KLEBER PAIVA TRUGILO

ANÁLISE DOS POLIMORFISMOS rs1800470 E rs1800471 DO *TGFB1* EM MULHERES HPV POSITIVAS E CONTROLES

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Dissertação apresentada ao Programa de Pós-graduação em Patologia Experimental da Universidade Estadual de Londrina como pré-requisito para obtenção do título de mestre.

Orientadora: Profa. Dra. Karen Brajão de Oliveira

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Londrina, 19 de abril de 2016.

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)

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RESUMO

A infecção pelo Papilomavírus Humano (HPV) e a manutenção de vias inflamatórias estão envolvidas na patogênese das lesões pré-malignas e do câncer cervical. No microambiente da infecção, várias citocinas são produzidas e influenciam direta e indiretamente a persistência das lesões cervicais e a progressão para o câncer. Neste contexto, o fator de transformação do crescimento β (TGFB), uma citocina reguladora do crescimento e diferenciação celular, possui um papel paradoxal e atua tanto na supressão como na progressão do tumor. Polimorfismos genéticos de base única (SNPs) que alteram a produção de TGFB, como c.29C>T (rs1800470) e c.74G>C (1800471), vêm sendo associados ao câncer. No entanto, a relação com as lesões provocadas pelo HPV e com o câncer cervical ainda não está bem estabelecida. Desta forma, o objetivo deste estudo foi avaliar a associação destes polimorfismos do *TGFB1* com a infecção por HPV e com as lesões intraepiteliais escamosas de baixo e alto grau (LSIL e HSIL). Para isso foram coletadas amostras de 350 mulheres para detecção de HPV por PCR e genotipagem dos polimorfismos c.29C>T e c.74G>C por PCR-RFLP; dados sócio-demográficos e de comportamento sexual foram obtidos através de aplicação de questionário. O DNA viral foi detectado em 172 (49,1%) mulheres com maior frequência entre as com idade ≤24 anos (p=0.006), solteiras (p=0.013), que nunca gestaram (p=0.007), nulíparas (p=0.008) e que desconheciam sobre o que é o HPV (p=0.017). No caso das lesões, LSIL foi mais frequente entre mulheres com idade ≤24 anos (p=0.036), enquanto HSIL esteve mais presente em mulheres que tiveram pelo menos 4 parceiros sexuais durante a vida (p<0.001). Tanto LSIL como HSIL tiveram alta frequência entre as fumantes (p=0.002). Em relação aos polimorfismos, pacientes HPV+ apresentaram maior frequência do genótipo c.74CG (p=0.004) e dos genótipos combinados c.29CC+CT/c.74CG (p=0.004) associados à maior susceptibilidade de contrair o vírus [OR=2.81 $CI_{95\%}$ (1.35-5.86); OR=3.14 $CI_{95\%}$ (1.42 – 6.94), respectivamente]. Por outro lado, utilizando um modelo de regressão logística ajustado, o genótipo c.29CC foi associado à HSIL (b=0.91, χ^2_{Wald} (df=1)=3.96, p=0.047) e elevou 2,48 vezes as chances de apresentar este tipo de lesão comparado ao genótipo c.29TT [OR=2.48 $CI_{95\%}$ (1.01-6.08)]. Desta forma, o presente trabalho demonstrou pela primeira vez que os polimorfismos c.74G>C e c.29C>T foram associados ao risco de infecção por HPV e ao desenvolvimento de HSIL, respectivamente. Por conseguinte, estes dois SNPs, fazendo parte da composição de um painel genotípico, poderiam ser utilizados como potenciais marcadores moleculares de prognóstico para estas doenças. Contudo, uma maior população de estudo é necessária para que sejam feitas análises adicionais a fim de validar estes resultados

Palavras-chave: Lesão intraepitelial escamosa de alto grau. Lesão intraepitelial escamosa de baixo grau. Diagnóstico molecular.

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ABSTRACT

Human Papillomavirus (HPV) infection and the maintenance of inflammatory pathways are involved in the pathogenesis of premalignant lesions and cervical cancer. Into the infection microenvironment, several locally produced cytokines directly and indirectly influence the persistence of cervical lesions and progression to cancer. In this context, the transforming growth factor β (TGFB), a regulator cytokine of growth and cellular differentiation, has paradoxical role, playing both tumor suppression and progression. Single nucleotide polymorphisms (SNP) that alter TGFB production, like c.29C>T (rs1800470) and c.74G>C (rs1800471), have been associated to cancer. However, their association with HPV lesions and cervical cancer has not been well established yet. Accordingly, the present study aimed to assess the association of these *TGFB1* polymorphisms with the HPV infection and low and high-grade squamous intraepithelial lesions (LSIL and HSIL). Samples were obtained from 350 women to HPV detection by PCR and c.29C>T and c.74G>C polymorphisms genotyping by PCR-RFLP; socio-demographical and sexual behavioral data were obtained by questionnaire. HPV DNA was detected in 172 (49.1%) patients, with frequency higher among women who were ≤24 years old (p=0.006), single (p=0.013), had not been pregnant (p=0.007) nor given birth (p=0.008), and did not know what the virus was (p=0.017). LSIL were more frequent in women ≤ 24 years old (p=0.036), while HSIL were more frequent among those who had ≥ 4 sexual partners in their lifetime (p<0.001). Lesions were also more frequent in smokers (p=0.002). c.74G>C and the combined c.29CC+CT/c.74GC genotype were more frequent in infected patients (35.1 % and 15.7 %) than in uninfected women (6.2 % and 14.7 %). Accordingly, these genotypes were associated with higher risk of HPV [OR=2.81 $CI_{95\%}$ (1.35-5.86), p=0.004; OR=3.14 $CI_{95\%}$ (1.42 – 6.94), p=0.004, respectively]. On the other hand, using an adjusted model of logistic regression, the c.29CC genotype were significantly associated with HSIL (b=0.91, λ^2_{Wald} (df=1)=3.96, p=0.047) and were 2.48 times more likely to cause HSIL compared to c.29TT genotype [adjusted OR=2.48 $CI_{95\%}$ (1.01-6.08)]. The present study demonstrated for the first time that the c.74G>C and c.29C>T polymorphisms were associated with the risk of HPV infection and HSIL, respectively. Therefore, both SNPs as part of a genotypic panel could be used as potential molecular markers of prognostic for these diseases. However, a larger study population is required for additional analyzes in order to validate these results.

Keywords: High-grade squamous intraepithelial lesion. Low-grade squamous intraepithelial lesion. Molecular diagnosis.

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

°C	*Celsius grade*
µM	*Micromolar*
Aa	*Amino acids*
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*
BaP	*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*
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*
kDa	*Kilodalton*
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*
mM	*Milimolar*
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*
nm	*Nanometer*
nM	*Nanomolar*
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*
pH	*Potential of hydrogen*
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*
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*
U	*Unit*
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*

SUMÁRIO

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, embora nem sempre a infecção apresente lesões clínicas (SCHIFFMAN et al., 2007).

Nas mulheres, o HPV possui papel central no desenvolvimento de lesões intraepiteliais cervicais e do câncer cervical. Neste último, este vírus está virtualmente presente em 100% dos casos (SCHIFFMAN et al., 2007). Dados do *HPV Information Centre* (2010), revelam que cerca de 11,4% das mulheres do mundo e 13,2% das sul-americanas que apresentam exame citológico normal, ou seja, sem anormalidades celulares na superfície epitelial da cérvix uterina, estão infectadas pelo HPV. Entre estas e também entre as brasileiras HPV positivas e com citologia normal, a prevalência da infecção é maior na faixa etária menor de 25 anos, a qual corresponde ao início da atividade sexual de ambos os sexos e ao período de maior taxa de aquisição do vírus.

Os tipos de HPVs considerados de alto risco oncogênico (HR-HPV) estão envolvidos na etiopatogenia do câncer e da maioria das lesões intraepiteliais cervicais (BRUNI et al., 2015; SCHIFFMAN et al., 2007). Dentre eles, o HPV16 e o HPV18 estão presentes em aproximadamente 5,4% das brasileiras com citologia normal, 30,6% com lesão intraepitelial escamosa de baixo grau (LSIL), 59,4% com lesões intraepiteliais de alto grau (HSIL) e 68,5% com câncer invasivo. Os HPVs de baixo risco oncogênico (LR-HPV), como HPV6 e HPV11, também podem causar doenças, entre elas a papilomatose respiratória juvenil recorrente e as verrugas genitais (BRUNI et al., 2015).

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). Sabe-se que a regressão ou progressão das lesões depende fortemente 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 polimorfismos no gene do TGFB1 a 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 associação ao câncer cervical ou às lesões provocadas pela infecção por HPV ainda não está bem estabelecida. Portanto, este trabalho pretende abordar de forma pormenorizada o papel do TGFB1 na carcinogênese cervical, avaliando a associação dos polimorfismos de base única (SNPs) rs1800470 (c.29C>T) e rs1800471 (c.74G>C) do gene *TGFB1* à infecção e às lesões provocadas pelo HPV.

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 *deoxyribonucleic acid* (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 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 de vida viral, E6 e E7 facilitam a manutenção estável de episssomas 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 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 episssomal. O genoma do vírus é amplificado e novas partículas são formadas para a propagação da infecção quando as células infectadas atingem um estado diferenciado (FEHRMANN et al., 2003; ZHENG; BAKER, 2006; 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 1 (displasia leve), 2 (displasia moderada) ou 3 (displasia severa/carcinoma *in situ*) (CIN1, 2 ou 3), 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) (BRASIL, 2011, 2012).

Embora a presença do vírus só 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 rastreio para prevenção do câncer de colo do útero no mundo todo.

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 BRASIL (2011, p. 19).
*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*.

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, promovendo a transformação tumoral.

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). Dos 47 tipos de HPVs que infectam a mucosa genital, todos do gênero Alphapapillomavirus (BZHALAVA et al., 2013), apenas 13 tipos são considerados HR-HPVs (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 e 68) (BZHALAVA et al., 2013; GUAN et al.,

2012). 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. Os LR-HPVs tipos 6 e 11 são os mais encontrados nas lesões genitais benignas, causando 90% dos condilomas (SCHIFFMAN et al., 2007; SMITH et al., 2007). Além do câncer de colo do útero, o HPV vem sendo 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 porcentagem 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 progridir para HSIL ou câncer invasivo como resultado da interação 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 e indiretamente as propriedades proliferativas e invasivas das células tumorais (AMEDEI; PRISCO; 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).

