FERNANDA COSTA BRANDÃO BERTI

INFLUÊNCIA DO POLIMORFISMO rs1800872 (c.-592C>A) DE INTERLEUCINA-10 NA INFECÇÃO POR HPV E SOBRE OS NÍVEIS PLASMÁTICOS E CERVICAIS DESTA CITOCINA EM MULHERES INFECTADAS PELO HPV

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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.

Orientador: Profa. Dra. Karen Brajão de Oliveira

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Londrina, 08 de setembro de 2016.

Dedico esta ao meu amado Deus e à minha família,
Refúgio e Apoio sempre presentes.

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"O que ninguém nunca viu nem ouviu, e o que jamais alguém pensou que podia acontecer, foi isso o que Deus preparou para aqueles que o amam."

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BERTI, Fernanda Costa Brandão. **Influência do polimorfismo rs1800872 (c.-592C>A) de Interleucina-10 na infecção por HPV e sobre os níveis plasmáticos e cervicais de IL-10 em mulheres infectadas pelo HPV.** 2016. 108 f. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2016.

RESUMO

O Papillomavirus Humano (HPV) destaca-se como um importante vírus transmitido sexualmente, envolvido no desenvolvimento de lesões intraepiteliais escamosas e do câncer cervical. Além do HPV, outros fatores influenciam tais desfechos clínicos, incluindo a Interleucina-10 (IL-10), citocina anti-inflamatória que favorece a imunossupressão cervical. A expressão e produção de IL-10 parece ser influenciada pelo HPV e por polimorfismos na região promotora do gene da *IL-10*, incluindo o rs1800872 (c.-592C>A). Assim, o presente estudo teve como objetivo avaliar a influência do polimorfismo c.-592C>A na infecção por HPV e sobre os níveis plasmáticos e cervicais de IL-10 em mulheres infectadas pelo HPV. Este estudo caso-controle incluiu 174 pacientes com detecção do genoma do HPV e 186 sem detecção deste, classificadas como controles. Raspados epiteliais cervicais foram obtidos a fim de determinar a presença do DNA do HPV por reação em cadeia da polimerase (PCR). Amostras de sangue periférico foram analisadas a fim de determinar o polimorfismo de *IL-10* por PCR seguida de análise de polimorfismo por comprimento dos fragmentos de restrição (RFLP). Níveis plasmáticos e cervicais de IL-10 foram avaliados por ensaio de imunoabsorção enzimática (ELISA). Maior frequência de HPV foi observada entre mulheres com <34 anos (p<0,001), solteiras (p=0,006) e que tiveram mais de 4 parceiros sexuais ao longo da vida (p=0,003). Assim como, entre mulheres que não tinham conhecimento sobre o HPV (p=0,042), que recebiam menos de 1 salário mínimo (p=0,013) e entre fumantes (p<0,001). O HPV foi mais prevalente entre portadoras do alelo A (p<0.001), confirmado por análise de regressão logística, incluindo idade, tabagismo, conhecimento sobre o HPV, número de parceiros sexuais ao longo da vida e estado civil como covariáveis. Heterozigotos [OR=2,081 95%CI (1,222 – 3,544), p=0,007] e homozigotos [OR=3,745 95%CI (1,695 – 8,271), p=0,001] para o polimorfismo c.-592C>A apresentaram aproximadamente 2 e 4 vezes mais risco, respectivamente, de terem HPV quando comparados a pacientes com genótipo CC. Os níveis cervicais de IL-10 foram maiores entre pacientes infectadas com HPV e portadoras do alelo polimórfico A (p=0,039), enquanto os níveis plasmáticos de IL-10 foram menores nestas pacientes (p=0,017) quando comparadas a pacientes CC. Análises de regressão logística mostraram que os níveis cervicais e plasmáticos de IL-10 não estiveram independentemente associados aos genótipos CA+AA (p=0,162 e p=0,647, respectivamente), nem à presença do HPV (p=0,061 e p=0,055, respectivamente), demonstrando que alterações nos níveis de IL-10 são possivelmente resultado da presença de ambos. Assim, o polimorfismo c.-592C>A de IL-10 mostrou-se independentemente associado à infecção por HPV e influenciou na persistência viral por meio de níveis cervicais aumentados de IL-10 em mulheres infectadas pelo HPV.

Palavras-chave: IL-10. rs1800872. HPV. IL-10 plasmática. IL-10 cervical.

BERTI, Fernanda Costa Brandão. **Influence of Interleukin-10 rs1800872 (c.-592C>A) polymorphism on HPV infection and over IL-10 plasmatic and cervical levels in HPV infected women.** 2016. 108 p. Dissertation (Master's degree in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2016.

ABSTRACT

Human Papillomavirus (HPV) stands as an important sexually transmitted virus, involved in squamous intraepithelial lesion and cervical cancer development. Besides HPV, other factors influence these clinical outcomes, including Interleukin-10 (IL-10), an important anti-inflammatory cytokine that favors cervical immunosuppression. IL-10 expression and production can be influenced by HPV itself and by polymorphisms on *IL-10* gene promoter region, including the rs1800872 (c.-592C>A). Therefore, the present study aimed to evaluate the influence of c.-592C>A polymorphism on HPV infection and over plasmatic and cervical levels of IL-10 in HPV infected women. This case control study included 174 patients with detected HPV genome and 186 without HPV genome, classified as controls. Cervical epithelial scrapings were obtained to determine HPV DNA presence by polymerase chain reaction (PCR). Peripheral blood samples were obtained to determine *IL-10* polymorphism by PCR followed by restriction fragment length polymorphism analysis (RFLP). IL-10 plasmatic and cervical levels were assessed by enzyme-linked immunosorbent assay (ELISA). Higher HPV frequency was observed within women that were <34 years old (p<0.001), single (p=0.006) and had more than 4 sexual partners during their lifetime (p=0.003). As well as among women who had no knowledge about HPV (p =0.042), who received less than 1 minimum wage (p=0.013) and among smokers (p<0.001). HPV was more prevalent among allele A carriers (p<0.001), confirmed by logistic regression analysis, including age, smoking status, knowledge about HPV, number of sexual partners during lifetime and marital status as confounders. Heterozygotes [OR=2.081 95%CI (1.222 – 3.544), p=0.007] and homozygotes [OR=3.745 95%CI (1.695 – 8.271), p=0.001] for c.-592C>A polymorphism presented, approximately, 2 and 4 time's greater odds of presenting HPV as compared to CC patients, respectively. IL-10 cervical levels were higher among HPV infected patients carrying polymorphic allele A (p=0.039), while IL-10 plasmatic levels were lower among those patients (p=0.017) when compared to CC patients. Logistic regression analyzes showed that IL-10 cervical and plasmatic levels were not independently associated to CA+AA genotypes (p=0.162 and p=0.647, respectively), neither to HPV´s presence (p=0.061 and p=0.055, respectively), thus demonstrating that changes in IL-10 levels are possibly a result of both HPV and allele A presence. In conclusion, *IL-10* c.-592C>A polymorphism was independently associated with HPV infection and influenced on viral persistence through increased IL-10 cervical levels in HPV infected women.

