their haplotype structures on intraepithelial lesions and cervical cancer development.

MATERIALS AND METHODS

ETHICAL APPROVAL

This study was approved by the Institutional Ethics Committee Involving Humans of the State University of Londrina, Londrina, PR, Brazil (CAAE 56738316.3.0000.5231). The purpose of the study and the procedures involved were explained to all participants, and written informed consent was obtained prior to sample collection and interview. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

PATIENTS AND SAMPLES

Biological materials (cervical secretion, blood samples, or formalin-fixed-parafin-embedded (FFPE) tumor tissues) were collected from 130 Brazilian women who underwent outpatient cytology testing, recruited from 2013 to 2017, or attended cervical cancer prevention programs at an ambulatory colposcopy facility of the Intermunicipal Consortium of Health of the Middle Paranapanema, at the University Hospital and Clinic Center of the State University of Londrina, at Basic Healthcare Units in Londrina—PR, Brazil, and at Cancer Hospital of Londrina. After cervical sample collection, cytobrushes were stored in 2 mL of TE buffer (10 mM Tris–HCl, 1 mM EDTA pH 8.0) at 4 °C until analysis. Peripheral blood was drawn into sterile syringes containing EDTA as anticoagulant, and stored at 4 °C until analysis. The cervical tumor tissue samples embedded in paraffin were provided by the Londrina Cancer Hospital.

HPV-infected patients (tested by PCR) were interviewed using a structured questionnaire and stratified in “no cervical lesion” (controls) and “cervical cancer”, based on cervical cytology or biopsy.

Clinical and pathological data of cervical cancer patients were available from the Londrina Cancer Hospital. Clinical staging was determined according to the International Federation of Gynecology and Obstetrics (FIGO) criteria. Pathological features analyzed included: histological classification and histopathological grade, according to World Health Organization (WHO) histological classification of tumors of the uterine cervix.

DNA EXTRACTION

Genomic DNA was obtained from: 1) cervical cytobrushes using DNAzol (Invitrogen™, Carlsbad, CA, USA); 2) peripheral blood using a Biopur Mini Spin Plus Kit (Biometrix, Curitiba, PR, Brazil); and 3) FFPE tumor tissues using PureLink™ Genomic DNA Mini Kit (Invitrogen™, Carlsbad, CA, EUA). All DNA extractions were carried out according to the manufacturer's instructions, and the products were stored at – 20 °C until use. DNA concentration was measured at 260 nm on a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and purity was assessed by the A260/A280 ratio.

HPV DETECTION BY PCR

HPV was detected by Polymerase Chain Reaction (PCR) using the primers MY09 (5′-CGTCCMAARGGAWACTGATC-3′) and MY11 (5′-GCMCAGGGWCATAAY-AATGG-3′), which were designed to amplify a conserved region of approximately 450 base-pairs (bp) in the HPV L1 gene (GenBank Accession number: AJ236888) (Bauer et al., 1991). A fragment of human b-globin with a length of 268 bp was co-amplified as an internal control using primers GH20 (5′-GAAGAGCCAAGGACAGGTAC-3′) and PC04 (5′-CAACTTCATCCACGTTCACC-3′) (Silva et al., 2012). Reactions without template DNA were used as a negative control to test for contamination, and DNA from HeLa cells, which are stably integrated with HPV18, was used as positive control. PCR products were electrophoresed on 10% polyacrylamide and stained with silver nitrate.

CERVICAL CYTOLOGY

Cytology samples were graded according to the Bethesda System (2001) at the Public Health System Laboratory. Patients were deemed to have low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL) or no lesions (NL) if cytology samples were normal, i.e., were not indicated as having low- or high-grade squamous intraepithelial lesions, cervical carcinomas, atypical squamous cells of undetermined significance, or other atypical squamous cells that cannot be excluded as high-grade squamous intraepithelial lesions (DA SILVA et al., 2012).

***TGFB1* VARIANTS GENOTYPING**

Genetic variants were analyzed by PCR followed by Restriction Fragment Length Polymorphism (RFLP) analysis, according to that described by Jin *et al.* (2004) with modifications. All PCR reagents were purchased from Invitrogen™ (Carlsbad, CA, USA), and all restriction enzymes were from New England Biolabs® (Ipswich, MA, USA). Briefly, two primers pairs were designed based on *TGFB1* gene reference sequence (NCBI gene bank accession number NG_013364.1): one encompassing the two promoter region variants (c.-1638G>A and c.-1347T>C) and the other encompassing the signal peptide variants (c.29C>T and c.74G>C) (JIN et al., 2004). The PCR conditions for the two reactions were the same. Both reactions were performed in a final volume of 25 µL using 2.5 µL of 10× PCR buffer, dNTP (0.1 mM), 0.2 µM of primers, $MgCl_2$ (1.0 mM), Taq DNA polymerase (1 U/reaction) and approximately 3 ng/µL of genomic DNA. PCR reactions were performed along with a negative control with no DNA addition to check for exogenous DNA contamination. The sequences of the primers flanking the promoter region polymorphisms were: 5′-GCAGTTGGCGAGAACAGTTG-3′ and 5′-CCAGAACGGA-AGGAGAGTCAG-3′, generating a 597 (bp) amplicon (annealing temperature: 59 °C). The restriction enzyme *HpyCH4IV* was used for enzymatic restriction of c.-1638G>A polymorphisms generating 402 and 195 bp fragments for G allele and *Bsu36I* restriction enzyme was used for c.-1347T>C genotyping, generating 488 and 109 bp fragments for C allele. Restriction conditions followed the manufacturer’s protocol. Primer sequences for the signal peptide region were: 5′-TTCCCTCGAGGCCCTCCTA-3′ and 5′-GCCGCAGCTTGGACAGGATC-3′. Annealing temperature was set at 62 °C. *MspA1I* restriction enzyme was used to determine c.29C>T genotypes, cleaving the 294 bp amplicon in 161, 67, 40 and 26 bp fragments for T allele, and in 149, 67, 40, 26 and 12 bp fragments for C allele. *BglI* restriction enzyme cleaved this same amplicon in 131, 103 and 60 bp fragments for G allele from c.74G>C polymorphism and in 163 and 131 bp fragments for C allele. Restriction conditions followed the manufacturer’s instruction. Amplicons and restriction fragments were analyzed by electrophoresis on polyacrylamide gel (10%) visualized after silver staining.

To confirm the genotyping method accuracy, one individual for each genotype from all genetic variants analyzed was sequenced in a 3500 Genetic Analyzer® (Applied Biosystems) using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Austin, TX, USA) and at least 5% of total sample were repeated for PCR–RFLP analysis, obtaining 100% of concordance between results.