O fator de transformação do crescimento β (TGFB) é uma citocina multifacetada que atrai atenção neste contexto de câncer e inflamação. Esta citocina é reconhecida por regular o comportamento celular e possuir um papel essencial na modulação do crescimento, maturação e diferenciação da célula e por também 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; 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 dependem da sua ligação aos seus receptores. Em humanos, são conhecidos o receptor tipo 1 [*Activin-like Kinase* (ALK) 1–7), o receptor tipo 2 [*Activin receptor* (ACTR) 2A e 2B, *Bone morphogenetic protein receptor* (BMPR) 2, *Anti-Mullerian hormone receptor* (AMHR) 2 e *Transforming growth factor β receptor* (TGFBR) 2] (MASSAGUÉ; GOMIS, 2006) e o receptor tipo 3 ou betaglicana, de função reguladora (MEYER et al., 2014). 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 propaga 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; MASSAGUÉ; GOMIS, 2006; MASSAGUÉ; SEOANE; WOTTON, 2005).

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 bastante 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 (MASSAGUÉ; SEOANE; WOTTON, 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 et al., 2014).

Além disso, 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). Da mesma forma, 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 células de linhagens 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). Ainda, 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 et al., 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, como mostrado em 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 et al., 2004; TARAPORE et al., 1997). Este complexo ativa a transcrição dos oncogenes virais E6 e E7(BALDWIN et al., 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 et al., 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. No entanto, o efeito do TGFB1 na expressão do HPV varia significativamente em diferentes linhagens celulares e, frequentemente, ocorre em células tumorais uma resistência parcial ao efeito inibitório desta citocina sobre o crescimento celular e a expressão dos genes precoces do HPV (WOODWORTH; NOTARIO; DIPAOLO, 1990). *In* vitro, uma progressiva resistência à inibição de crescimento pelo TGFB foi observada durante o processo de malignização. Enquanto o efeito anti-proliferativo do TGFB predomina nas fases iniciais da transformação, ao final, esta citocina provoca *epithelial-to-mesenchymal transition* (EMT) e motilidade celular. 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).

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.

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

3.2 POLIMORFISMOS rs1800470 (c.29C>T) E rs1800471 (c.74G>C) NO GENE *TGFB1*

Polimorfismos são variações na sequência de DNA, herdadas de pai para filho e que estão presentes na população numa frequência igual ou superior a 1% (KARKI et al., 2015). 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, os polimorfismos podem influenciar na expressão gênica e na produção, estrutura e função de proteínas (BALASUBRAMANIAN et al., 2004).

Dentre os polimorfismos genéticos, os polimorfismos de base única (SNPs) são os mais comuns. Estes são caracterizados pela substituição de um único par de bases numa posição particular da sequência de nucleotídeos. Os SNPs são utilizados como “assinaturas genéticas” na população para estudar a predisposição a certas características que os indivíduos apresentam, incluindo as doenças (BALASUBRAMANIAN et al., 2004; KARKI et al., 2015).

Vários polimorfismos foram descritos para o *TGFB1,* dentre eles os SNPs em região que codifica o peptídeo sinal rs1800470 e rs1800471. O SNP

rs1800470 (c.29C>T ou Pro10Leu) ocorre em região codificante (c.) do DNA, a +29bp posteriores ao sítio de início da tradução (+1), no códon 10 do peptídeo sinal (SHAH et al., 2006). Neste local, a base C (citosina) do alelo ancestral (c.29C) pode ser substituída pela base T (timina), gerando o alelo variante (c.29T). Esta troca resulta na substituição de uma prolina (Pro) por uma leucina (Leu) na proteína. Em relação ao SNP rs1800471 (c.74G>C ou Arg25Pro), a substituição de uma guanina (G) por uma citosina (C) no códon 25 acarreta, na proteína, a troca de uma arginina (Arg) por uma prolina (Pro).

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 29. As trocas Pro10Leu (rs1800470) e Arg25Pro (rs1800471) estão localizadas, respectivamente, na região que codifica o núcleo hidrofóbico e próximo à extremidade 3' deste núcleo (CAMBIEN et al., 1996). 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., 2000).

Os alelos ancestrais 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).

Estes polimorfismos vêm sendo relacionados a alguns tipos de cânceres (Tabela 2). Desta forma, considerando-se a importância epidemiológica da infecção pelo HPV e seu papel no desenvolvimento das lesões intraepiteliais cervicais e progressão para o câncer, somada ao fato de que a relação dos polimorfismos de TGFB não está bem estabelecida com a infecção pelo HPV ou mesmo com o desenvolvimento dessas lesões, existe a necessidade de estudos adicionais para que se defina melhor a associação dos SNPs c.29C>T e c.74G>C na patogênese da infecção e lesões causadas pelo HPV.

Tabela 2 – Cânceres associados aos SNPs c.29C>T e c.74G>C do *TGFB1*

SNP	Tipo de câncer	Referência
c.29C>T (rs1800470)	Mama	DUNNING et al., 2003 ZIV et al., 2001 POOJA et al., 2013
	Pulmão	CHEN, 2014
	Nasofaríngeo	WEI et al., 2007b
	Cárdia	GUO et al., 2011
	Boca	CARNEIRO et al., 2013
	Orofaríngeo	GUAN et al., 2010
	Esôfago	WEI et al., 2007a
	Próstata	LI et al., 2004
c.74G>C (rs1800471)	Mama	POOJA et al., 2013 SHU et al., 2004

Objetivos

4 OBJETIVOS

4.1 Objetivo Geral

Este trabalho teve como objetivo geral analisar os polimorfismos rs1800470 (c.29C>T) e rs1800471 (c.74G>C) do gene *TGFB1* em mulheres HPV positivas e controles atendidas pelos programas de prevenção ao câncer de colo do útero do setor público de saúde da região norte do Paraná.

4.2 Objetivos Específicos

- Realizar a detecção do HPV, por PCR, 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;

- Avaliar a associação do perfil sociodemográfico e comportamento sexual das pacientes com a infecção por HPV e com a presença de LSIL e HSIL;

- Comparar a ocorrência dos polimorfismos c.29C>T e c.74G>C do *TGFB1* nas pacientes e controles;

- Avaliar a susceptibilidade à infecção por HPV e ao desenvolvimento de LSIL e HSIL associada aos genótipos dos polimorfismos c.29C>T e c.74G>C do *TGFB1*.

Produção Científica

5 PRODUÇÃO CIENTÍFICA

5.1 ARTIGO 1

ROLE OF TGFB1 IN CERVICAL CARCINOGENESIS

ABSTRACT

Transforming growth factor β (TGFB) plays an important role in cervical carcinogenesis. Levels of this cytokine and its mRNA gradually increase according to normal tissue progresses to malignancy, showing close relation to Human Papillomavirus (HPV) infection severity. High-risk HPVs (HR-HPVs) are involved in about all cervical cancer cases. However, HPV alone is not sufficient for malignant progression. Effects of a chronic inflammation linked to persistent HPV infection and the interaction of immune components with the infected microenvironment may contribute to cancer development. TGFB, in the beginning of infection, may regulate the infected cells proliferation and virus amplification by cellular growth inhibitory action and downregulating the HPV long control region (LCR) transcriptional activity, which decrease early genes expression. When infected cells progress to malignant transformation, they lose the TGFB growth inhibitory response and present only partial repression of E6 and E7 expression. In this context, TGFB provokes epithelial-to-mesenchymal transition (EMT), cell motility, and angiogenesis. High levels of TGFB in tumor microenvironment could be explained through TGFB producer stromal cells attendance and through upregulation of TGFB expression in cervical cancer cells stimulated by stromal cells, and E6 and E7 oncoproteins.

Keywords: Human Papillomavirus; Cervical Cancer; HPV oncoproteins.

INTRODUCTION

Cervical cancer is the third most common type of cancer among Brazilian women (BRASIL, 2015) and the fourth worldwide (TORRE et al., 2015). Human Papillomavirus (HPV) is strongly associated to this disease, and its prevalence in cervical cancer is reported to 99.7% when gold standard HPV detection techniques are employed (SMITH et al., 2007; WALBOOMERS et al., 1999).

More than 200 HPV types are known (BZHALAVA; EKLUND; DILLNER, 2015) and according to the carcinogenic potential of each one they are classified as high-risk (HR-HPV), undetermined-risk (UR-HPV) and low-risk (LR-HPV). Up to now, 13 types are considered HR-HPV (BZHALAVA et al., 2013; GUAN et al., 2012) and two of which, HPV16 and HPV18, both account for 70% of cervical

cancer cases (SCHIFFMAN et al., 2007; SMITH et al., 2007). This virus can also be associated with cancer of vagina (ALEMANY et al., 2014), vulva (SIRIAUNKGUL et al., 2014), penis (LEBELO et al., 2014), anus (ALEMANY et al., 2015), neck and head (CORRENTI; RIVERA; CAVAZZA, 2004; GUDLEVICIENE et al., 2009), kidney (FARHADI et al., 2014) and breast (FU et al., 2015).

Nevertheless, only a subset of individuals exposed to HPV develops persistent infection which could progress to high-grade squamous intraepithelial lesion (HSIL) and cervical cancer. This suggests that multiple genetic events may contribute to the occurrence of HPV persistent infection and progression of lesions. In this context, TGFB, a potent modulator of cell growth, maturation, and differentiation (KUBICZKOVA et al., 2012), could be considered as an important cytokine into infection microenvironment. Therefore, this review addresses the role of TGFB1 in cervical carcinogenesis and the relationship between this cytokine and HPV in the development of cervical cancer.

HPV AND CERVICAL CANCER

HPV belongs to the *Papillomaviridae* family and it is a nonenveloped virus comprised of a capsid and double-stranded circular deoxyribonucleic acid (DNA). The viral genome is around 8000bp and typically consists in eight open reading frames (ORF's) and a non-coding segment named long control region (LCR). The ORF's may be divided into early (E) and late (L) genes. Whereas early genes encode the viral replication related-proteins (E1, E2, E4, E5, E6 and E7), the late genes encode the capsid proteins (L1 and L2) which are required for virion assembly (DOORBAR, 2006; KIRNBAUER et al., 1992).