Keywords: IL-10. rs1800872. HPV, Plasmatic IL-10. Cervical IL-10.

LISTA DE ABREVIATURAS E SIGLAS

aa	*Amino acids*
$AgNO_3$	*Silver nitrate*
APC	*Antigen presenting cell*
ATF	*Activating transcription factor*
ATM-ATR	*Ataxia Telangiectasiamutated–ATM and RAD3-related DNA damage repair pathway*
B	*B cell*
BaP	*Benzo[a]pyrene*
Bcl-XL	*B-cell lymphoma-extra large*
Bcl-2	*B-cell lymphoma 2*
bp	*Base pairs*
CD	*Cluster of differentiation*
CDC1	*Cluster of differentiation 1*
CDC4	*Cluster of differentiation 4*
CDC8	*Cluster of differentiation 8*
CDC14	*Cluster of differentiation 14*
CDC80	*Cluster of differentiation 80*
CDC86	*Cluster of differentiation 86*
CDC141	*Cluster of differentiation 141*
CDK	*Cyclin-dependent kinase*
°C	*Celsius grade*
CEP/UEL	*Institutional Ethics Committee Involving Humans of the State University of Londrina*
CIN	*Cervical intraepithelial neoplasia*
CIN 1	*Cervical intraepithelial neoplasia grade 1*
CIN 2	*Cervical intraepithelial neoplasia grade 2*
CIN 3	*Cervical intraepithelial neoplasia grade 3*
CIS	*Carcinoma in situ*
CISMEPAR	*Consórcio Intermunicipal de Saúde do Médio Paranapanema*
c-MYC	*v-MYC avian myelocytomatosis viral oncogene homolog*
CSIF	*Cytokine synthesis inhibitory factor*

CTL	*Cytolytic T lymphocyte*
DC	*Dendritic cell*
DNA	*Deoxyribonucleic acid*
dNTP	*Deoxynucleotide*
E	*Early region*
E2F	*E2 promoter-binding factor*
E6AP	*E6-associated protein*
EDTA	*Ethylenediaminetetraacetic acid*
EGFR	*Epidermal growth factor receptor*
ELISA	*Enzyme-Linked Immunosorbent Assay*
GM-CSF	*Granulocyte macrophage - Colony-stimulating factor*
HeLa	*Cell line of cervical adenocarcinoma containing integrated HPV18 genome*
HLA-1	*Human leukocyte antigen class I*
HLA-G	*Human leukocyte antigen G*
HPV	*Human Papillomavirus*
HPV-AR	*HPV de Alto Risco*
HPV-BR	*HPV de Baixo Risco*
HPV-RI	*HPV de Risco Indeterminado*
HpyCH4V	*Helicobacter pylori CH4V*
HR-HPV	*High-risk HPV*
HSIL	*High-grade squamous intraepithelial lesions*
ICAM-1	*Intercellular Adhesion Molecule 1*
IDO	*Indoleamine 2,3-dioxygenase*
IFN	*Interferon*
IFN-γ	*Interferon gamma*
IL	*Interleukin*
IL-1β	*Interleukin-1 beta*
IL-2	*Interleukin-2*
IL-2R	*Interleukin-2 Receptor*
IL-5	*Interleukin-5*
IL-6	*Interleukin-6*
IL-10	*Interleukin-10*

IL-10R	*Interleukin-10 Receptor*
IL-10R1	*Interleukin-10 Receptor subunit 1*
IL-10R2	*Interleukin-10 Receptor subunit 2*
IL-12	*Interleukin-12*
IL-19	*Interleukin-19*
IL-22	*Interleukin-22*
IL-24	*Interleukin-24*
JAK	*Janus kinase 1*
kDa	*Kilodalton*
L	*Late region*
LC	*Langerhans cell*
LCR	*Long control region*
LIE	*Lesão intraepitelial escamosa*
LIEAG	*Lesão intraepitelial escamosa de alto grau*
LIEBG	*Lesão intraepitelial escamosa de baixo grau*
LR-HPV	*Low-risk HPV*
LSIL	*Low-grade squamous intraepithelial lesions*
M2	*Type II macrophages*
MAP-kinase	*Mitogen-Activated Protein Kinase*
MCP-1	*Monocyte chemotactic protein-1*
$MgCl_2$	*Magnesium chloride*
MHC	*Major histocompatibility complex*
MHC I	*Major histocompatibility complex classs I*
MHC II	*Major histocompatibility complex class II*
MHC	*Major histocompatibility complex*
µM	*Micromolar*
mL	*Milliliter*
mM	*Millimolar*
MMP	*Matrix metalloproteinase*
MMP-9	*Matrix metalloproteinase 9*
mRNA	*Messenger ribonucleic acid*
NF-κB	*Nuclear factor kappa-light-chain-enhancer of activated B cells*
ng	*Nanogram*

NIC	*Neoplasia intraepitelial cervical*
NIC I	*Neoplasia intraepitelial cervical grau I*
NIC II	*Neoplasia intraepitelial cervical grau II*
NIC III	*Neoplasia intraepitelial cervical grau III*
NK	*Natural killer cell*
NKT	*Natural killer T cell*
nm	*Nanometer*
nM	*Nanomolar*
NSIL	*Non-squamous intraepithelial lesions*
nt	*Nucleotide*
ORF	*Open reading frames*
p21	*Cyclin-dependent kinase inhibitor 1A*
p27	*Cyclin-dependent kinase inhibitor 1B*
p53	*Tumor Supressor p53*
p97	*Epithelium-specific early promoter 97*
PBMC	*Peripheral blood mononuclear cells*
PCR	*Polymerase chain reaction*
pg	*picogram*
%	*Porcent*
pRb	*Retinoblastoma protein*
RFLR	*Restriction fragment length polymorphism*
RNA	*Ribonucleic acid*
Rsal	*Rhodopseudomonas sphaeroides*
SBE	*STAT-Binding Elements*
SIL	*Squamous intraepithelial lesions*
Ski	*Sloan-Kettering Institute proto-oncoprotein*
SNP	*Single nucleotide polymorphisms*
SOCS3	*Suppressor of cytokine signaling 3*
Sp1	*Specificity protein 1 transcription factor*
STAT31	*Signal transducer and activator of transcription 1*
STAT3	*Signal transducer and activator of transcription 3*
STAT5	*Signal transducer and activator of transcription 5*
TCD8+	*Type 2 T helper*