HAPLOTYPE ANALYSIS

The TGFB1 haplotypes were inferred based on the genotypes of all study participants using PHASE software version 2.1.1 (STEPHENS; SCHEET, 2005; STEPHENS; SMITH; DONNELLY, 2001). The web-based application SNPstats (Catalan Institute of Oncology, Barcelona, Spain) (https://www.snpstat.net) was used to analyze linkage disequilibrium between *TGFB1* genetic variants (MACHIELA; CHANOCK, 2015; SOLE et al., 2006).

STATISTICAL ANALYSES

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 *TGFB1* variants inheritance models between controls and case groups (i.e., cervical status). Bonferroni correction was used as a post hoc test to avoid false-positive (type I error) findings arising from multiple comparisons. The Kolmogorov–Smirnov test was performed to assess the normality of distribution. We assessed the differences in continuous variables between groups using the Mann–Whitney test. Categorical variables were expressed as absolute number (n) and percentage (%) and continuous variables were expressed as median and interquartile range (IQR). Departures from Hardy–Weinberg equilibrium and frequency differences between groups were evaluated by the Chi-square test. Binary logistic regression controlled by confounders (for age, age in the first sexual intercourse, smoking status, and ethnicity) 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., cervical status) as dependent variable. Adjusted odds ratios (ORs) and 95% confidence intervals (CI) were estimated. All tests were two-tailed, with a p value (P) < 0.05 considered statistically significant. Statistical analyses were carried out using SPSS Statistics 25.0 software (SPSS, Inc., Chicago, IL, USA).

RESULTS

In this case control study, the patients' groups (NL and cancer) were initially characterized for age (years), age at first sexual intercourse (years), smoking status and ethnicity as summarized in Table 1. Groups differed in age (P<0.001) with cancer

group median age of 51 (IQR= 25) years being higher than median of 35 (24) years of the NL groups. Stratifying the age in ranges, there is a higher proportion of women 55 years-old and older in the cancer group than in controls (*P*< 0.001). Another difference was found in smoking status, with a lower proportion of non-smoking women in the cancer group than in the control group (*P*= 0.039).

Clinicopathological characteristics of cervical tumors as histological classification, histopathological grade and staging were analyzed for the frequency in the cancer group (Table 2). Most cervical cancer patients presented squamous cell carcinoma (85.1%), moderately differentiated tumor (59.4%), and stages "II" (42.6%) and III (40.4%).

DISTRIBUTION OF *TGFB1* SNVS ALLELES, GENOTYPES AND HAPLOTYPES ACCORDING TO CERVICAL STATUS.

All 130 patients were genotyped for *TGFB1* SNVs c.-1638 G>A, c.-1347 T>C, c.29 C>T, and c.74 G>C (Table 3). Genotype frequencies were under Hardy-Weinberg Equilibrium (*P*>0.05) in all groups and higher linkage disequilibrium were observed between c.-1347 T>C and c.29 C>T (D'= 0.90, r^2= 0.58). The variant allele frequency were lower in c.-1638 G>A and c.74 G>C with 5.9 and 7.0% (allele A) and 5.7 and 5.9% (allele C), respectively. On the other hand, the variant allele frequency were higher in c.-1347 T>C (63.3 and 55.9%) and similar to wild allele in c.29 C>T (53.2 and 51.0%). None of the SNVs were associated with cancer.

Seven haplotype structures were inferred and their counting in the study-population and distribution in "no cervical lesion" and "cancer" groups are presented in Table 4. No differences were found in haplotype variability between groups, neither in the distribution of each haplotype.

TGFB1 SNVS GENOTYPE AND HAPLOTYPE MODELS AND SUSCEPTIBILITY TO CERVICAL CANCER.

Susceptibility to cervical cancer was evaluated for the presence of *TGFB1* genetic variations by binary logistic regression adjusted for age, age in the first sexual intercourse, smoking status, and ethnicity, with "no cervical lesion group" as reference (Table 5).

Genotypes in co-dominant, dominant and recessive models were assessed, however no significant association had been found to any SNV.

Regarding haplotypes, five structures with frequencies above 5% were evaluated (GCTG, GTCG, ACTG, GCCC and GCCG). SNV alleles are represented in haplotype structures according to their position in the *TGFB1* gene, following the order: c.-1638 G>A, c.-1347 T>C, c.29 C>T and c.74 G>C. The dominant model was tested for all haplotypes and the recessive model was tested for the two most frequent haplotypes (GCTG and GTCG).

Only the GTCG haplotype in both dominant and recessive models increased the susceptibility to cervical cancer (OR= 2.48, $CI_{95\%}$= 1.05-5.83 and OR= 4.73, $CI_{95\%}$= 1.18-19.02, respectively). A protective tendency for cervical cancer was also observed in the GCTG dominant model (OR= 0.42, $CI_{95\%}$=0.17-1.04, *P*= 0.062).

EFFECT OF *TGFB1* GENETIC VARIATIONS ON CLINICOPATHOLOGICAL CHARACTERISTICS OF CERVICAL CANCER.

Genotypes and haplotypes models were tested to verify if there were any effects on clinicopathological characteristics of cervical tumors (the same presented in Table 2). No significant association of *TGFB1* variation was observed with histological classification, histopathological grade, or staging.

DISCUSSION

To the best of our knowledge, this study was the first to perform association analyses between *TGFB1* haplotype structures and cervical status in HPV-infected patients with normal cervix and invasive tumors.

In this case control study, cervical cancer patients aged higher than the other groups. However, it is in concordance with the global statistics, in which nearly half of the cases is diagnosed with cervical cancer up to 50 years old (DE MARTEL et al., 2017). Generally, cervical cancer development takes time, and is associated with the host's inability to keep a check on HPV gene expression. During the ordered productive life cycle, E6 and E7 HPV oncogenes are carefully regulated. Probably, as their expression increases in the basal layer, normal cellular controls are progressively compromised, leading to cervical intraepithelial neoplasia. With this process, the HPV ability to complete its life cycle and to produce infectious virions at the epithelial surface is progressively lost. Deregulated viral gene expression can go unchecked in the immune-tolerant microenvironment. The failure of the immune system is generally

characterized by only low numbers of circulating antigen-specific T cells and an abundance of $CD25^{+}$ T-regulatory cells (Tregs). Tregs, in turn, contribute to an immunosuppressed microenvironment with cytokines such as IL10 and TGFB at elevated levels (DOORBAR, 2018; HIBMA, 2012).