The E1 and E2 proteins are involved with the initiation of viral replication. At elevated levels, E2 downregulates E6 and E7 expression, decreasing these oncoproteins production, and activates late genes expression (HEBNER; LAIMINS, 2006; JOHANSSON; SCHWARTZ, 2013). The E4 protein appears to contribute to greater efficiency of the viral genome amplification and also facilitate the release of newly formed virions (DOORBAR, 2013). E5 is an oncoprotein associated with increased E6 and E7-induced malignant transformation, upregulation of epidermal growth factor receptor (EGFR) signaling pathway, angiogenesis and antiapoptotic effect (KIM et al., 2010). The viral E6 and E7 oncoproteins are

necessary for malignant conversion. The abilities of high-risk HPV E6 and E7 proteins to target a number of negative regulators of the cell cycle, primarily tumor-suppressor protein 53 (p53) and retinoblastoma protein (pRb), respectively, have been suggested as a mechanism by which these viral proteins induce tumors (YIM; PARK, 2005). The L1 and L2 structural proteins are two coat proteins that comprise the virus shell. L1 is the primary structural element and L2 is the minor component of the capsid. Both proteins play an important role in mediating efficient virus infectivity (DOORBAR, 2006; MODIS; TRUS; HARRISON, 2002).

The HPV transmission mainly occurs by skin-to-skin or mucosa-to-mucosa contact (ROBERTS et al., 2007; SCHIFFMAN et al., 2007). The virions infect basal layers cells of epithelium, generally through micro-wounds, and enter cells via an unidentified receptor. After episomal establishment in the nucleus, the genomes are amplified, and new virus particles are formed and released while infected cells differentiate (FEHRMANN; KLUMPP; LAIMINS, 2003; FERNANDES et al., 2015; HEBNER; LAIMINS, 2006; ZHENG; BAKER, 2006). In the cervix, HPV can be cleared after a transient infection or remain persistent which is highly linked to precancerous lesions. In a pre-malignant stage, the abnormalities found in histopathological exam or in the cervical citology (Papanicolaou smear test) can, respectively, characterize the severity of these lesions in cervical intraepithelial neoplasia grade 1, 2 or 3 (CIN 1, 2 or 3) or low-grade or high-grade squamous intraepithelial lesions (LSIL or HSIL). CIN1 (mild dysplasia) corresponds to LSIL; CIN2 (moderate dysplasia) and CIN3 (severe dysplasia / carcinoma *in situ*) are considered HSIL (SCHIFFMAN et al., 2007; WOODMAN; COLLINS; YOUNG, 2007). Cervical cancers develop when cellular changes of pre-malignant lesions achieve malignant features, leaving the *in situ* character to acquire an invasive one. At this stage, the viral DNA is usually integrated into the host genome, causing loss of E2 and consequent breakage of negative feedback which controls the oncogenes expression. As a result, E6 and E7 become actively expressed which contribute to tumor transformation (FEHRMANN; LAIMINS, 2003; SCHIFFMAN et al., 2007; SCHWARZ et al., 1985; STOLER et al., 1992; WOODMAN; COLLINS; YOUNG, 2007).

It is well established that the presence of some HPV types can cause persistent infections, leading to cervical cancer (CASTRO-VÁSQUEZ; ARELLANO-GÁLVEZ, 2010; HO et al., 1995; WOODMAN; COLLINS; YOUNG, 2007)(HO et al.,

1995; WOODMAN; COLLINS; YOUNG, 2007). Most of the cervical HPV infections are cleared or suppressed within 1-2 years of exposure (SCHIFFMAN et al., 2007; STANLEY, 2009). The HPV clearance is a result of specific immunological reactions, which require competent humoral and cell-mediated immune mediators (DENIS; HANZ; ALAIN, 2008; STANLEY, 2009; SYRJÄNEN, 2007; TORRES-POVEDA et al., 2014). However, the majority of HPV infections caused by HR-HPV progress to cervical intraepithelial neoplasia or cancer according to the immune cells and molecules in lesion microenvironment (PATEL; CHIPLUNKAR, 2009).

INFLAMATORY MICROENVIRONMENT IN CANCER

In the 19th century, Rudolf Virchow observed the presence of leukocytes within tumors providing the first indication of a possible link between inflammation and cancer (GRIVENNIKOV; GRETEN; KARIN, 2010). A role for inflammation in tumorigenesis is now generally accepted, and it has become evident that an inflammatory microenvironment is an essential component of all tumors, including some in which a direct causal relationship with inflammation is not yet proven (GRIVENNIKOV; GRETEN; KARIN, 2010; MANTOVANI et al., 2008).

The cancer development is highly associated with the process of chronic inflammation, which is responsible for the cascade of signals that recruits cells of the acquired immune response. In this cascade, cytokines and chemokines stimulate the maturation of the cells (such as dendrict cells) and the migration of leukocyte for the inflammation site (COUSSENS; WERB, 2002). Chronic inflammation contribute to tumorigenesis at all stages: cancer initiation by generating genotoxic stress, cancer promotion by inducing cellular proliferation, and cancer progression by enhancing angiogenesis and tissue invasion (CHOW; MÖLLER; SMYTH, 2012).

Cytokines and chemokines have a crucial role in cancer-related inflammation with consequent, direct, and indirect effects on the proliferative and invasive properties of tumor cells (AMEDEI; PRISCO; D'ELIOS, 2013; DE OLIVEIRA et al., 2014)

Cytokines are released in response to a diverse range of cellular stresses, including carcinogen-induced injury, infection and inflammation. The cytokines produced in the tumor microenvironment play an important role in cancer

pathogenesis, and they can function to inhibit tumor development and progression but cancer cells can respond to host-derived cytokines that promote growth, attenuate apoptosis and facilitate invasion and metastasis (DRANOFF, 2004). Consequently host reactions to cellular stress can impact on several stages of cancer formation and progression. Therefore cytokines participation in tumor pathogenesis remains incompletely understood.

Multiple factors in the tumor microenvironment play a role in tumor cell behavior. Stimuli from tumor and immune cells cause cancer cells to dedifferentiate into cells with the ability to self-renew and to eventually migrate to distant sites (SHIGDAR et al., 2014). Whether the immune reaction is pro or anti-tumor depends on which cell type is in abundance and the status of activation (GRIVENNIKOV; GRETEN; KARIN, 2010).

Dissection of the diversity of cancer-related inflammation is instrumental to the design of therapeutic approaches that target cancer-related inflammation (BALKWILL; MANTOVANI, 2012).

In this context we emphasize transforming growth factor β (TGFB), one of the cytokines responsible for regulation of cell behavior, playing pivotal role in modulation of cellular growth, maturation, differentiation, and cancer progression (ZARZYNSKA, 2014).

TGFB

The TGFB superfamily comprises over 30 dimeric proteins with conserved structures (DELA CRUZ; REIS, 2015), with a molecular weight of approximately 25 kDa (ROBERTS; SPORN, 1993), including TGFBs proteins, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activins (ACTs), inhibins (INHs), and glial-derived neurotrophic factors (GDNFs), as well as some proteins not included in the above families, such as Müllerian inhibiting substance (MIS) also known as anti-Müllerian hormone (AMH), left-right determination factor (LEFTY), and nodal growth differentiation factor (NODAL) (JAVELAUD; MAUVIEL, 2004; MASSAGUÉ; GOMIS, 2006; PONIATOWSKI et al., 2015).

TGFB superfamily molecules are secreted signaling molecules that regulate a plethora of cellular responses, such as proliferation, differentiation, migration and apoptosis. TGFB family members have critical roles during

embryogenesis and in maintaining tissue homeostasis during adult life (SHI; MASSAGUÉ, 2003; TAN; ALEXE; REISS, 2009; TEN DIJKE, 2000).

In mammalian there are three closely related TGFB isoforms: TGFB1, TGFB2, and TGFB3 that arose by duplication of a common ancestor (PAPAGEORGIS, 2015; VAIDYA; KALE, 2015) each encoded by a different gene. The *TGFB1* gene is located in 19q13 chromosome, *TGFB2* in 1q41 and *TGFB3* in 14q24 (KRSTIC; SANTIBANEZ, 2014; LEBRUN, 2012). Similarity is the most striking in the C-terminal domain (64–82%), with nine conserved cysteine residues forming four intra-chain and one inter-chain disulfide bonds. Despite this high sequence homology, analysis of the *in vivo* functions of the three isoforms by gene knockouts reveal striking differences, illustrating their non-redundancy. Overall, TGFB1 is the most abundant isoform, with the largest sources of TGFB1 being platelets (20 mg/kg) and bone (200 μg/kg) (VAIDYA; KALE, 2015). In addition, in humans, virtually almost all cells can synthesize it, primarily platelets, regulatory T cells (Treg), macrophages/monocytes, lymphocytes, fibroblasts, epithelial cells, and dendritic cells (KAJDANIUK et al., 2013).

Primarily these cytokines are expressed in inactive form, and are processed at extracellular matrix by enzymes and others convertases producing an active dimer cytokine (PIEK; HELDIN; TEN DIJKE, 1999) that is stabilized by hydrophobic interactions, which are further strengthened by an intersubunit disulfide bridge (IKUSHIMA; MIYAZONO, 2011; SCHLUNEGGER; GRÜTTER, 1992). Furthermore, TGFB can be activated by low pH levels in the local environment (LYONS; KESKI-OJA; MOSES, 1988) or upon irradiation-induced reactive oxygen species (ROS) production (BARCELLOS-HOFF et al., 1994; PAPAGEORGIS, 2015).

The various biological activities of TGFB isoforms are mediated by specific cell surface receptors in responsive cells. The human genome encodes seven type 1 receptors [Activin-like Kinase (ALK) 1–7) and five type 2 receptors [Activin receptor (ACTR) 2A e 2B, Bone morphogenetic protein receptor (BMPR) 2, Anti-Mullerian hormone receptor (AMHR) 2 e Transforming growth factor β receptor (TGFBR) 2] that are paired in different combinations as receptor complexes for the various members of the TGFB family (MASSAGUÉ; GOMIS, 2006).

TGFB ligands bind to their cognate receptor complexes at the cell surface; these consist of two type 1 (TGFBR1 also known as ALK5) and two type 2 serine/threonine kinase receptors (TGFBR2) (DERYNCK; AKHURST, 2007;

MASSAGUÉ; GOMIS, 2006). In these complex, type 2 receptors phosphorylate the type 1 components, which then propagate the signal (MASSAGUÉ, 2012; WRANA et al., 1994) through activation of both the small mothers against decapentaplegic protein (SMAD) and non-SMAD signaling pathway, activating gene expression (JOSHI; CAO, 2010; SHI; MASSAGUÉ, 2003). In the absence of phosphorylation, SMADs are transcriptionally inert.