T	*T cell*
TAM	*Tumor associated macrophages*
TAMC	*Tumor associated myeloid cell*
TE	*Tris-HCl-EDTA*
TGF-β1	*Transforming growth factor beta 1*
Th1	*Type 1 T helper*
Th2	*Type 2 T helper*
Th17	*Type 17 T helper*
TLR	*Toll-like receptor*
TLR-3	*Toll-like receptor 3*
TLR-9	*Toll-like receptor 9*
TNF-α	*Tumor necrosis factor alpha*
TNFR1	*Tumor necrosis factor receptor 1*
Tr1	*Type 1 T regulatory*
Treg	*Regulatory T cell*
U	*Unit*
UBE3A	*Ubiquitin-protein ligase E3A*
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*
TyK2	*Tyrosine kinase 2*

SUMÁRIO

INTRODUÇÃO

1 INTRODUÇÃO

Determinados vírus apresentam a habilidade de transformar células infectadas em células tumorais benignas ou malignas, estimulando o crescimento e a sobrevivência celular por diferentes mecanismos, estando diretamente envolvidos no desenvolvimento de um amplo espectro de manifestações clínicas em humanos, incluindo o câncer. Entre estes inclui-se o Papilomavírus Humano (HPV), responsável por causar desde infecções não aparentes até neoplasias malignas (SCHOTTENFELD; BEEBE-DIMMER, 2015).

Em mulheres, o HPV está ativamente envolvido no desenvolvimento de lesões intraepiteliais escamosas (LIE), de baixo grau (LIEBG) e de alto grau (LIEAG), bem como no desenvolvimento do câncer cervical. Lesões do tipo LIEBG estão associadas à intensa replicação viral e alterações discretas nos queratinócitos, sendo incapazes de progredir diretamente para um quadro de carcinoma invasivo, normalmente regredindo espontaneamente. Esporadicamente, a progressão para LIEAG pode ocorrer, acompanhada de desregulação progressiva do ciclo celular, resultando em aumento da proliferação celular, redução da replicação viral e bloqueio de maturação do epitélio, o que por sua vez, pode resultar na transformação celular e ao estabelecimento do câncer cervical (KAJITANI, SCHWARTZ, 2015). O HPV encontra-se virtualmente presente em 100% dos casos de câncer cervical, sendo considerado fator crucial neste processo (SCHIFFMAN et al., 2007).

Até o presente momento, mais de 200 tipos de HPV foram identificados e aproximadamente 150 tipos sequenciados (NGUYEN; RAMÍREZ-FORT; RADY, 2014). Dentre estes, os tipos considerados de alto risco oncogênico (HPV-AR) estão envolvidos na etiopatogênese do câncer cervical e da maioria das lesões intraepiteliais cervicais (SCHIFFMAN et al., 2007), destacando-se o HPV16 e o HPV18. Em contrapartida, os HPV de baixo risco oncogênico (HPV-BR), como HPV6 e HPV11, são responsáveis por causar quadros clínicos menos severos, como o desenvolvimento de verrugas genitais (BRUNI et al., 2015).

Após a infecção pelo HPV e, consequente proliferação viral, uma resposta imune integrada tende a ocorrer, tendo papel crucial na eliminação do vírus e na resolução da infecção. Embora essa resolução ocorra na maioria dos casos, raramente, o vírus não é eficazmente eliminado, e a infecção pode persistir durante

vários anos (AMADOR-MOLINA et al., 2013). Em média, mais da metade dos casos são assintomáticos e tendem a ser resolvidos dentro de 6-8 meses. Entre a outra metade, aproximadamente 90% dos casos são resolvidos em 1-3 anos após adquirida infecção, enquanto 10% persistem e cerca de 1% progride para quadro de câncer cervical (WINER et al., 2011). Assim, a persistência da infecção pelo HPV é um fator vital para a malignização destes queratinócitos, processo que leva de anos a décadas para ocorrer (KAJITANI, SCHWARTZ, 2015). A persistência viral tem sido cada vez mais associada a outros fatores externos ao vírus, estando fortemente associada ao estado imunológico do indivíduo, e à maneira como o mesmo responde à infecção viral (SCHIFFMAN et al., 2007).

Diversos componentes do sistema imune estão envolvidos na persistência do HPV, estando associados ao desenvolvimento de lesões intraepiteliais cervicais, assim como ao câncer cervical. Entre eles, a Interleucina-10 (IL-10) se destaca como um importante fator anti-inflamatório, levando à evasão do sistema imune através do favorecimento de um estado imunossupressor. No microambiente cervical, durante diversos estágios da infecção pelo HPV, a produção de IL-10 parece ser mantida e sustentada por distintas populações celulares, tendo papel importante no desfecho do quadro clínico (WANG et al., 2013). No que concerne ao seu envolvimento no desenvolvimento do câncer, o papel desta citocina ainda não está bem estabelecido. Se por um lado, sua ação imunossupressora encontra-se bem estabelecida, por outro, alguns trabalhos mostram uma possível ação imunoestimulante da IL-10. Apesar da incerteza, é indiscutível a modulação exercida pela IL-10 no favorecimento de um ambiente imunossupressor, presente nas pacientes com lesões intraepiteliais cervicais ou câncer cervical (SYRJÄNEN et al., 2009).

Ao longo das últimas décadas, diversos estudos têm demonstrado a associação de polimorfismos no gene da IL-10 a doenças distintas, incluindo o câncer cervical (NI et al., 2013). Todavia, a associação entre estes polimorfismos e maior susceptibilidade à infecção pelo vírus HPV, assim como a influência destes sobre os níveis de IL-10 produzidos ainda precisa ser melhor esclarecida. Em vista disso, o presente trabalho pretende avaliar a influência do polimorfismo rs1800872 (c.-592C>A) do gene da IL-10 na infecção por HPV e sobre os níveis plasmáticos e cervicais desta citocina em mulheres infectadas por este vírus e controles.