Being a multifunctional cytokine that regulates cell behavior with a pivotal role in the cell growth, maturation, and differentiation (ZARZYNSKA, 2014), TGFB1 seems to be also implicated in cervical cancer, suggesting an association with HPV infection severity and malignancy (ALCOCER-GONZÁLEZ et al., 2006; GUAN et al., 2010). Thus, the *TGFB1* sequence variants c.-1638G>A, c.-1347T>C, c.29C>T c.74G>C may be a factor that impact on TGFB1 participation in the cervical cancer development and progression.

The c.-1638G>A SNV is located in the enhancer region 1. Regulatory activity is related with binding to the cAMP response element binding protein (CREB) family. When guanine is altered to adenine at this locus, lower is the affinity to CREB proteins and lower is the TGFB1 production (GRAINGER, 1999). Other SNV in the regulatory region is the c.-1347C>T. It is located in the first negative regulatory region and T allele is associated with higher TGFB1 levels. Two proposed explanations are the loss of negative regulation and the increased binding of transcription factor Yin-Yang 1 (YY1). Hence, both of them would culminate with higher transcriptional activity (CEBINELLI et al., 2016).

The c.29C>T and c.74G>C are *TGFB1* signal peptide SNVs that cause amino acid substitutions, proline to leucine and arginine to proline exchanges at positions 10 and 25, respectively. Modifications in amino acid composition of the signal peptide could affect its polarity and alter the rates of protein export to endoplasmic reticulum (WOOD et al., 2000b). The 29C and 74G alleles have been associated to increased TGFB1 serum levels (AWAD et al., 1998; TAUBENSCHUSS et al., 2013; YOKOTA et al., 2000).

None of the SNVs was significantly associated with cervical status. However, the variant effect can be undetectable when analyzed as a single marker but evident in haplotype structures. Additionally, more than one functional locus could be existing, and associations with phenotype would only be elicited when combinations of specific loci (i.e. haplotypes) were considered (BREMER et al., 2008; SCHIRMER et al., 2011; VORMFELDE; BROCKMÖLLER, 2007). Thus, methods based on haplotypes comprising multiple SNVs on the same inherited chromosome may provide additional

power for mapping disease genes and also provide insight on factors influencing the dependency among genetic markers (LIU; ZHANG; ZHAO, 2008).

Although haplotype structures count has differed neither in the variability between-groups nor in the distribution of each one, the GTCG in both dominant (carriers) and recessive (homozygous patients) models increased the susceptibility to cervical cancer while GCTG in dominant model offered protective tendency. Vitiello and colleagues (2018) evaluated the same *TGFB1* genetic variations in Brazilian women with breast cancer and controls. The haplotypes distribution in their control group was similar to present controls, even the second being HPV-infected women. This agreement may represent with better confidence the distribution of haplotypes in this population.

GTCG haplotype combines the TGFB1 high-producer alleles -1638G, -1347T, 29C, and 74G. High levels of TGFB in the tumor have been correlated with neoplasm progression and considered a poor prognosis marker in some cancer types such as gastric carcinoma, colorectal cancer, bladder carcinoma, prostate cancer, breast cancer, lung cancer, esophageal adenocarcinoma, and melanoma as reviewed by Krstic and Santibanez (2014). In cervical cancer context, patients with invasive tumor presented high levels of TGFB compared with healthy females (CHEN et al., 2013). Furthermore, during lesion progression, increased TGFB1 mRNA expression was also observed (PEGHINI et al., 2012).

Tumors generally produce and secrete a large amount of TGFB that not only affect the tumor cells themselves but also the surrounding stroma by inhibiting cell adhesion, inducing immunosuppression and angiogenesis, and by promoting the degradation of the extracellular matrix, further contributing to the metastatic process (NEEL; HUMBERT; LEBRUN, 2012). In addition, the presence of Tregs and Th17 cells infiltrated into tumors can contribute to tumor progression. Tregs suppress Th1 and Th2 responses and induce inflammatory Th17 cell-mediated responses. Indeed, TGFB play a central role inducing $CD4^{+}$ T cells to differentiate in Treg and Th17 phenotypes and acting as Treg effector cytokine in the immunosuppression. In turn, Th17 response has been implicated to coordinate the chronic inflammatory responses that contribute to the tumor growth. Probably, a microenvironment with chronic inflammation and Th1/Treg ratio imbalance could be a critical mechanism for tumor cell evasion of the immune surveillance and tumor progression (CHEN et al., 2013; FERNANDES et al., 2015).

Therefore, whether the amount of intralesion TGFB is so crucial to tumor development, genetic variations associated with higher levels of TGFB as GTCG haplotype could predispose HPV-infected patients to have cervical cancer while low producer as GCTG would confer protection. Possibly because of few patients with cervical cancer, *TGFB1* variations were not related to prognosis characteristics in this study.

To our knowledge, this is the first time that *TGFB1* haplotype were associated with cervical cancer: GTCG carriers or just GTCG homozygous patients were more susceptible to have cervical cancer. Although further studies are warranted to confirm the results, this study suggests c.-1638G>A, c.-1347T>C, c.29C>T and c.74G>C haplotype structures in the *TGFB1* gene as candidate susceptibility markers to cervical cancer in HPV-infected women.

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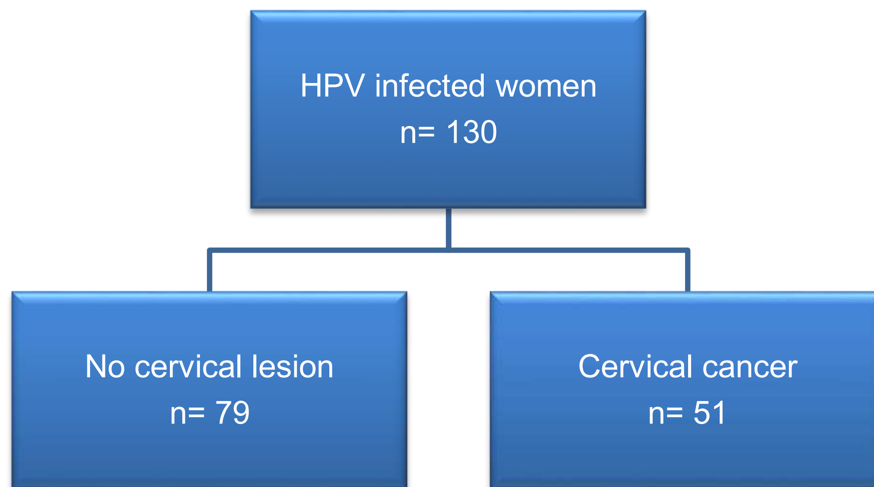

Figure 1 Study flow chart.