TGFB SIGNALING

After activation type 1 receptor, in turn, phosphorylates receptor-bound SMAD (SMAD2/3) transcription factors at the carboxy-terminal SXS motif, releasing them from retention in the cytoplasm and allowing them to translocate into the nucleus. SMAD4 acts as a common partner of activated SMADs to help to execute their function. SMAD proteins continuously undergo nucleocytoplasmic shuttling by interacting with nuclear-pore complexes. Once in the nucleus, activated SMAD proteins form final complexes that regulate target gene transcription, generating approximately three hundred early gene responses that can promote both gene activation or repression responses (MASSAGUÉ; GOMIS, 2006).

SMADs regulate different genes in different cell types through its association with different protein partners (MASSAGUÉ, 2000), resulting in a combination that targets a particular subset of genes depending on the DNA binding specificity of it, and recruits either transcriptional co-activators or co-repressors. These protein partners include forkhead box (FOX), homeobox (HOX), Runt-related transcription factor (RUNX), E2 promoter-binding factor (E2F), activator protein 1 (AP1), cyclic-AMP response element binding protein (CREB)/ activating transcription factor (ATF), Zinc-finger and other families. The availability of such cofactors depends on the cell type, partly determining the gene responses to TGFB in each cell type (FENG; DERYNCK, 2005; MASSAGUÉ; GOMIS, 2006; MASSAGUÉ; SEOANE; WOTTON, 2005).

TGFB can also employ SMAD-independent pathways as downstream effectors that are collectively referred to as ‘non-canonical’ TGFB signaling (MASSAGUÉ, 2012) by inducing other pathways such as extracellular signal-regulated MAP kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) (MULDER, 2000; VAIDYA; KALE, 2015),

phosphoinositide 3-kinase (PI3K)-“Ak” thymoma protein (AKT)- mechanistic target of rapamycin complex (mTOR) pathway (LAMOUILLE; DERYNCK, 2007), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and wingless-type MMTV integration site family (WNT)/β-catenin pathway (BAKIN et al., 2002; GINGERY et al., 2008), ubiquitin ligase tumor necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) and TGFB-associated kinase 1 (TAK1) and the small guanosine triphosphatases (GTPases) rat sarcoma viral oncoprotein homolog (RAS)-homolog protein (RHO), RAS-related C3 botulinum toxin substrate (RAC), and cell division cycle (CDC) 42, and the RAS-ERK-MAPK pathway (MU; GUDEY; LANDSTRÖM, 2012).

In addition to the type 1 and type 2 receptors, the type 3 TGFB receptor (TGFBR3, or betaglycan) is a critical regulator of TGFB signaling (MEYER et al., 2014). Loss or reduced expression of the TGFBR3 has been demonstrated in multiple human cancers like ovarian cancer (HEMPEL et al., 2007), breast cancer (DONG et al., 2007) and non-small cell lung cancer (FINGER et al., 2008). TGFBR3 has also been shown to be an important regulator of cell migration, invasion, cell growth, and angiogenesis in both *in vitro* and *in vivo* cancer models (GATZA; OH; BLOBE, 2010).

TGFBR3 mediates both ligand dependent and independent effects. TGFBR3 can mediate cell migration in a β-arrestin2 dependent manner through the activation of CDC42 in a ligand independent manner. TGFBR3 activates p38 in both a ligand independent and dependent manner. TGFBR3 undergoes ectodomain shedding producing soluble TGFBR3, which can bind to and sequester ligand, inhibiting TGFB signaling. TGFBR3 also presents ligand to TGFBR2, which phosphorylates TGFBR2, and recruits and phosphorylates TGFBR1, causing phosphorylation of the R-SMADs allowing interaction with Co-SMAD4, mediating nuclear translocation of the SMAD complex and regulation of transcriptional activity. Interaction of TGFBR3 with β-arrestin2 results in the internalization of the TGFBR3/TGFBR2/β-arrestin2 complex and subsequent downregulation of TGFB signaling. TGFBR3 also negatively regulates NFκB signaling in a β-arrestin2 dependent manner. Interaction of TGFBR3 with GAIP-interacting protein C-terminus (GIPC) stabilizes TGFBR3 at the cell membrane and enhances TGFB signaling, as well as mediating effects on migration and invasion (GATZA; OH; BLOBE, 2010)

Within this basic signal transduction pathway, a negative regulatory feedback loop is intricately built that involves primarily three types of proteins: inhibitory SMADs (I-SMADs), ubiquitin ligases of the Smad ubiquitination-related factor (SMURF) family and phosphatases (MASSAGUÉ; SEOANE; WOTTON, 2005; PARDALI; MOUSTAKAS, 2007). The I-SMAD, SMAD6 and SMAD7, negatively regulate TGFB signaling by competing with R-SMADs for receptor or Co-SMAD interaction and by targeting the receptors for degradation (SHI; MASSAGUÉ, 2003). TGFB/SMAD signaling rapidly induces *I-SMAD* and *SMURF* gene expression. SMAD7 is then recruited to the TGFB receptor complex and competitively inhibits the phosphorylation of R-SMADs by TGFBR1. SMAD7 also recruits phosphatases that dephosphorylate and inactivate the receptor complex and directly binds and activates the catalytic activity of SMURF ubiquitin ligases, leading to ubiquitylation of TGFBR1 and thus promoting endocytosis and final lysosomal degradation of the receptor–ligand complex (PARDALI; MOUSTAKAS, 2007).

Since TGFB participates in fetal development, control of cell growth and differentiation, induction of fibrosis and wound healing, immunosuppression, angiogenesis, and inflammatory processes (KAJDANIUK et al., 2013), dysregulated expression and activity of TGFB, TGFBR1/2 and SMADs, and TGFBR3 contributes to a broad variety of pathologies, among them, cancer (DRABSCH; TEN DIJKE, 2012; FABREGAT et al., 2014; GATZA; OH; BLOBE, 2010).

TGFB IN CANCER

The role of TGFB1 in cancer progression has been shown to be multifaceted, depending on the tumor stage acting both as a tumor suppressor and as a promoter of tumor metastasis (LEBRUN, 2012; ZARZYNSKA, 2014).

This cytokine acts as a potent growth inhibitor since it has been shown to inhibit epithelial cell cycle progression and promote apoptosis that together significantly contribute to the tumor suppressive role during carcinoma initiation and progression (ZARZYNSKA, 2014).

TGFB exerts strong cytostatic effects and induces cell cycle arrest in the G1 phase by increasing the expression of the small cyclin-dependent kinase inhibitors p15, p21, and p27. TGFB-induced cell cycle arrest also relies on the downregulation of the oncogene v-MYC avian myelocytomatosis viral oncogene

homolog (c-MYC) through SMADs and repressor E2F4/5 (LEBRUN, 2012). TGFB also induces apoptosis through the E2F1-pRb-p300/CREB-binding protein-associated factor (P/CAF) central pathway that leads to gene transcription of multiple TGFB proapoptotic target genes in various types of normal and cancer cells (KORAH et al., 2012). TGFB also promotes apoptosis in an stress-activated protein kinases (SAPK)-dependent manner by inducing pro-apoptotic target gene expression [B-cell lymphoma 2 (BCL2)-modifying factor (BMF), BCL-2-interacting mediator of cell death (BIM), and BCL2-associated X protein (BAX)] and by repressing antiapoptotic gene expression [B-cell lymphoma-extra large (BCL-Xl) e BCL2] (OHGUSHI et al., 2005), further inducing mitochondrial release of cytochrome C and subsequent activation of caspases 9 and 3 (CHIPUK et al., 2001).

TGFB also exerts its tumor suppressive effects through inhibition of cell immortalization in normal and cancer cells regulating the telomerase (hTERT) gene promoter through SMAD3, ERK1/2, and p38 pathways kinase, preventing cell immortalization by the inhibition of telomerase expression (LACERTE et al., 2008).

However, as tumors progress, tumor cells may lose their growth-inhibitory response to TGFB and may instead respond inducing other effects that contribute to tumor progression (FABREGAT et al., 2014), because of its prominent role in the regulation of cell growth, differentiation and migration (PICKUP; NOVITSKIY; MOSES, 2013). This switch helps the tumor to use TGF-β as an oncogenic factor inducing tumor motility, invasion, metastasis and epithelial-to-mesenchymal transition (EMT) (KUBICZKOVA et al., 2012).

In mammalian epithelial cell cultures, TGFB initiates cell scattering and cytoskeletal reorganization, transforming a tightly organized epithelial sheet into a motile population of cells. TGFB induces not only EMT but also the further differentiation of fibroblasts into myofibroblasts that are contractile and have an elevated expression of pro-metastatic factors, such as matrix metalloproteinases (MMP), interleukin (IL)-8, vascular endothelial growth factor and the chemokine receptor CXCR4 leading to enhanced migration, invasion, and intravasation or extravasation in or out of the circulatory system (DERYNCK; AKHURST, 2007).

Furthermore TGFB is the most powerful physiological immunosuppressor in mammals (GORELIK; FLAVELL, 2002) decreasing tumor cell recognition and clearing (GIGANTE; GESUALDO; RANIERI, 2012; PICKUP; NOVITSKIY; MOSES, 2013). TGFB elicits strong immunosuppressive effects by

inhibiting the functions of different immune cell types. It has long been known that TGFB inhibits the proliferation of and suppresses the antitumor functions of CD4+ or CD8+ T cells, natural killer (NK) cells both *in vitro* and *in vivo* (GORELIK; FLAVELL, 2001; ROOK et al., 1986), it is also capable of inducing apoptosis in B cells (RAMESH; WILDEY; HOWE, 2009).

TGFB can also promote tumor growth by inducing polarization of macrophages and neutrophils from the cancer cell-attacking type 1 to the type 2, which exhibits significantly reduced effector function and produces inflammatory cytokines, like IL-6, IL-11, and TGFB (FRIDLENDER et al., 2009; MANTOVANI et al., 2002).

Recent work has also shown that TGFB mediates the immunosuppressive differentiation of T cells (FLAVELL et al., 2010). Treatment of naive T cells with TGFB induces the expression of the transcription factor forkhead box protein P3 (FOXP3), which drives the phenotypical conversion of a naive T cell to a Treg cell (CHEN et al., 2003; TONE et al., 2008). These studies collectively establish a critical role for TGFB in suppressing host immune system to facilitate cancer progression (PAPAGEORGIS, 2015).