2 PAPILOMAVÍRUS HUMANO (HPV)

Os papilomavírus compõem um grupo heterogêneo de vírus, ocorrendo em animais, principalmente em mamíferos, sendo associado a diversas manifestações clínicas (GALLOWAY; LAIMINS, 2015; DE VILLIERS et al., 2004). Os HPV são representantes que infectam o homem e, dentre os diversos agentes infecciosos transmitidos sexualmente, destacam-se como os mais comuns (GROSS, 2014; YARBROUGH; BURNHAM, 2016). A família *Papillomaviridae* agrupa vírus com DNA dupla-fita, pequenos e circulares. Os virions têm aproximadamente 50-52 nm de diâmetro, contendo o genoma viral aproximadamente 8000 pares de bases. Este é constituído por um segmento longo não-codificante denominado de *long control region* (LCR) ou *upstream regulatory region* (URR), envolvido na transcrição e no controle de replicação; por oito ou nove sequências de leitura aberta, denominadas de *open reading frames* (ORF), divididas em duas regiões, uma de expressão precoce (E) e outra de expressão tardia (L) (DOORBAR et al., 2015; GALLOWAY; LAIMINS, 2015).

A região E codifica as proteínas virais E1, E2, E4, E5, E6 e E7, que são necessárias para a replicação do DNA viral. Em conjunto, as proteínas E1, E2 e E4 são responsáveis pela amplificação e liberação viral, enquanto E5, E6 e E7 exercem efeito pró-tumoral. Por sua vez, a região L codifica as proteínas virais estruturais, L1 e L2, ambas expressas apenas em células infectadas superficiais, as quais liberarão novos virions. Enquanto L1 é a proteína principal do capsídeo, L2 é componente menor, sendo responsável por recrutar L1 para a montagem de novos virions. A expressão de L1 e L2, em geral, ocorre nas células diferenciadas, na parte superior do epitélio (BRAVO; FÉLEZ SANCHEZ, 2015; NGUYEN; RAMÍREZ-FORT; RADY, 2014).

Todos os HPV compartilham alguns elementos conservados, incluindo a presença da LCR, as proteínas de expressão precoce E1 e E2, assim como de expressão tardia L1 e L2 (GARCIA-VALLVE; ALONSO; BRAVO, 2005; DE VILLIERS et al., 2004). E apesar de diferentes membros da família *Pappilomaviridae* apresentarem proteínas homólogas, efeitos biológicos distintos são observados quando tipos diferentes de HPV são confrontados, especialmente quanto ao risco carcinogênico ou ao gênero envolvido (CHIESA et al., 2016; GALLOWAY; LAIMINS, 2015).

De acordo com seu potencial carcinogênico, os HPV podem ser classificados como HPV de risco indeterminado (HPV-RI), HPV-BR ou HPV-AR. Infecções com HPV-BR, também denominados de HPV não oncogênicos, causam anormalidades benignas, como verrugas genitais e papilomas laríngeos (MUÑOZ et al., 2003), enquanto HPV-AR atuam como agentes carcinogênicos biológicos (SCHIFFMAN et al., 2007). Ainda, as proteínas E6 e E7 dos HPV-AR e as produzidas por HPV-BR diferenciam-se entre si, promovendo efeitos biológicos distintos (DOORBAR et al., 2015).

Por sua vez, considerando o critério filogenético, os HPV são agrupados separadamente, levando à definição de níveis taxonômicos diferentes. Em geral, é possível definir um tipo distinto de HPV através da sequência L1, uma vez que este é o gene mais conservado entre todo o genoma viral. Se a sequência de L1 variar mais do que 10% de qualquer tipo caracterizado, um novo tipo de HPV é definido. Adicionalmente, se a sequência de L1 variar de 2-10% ou menos de 2%, um novo subtipo ou variante, respectivamente, é caracterizado (DE VILLIERS et al., 2014; CHEN et al., 2005). Ainda, com base em determinadas propriedades biológicas e organização do genoma, cinco gêneros de HPV foram estabelecidos: alfa-, beta-, gama-, mu- ou nu- papilomavírus, englobando 28 espécies e aproximadamente 40 tipos responsáveis por infectar o trato genital (DE VILLIERS et al., 2014; NGUYEN; RAMÍREZ-FORT; RADY, 2014).

Os alfa-HPV normalmente infectam a mucosa e estão associados a cânceres anogenitais, sendo que dentre estes encontram-se vários HPV-AR, como o HPV 16, 18, 31, 33 e 58, apesar de alguns HPV-BR também se incluírem neste grupo, tais como o HPV 42 e 54. Por sua vez, os beta-HPV infectam o epitélio cutâneo, sendo que estudos recentes têm demonstrado a participação destes, como cofatores, no desenvolvimento de câncer de pele não-melanoma (GALLOWAY; LAIMINS, 2015).

Estes vírus são geralmente transmitidos pelo contato pele-pele, pele-mucosa ou mucosa-mucosa, apresentando tropismo por células epiteliais, em diversas localizações anatômicas, infectando tanto o epitélio mucoso, quanto o cutâneo (KAJITANI, SCHWARTZ, 2015). Apesar da relevância da transmissão sexual, outras formas de transmissão são conhecidas, como a transmissão vertical e transmissão por autoinoculação ou por fômites. Geralmente, a infecção se dá pela entrada de partículas virais através de lesões ou microlesões na superfície do

epitélio. A fim de inserir efetivamente o genoma viral no núcleo da célula infectada, a infecção precisa ocorre em uma célula basal ou com atividade mitótica intensa (MOSCICKI et al., 2012).

2.1 HPV E DESENVOLVIMENTO DE LESÕES CERVICAIS

Na cérvice uterina, a medida em que ocorre a substituição do epitélio glandular pelo epitélio escamoso, uma grande área de tecido escamoso metaplásico é formada, caracterizando a zona de transformação, a qual é especialmente vulnerável à infecção pelo HPV (DOORBAR et al., 2015). Apesar da infecção pelo HPV apresentar baixa frequência de persistência, quando esta ocorre, anormalidades na cérvice uterina são induzidas, consequentemente, aumentando o risco de desenvolvimento de lesões precursoras (BRAATEN; LAUFER, 2008; WOODMAN; COLLINS; YOUNG, 2007).