Table 1 Age, smoking status and ethnicity regarding cervical status of HPV infected patients.

Characteristics	NL (n=79)	Cancer (n=51)	*P*
Age (years)			
Median (IQR)	35 (24)	51 (25)	**<0.001**
Range [n (%)]			**<0.001**
<25	14 (17,7)	1 (2,0)	
25 – 34	25 (31,6)	5 (9,8)	
35 – 44	15 (19,0)	15 (29,4)	
45 – 54	12 (15,2)	8 (15,7)	
≥ 55	13 (16,5)	22 (43,1)	
Age at first sexual intercourse (years) [median (IQR)]	17 (4)	17 (3)	0.729
Smoking status [n (%)]			**0.039**
No	61 (77,2)	27 (56,3)	
Yes	15 (19,0)	16 (33,3)	
Former smoker	3 (3,8)	5 (10,4)	
Ethnicity* [n (%)]			0.906
Caucasian	42 (53,2)	25 (52,1)	
Non-Caucasian	37 (46,8)	23 (47,9)	

Continuous data were analyzed by Mann-Whitney test, while contingence table by two-sided χ^2 test. *P*<0.05 considered significant. NL, no cervical lesion; IQR, interquartile range. *Self-reported ethnicity information.

Table 2 Clinicopathological characteristics of cervical tumors.

Characteristics	Cervical cancer group [n (%)]
Histological classification*	
Squamous cell carcinoma	40 (85.1)
Adenocarcinoma	6 (12.8)
Neuroendocrine	1 (2.1)
Unknown	4
Histopathological grade	
Well differentiated	6 (18.8)
Moderately differentiated	19 (59.4)
Poorly differentiated	7 (21.9)
Unknown	19
Staging**	
I	5 (10.6)
II	20 (42.6)
III	19 (40.4)
IV	3 (6.4)
Unknown	4

* World Health Organization (WHO) histological classification of tumors of the uterine cervix.
**International Federation of Gynecology and Obstetrics (FIGO) staging of cervical carcinomas.

Table 3 *TGFB1* genetic variations in cervical status of HPV infected patients.

TGFB1 SNV	NL (n=79)	Cancer (n=51)	*P*
c.–1638 G>A			0.721
GG	68 (86.1)	45 (88.2)	
GA	11 (13.9)	6 (11.8)	
Allele G	147 (93.0)	96 (94.1)	0.731
Allele A	11 (7.0)	6 (5.9)	
c.–1347 T>C			0.470
TT	10 (12.7)	10 (19.6)	
TC	38 (48.1)	25 (49.0)	
CC	31 (39.2)	16 (31.4)	
Allele T	58 (36.7)	45 (44.1)	0.233
Allele C	100 (63.3)	57 (55.9)	
c.29 C>T			0.863
CC	17 (21.5)	13 (25.5)	
CT	40 (50.6)	24 (47.1)	
TT	22 (27.9)	14 (27.4)	
Allele C	74 (46.8)	50 (49.0)	0.731
Allele T	84 (53.2)	52 (51.0)	
c.74 G>C			0.948
GG	70 (88.6)	45 (88.2)	
GC	9 (11.4)	6 (11.8)	
Allele G	149 (94.3)	96 (94.1)	0.950
Allele C	9 (5.7)	6 (5.9)	

Data presented as absolute number and percentage. Two-sided χ^2 test, with $P<0.05$ considered significant. SNV, single nucleotide variant; NL, no cervical lesion.

Table 4 Comparison of the frequency of *TGFB1* haplotype structures in cervical status of HPV infected patients.

TGFB1 Haplotypes	All	Cervical status		*P***
		No lesion	Cancer	
GCTG	109 (41.9)	70 (44.3)	39 (38.2)	0.333
GTCG	94 (36.1)	55 (34.8)	39 (38.2)	0.574
ACTG	17 (6.5)	11 (7.0)	6 (5.9)	0.731
GCCC	14 (5.4)	9 (5.7)	5 (4.9)	0.782
GCCG	16 (6.2)	10 (6.3)	6 (5.9)	0.884
GTTG	9 (3.5)	3 (1.9)	6 (5.9)	0.086
GCTC	1 (0.4)	0	1 (1.0)	0.212
*P**	-	-	0.501	

Haplotype counting presented as absolute number and percentage. Two-sided χ^2 test, with $P<0.05$ considered significant. *Obtained by comparison between cancer group and no lesion group. **Obtained by comparison of the frequency of a haplotype structure and the sum of the frequencies of the other haplotypes between cervical status groups. SNVs alleles in haplotype structures follow the order: c.-1638G>A, c.-1347T>C, c.29C>T and c.74G>C.

Table 5 Susceptibility to cervical cancer according *TGFB1* genetic variations.

TGFB1 SNVs		Adjusted odds ratio [OR ($CI_{95\%}$)] Cervical cancer	*P*
c.–1638 G>A			
	GA vs GG	0.97 (0.26-3.58)	0.967
c.–1347 T>C			
	TC vs TT	0.57 (0.18-1.83)	0.346
	CC vs TT	0.79 (0.22-2.81)	0.717
	Dominant	0.64 (0.21-1.94)	0.433
	Recessive	1.21 (0.49-3.01)	0.678
c.29 C>T			
	CT vs CC	0.75 (0.28-2.05)	0.753
	TT vs CC	1.11 (0.35-3.55)	0.862
	Dominant	0.85 (0.33-2,19)	0.744
	Recessive	1.34 (0.52-3.48)	0.548
c.74 G>C			
	GC vs GG	1.10 (0.30-4.05)	0.884
GCTG			
	Dominant	0.42 (0.17-1.04)	0.062
	Recessive	0.53 (0.19-1.45)	0.217
GTCG			
	Dominant	**2.48 (1.05-5.83)**	**0.038**
	Recessive	**4.73 (1.18-19.02)**	**0.028**
ACTG			
	Dominant	0.54 (0.13-2.19)	0.386
GCCC			
	Dominant	0.66 (0.16-2.77)	0.568
GCCG			
	Dominant	0.70 (0.17-2.86)	0.623

Binary logistic regression adjusted for age, age in the first sexual intercourse, smoking status, and ethnicity, with “no cervical lesion group” as reference. $CI_{95\%}$, 95% confidence interval. SNVs alleles in haplotype structures follow the order: c.-1638G>A, c.-1347T>C, c.29C>T and c.74G>C. $P<0.05$ considered significant.