The role of TGFB as a potent tumor suppressor is further highlighted by the fact that many inactivating mutations in TGFB receptors and SMAD genes have been found to be an underlying cause for human cancer (BLOBE; SCHIEMANN; LODISH, 2000; MASSAGUÉ, 2008; SIEGEL; MASSAGUÉ, 2003). Furthermore loss of TGFB signaling components in human cancer has been associated with poor prognosis as a result of increased progression and metastasis in many cancers as head and neck squamous cell carcinoma (LU et al., 2006), breast tumor (PAIVA et al., 2012), lung adenocarcinoma and squamous cell carcinoma (LEVY; HILL, 2006; MALKOSKI et al., 2012; PICKUP; NOVITSKIY; MOSES, 2013) and cervical carcinoma (KI et al., 2009), the latter being especially addressed in this review.

TGFB AND CERVICAL CANCER

In all steps of cervical cancer pathogenesis, TGFB appears to be involved. This cytokine levels and its messenger ribonucleic acid (mRNA) gradually increase according to progression from normal tissue to malignancy (GARCÍA-

ROCHA et al., 2015; KI et al., 2009; PEGHINI et al., 2012; XU et al., 2006) suggesting an association with HPV infection severity.

Evidence has shown that the stroma around cancer cells plays an important role in cancer progression. Cancer-associated fibroblasts (CAFs) exhibit morphological phenotypes of myofibroblasts and are known to promote cancer progression through interactions with adjacent cancer cells. TGFB is a key cytokine mediating such interactions (NAGURA et al., 2015). Previously, it was demonstrated that cervical cancer cells, via the paracrine effect of TGFB1, were capable of augmenting the intratumoral stroma and decreasing the tumor infiltrate (HAZELBAG et al., 2002). Moreover, a study using mesenchymal stromal cells derived from cervical carcinoma cocultured with human cervical adenocarcinoma cell line with integrated HPV18 genome (HeLa) and cervical carcinoma metastasis cell line with integrated HPV16 and HPV18 genomes (CaSki) provides evidences that tumor mesenchymal stromal cells can induce significant expression of TGFB1 by cervical cancer cells (GARCÍA-ROCHA et al., 2015). Recently, it was found that interaction between cervical squamous cell carcinoma (CSCC) and CAFs activates TGFB locally mediated by thrombospondin-1 (TSP-1) resulting in up-regulation of phosphorylated SMAD3 (pSMAD3) in CSCC cells, ultimately promoting invasion. Expression of pSMAD3 in the boundary area of CSCC indicates TGFB activation at the tumor front, and is highly likely to be accompanied by lymph node metastasis (NAGURA et al., 2015). Together, these mechanisms show to be greatly important to tumor growth and metastasis, probably facilitating the tumor cells escape from the host immune surveillance.

In addition, HPV itself acts to evade the host immune response through close interaction with TGFB1 (GUAN et al., 2010). A previous study reported that TGFB1 expression and production by cervical carcinoma cell line with integrated HPV16 (SiHa), CaSki and HeLa cells were higher than in that cells without HPV genomes and were positively correlated with the presence of E7 (XU et al., 2006). Additionally, after HPV infection of basal epithelia cervical cells, E6 and E7 oncoproteins are expressed, and induce TGFB1 expression throughout the specificity protein 1 transcription factor (Sp1). The E6-Sp1 and E7-Sp1 complex can migrate into the nucleus and induce the TGFB1 gene expression (PERALTA-ZARAGOZA et al., 2006; TORRES-POVEDA et al., 2014). TGFB1 can play an important role in the downregulation of human leukocyte antigen class 1 (HLA-I) molecules on cervical

cancer cells and protects these cells against specific cytolytic T lymphocytes (CTL) lysis (GARCÍA-ROCHA et al., 2015), down-regulates IL-2 receptor signaling in T cells and IL-12 expression by antigen presenting cell (APC) and induces the expression of IL-10 by macrophages, contributing to immunosuppression during cervical carcinogenesis (PERALTA-ZARAGOZA et al., 2006; TORRES-POVEDA et al., 2014).

However, it has been shown that TGFB1 suppresses the LCR-driven transcriptional activity and downregulates at the transcriptional level the HPV16 early genes expression in HPV-immortalized human genital epithelial cells (WOODWORTH; NOTARIO; DIPAOLO, 1990) and also downregulates E6 and E7 expression in CaSki cells in which was reported recue of p53 expression, pRb response pathway, and cellular-induced senescence (DONALISIO et al., 2008). LCR transcriptional activity is regulated by the NF1/Ski complex which is formed by interaction between nuclear factor-1 (NF1) and Sloan-Kettering Institute proto-oncoprotein (Ski) (BALDWIN et al., 2004; TARAPORE et al., 1997). This complex activates the viral oncogenes E6 and E7 transcription (BALDWIN et al., 2004; KOWLI et al., 2013). TGFB1 leads to NF1/Ski impairment, resulting in HPV-LCR transcriptional activity inhibition and hence E6 and E7 levels suppression (BALDWIN et al., 2004; KOWLI et al., 2013).

Autocrine regulation of HPV16 gene expression by TGFB1 could represent one component of an intracellular surveillance system directed against HPV transcription. Furthermore, the effects of TGFB1 on HPV expression vary significantly in different cell lines, and often the resistance to the inhibitory effects of TGFB1 on cell growth and HPV early gene expression that develops in tumorigenic cells is only partial (WOODWORTH; NOTARIO; DIPAOLO, 1990). *In vitro*, progressive resistance to growth inhibition by TGFB was observed across the transformation process. Whereas the TGFB growth inhibitory effects predominate at the beginning, in the end, TGFB provokes EMT and cell motility. Resistance to anti-growth action of TGFB in HPV transformed cells can be explained by the 50% reduction in SMAD signaling associated with a complete loss of expression of growth inhibitory genes (KOWLI et al., 2013) and the partial loss of TGFBR1 (MI et al., 2000). In addition, TGFB1 may have an important effect on the progression of premalignant cervical lesions through the induction of telomere dysfunction and hence chromosomal instability (DENG et al., 2008), and it may be involved in tumor

progression in the later stage of angiogenesis when associated with Endoglin, a co-receptor for TGFB1 in vascular endothelial cells (LIN et al., 2012).

In conclusion, HPV is necessary but not sufficient to cause cervical cancer. The participation of host immune response is greatly important to determine regression or progression from infection to malignancy. In this context, The TGFB plays a dual role in cervical carcinogenesis as summarized in Figure 1. At the beginning, TGFB acts on the control of viral early genes transcription and inhibits epithelial cell growth during transforming process initiation. Lately, it may induce EMT and facilitate invasion and metastasis. Additionally, the association between TGFB high production and cervical lesion severity suggests the TGFB amount within the lesion microenvironment could be a prognostic factor to cancer development. Therefore, an improved understanding of the role of TGFB in cervical carcinogenesis as well as the TGFB signaling pathways involved in this process, could contribute to find new strategies that would assist to fight against cervical cancer.

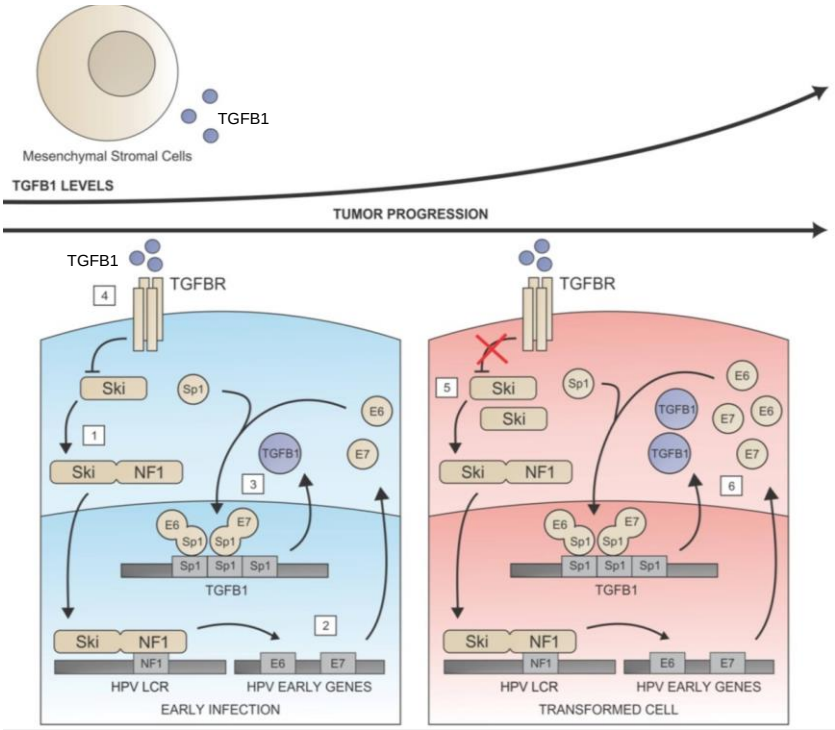

Figure 1 Regulation of HPV early genes and *TGFB1* expression by TGFB1 and E6/E7 oncoproteins. 1) NF1/Ski complex, formed by Nuclear factor-1 (NF1) and oncoprotein Ski, upregulates the HPV long control region (LCR) transcriptional activity, 2) and increases the viral oncogenes E6 and E7 expression. 3) E6 and E7 oncoproteins induce TGFB1 expression throughout the Sp1 transcription factor. 4) TGFB1 leads to NF1/Ski impairment, resulting in HPV-LCR transcriptional activity inhibition and hence E6 and E7 levels suppression, in the beginning of infection. 5) In a transformed cell, Ski is present in great amount, increasing NF1/Ski complex formation, which leads, along with the HPV DNA integration, to higher early genes expression. 6) High levels of E6 and E7 increase TGFB1 production. Mesenchymal stromal cells and regulatory T cells (Tregs) contributes to rise TGFB1 into the tumor microenvironment.

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5.2 ARTIGO 2

POLYMORPHISMS IN THE TGFB1 SIGNAL PEPTIDE INFLUENCE HUMAN PAPILLOMAVIRUS INFECTION AND DEVELOPMENT OF CERVICAL LESIONS

ABSTRACT

Purpose

To assess the effect of c.29C>T and c.74G>C polymorphisms in the *TGFB1* signal peptide on HPV infection and development of cervical lesions.