A progressão para o câncer cervical, a partir de um estado pré-invasivo até o câncer invasivo propriamente dito, é um processo lento, levando anos ou até mesmo décadas para ocorrer. Uma vez que a infecção pelo HPV ocorre, este começa a se replicar e a induzir alterações displásicas na célula hospedeira, as quais podem levar ao desenvolvimento de lesões intraepiteliais cervicais, as quais são consideradas lesões precursoras pré-malignas (RAMAKRISHNAN; PARTRICIA; MATHAN, 2015). Ao longo do tempo, a classificação dessas lesões evoluiu, com distintos termos sendo adotados por diferentes sistemas de classificação, tanto histológicos quanto citológicos, os quais evoluíram separadamente. Enquanto a classificação histológica caracteriza o processo neoplásico e auxilia no tratamento, a citologia tem papel importante na prevenção do câncer cervical (SCHIFFMAN; SOLOMON, 2013).

Diversos sistemas de classificação histológica foram adotados, dentre eles o sistema que considera diferentes graus de neoplasia intraepitelial cervical (NIC), sendo esta caraterizada pela presença de arquitetura celular anormal, incluindo irregularidades nucleares, razão núcleo/citoplasma alterada e mitoses anormais e/ou arquitetura epitelial anormal, com diferenciação, atividade mitótica e orientação celular alteradas. Este sistema considera diferentes graus de displasia intraepitelial, com NIC1 apresentando displasia leve e apenas um terço do epitélio anormal, NIC 2 apresentando displasia moderada e até dois terços do epitélio

anormal e NIC 3 apresentando displasia severa ou carcinoma *in situ* (CIS), estando toda a extensão do epitélio comprometida (BRAATEN; LAUFER, 2008).

Em relação à classificação citológica, o sistema Bethesda tem sido o mais adotado, considerando dois graus distintos de LIE, incluindo LIEBG e LIEAG. A presença de LIEBG está associada à alta taxa de replicação viral e apenas leves alterações no crescimento das células epiteliais, regredindo na maioria dos casos, espontaneamente. Diferentemente, a presença de LIEAG é marcada por intensa e progressiva desregulação do ciclo celular, causada pelo HPV, levando a aumento na proliferação celular e redução ou parada na maturação celular (APGAR; ZOSCHNICK; WRIGHT, 2003; DAVEY et al., 2004).

Dessa forma, o câncer cervical inicia-se com o desenvolvimento de LIEBG, que ocorre como resultado da infecção por um HPV-AR associada à eliminação viral ineficiente. Por razões distintas, se a taxa de eliminação do HPV é reduzida, o risco de progressão para LIEAG aumenta, e uma vez estabelecido este grau de lesão, alterações no ciclo celular podem tornar-se irreversíveis, conduzindo a célula infectada a um fenótipo transformado (MOSCICKI et al., 2012; ZSEMLYE, 2008).

2.2 HPV E CÂNCER CERVICAL

Diversos fatores estão envolvidos na carcinogênese cervical, sendo a presença de um HPV-AR fator indispensável (FORMAN, 2012), sendo o HPV16 o tipo mais predominante, estando presente em mais de 50% dos mais de 500.000 casos de câncer cervical diagnosticados anualmente (MUÑOZ et al., 2003), sendo conjuntamente com o HPV18, responsável pela grande maioria de casos de câncer cervical (WOODMAN; COLLINS; YOUNG, 2007). Apesar do HPV16 apresentar elevado potencial carcinogênico, estima-se que menos de 5% dos indivíduos infectados venham a desenvolver câncer ao longo de suas vidas (GOLDIE et al., 2003), sendo o mesmo padrão observado para outros HPV-AR (GALLOWAY; LAIMINS, 2015).

Os HPV-AR apresentam diferenças notáveis em relação a outros tipos de HPV, as quais estão intrinsecamente relacionadas a habilidade destes em levar a célula hospedeira à malignização. Além disso, diferenças no efeito biológico promovido pela ação de suas oncoproteínas, torna-os mais eficazes em favorecer a

desrregulação do ciclo celular, aumentando a proliferação e sobrevivência celular (MCLAUGHLIN-DRUBIN; MÜNGER, 2009).

2.2.1 Oncoproteínas Virais

Dentre as proteínas produzidas pelo HPV, E5, E6 e E7 estão diretamente relacionadas com a iniciação e progressão do câncer cervical. E5 atua modulando a atividade de diversas proteínas celulares, participando do efeito transformador do HPV, e atua aumentando a atividade de E6 e E7, promovendo a hiperproliferação das células infectadas e contribuindo para a progressão tumoral. Estas proteínas também desempenham papel essencial no ciclo viral, estimulando a sinalização pelo receptor de fator de crescimento epidermal (EGFR), contribuindo para a evasão da apoptose e afetando a expressão de moléculas do complexo principal de histocompatibilidade (MHC), afetando a comunicação célula-célula (DIMAIO; PETTI, 2013).

Por sua vez, E6 exerce efeito sobre diversas vias de sinalização na célula infectada, promovendo múltiplos efeitos incluindo a perda do reparo do DNA, inibição da apoptose, imortalização e perda da polaridade celular. A oncoproteína E6 se liga à p53, recrutando a ubiquitina ligase UBE3A (E6AP), levando à poliubiquitinação de p53 e degradação pelo proteassoma. Ainda, esta oncoproteína interage com vários fatores de transcrição, ativando a expressão da unidade catalítica da telomerase, prevenindo o encurtamento dos telômeros, conduzindo a célula ao estado de imortalização. Adicionalmente, E6 inibe a apoptose via interação com diversas moléculas, incluindo o receptor do fator de necrose tumoral alfa (TNFR1) e a caspase 8, e ainda através da degradação de proteínas pró-apoptóticas (DOORBAR et al., 2015; GALLOWAY; LAIMINS, 2015; WALLACE; GALLOWAY, 2015).