Conclusões

6 CONCLUSÕES

ARTIGO 1

- A infecção pelo HPV foi mais frequente entre mulheres abaixo de 35 anos de idade e que iniciaram a vida sexual antes dos 18 anos.
- As frequências dos alelos –1347T e 29C e do genótipo 29CC foram maiores entre as mulheres infectadas pelo HPV do que entre as não infectadas.
- Os genótipos –1347TT e –1347CT+TT, assim como 29CT, 29CC e 29CT+CC foram associados com maior suscetibilidade à infecção pelo HPV quando comaparados, respectivamente a –1347CC e 29TT.
- Os haplótipos com frequência maior que 5% na população de estudo foram, em ordem decrescente, **4* (GCTG), **3* (GTCG), **5B* (ACTG) e **2* (GCCC).
- O haplótipo **4* foi mais frequente no grupo de mulheres infectadas do que não infectadas.
- Mulheres com o haplótipo **4* em homozigose foram menos suscetíveis à infecção do que mulheres não portadoras deste haplótipo.
- Mulheres **3/*3* + **3/*4* foram mais suscetíveis ao HPV do que mulheres **4/*4*.
- Os níveis plasmáticos e cervicais de TGFB1 foram maiores entre as mulheres infectadas do que não infectadas, enquanto apenas os níveis plasmáticos estiveram aumentados nas pacientes infectadas com lesão de baixo grau comparados com as pacientes infectadas sem lesão.
- Pacientes infectadas **3*/outros e **3/*3* + **3*/outros apresentaram menores níveis plasmáticos de TGFB1 do que as pacientes infectadas não portadoras deste haplótipo.

ARTIGO 2

- O câncer de colo do útero é mais frequente em mulheres com idade mais avançada e tabagistas.
- Os haplótipos com frequência maior que 5% na população de estudo foram GCTG, GTCG, ACTG, GCCC e GCCG.

- O haplótipo GTCG aumentou a suscetibilidade para o câncer cervical tanto para as portadoras deste haplótipo (GTCG/GTCG + GTCG/Outros) quanto para as mulheres homozigotas (GTCG/GTCG).

7 CONSIDERAÇÃO FINAL

O câncer de colo do útero é uma doença de etiologia multifatorial e que tem a infecção pelo HPV como evento necessário, porém insuficiente, para a carcinogênese. Ainda não está esclarecido o que leva apenas uma pequena parcela das mulheres infectadas por HPVs de alto risco oncogênico a progredirem de uma infecção para o câncer invasivo. A busca por fatores, tanto extrínsecos como intrínsecos, que pudessem colaborar com o entendimento deste processo levou este grupo de pesquisa a estudar aspectos imunogenéticos dentro do contexto desta doença.

Assim, de forma inédita, o presente trabalho demonstra que haplótipos das variações genéticas c.–1638G>A, c.–1347T>C, c.29C>T e c.74G>C da citocina multifacetada TGFB1 possuem um grande potencial de serem candidatos a *loci* de suscetibilidade para a infecção pelo HPV e para o câncer de colo do útero. Contudo, estudos adicionais são necessários para validação destes resultados.

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Apêndices

APÊNDICE A

Termo de Consentimento Livre e Esclarecido

“Prevalência e genotipagem de HPV e sua possível associação com os genes de citocinas, quimiocinas e seus receptores em nível de DNA, RNA e proteína: implicações no microambiente tumoral.”

Prezado(a) Senhor(a):

Gostaríamos de convidá-lo (a) a participar da pesquisa **“Prevalência e genotipagem de HPV e sua possível associação com os genes de citocinas, quimiocinas e seus receptores em nível de DNA, RNA e proteína: implicações no microambiente tumoral.”**, realizada no **“Laboratório de Genética Molecular e Imunologia, Departamento de Ciências Patológicas da Universidade Estadual de Londrina”.** O objetivo da pesquisa é avaliar a presença do vírus em mulheres atendidas em programas de prevenção ao câncer cervical do setor público de saúde da região norte do Paraná, por meio de metodologia específica e sensível, visando também à associação de dados demográficos, para análise dos fatores de risco que contribuem para a exposição da população ao vírus, bem como os determinantes de sua manutenção. Adicionalmente objetiva-se compreender o papel do sistema imune no controle e iniciação tumoral, bem como na sua formação, crescimento e progressão, em especial avaliar a interação tumor-hospedeiro em pacientes portadoras do vírus HPV e no desenvolvimento do câncer cervical. A sua participação é muito importante e ela se daria da seguinte forma: **doação de 5mL de sangue periférico coletado por punção venosa e doação do swab cérvico-vaginal utilizado para confecção das lâminas para o exame preventivo para anállises moleculares, bem como responder um questionário sociodemográfico.** Gostaríamos de esclarecer que sua participação é totalmente voluntária, podendo você: recusar-se a participar, ou mesmo desistir a qualquer momento sem que isto acarrete qualquer ônus ou prejuízo à sua pessoa. Informamos ainda que as informações serão utilizadas somente para os fins desta pesquisa e serão tratadas com o mais absoluto sigilo e confidencialidade, de modo a preservar a sua identidade.
As amostras biológicas (sangue periférico e secreção cérvico-vaginal) serão utilizados para extração de DNA e RNA para análises moleculares e imunológicas. Estes materiais serão obtidos em pequenas quantidades portanto não haverá sobra de material biológico.
Os benefícios esperados são a detecção precoce do vírus HPV em mulheres atendidas em programas de prevenção ao câncer de colo de útero do setor público de saúde da região norte do Paraná. Informamos que a paciente que se dispor a participar do projeto não sofrerá desconfortos nem riscos à saúde, não havendo qualquer prejuízo às mesmas. Informamos que a senhora não pagará nem será remunerada por sua participação. Garantimos, no entanto, que todas as despesas decorrentes da pesquisa serão ressarcidas, quando devidas e decorrentes especificamente de sua participação na pesquisa.
Caso você tenha dúvidas ou necessite de maiores esclarecimentos pode nos contactar **Karen Brajão de Oliveira, Laboratório de Genética Molecular e Imunologia, Departamento de Ciências Patológicas, Universidade Estadual de Londrina, 3371-4267, karen.brajao@uel.br),** ou procurar o Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina, na Avenida Robert Kock, nº 60, ou no telefone 33712490. Este termo deverá ser preenchido em duas vias de igual teor, sendo uma delas, devidamente preenchida e assinada entregue a você.

Londrina, ___ de ________de 201__.

Pesquisador Responsável ___________________________
Profª. Drª. Karen Brajão de Oliveira
RG:: 6.538.742-5

___________________________________ (nome por extenso do sujeito de pesquisa), tendo sido devidamente esclarecido sobre os procedimentos da pesquisa, concordo em participar **voluntariamente** da pesquisa descrita acima.