Patients and Methods

In this case-control study, cervical swabs and blood samples were obtained from 350 outpatient women, along with socio-demographic and sexual behavioral data. The study population was stratified by lesion grade, as well as by absence or presence of HPV DNA, as tested by PCR. *TGFB1* signal peptide polymorphisms were genotyped by PCR-restriction fragment length polymorphism.

Results

HPV DNA was detected in 172 (49.1 %) patients, with frequency higher among women who were ≤ 24 years old (p = 0.006), single (p = 0.013), had not been pregnant (p = 0.007) nor given birth (p = 0.008), and did not know what the virus was (p = 0.017). Low-grade squamous intraepithelial lesions were more frequent in women ≤ 24 years old (p = 0.036), while high-grade lesions were more frequent among those who had ≥ 4 sexual partners in their lifetime (p < 0.001). Lesions were also more frequent in smokers (p = 0.002). c.74G>C and the combined c.29CC+CT/c.74GC genotype were more frequent in infected patients (35.1 % and 15.7 %) than in uninfected women (6.2 % and 14.7 %). Accordingly, these genotypes were associated with higher risk of HPV infection, with odds ratio and 95 % confidence interval 2.81 and 1.35-5.86 (p = 0.004) for c.74G>C and 3.14 and 1.42-6.94 (p = 0.004) for the combined genotype. High-grade lesions were also 2.48 times more likely to occur in c.29CC patients than in c.29TT patients, with adjusted odds ratio 2.48 and 95 % confidence interval 1.01-6.08 (p = 0.047).

Conclusion

Although further studies are necessary, the data demonstrate that c.74G>C and c.29C>T polymorphisms are significantly associated with risk of HPV infection and high-grade squamous intraepithelial lesions, respectively. Thus, *TGFB1* signal peptide polymorphisms are potential susceptibility markers.

Keywords: Susceptibility marker; High-grade squamous intraepithelial lesions; Low-grade squamous intraepithelial lesions; rs1800470; rs1800471.

INTRODUCTION

Human papillomavirus (HPV), more precisely high-risk HPV, is strongly associated with cervical intraepithelial lesions and cancer.[1,2] HPV is mainly transmitted by skin or mucosa contact, which enables virions to infect basal layer cells in the epithelium via micro-wounds. In the cervix, most HPV infections are cleared or suppressed by cell-mediated immunity within 1–2 years. However, persistent infections are strongly linked to the development of precancerous squamous intraepithelial lesions, which are typically scored as low-grade or high-grade according to the Bethesda System (2001).[3,4]

Infection with high-risk HPV is necessary but not sufficient for cell immortalization and subsequent malignancy. Genetic modifications in the host due to viral DNA integration, chemical, and physical mutagens may also contribute to these processes.[5] In addition, exogenous and endogenous factors like tobacco use, parity, oral contraceptive use,[6] immune system impairment, and immunological interactions at the site of infection[7] may all influence progression from HPV infection to high-grade cervical lesions.

Transforming growth factor-β (TGFB), one of several cytokines that regulate cell growth, maturation, and differentiation, is also implicated in cervical cancer, suggesting an association with HPV infection severity, malignancy, or both.[10,11] We note, however, that TGFB may either suppress tumors or promote metastasis depending on tumor stage.[8,9] Further, sequence variations in *TGFB* and its receptors may alter expression and activity.[12–16] For instance, several single nucleotide polymorphisms (SNPs) have been described in *TGFB1*, including c.29C>T (rs1800470) and c.74G>C (rs1800471) in the signal peptide, which are associated with breast,[13,17–19] oropharyngeal,[10,20] and lung cancers.[21] We assess, for the first time, the significance of c.29C>T and c.74G>C polymorphisms in HPV infection and development of cervical lesions.

MATERIALS AND METHODS

Ethical Approval

This study was approved by the Institutional Ethics Committee Involving Humans at State University of Londrina, Londrina – PR, Brazil (CEP/UEL 133/2012; CAAE 05505912.0.0000.5231). 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.

Patients and Samples

In this case-control study, we analyzed 350 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 University Hospital and Clinic Center of State University of Londrina, and at two Basic Healthcare Units in Londrina – PR, Brazil. After sample collection, cytobrushes were stored in 2 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) at -20 °C until analysis. Peripheral blood was drawn into sterile syringes containing EDTA as anticoagulant, and stored at -20 °C until analysis. Patients were interviewed using a structured questionnaire to collect socio-demographic and sexual behavioral data. Participants were stratified based on presence or absence of HPV DNA, as tested by PCR, and based on lesion grade, as determined by cervical cytology.

Cervical Cytology

Cytology samples were graded according to the Bethesda System (2001) at the Public Health System Laboratory. Patients were deemed to have no lesions 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.[22]

DNA Extraction

Genomic DNA was obtained from cervical cytobrushes using DNAzol (Invitrogen Inc., 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 Biopur Mini Spin Plus Kit (Biometrix, Curitiba – PR, Brazil). DNA concentration was measured at 260 nm on a NanoDrop 2000c® Spectrophotometer (Thermo Fisher Scientific, USA), and purity was assessed by A260/A280 ratio.

HPV Detection by PCR

HPV was detected by PCR using the primers MY09 (5'-CGTCCMAARGGAWACTGATC-3') and MY11 (5'-GCMCAGGGWCATAAYAATGG-

3'), which are designed to amplify a conserved region of approximately 450 bp in the HPV L1 gene (GenBank Accession number: AJ236888).[23] This method was selected because it targets very small fragments, and consequently is more sensitive than several other molecular techniques.[22] A fragment of human b-globin, approximately 268 bp, was co-amplified as internal control, using primers GH20 (5'-GAAGAGCCAAGGACAGGTAC-3') and PC04 (5'-CAACTTCATCCACGTTCACC-3'). Reactions without template DNA were used as 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 .

Genotyping of TGFB1 Signal Peptide Polymorphisms

TGFB1 signal peptide polymorphisms were genotyped by PCR-restriction fragment length polymorphism according to Wood et al.[15] In this method, a single amplicon is digested with *MspA1-I* and *Bgl-I* to detect c.29C>T and c.74G>C polymorphisms, respectively. Digestion with *MspA1-I* produces fragments of 149, 67, 40, 26, and 12 bp for the C allele at the c.29C>T site, and 161, 67, 40 and 26bp for allele T. On the other hand, digestion with *Bgl-I* generates 131, 103, and 60 bp for the G allele at the c.74G>C site, and 163 and 131 bp for allele C (Fig 1).

Statistical Analysis

Differences in socio-demographic and sexual behavioral data between infected and uninfected women were examined using contingency tables and Pearson's χ^2 test. Allele frequency was calculated as [1(h+2H)]/2N, where h represents the heterozygous genotype, H is the homozygous genotype, and N is the sample size for each population. Hardy-Weinberg equilibrium in infected and uninfected women was tested using χ^2 test. Differences in the distribution of genotypes were assessed by χ^2 between infected and infected women, and among women with or without low- and high-grade squamous intraepithelial lesions. Effect size was estimated using odds ratio with 95 % exact confidence intervals. Adjusted odds ratio with 95 % confidence interval was calculated to estimate the association between polymorphisms and lesion grade based on a multinomial logistic regression model adjusted for confounding factors including HPV status, age, and tobacco use.

Data were analyzed in SPSS Statistics 22.0 (SPSS Inc., Chicago, Illinois, USA). A *p* value < 0.05 was considered statistically significant.

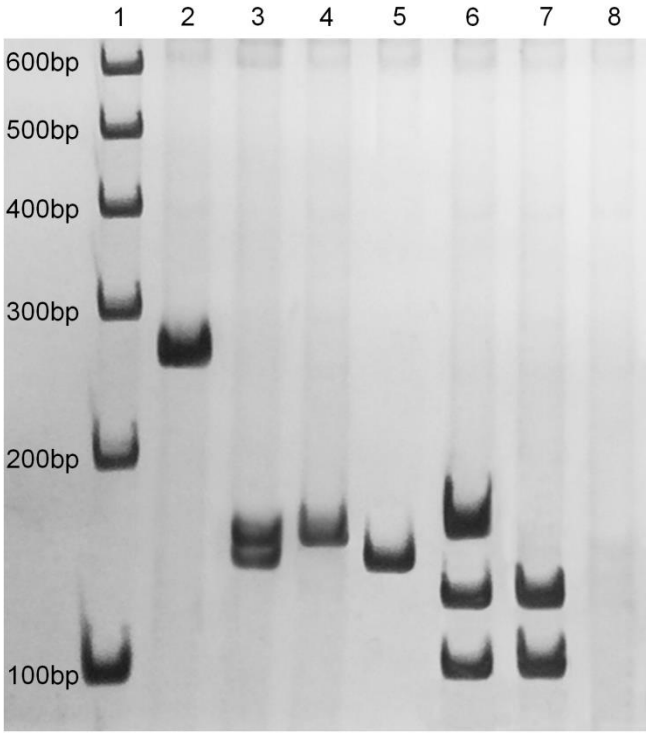

Figure 1 Eletrophoretic profile of *TGFB1* signal peptide polymorphisms: 1- Ladder (100bp); 2- *TGFB1* amplicon (294bp); c.29C>T genotypes [3- CT (161-149bp); 4- TT (161bp); 5- CC (149bp)]; c.74G>C genotypes [6- GC (163-131-103bp)]; 7- GG (131-103bp); 8- Negative control of amplification.

RESULTS

The study population consisted of 350 women, of whom 172 (49.1 %) tested positive for HPV DNA, and 178 (50.9 %) did not. Mean age was 39.06 ± 12.44 years, with median 38 years. Infected women were significantly younger ($p < 0.05$) at 36.56 ± 12.94 years on average (median 35 years) than uninfected women, who were 41.47 ± 11.46 years on average with median 41.5 years.

Socio-demographic and Sexual Behavioral Data

Socio-demographic and sexual behavioral data are listed in Table 1 and Table 2, respectively. HPV infection was frequently observed in women who were ≤ 24 years old ($p = 0.006$), single ($p = 0.013$), and had not been pregnant ($p = 0.007$) nor given birth ($p = 0.008$). In addition, HPV infection was more common among patients who did not know what the virus was ($p = 0.017$). Notably, low-grade

squamous intraepithelial lesions were more frequent in women 24 years or less (p = 0.036), while high-grade intraepithelial lesions were more common among women who had at least four sexual partners in their lifetime (p < 0.001). Low- and high-grade lesions were also more frequent among women who smoked (p = 0.002).