Da mesma forma que E6, E7 também interage com distintas vias de sinalização celular, promovendo hiperproliferação celular, instabilidade genômica, inibição da apoptose, entre outros efeitos. Esta se liga à proteína do retinoblastoma (pRB), liberando o fator de transcrição E2F, que se encontra normalmente ligado à pRB, promovendo progressão do ciclo celular. Além disso, E7 inativa os inibidores de quinases dependentes de ciclinas (CDK), como p21 (*Cyclin-dependent kinase inhibitor 1A*) e p27 (*Cyclin-dependent kinase inhibitor 1B*), ativando as ciclinas E e A,

respectivamente, levando à desregulação do ciclo celular e favorecendo a hiperproliferação. Adicionalmente, E7 favorece um estado de instabilidade genômica por diferentes mecanismos, incluindo estímulo à síntese anormal do centrossomo. E7 induz rapidamente a amplificação do centrossomo, favorecendo a ocorrência de erros e levando à duplicação aberrante do mesmo. Além disso, E7 parece interagir com a γ-tubulina, molécula evolvida na nucleação de microtúbulos e que tem papel importante na regulação de centrossomos, podendo esta interação também resultar na síntese anormal de centrossomos. Ainda, E7 parece favorecer a instabilidade genômica através da indução de dano ao DNA. E apesar de E7 ativar a via de reparo ATM-ATR (*Ataxia Telangiectasia mutated–ATM and RAD3-related DNA damage repair pathway*), essa interação acaba levando ao reparo ineficiente do DNA, contribuindo, consequentemente, para o acúmulo de alterações cromossômicas (MOODY; LAIMINS, 2010; PARK et al., 2014). Constantemente, novas interações entre estas oncoproteínas e outras proteínas celulares têm surgido, como o envolvimento de E6 e E7 na ativação da via Wnt/β-catenina, via de sinalização crucial no desenvolvimento do câncer. Esta está envolvida no desenvolvimento, proliferação, diferenciação e adesão celular, sendo que algumas proteínas do HPV, incluindo E6 e E7, parecem estimulá-la, levando à desregulação de processos moleculares críticos na célula (BELLO et al., 2015).

2.2.2 Integração do Genoma Viral

Adicionalmente aos efeitos provocados pelas oncoproteínas do HPV, a integração do genoma viral ao genoma da célula hospedeira parece favorecer o processo neoplásico, promovendo crescimento e sobrevivência celular, através do aumento da expressão destas oncoproteínas, alteração na expressão de genes reguladores de controle e da proliferação do ciclo celular, assim como mudanças no padrão de metilação e transcrição (RUSAN; LI; HAMMERMAN, 2015). Apesar de ainda incerto o momento de integração do genoma viral, acredita-se que esta ocorra durante a progressão para LIEAG, uma vez que, na maioria dos casos de LIEAG esta é observada (HUDELIST et al., 2004; LI et al., 2008).

A integração do genoma viral mostra-se como passo crucial na carcinogênese cervical, uma vez que esta rompe a ORF E2, na maioria dos casos. Como consequência, a produção desta proteína reguladora é abolida, permitindo a

expressão aumentada de E6 e E7. Apesar de ainda incerto, alguns autores acreditam que a inserção do genoma ocorra de maneira não randômica, ocorrendo em locais frágeis ou próximo a estes (MULLER et al., 2012; MULLER; DEMERET, 2012).

Diferentes mecanismos pelos quais a integração do HPV leva à desregulação de genes essenciais ao controle do ciclo celular foram descritos, incluindo perda de função pela integração a determinado gene (SCHMITZ et al., 2012) e amplificação e subsequente expressão aumentada de genes específicos, incluindo certos oncogenes, como o c-*MYC* (*v-MYC avian myelocytomatosis viral oncogene homolog*) (FERBER et al., 2003; OJESINA et al., 2014).

Adicionalmente, alterações epigenética (que não alteram a sequência do DNA), em especial metilação, parecem desempenhar um papel relevante na carcinogênese cervical relacionada ao HPV. Diversos estudos vêm demonstrando aumento significativo na metilação de genes diversos na célula hospedeira, em pacientes que apresentam diferentes graus de lesão cervical quando comparados a pacientes sem lesão (WENTZENSEN et al., 2009; WHEELER, 2013). Ainda, padrões distintos de metilação têm sido descritos nestes diferentes graus de lesão cervical (MIRABELLO et al., 2013), assim como em tumores cervicais com integração do genoma viral, quando comparados a casos onde esta não ocorreu. No entanto, razões para a ocorrência destas alterações epigenéticas, assim como o mecanismo através do qual a integração do genoma viral influencia do padrão de metilação observado, ainda são incertas (RUSAN; LI; HAMMERMAN, 2015). De qualquer forma, nota-se que há certa associação entre o estado físico do HPV e o estágio da infecção por este (AKEEL, 2015).

2.2.3 Cofatores na Carcinogênese Cervical

Incontestavelmente, a infecção pelo HPV se apresenta como principal fator de risco para o desenvolvimento do câncer cervical. Todavia, sabe-se que a presença deste vírus, isoladamente, não é suficiente para causar este tipo de câncer, especialmente baseado no fato de que apenas uma minoria de mulheres infectadas por um HPV-AR desenvolve neoplasia maligna. Dessa forma, o surgimento do câncer cervical tende a ser resultado da ação conjunta de diversos fatores (DOORBAR et al., 2012; NYGUEN, RAMÍREZ-FORT, RADY, 2014).

Alguns fatores comportamentais, que favorecem a transmissão viral, estão associados a maior risco de câncer cervical, especialmente aqueles associados a práticas sexuais, como idade precoce da primeira relação sexual (PLUMMER; PETO; FRANCESCH, 2012), multiplicidade de parceiros sexuais (CASTELLSAGUÉ; BOSCH; MUÑOZ, 2003) e não uso de contraceptivos de barreira (HARIRI; WARNER, 2013; LAM et al., 2104; PIERCE CAMPBELL et al., 2013). Adicionalmente a estes fatores comportamentais, outros fatores relacionados ao hospedeiro estão também associados à progressão de lesões cervicais e à transformação maligna, assim como a carga viral, idade, hábito tabagista, coinfecção com outros patógenos, susceptibilidade genética, e estado imunológico (MOSCICKI et al., 2012; TAN; ANKATHIL, 2015).

Dentre as moléculas do sistema imune, que participam ativamente no desenvolvimento de lesões cervicais e na progressão para o câncer, destaca-se a IL-10, uma citocina imunorreguladora, que atua no microambiente cervical favorecendo a imunossupressão deste. Além de exercer seus efeitos intrínsecos, esta citocina parece interagir com o HPV, amplificando o efeito imunossupressor. Assim, favorecendo a persistência viral e progressão da infecção (SYRJÄNEN et al., 2009).