Assinatura (ou impressão dactiloscópica):___________________________
Data:__________________

APÊNDICE B

Nº LAB

QUESTIONÁRIO SOCIOEPIDEMIOLÓGICO

Data: ____/____/_____ Reg. N° ______________________________

1. Conhece o HPV???
( ) Nunca ouvi falar
( ) Já ouvi falar mas não sei o que é
( ) Conheço

2. Idade ________ anos DN, _______________

3. Etnia: __________________________
Branca / parda / negra / asiática / indígena

4. Sua renda mensal (em salário mínimo) é de?
( ) Até 1 Salário ( ) De 1 à 3 salários
( ) De 3 à 5 salários ( ) De 5 à 7 salários
( ) De 7 à 10 salários

5. Você fuma?
( ) Não ( ) Sim **Tempo:** ____________

6. Qual o seu grau de escolaridade?
( ) Fundamental Incompleto
( ) Fundamental Completo
( ) Médio Incompleto ( ) Médio completo
( ) Superior incompleto ( ) Sup. completo

7. Estado Civil:
( ) Solteira ( ) Casada
( ) Divorciada ( ) Viúva

8. Qual sua profissão?
____

9. Faz o uso de algum método contraceptivo?
( ) Não ( ) Sim **Qual:** _________________

10. Tipo de Parto:
( ) Normal ( ) Cesária

11.Nºde gestações: _____________

12. Números de Partos:
( ) Nenhum ( ) Um
( ) Dois ( ) Três
( ) Quatro ou mais

13. Idade da 1ª relação sexual: ________anos

14. Idade da 1ª menstruação: ________ anos

15.Número de parceiros sexuais durante a vida:

____

16. Número de parceiros sexuais nos últimos 6 meses: ___________________ .

17. Já realizou outros exames preventivos?
( ) Sim ( ) Não

18. Exames de prevenção realizados no passado apresentaram algum tipo de alteração?
( ) Sim ( ) Não
( ) Não me lembro
Em caso de resposta "Sim" favor descrever a alteração: _________________________________

19. Já contraiu alguma infecção ginecológica
( ) Não ( ) Sim ( ) não sei informar
Em caso de resposta ``SIM``, se possível descrever qual:________________________

20. Já esteve infectada pelo HPV?
( ) Sim ( ) Não ( ) Não sei informar

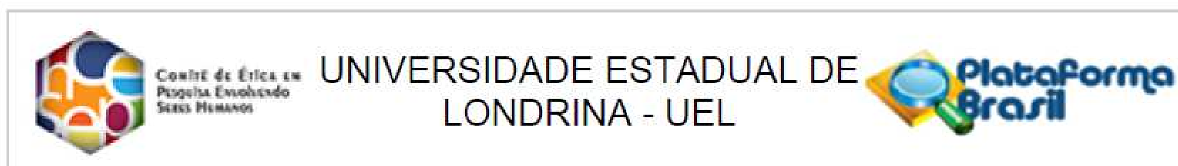
21. Conhece as formas de transmissão ou formas de contrair o vírus?
( ) Não ( ) Sim Qual ou quais:
____

22. Existem casos de câncer de colo de útero em sua família?
( ) Sim ( ) Não
Em caso de resposta ``SIM`` descrever o grau de parentesco: ______________________________

Pesquisador: ________________________

Anexos

ANEXO A



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: TGFB: possível marcador molecular de susceptibilidade para progressão das lesões cervicais induzidas pelo Papilomavírus Humano (HPV)

Pesquisador: Karen Brajão de Oliveira

Área Temática:

Versão: 1

CAAE: 56738316.3.0000.5231

Instituição Proponente: CCB - Departamento de Ciências Patológicas

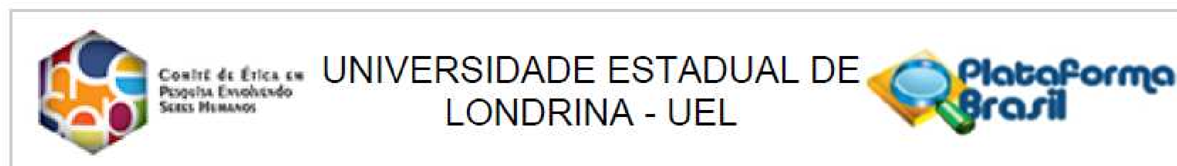
Patrocinador Principal: Fundação Araucária

DADOS DO PARECER

Número do Parecer: 1.590.141

Apresentação do Projeto:

Trata-se de estudo experimental, que será realizado com mulheres, 18 anos, atendidas em UBSs de Londrina e no Hospital do Câncer de Londrina (HCL). A adesão das pacientes será voluntária, após receberem informações e explicações sobre o estudo e seus objetivos. Aquelas que aceitarem participar firmarão a concordância, mediante leitura e assinatura do TCLE. O presente estudo pretende avaliar a presença do HPV em mulheres atendidas em programas de prevenção ao câncer cervical do setor público de saúde da região norte do Paraná e no Hospital do Câncer de Londrina por meio de metodologia específica e sensível, a PCR, visando também à associação de dados demográficos e análise dos fatores de risco que contribuem para a exposição da população ao HPV, bem como os determinantes da manutenção da infecção. Objetiva-se, ainda, avaliar a influência destes quatro polimorfismos do gene TGFB1 e do polimorfismo do gene TGFBR2, sozinhos e combinados, em mulheres infectadas pelo HPV em vários estágios de desenvolvimento de lesões, a fim de elaborar um painel de genótipos que poderiam vir a ser utilizados como marcadores moleculares de susceptibilidade para a infecção pelo HPV e também para a carcinogênese cervical na prática clínica.



Objetivo da Pesquisa:

Este projeto tem como objetivo geral verificar a presença do vírus em mulheres (pacientes e controles) atendidas pelos programas de prevenção ao câncer de colo do útero do setor público de saúde da região norte do Paraná e pelo Hospital do Câncer de Londrina, identificar os tipos virais mais prevalentes na região, bem como avaliar o papel do eixo fator de crescimento transformante 1 (TGFB1) e seu receptor tipo 2 (TGFBR2) na infecção por HPV e no desenvolvimento das lesões intraepiteliais escamosas de baixo e alto grau (LIEBG e LIEAG) e do câncer invasivo. E também realizar palestras educativas para a população e para a equipe médica. Objetivo Secundário: - Realizar a detecção e posterior tipagem do HPV em amostras de pacientes do sexo feminino atendidas pelos programas de prevenção ao câncer de colo do útero do Sistema Único de Saúde (SUS) na região norte do Paraná e no Hospital do Câncer de Londrina;- Avaliar a prevalência do HPV e de seus tipos em mulheres atendidas pelos programas de prevenção ao câncer de colo do útero do Sistema Único de Saúde (SUS) na região norte do Paraná e no Hospital do Câncer de Londrina;- Avaliar o perfil sociodemográfico das mulheres atendidas pelos programas de prevenção ao câncer de colo do útero do Sistema Único de Saúde (SUS) na região norte do Paraná e no Hospital do Câncer de Londrina;- Correlacionar a infecção pelo HPV e a presença das lesões provocadas por ele com dados sociodemográficos das mulheres atendidas, como faixa etária, estado civil, grau de escolaridade, renda familiar e hábito tabagista entre outros;- Correlacionar a infecção por HPV e a presença das lesões provocadas por ele com as variáveis sexuais e reprodutivas, como menarca, idade da primeira relação sexual, uso de métodos contraceptivos, número de parceiros sexuais ao longo da vida e nos últimos seis meses, número de partos e tipo(s) de parto(s) realizado(s) e a frequência de realização de exames preventivos.Avaliar a frequência dos alelos, genótipos e haplótipos dos polimorfismos rs1800468 (-1638G>A), rs1800469 (-1347C>T), rs1800470 (+29T>C) e rs1800471 (+74G>C) do TGFB1 e rs3087465 (-875G>A) do TGFBR2 na população feminina da região Norte do Paraná;- Avaliar o risco de infecção por HPV e desenvolvimento de LIEBG, LIEAG e câncer cervical associado aos alelos, genótipos e haplótipos dos polimorfismos rs1800468 (1638G>A), rs1800469 (-1347C>T), rs1800470 (+29T>C) e rs1800471 (+74G>C) do TGFB1 e rs3087465 (-875G>A) do TGFBR2 na população feminina da região Norte do Paraná;- Analisar a secreção de TGFB1 no sangue periférico de mulheres atendidas pelos programas de prevenção ao câncer de colo do útero do Sistema Único de Saúde (SUS) na região norte do Paraná e correlacioná-la aos alelos, genótipos e haplótipos dos polimorfismos rs1800468 (-1638G>A), rs1800469 (-1347C>T), rs1800470 (+29T>C) e rs1800471 (+74G>C) do TGFB1 e rs3087465 (-875G>A) do TGFBR2 e com a infecção por HPV e desenvolvimento de LIEBG,

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LIEAG e câncer cervical.- Realizar minicursos e seminários a fim de transferir as experiências metodológicas de diagnóstico molecular para alunos de iniciação científica e pós-graduandos.- Realizar palestras e oficinas sobre as formas de transmissão, tratamento e prevenção do câncer de colo de útero, para as mulheres atendidas pelos programas de prevenção ao câncer de colo de útero que realizam exames de prevenção ao câncer de colo de útero rotineiramente nas UBS, bem como toda a comunidade que frequenta as UBSs.- Realizar palestras aos médicos do Hospital do Câncer de Londrina, a respeito da participação do sistema imunológico na progressão de lesões cervicais e no desenvolvimento do câncer cervical, abordando a possível participação do TGB1 como marcador de prognóstico das lesões.
Metodologia Proposta: O estudo será realizado com mulheres, 18 anos, atendidas em UBSs de Londrina e no Hospital do Câncer de Londrina (HCL). A adesão das pacientes será voluntária, após receberem informações e explicações sobre o estudo e seus objetivos. Aquelas que aceitarem participar firmarão a concordância, mediante leitura e assinatura do TCLE, e participarão de uma entrevista baseada em um questionário sóciodemográfico (anexo 1). As amostras ginecológicas serão obtidas no momento do exame ginecológico, de acordo com o procedimento clínico que a mulher será submetida. Assim, mulheres em exame preventivo de rotina nas UBSs terão células, do epitélio cervical uterino, coletadas no momento do exame pela enfermeira da UBS. Para tanto as escovas cervicais utilizadas na confecção do esfregaço, que seriam descartadas, serão acondicionadas em tubos falcon contendo 2 mL de solução tampão TE, e então transportadas sob refrigeração até o Laboratório de Genética Molecular – Imunologia da UEL onde serão processadas para extração do DNA/RNA. Em relação às mulheres em atendimento no HCL, poderão ser coletados diferentes tipos de material ginecológico, pelo médico responsável, de acordo com o procedimento ao qual a mulher será submetida, desta forma poderão ser coletados: 1) amostra de células, oriundas do epitélio cervical uterino, coletadas utilizando-se escovas cervicais estéreis; 2) amostra proveniente de material de biópsia; 3) fragmento do tecido tumoral fresco, quando a mulher for submetida a ressecção cirúrgica do tumor, 4) cortes de bloco de parafina de tecido tumoral; todos os materiais serão separados posteriormente a realização das lâminas para análise patológica, pelo médico(a) patologista do HCL. Todos os médicos que participarão destas etapas fazem parte da equipe de trabalho deste projeto. No momento logo após a abordagem das mulheres nas UBSs, ou no HCL, mediante assinatura do TCLE, amostras de sangue periférico serão obtidas por punção venosa, por profissionais capacitados, membros da equipe do projeto, em tubos contendo EDTA, e os mesmos serão transportados sob refrigeração até o Laboratório de Genética Molecular e Imunologia da Universidade Estadual de Londrina, onde serão processados

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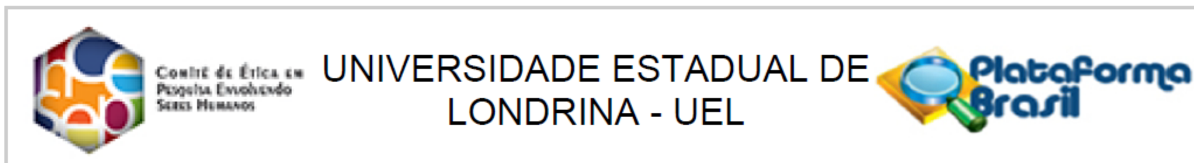
para separação do plasma e obtenção do DNA, os quais serão armazenados a -20°C, para as análises de polimorfismos e dosagens de citocinas.O DNA do sangue periférico será isolado pelo Biopur Kit de Extração Mini Spin Plus (Biometrix), e o do material ginecológico utilizando-se o reagente DNAzol Reagent (invitrogen). Os DNAs serão quantificados por espectrofotometria, e utilizar-se-ão amostras que apresentem razões (A260/A280) 1,7.O diagnóstico molecular do HPV será realizado por PCR, com os primers MY09 e MY11. O gene da ß-globina humana será utilizado como controle interno da reação utilizando-se os primers GH20 e PC04. As reações serão efetuadas com um controle negativo, e um controle positivo que consiste em DNA obtido a partir da linhagem celular HeLa, que contem o DNA do HPV18 inserido em seu genoma.As amostras positivas para o HPV serão submetidas à clivagem com enzima HpyCH4V, para identificação dos tipos de HPV, conforme descrito por Santiago et al. (2006). Os fragmentos serão analisados por eletroforese em poliacrilamida 10%, 20x20cm (100V-8h), corado por AgNO3. Para identificação dos polimorfismos de TGFB e TGFBR2 será utilizada a técnica de PCR-RFLP, utilizando-se primers específicos. Os produtos de amplificação serão submetidos à clivagem pelas enzimas Tai-I, Eco81-I, MspA1-I, Bgl-I e HpyCh4-III para análise dos polimorfismos rs1800468, rs1800469, rs1800470, rs1800471, e rs3087465, respectivamente. A análise dos genótipos se dará por eletroforese em poliacrilamida 10% 10x10cm corado por AgNO3. O nível plasmático de TGFB1 será avaliado pela técnica de ELISA. As análises estatísticas serão realizadas utilizando-se o programa SPSS Statistics 22.0. O nível de significância adotado será de $p<0,05$.