TGFB1 Signal Peptide Polymorphisms and HPV Infection

One patient was excluded from analysis of *TGFB1* polymorphisms due to genotyping failure (N = 349). We found that *TGFB1* signal peptide polymorphisms were in Hardy-Weinberg equilibrium (p > 0.05) in infected and uninfected women. Among uninfected women, 64 (36.2 %) were genotype TT at the c.29C>T site, while 83 (46.9 %) and 30 (16.9 %) were genotype CT and CC, respectively. Among infected women, 50 (29.1 %) were genotype TT, 81 (47.1 %) were genotype CT, and 41 (23.8 %) were genotype CC. The distribution of c.29C>T genotypes was comparable between these groups (p = 0.185).

However, the distribution of c.74G>C polymorphisms was significantly different between infected and uninfected women (p = 0.004). In particular, 166 (93.8 %) uninfected and 145 (84.3 %) infected women were genotype GG, while 11 (6.2 %) uninfected and 27 (15.7 %) infected patients were genotype GC. The CC genotype was not observed. Indeed, infected patients were more likely to have the variant allele C than controls (p < 0.05). Accordingly, these women were at increased risk of infection, with odds ratio 2.81 and 95 % confidence interval 1.35-5.86. Higher susceptibility was also observed with the combined genotypes c.29CC+CT/c.74GC and c.29TT/c.74GG. For instance, infected patients were more likely to be genotype c.29CC+CT/c.74GC than uninfected women (p = 0.004), and were 3.14 times more likely to be infected than c.29TT/c.74GG genotypes, with 95 % confidence interval 1.42-6.94 (Table 3).

TGFB1 Signal Peptide Polymorphisms and Cervical Lesions

Based on cervical cytology, 343 women were included in further analysis, of whom 259 did not have cervical lesions, while 22 and 62 had low- and high-grade squamous intraepithelial lesions, respectively (Table 4). Four of the excluded patients were diagnosed with cervical cancer, another two were excluded because of missing cervical cytology results, and one was excluded due TGFB1 amplification failure. Significant differences in the distribution of c.29C>T

polymorphisms were noted between women without lesions and those with high-grade squamous intraepithelial lesions (p = 0.042). Based on a multinomial logistic regression model adjusted for HPV status, age, and tobacco use, the CC genotype was found to be associated with high-grade lesions, with b 0.91, χ^2_{Wald} (df = 1) 3.96, and p = 0.047. Indeed, the CC genotype increases the chances of developing high-grade lesions 2.48 times compared to the TT genotype, with 95 % confidence interval 1.01-6.08. In contrast, c.74G>C polymorphisms were not significantly associated with lesion grade (p > 0.05, Table 5).

DISCUSSION

HPV was detected by PCR in 49.1 % of the study population. Socio-demographic and sexual behavioral profiles demonstrated that HPV was more frequent among women who were not aware of the virus (p = 0.017), suggesting that lack of information about HPV and how to avoid exposure probably predisposes women to infection. HPV was also more frequent in younger women ≤ 24 years old (p = 0.006), who, in this study, were also typically single (p = 0.013) and had never been pregnant (p = 0.007) nor had given birth (p = 0.008). Indeed, young age is a well-known independent factor associated with HPV infection,[24] perhaps due to increased sexual activity at this age, as well as to cervical ectopy, an anatomical feature in young women that exposes the columnar epithelium in the ectocervix and renders the tissue more vulnerable to infection.[24,25]

While high-grade squamous intraepithelial lesions are expected to be more frequent also in the same younger age group at higher risk of HPV, low-grade lesions were found to be proportionally higher instead (p = 0.036). Similarly, Nunes et al.[26] reported that high-grade lesions were more prevalent in older women than in younger women, suggesting either that HPV infections are spontaneously cleared more often in women under 30 and do not progress to high-grade lesions, or that high-grade lesions regress in younger women. Interestingly, a high number of sexual partners over the lifetime was not associated with HPV infection in our cohort, in contrast to previous studies. Nevertheless, a high number of sexual partners can reasonably be expected to increase the risk of infection, and hence of high-grade lesions.

Notably, low- and high-grade lesions were more frequent in women who self-reported as smokers than in non-smokers (p = 0.002), in agreement with

previous reports indicating that cigarette use synergizes with HPV infection to promote malignant progression of cervical lesions.[27,28]. In particular, tobacco carcinogens such as benzo[*a*]pyrene have been detected in the cervical mucus of women who smoke and who develop cervical dysplasias.[29] Indeed, benzo[*a*]pyrene was shown to ehance virus replication and persistence in a dose-dependent manner, or otherwise to correlate with HPV genome amplification, potentially resulting in increased expression of the E6 and E7 oncoproteins.[28]

Nevertheless, only a subset of individuals exposed to HPV develops persistent infection that may progress to high-grade lesions and cervical cancer, implying that other genetic factors may contribute to the process. Such genetic factors may include sequence variations in *TGFB1*, since TGFB is a potent modulator of cell growth, maturation, and differentiation, and an important cytokine in the infection microenvironment.[9,30] Thus, we assessed, for the first time using molecular techniques, whether the *TGFB1* signal peptide polymorphisms c.29C>T and c.74G>C are associated with HPV infection and development of cervical lesions.

We found that the c.74GC genotype (p = 0.004) and the combined c.29CC+CT/c.74GC genotype (p = 0.004) was more prevalent among infected women, in agreement with Gua et al.,[10] who reported that women with the c.29CC+CT and c.74GC+CC genotypes are predisposed to infection with HPV16. One possibility is that these polymorphisms affect the ability of the host to clear cells infected with HPV. We note that c.29C>T and c.74G>C genotypes by themselves were similarly distributed in infected and unifected women in our cohort, and thus analysis of combined polymorphisms was more informative, as has been noted.[31] Hence, a panel of polymorphisms may be more reliable markers of susceptibility to HPV infection and other disease.

Remarkably, only c.29C>T polymorphisms were associated with lesion grade. However, other factors such as HPV status, age, and tobacco use may have confounded this result. Therefore, multinomial logistic regression was used to adjust for these confounding factors, and adjusted results confirmed that women with the c.29CC genotype are 2.5 times more likely to develop high-grade squamous intraepithelial lesions compared to women with the c.29TT genotype (p = 0.047).

One overarching possibility is that c.29C>T and c.74G>C polymorphisms profoundly impact TGFB1 production, which was found to gradually increase during progression from normal tissue to cervical cancer.[33-35] For instance,

the c.74G and c.29C alleles upregulate TGFB1 production *in vitro,*[13,36,37], and the latter is associated with high serum levels.[38-40] Since TGFB1 may auto regulate HPV gene expression in infected genital epithelial cells[41], we believe that women who produce TGFB1 more abundantly due to a c.74GG genotype may have some protection against HPV infection. Conversely, women with the c.74C genotype are at an elevated risk of HPV infection, as we observed in our cohort, presumably because this genotype suppresses TGFB production.[37] We note that TGFB may additionally control HPV replication early in infection by cell cycle arrest in the G1 phase, a process that depends on inducing the small cyclin-dependent kinase inhibitors p15, p21, and p27, and downregulating the oncogene c-myc.[8]

However, in the event that HPV infection persists and lesions develop, increased TGFB production due to a c.29CC genotype may then worsen prognosis, since TGFB is also the most powerful immunosuppressor in mammals.[42] In particular, TGFB suppresses the differentiation of T cells,[43] and induces naive T cells to express the transcription factor forkhead box protein P3, which drives conversion to a regulatory T cell.[44,45] Notably, regulatory T cells that produce TGFB abound in high-grade squamous intraepithelial lesions, cervical carcinomas, and draining lymph nodes.[46] Therefore, the *TGFB1* c.29CC genotype may drive HPV progression due to increased immunosuppression.

Although the small number of patients with low-grade squamous intraepithelial lesions and the exclusion of patients with cervical cancer may have limited sensitivity, the strengths of our analysis lie in the analysis of combined polymorphisms and in adjusting for potential confounding factors including HPV, age, and smoking. Thus, while further studies with a larger number of patients are warranted to confirm results, we demonstrated, for the first time, that the c.74G>C and c.29C>T polymorphisms are significantly associated with susceptibility to HPV infection and high-grade squamous intraepithelial lesions, respectively, and could be used as potential susceptibility markers.

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Table 1. Socio-demographic Data

Variable	Uninfected		HPV-infected		*p* value*
	n	(%)	N	(%)	
Knowledge of HPV					0.017
No	32	(18.5)	46	(31.7)	
Have ever heard	97	(56.1)	73	(50.3)	
Yes	44	(25.4)	26	(17.9)	
Age (years)					0.006
≤ 24	13	(7.3)	31	(18.0)	
25 – 34	46	(25.8)	53	(30.8)	
35 – 44	45	(25.3)	40	(23.3)	
45 – 54	50	(28.1)	38	(16.3)	
≥ 55	24	(13.5)	20	(11.6)	
Ethnicity					0.178
Caucasian	91	(52.9)	67	(46.9)	
Brown	59	(34.3)	63	(44.1)	
Black	22	(12.8)	13	(9.1)	
Monthly income[a]					0.137
< 1 minimum wage	42	(25.8)	43	(32.8)	
1 – < 3 minimum wages	107	(65.6)	77	(58.8)	
3 – < 5 minimum wages	10	(6.1)	11	(8.4)	
≥ 5 minimum wages	4	(2.5)	0	(0.0)	
Smoking status					0.168
No	144	(81.8)	118	(75.6)	
Yes	32	(18.2)	38	(24.4)	
Education[b]					0.228
Incomplete elementary	58	(33.7)	43	(29.9)	
Complete elementary	15	(8.7)	21	(14.6)	
Incomplete secondary	21	(12.2)	24	(16.7)	
Complete secondary	58	(33.7)	45	(31.3)	
Incomplete higher education	6	(3.5)	6	(4.2)	
Complete higher education	14	(8.1)	5	(3.5)	
Marital status					0.013
Single	18	(10.1)	39	(23.2)	
Married / Civil partner	128	(71.9)	103	(61.3)	
Divorced	23	(12.9)	18	(10.7)	
Widowed	9	(5.1)	8	(4.8)	

[a]Based on Brazilian minimum wage, approximately US$ 220.00.
[b]Based on Brazilian educational system.
*By two-sided χ^2 test, with $p < 0.05$ considered significant (SPSS Inc., Chicago, Illinois, USA).