3 INTERLEUCINA-10 (IL-10)

O sistema imune apresenta uma habilidade especial de controlar e modular diferentes atividades exercidas por populações celulares distintas, e este efeito se dá através da ação de citocinas, ativando diferentes vias de sinalização nestas células. Entre as diversas citocinas imunorreguladoras, envolvidas em processos fisiológicos, assim como patológicos, encontra-se a IL-10, uma citocina anti-inflamatória, responsável por causar imunossupressão, e consequente, evasão do sistema imune (GOPAL, 2015).

A IL-10 foi isolada e caracterizada inicialmente na década de 80, sendo originariamente denominada fator inibitório da síntese de citocinas (CSIF). Ela pertence à superfamília da IL-10, junto com outros representantes, incluindo Interleucina-19 (IL-19), Interleucina-20 (IL-20), Interleucina-22 (IL-22) e Interleucina-24 (IL-24), sendo composta por 178 aminoácidos, sendo homodimérica e apresentando peso molecular de aproximadamente 37 kDa (MANNINO et al., 2015; PESTKA et al., 2004). A IL-10 é codificada por um gene localizado no cromossomo 1 (1q31-1q32), composto por 5 éxons e 4 íntrons, sendo este altamente polimórfico, com pelo menos 40 polimorfismos de base única (SNP) identificados, incluindo três na região promotora (c.−1082G>A; c.−819T>C; e c.−592C>A) (DING et al., 2013; NI et al., 2013), gerando níveis distintos de expressão de IL-10. Sendo assim, os níveis de IL-10 parecem ser influenciados por fatores genéticos (FERNANDES et al., 2015; NI et al., 2013).

A regulação da produção de IL-10 por células imunes mostra-se complexa, e exercida tanto no nível transcricional, quanto pós-transcricional (SARAIVA; O'GARRA, 2010). Acredita-se que células imunes constitutivamente transcrevam o gene da *IL-10*, regulando sua expressão pós-transcricionalmente, objetivando encurtar o tempo de resposta, uma vez que a IL-10 é uma importante citocina imunomodulatória, prontamente requerida em algumas situações (MANNINO et al., 2015; POWELL et al., 2000). Diversas populações celulares produzem IL-10, incluindo linfócitos T auxiliares do tipo 2 (Th2), linfócitos T reguladores (Treg), linfócitos T reguladores do tipo 1 (Tr1) (um subtipo de célula T reguladora), linfócitos T auxiliares do tipo 17 (Th17) e linfócitos T CD8+ (TCD8+). Adicionalmente, alguns subtipos de células dendríticas (DC), macrófagos, células B,

células natural killers (NK) e queratinócitos produzem IL-10, assim como células tumorais (O'GARRA; VIEIRA, 2004; SARAIVA; O'GARRA, 2010).

A IL-10 atua em diferentes tipos celulares, através da ligação com seu receptor (IL-10R), caracterizado como um receptor transmembrana tetramérico, composto por duas subunidades principais (IL-10R1) e duas subunidades acessórias (IL-10R2) (PESTKA et al., 2004). Diversas vias de sinalização intracelular são ativadas pela IL-10, em diferentes tipos celulares, com destaque para a via JAK1/Tyk2/STAT3 (J*anus kinase 1/ Tyrosine kinase 2/ Signal transducer and activator of transcription 3*), sendo esta a mais comumente ativada em células mieloides (HU et al., 2007; MOORE et al., 2001). A via JAK1/Tyk2/STAT3 é ativada quando a IL-10 homodimérica se liga aos domínios extracelulares das subunidades IL-10R1, promovendo a fosforilação de quinases associadas ao receptor, incluindo JAK1 e Tyk2, constitutivamente associados à IL-R1 e IL-R2, respectivamente. Uma vez fosforiladas, essas quinases fosforilam resíduos de tirosina, localizados no domínio intracelular de IL-10R1, o qual atua como sítio de ancoragem para o fator de transcrição STAT3 latente. Após se ligar a estes sítios, STAT3 é fosforilado por JAK1 e Tyk2, ocorrendo sua homodimerização e translocação para o núcleo, ligando-se a motivos SBE (*STAT-Binding Elements*), presentes em regiões promotoras de diversos genes responsivos à IL-10, como o gene supressor de sinalização de citocina 3 (SOCS3), que codifica uma proteína supressora a qual promove diferentes efeitos intracelulares (DONNELLY; DICKENSHEETS; FINBLOOM, 1999; WILLIAMS et al., 2004). Dentre estes estão a inibição da expressão de diversas citocinas inflamatórias, através da inibição da via de MAP-quinases (*Mitogen-Activated Protein Kinase*), afetando a translocação nuclear do fator nuclear κB (NF-kB), consequentemente, inibindo a síntese de Interleucina 6 (IL-6), fator de necrose tumoral alfa (TNF-α) e Interleucina 1 beta (IL-1β) (BERLATO et al., 2002; MURRAY, 2006). Adicionalmente, STAT3 estimula a transcrição de genes antiapoptóticos, como *Bcl-XL* (*B-cell lymphoma-extra large*) e *Bcl-2* (*B-cell lymphoma 2*), e também de genes envolvidos na progressão do ciclo celular (DONNELLY; DICKENSHEETS; FINBLOOM, 1999). Além da fosforilação de STAT3, a ligação IL-10/IL-10R também parece resultar na fosforilação de STAT1 (*Signal transducer and activator of transcription 1*) e STAT5 (*Signal transducer and activator of transcription 5*), em monócitos e células T (FINBLOOM; WINESTOCK, 1995; WEHINGER et al, 1996).

A IL-10 é considerada o protótipo de citocina anti-inflamatória, modulando a síntese de citocinas e exercendo efeitos em células imunes residentes ou circulantes. Originalmente foi descrita como uma citocina Th2, participando da regulação da resposta imune em diversos níveis. Conhecida por seu efeito regulador sobre equilíbrio Th1/Th2, esta citocina é capaz de inibir tanto a resposta Th1, quanto Th2, com efeito predominante sobre a subpopulação Th1 (FIORANELLI; GRAZIA, 2014). Contudo a IL-10 tem sido associada também a respostas reguladoras e respostas envolvidas na tolerância imune (SARAIVA; O'GARRA, 2010; O'GARRA; VIEIRA, 2004).