Avaliação dos Riscos e Benefícios:

Os riscos desta pesquisa são mínimos, pois os únicos procedimentos extras aos quais as mulheres serão submetidas são: 1) a realização de questionário sociodemográfico, que será aplicado em local reservado, por membros treinados da equipe, a fim de evitar constrangimentos a participante, que poderá se recusar a responder as perguntas caso não se sinta confortável; 2) a realização de coleta de sangue poderá gerar certo desconforto, dor, hematoma no local, e raramente desmaios, contudo serão realizadas por profissionais capacitados a fim de minimizar estes possíveis riscos, e serão realizadas nas UBSs ou no HCL, em sala apropriada. Em relação à coleta de material ginecológico, não há riscos diretos da participação nesta pesquisa, tendo em vista que todas as mulheres serão submetidas aos procedimentos descritos, para seu diagnóstico e tratamento, independentemente de sua participação neste estudo. Contudo caso ocorra algum tipo de desconforto a participante será prontamente atendida e amparada por mim (pesquisadora responsável) e / ou pela equipe de trabalho do projeto.



Benefícios: Como benefícios diretos à participante, será realizado o diagnóstico molecular de HPV e sua tipagem, por meio de metodologia sensível e específica, que permite diagnóstico precoce da infecção. E como benefícios indiretos espera-se contribuir para uma maior compreensão dos mecanismos imunopatológicos envolvidos microambiente da infecção e das lesões provocadas pelo HPV, contribuindo assim para a elaboração de um painel de genótipos que poderiam vir a ser utilizados como marcadores moleculares de susceptibilidade para a infecção pelo HPV e também para a carcinogênese cervical na prática clínica.

Comentários e Considerações sobre a Pesquisa:

Projeto de grande relevância, uma vez que ao identificar a frequÊncia de infecções pelo HPV e dos tipos mais prevalentes em mulheres atendidas por programas de prevenção ao câncer de colo uterino e pelo Hospital do Câncer de Londrina, será possível obtermos dados a respeito da incidência do virus de acordo com as características socioepidemiológicas, que podem contribuir para a exposição das mulheres ao vírus, bem como os determinantes de sua manutenção, contribuindo desta forma para a elaboração de medidas eficazes de prevenção e promoção de saúde dentre as mulheres atendidas pela rede pública de saúde.Por meio da análise dos polimorfismos de TGFB1 e de seu receptor TGFBR2, pretendemos avaliar os determinantes inerentes aos indíviduos que podem contribuir tanto para a infecção pelo HPV, como também para a regressão ou progressão das lesões provocadas pelo mesmo. Permitindo desta forma a elaboração de um painel de genótipos, que poderia vir a ser utilizado biomarcador de susceptibilidade para o desenvolvimento de lesões cervicais e carcinoma cervical, na prática clínica.

Considerações sobre os Termos de apresentação obrigatória:

Folha de rosto está preenchida adequadamente.

Apresenta as autorizações da Secretaria Municipal de Saúde e do HCL

Atesta que os profissionais que realizarão as coletas de sangue apresentam condições /formação adequada pra realizá-las.

Orçamento previsto no valor de R$ 76.260,00 e informa que o mesmo será realizado desde que seja contemplado em editais de fomento, tendo sido submetido ao doPPSUS-Fundação Araucária.

Prevê o início da coleta de dados a partir de 12/2016.

Como Critério de Inclusão, informa que participarão da pesquisa Mulheres com idade igual ou superior a 18 anos, atendidas em programas de prevenção ao câncer de colo de útero de unidades básicas de saúde (UBS) de Londrina, ou para tratamento de Câncer de colo de útero no Hospital de

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Câncer de Londrina e como ritério de Exclusão: Mulheres com idade inferior a 18 anos.
O TCLE está descrito de forma acessível, bastante claro e informa a participante que o material biológico será armazenado no Laboratório Genética Molecular e Imunologia da UEL para uso em pesquisas futuras, garantindo todas condições de sigilo e confidencialidade.
Apresenta também o TSC devidamente assinado.

Conclusões ou Pendências e Lista de Inadequações:

Não há pendências.

Considerações Finais a critério do CEP:

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_723786.pdf	02/06/2016 11:07:54		Aceito
Outros	DECLARACOES_COLETA_SANGUE.pdf	24/05/2016 20:16:13	Karen Brajão de Oliveira	Aceito
Outros	TSC.pdf	24/05/2016 20:15:39	Karen Brajão de Oliveira	Aceito
Declaração de Instituição e Infraestrutura	Autorizacao_SMS.pdf	24/05/2016 20:15:13	Karen Brajão de Oliveira	Aceito
Folha de Rosto	Folha_de_Rosto.pdf	24/05/2016 20:14:52	Karen Brajão de Oliveira	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.doc	22/05/2016 13:53:14	Karen Brajão de Oliveira	Aceito
Declaração de Instituição e Infraestrutura	declaraco_HCL.pdf	22/05/2016 13:15:15	Karen Brajão de Oliveira	Aceito
Orçamento	Orcamento.pdf	22/05/2016 13:14:22	Karen Brajão de Oliveira	Aceito
Projeto Detalhado / Brochura Investigador	BRAJAO_Projeto_PPSUS_CEP.doc	22/05/2016 13:12:56	Karen Brajão de Oliveira	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

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Não

LONDRINA, 14 de Junho de 2016

Assinado por:
Alexandrina Aparecida Maciel Cardelli
(Coordenador)

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