Table 2. Sexual Behavioral Characteristics

Variable	Uninfected		HPV-infected		*p* value*
	n	(%)	N	(%)	
Contraception					0.135
No	103	(58.5)	91	(55.8)	
Yes, hormonal	60	(34.1)	54	(33.1)	
Yes, condom	13	(7.4)	13	(8.0)	
Yes, both	0	(0.0)	5	(3.1)	
Number of full-term pregnancies					0.007
0	13	(7.3)	30	(17.5)	
1	29	(16.3)	43	(25.1)	
2	56	(31.5)	38	(22.2)	
3	45	(25.3)	36	(21.1)	
4	19	(10.7)	12	(7.0)	
≥ 5	16	(9.0)	12	(7.0)	
Parturition					0.008
No	15	(8.4)	35	(20.5)	
Natural birth	70	(39.3)	68	(39.8)	
Cesarean birth	59	(33.1)	43	(25.1)	
Both	34	(19.1)	25	(14.6)	
Abortion					0.654
No	130	(78.8)	114	(80.9)	
Yes	35	(21.2)	27	(19.1)	
Age at first sexual intercourse (years)					0.095
≤17	93	(52.8)	99	(61.9)	
≥18	83	(47.2)	61	(38.1)	
Age at menarche (years)					0.152
≤11	37	(20.9)	42	(26.1)	
12	43	(24.3)	47	(29.2)	
13	48	(27.1)	28	(17.4)	
≥14	49	(27.7)	44	(27.3)	
Sexual partners during lifetime					0.062
1	68	(39.3)	39	(26.9)	
2 – 3	48	(27.7)	51	(35.2)	
≥4	57	(32.9)	55	(37.9)	
Sexual partners in past 6 months					0.442
0	22	(12.7)	23	(15.8)	
1	148	(85.5)	118	(80.8)	
≥2	3	(1.7)	5	(3.4)	

*By two-sided χ^2 test, with $p < 0.05$ considered significant (SPSS Inc., Chicago, Illinois, USA).

Table 3. *TGFB1* Genotypes and Susceptibility to HPV Infection

***TGFB1* genotype**		**HPV− (N = 177)[a]**		**HPV+ (N = 172)[b]**		**OR****	**$CI_{95\%}$*****	***p* value***
		n	**(%)**	**n**	**(%)**			
rs1800470								0.185
(c.29C>T)	TT	64	(36.2)	50	(29.1)	1.00	reference	
	CT	83	(46.9)	81	(47.1)	1.25	0.77 – 2.02	
	CC	30	(16.9)	41	(23.8)	1.75	0.96 – 3.18	
rs1800471								0.004
(c.74G>C)	GG	166	(93.8)	145	(84.3)	1.00	reference	
	GC	11	(6.2)	27	(15.7)	2.81	1.35 – 5.86	
	CC	0	(0.0)	0	(0.0)	-	-	
rs1800470/rs1800471[1]								0.004
	TT/GG	64	(85.3)	50	(64.9)	1.00	reference	
	CC+CT/GC	11	(14.7)	27	(35.1)	3.14	1.42 – 6.94	

*By two-sided χ^2 test, with $p < 0.05$ considered significant (SPSS Inc., Chicago, Illinois, USA).
**Odds ratio and ***95 % confidence interval (SPSS Inc., Chicago, Illinois, USA).
[1]Combined *TGFB1* genotypes.
[a] Hardy-Weinberg equilibrium χ^2: rs1800470 = 0.122, $p > 0.05$; rs1800471 = 0.182, $p > 0.05$.
[b]Hardy-Weinberg equilibrium χ^2: rs1800470 = 0.531, $p > 0.05$; rs1800471 = 1.248, $p > 0.05$.

Table 4. Distribution of *TGFB1* Genotypes by Lesion Grade

***TGFB1* genotype**		**NL[a] (N = 259)**		**LSIL[b] (N = 22)**		**HSIL[c] (N = 62)**		***p* value***
		n	**(%)**	**n**	**(%)**	**n**	**(%)**	
rs1800470								0.042
(c.29C>T)	TT	95	(36.7)	6	(27.3)	13	(21.0)	
	CT	120	(46.3)	11	(50.0)	29	(46.8)	
	CC	44	(17.0)	5	(22.7)	20	(32.3)	
rs1800471								0.149
(c.74G>C)	GG	235	(90.7)	19	(86.4)	51	(82.3)	
	GC	24	(9.3)	3	(13.6)	11	(17.7)	
	CC	0	(0.0)	0	(0.0)	0	(0.0)	

*By two-sided χ^2 test, with $p < 0.05$ considered significant (SPSS Inc., Chicago, Illinois, USA).
[a]No lesion, [b]low-grade squamous intraepithelial lesion, and [c]high-grade squamous intraepithelial lesion.

Table 5. *TGFB1* Genotypes and Susceptibility to Low- and High-grade Squamous Intraepithelial Lesions

Cervical lesion	*TGFB1* genotypes	χ^2_{Wald} (df=1)		*p* value		Odds Ratio $CI_{95\%}$			
		crude*	adjusted**	crude*	adjusted**	crude*		adjusted**	
LSIL[a]	rs1800470 (c.29C>T)								
	TT	-	-	-	-	1.00	reference	1.00	reference
	CT	0.50	1.40	0.479	0.237	1.45	0.52 – 4.07	2.08	0.62 – 7.00
	CC	0.86	1.04	0.353	0.307	1.80	0.52 – 6.21	2.06	0.51 – 8.30
	rs1800471 (c.74G>C)								
	GG	-	-	-	-	1.00	reference	1.00	reference
	GC	0.44	0.56	0.507	0.452	1.55	0.43 – 5.60	1.69	0.43 – 6.67
HSIL[b]	rs1800470 (c.29C>T)								
	TT	-	-	-	-	1.00	reference	1.00	reference
	CT	2.48	2.13	0.115	0.144	1.77	0.87 – 3.58	1.80	0.82 – 3.97
	CC	9.00	3.96	0.003	0.047	3.32	1.51 – 7.28	2.48	1.01 – 6.08
	rs1800471 (c.74G>C)								
	GG	-	-	-	-	1.00	reference	1.00	reference
	GC	3.57	0.85	0.059	0.357	2.11	0.97 – 4.58	1.52	0.62 – 3.72

*Multinomial logistic regression, with "no lesion" as reference and $p < 0.05$ considered significant (SPSS Inc., Chicago, Illinois, USA).
**Multinomial logistic regression adjusted for HPV status, age, and tobacco use, with "no lesion" as reference and $p < 0.05$ considered significant (SPSS Inc., Chicago, Illinois, USA).
[a]Low-grade squamous intraepithelial lesion, and [b]high-grade squamous intraepithelial lesion.

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Conclusões

6 CONCLUSÕES

ARTIGO 1

- O TGFB influencia na infecção por HPV:
    - diminuindo a amplificação viral nos estágios iniciais via supressão da atividade transcricional da região reguladora, LCR, do genoma do vírus;
    - contribuindo tardiamente (HSIL e câncer cervical) para a imunossupressão do microambiente, favorecendo a evasão das células infectadas e a progressão para o câncer invasivo e metástase.
- Os níveis locais de TGFB se elevam conforme aumenta o grau de lesão.
- Células estromais mesenquimais presentes no tumor e as próprias células tumorais contribuem para o aumento de TGFB no microambiente.

ARTIGO 2

- Mulheres mais jovens, solteiras, que nunca gestaram, nulíparas e que desconhecem o HPV se mostraram mais susceptíveis à infecção por este vírus.
- A pouca idade, a multiplicidade de parceiros sexuais e o hábito tabagista estiveram associados ao desenvolvimento de lesões cervicais provocadas pelo HPV.
- O polimorfismo c.29C>T não mostrou diferença significativa entre os grupos HPV+ e controles. O genótipo c.74GC e os genótipos combinados c.29CC+CT/c.74GC apresentaram associação ao aumento da susceptibilidade de contrair o vírus.
- Não foi observada diferença significativa entre o polimorfismo c.74G>C e os grupos sem lesão (controle), com LSIL e HSIL. O genótipo c.29CC foi associado ao aumento da susceptibilidade de desenvolver HSIL.

7 CONSIDERAÇÃO FINAL

Os polimorfismos c.74G>C (rs1800471) e c.29C>T (rs1800470) se mostraram como potenciais marcadores de susceptibilidade para a infecção por HPV e para o desenvolvimento de HSIL, respectivamente. Por conseguinte, estes dois SNPs poderiam fazer parte da composição de um painel genotípico e assim serem utilizados como marcadores moleculares de prognóstico para estas doenças, na prática clínica. Contudo, uma maior população de estudo é necessária para que sejam feitas análises adicionais a fim de validar estes 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

Autorização do Comitê de Ética em Pesquisa Envolvendo Seres Humanos/Uel

Universidade Estadual de Londrina

PARANÁ
GOVERNO DO ESTADO

COMITÊ DE ÉTICA EM PESQUISA ENVOLVENDO SERES HUMANOS
Universidade Estadual de Londrina
Registro CONEP 5231

Parecer CEP/UEL:	133/2012
CAAE:	05505912.0.0000.5231
Processo:	19275/2012
Pesquisador(a):	Karen Brajão de Oliveira
Unidade/Órgão:	CCB – Departamento de Ciências Patológicas

Prezado(a) Senhor(a):

O "**Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina**" (Registro CONEP 5231) – de acordo com as orientações da Resolução 196/96 do Conselho Nacional de Saúde/MS e Resoluções Complementares, avaliou o projeto:

"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."

Situação do Projeto: **Aprovado**

Informamos que deverá ser comunicada, por escrito, qualquer modificação que ocorra no desenvolvimento da pesquisa, bem como deverá ser encaminhado ao CEP/UEL relatório final da pesquisa, conforme prevê a Resolução 196/96 do Conselho Nacional de Saúde/MS e Resoluções Complementares.

Londrina, 28 de agosto de 2012.

Profa. Dra. Alexandrina Aparecida Maciel Cardelli
Coordenadora do Comitê de Ética em Pesquisa Envolvendo Seres Humanos
Universidade Estadual de Londrina

Profª Drª Paula Mariza Zedu Alliprandini
Vice-Coord. do Comitê de Ética em Pesquisa
Envolvendo Seres Humanos
Universidade estadual de Londrina