Diversos efeitos em uma ampla variedade de células são exercidos pela IL-10, desempenhando papel anti-inflamatório, imunossupressor e antiproliferativo. Entre eles está a inibição de citocinas inflamatórias normalmente sintetizadas por macrófagos ativados e células mononucleares, como Interleucina 2 (IL-2), Interleucina 12 (IL-12) e Interferon-γ (IFN-γ) (inibindo a polarização Th1 e a ativação de células NK) e TNF-α, IL-1β, IL-6 (reduzindo a infiltração celular e inflamação) (BOLPETTI et al., 2010; TORRES-POVEDA et al., 2014). A IL-10 ainda é responsável pela regulação negativa da expressão de moléculas de MHC (MHC 1 e MHC 2), assim como de moléculas coestimulatórias (CD80 e CD86), moléculas de adesão intercelular (ICAM-1), além de interferir na maturação de DC, exercendo efeito inibitório em sua diferenciação, impedindo a apresentação de antígenos, direta e indiretamente (SABAT et al., 2010). Esta população celular parece ser o maior alvo da IL-10 (GRÜTZ, 2005), e acredita-se que o efeito imunossupressor desta é principalmente mediado por células Treg (DENNIS et al., 2013; SABAT et al., 2010; TAYLOR et al., 2006). Além disso, a IL-10 atua como regulador negativo entre a resposta imune inata e a adaptativa, e parece regular a angiogênese, inibindo-a (GOPAL, 2015).

3.1 IL-10 E INFECÇÃO POR HPV

A relação entre a IL-10 e a infecção pelo HPV está baseada, principalmente, no efeito imunomodulador exercido por esta citocina, diminuindo a resposta imune contra o vírus e favorecendo a persistência viral, que está intimamente associada ao desenvolvimento de lesões intraepiteliais cervicais e à progressão para o câncer cervical (SCHIFFMAN; SOLOMON, 2013; WOODMAN;

COLLINS; YOUNG, 2007). Adicionalmente, IL-10 e HPV parecem interagir, amplificando a imunossupressão na cérvice uterina, com IL-10 induzindo a expressão das protéínas E6 e E7, enquanto E2, E6 e E7 induzem a expressão de IL-10 (TORRES-POVEDA et al., 2014).

A IL-10 afeta a transcrição do promotor precoce do HPV (p97), localizado na região URR, aumentando sua transcrição e, por conseguinte, a expressão de E7 via STAT3 (ARANY; GRATTENDICK; TYRING, 2002), e acredita-se, que de maneira similar, de E6 (TORRES-POVEDA et al., 2014). Dessa forma, no microambiente cervical na presença do HPV, a IL-10 atua não apenas como um fator imunomodulador, mas adicionalmente como um regulador da transcrição do HPV (WANG et al., 2013).

Por outro lado, algumas proteínas do HPV - E6, E7 e E2 – têm a habilidade de se ligar à região promotora do gene da *IL-10*, promovendo aumento na expressão e produção desta citocina. Fisiologicamente os níveis cervicais de IL-10 tendem a estar discretamente aumentados, se elevando ainda mais após a infecção pelo HPV. Após a infecção, especialmente com um HPV-AR, os níveis de IL-10 tendem a aumentar ainda mais, especialmente nos casos de lesão ou câncer cervical (DANIILIDIS et al., 2016; NYGUEN, RAMÍREZ-FORT, RADY, 2014). Alguns trabalhos demonstram que os níveis de IL-10, na cérvice uterina, estão diretamente associados ao grau da lesão e estão intimamente relacionados à presença do vírus (BERMÚDEZ-MORALES et al., 2008; BHAIRAVABHOTLA et al., 2007).

Além de regular a transcrição e replicação do genoma viral, a proteína E2 atua sobre o gene da IL-10, ligando-se à região reguladora deste gene, induzindo aumento na atividade de seu promotor e, por consequência, na expressão de IL-10 (BERMÚDEZ-MORALES et al., 2011). Por sua vez, alguns autores acreditam que E6 e E7 também estimulem o aumento na expressão de IL-10, de maneira similar à exercida sobre o gene do Fator de Transformação do Crescimento Beta 1 (TGF-β1). E6 e E7 ligam-se ao fator de transcrição Sp1 (*Specificity protein 1 transcription factor*), o qual se liga à determinada sequência presente no gene do TGF-β1, aumentando a expressão e produção desta citocina. E como o gene da IL-10 compartilha esta mesma sequência de ligação ao fator de transcrição Sp1, acredita-se que a ligação de E6-Sp1 e E7-Sp1 estimule o aumento na expressão e produção de IL-10 (PERALTA-ZARAGOZA et al., 2006; TORRES-POVEDA et al., 2014). Adicionalmente, IL-10 e TGF-β1 interagem entre si, com IL-10 induzindo o

aumento da produção de TGF-β1, e vice-versa. Ainda, como a IL-10 aumenta E6 e E7, esta acaba, indiretamente, aumentando a produção de TGF-β1 (FERNANDES et al., 2015).

Assim, supõe-se que neste microambiente, as proteínas virais E2, E6 e E7 aumentam a expressão de IL-10 e de TGF-β1 (E6 e E7), assim como a IL-10 estimula a expressão de E6 e E7, gerando uma alça de retroalimentação, além da interação direta ou indireta entre IL-10 e TGF-β1. Resultando em um microambiente altamente imunossuprimido na cérvice uterina, o que pode favorecer a progressão de uma simples infecção por HPV até o desenvolvimento do câncer cervical (TORRES-POVEDA et al., 2014).

3.2 IL-10 E DESENVOLVIMENTO DE LESÕES CERVICAIS

Desde o início da infecção pelo HPV, os níveis de IL-10 tendem a aumentar no microambiente cervical, resultado da ação das oncoproteínas do HPV sobre o gene da IL-10. Mantendo-se este padrão aumentado de expressão de IL-10, juntamente com efeitos imunossupressores adicionais causados pelo HPV e pela ação de outras citocinas, torna-se mais provável a persistência viral e evasão da resposta imunológica, propiciando o desenvolvimento de lesões cervicais. E uma vez havendo progressão para LIEBG, e em alguns casos para LIEAG, a integração do genoma viral, assim como a transformação e a imortalização destas células epiteliais podem ocorrer, favorecendo a progressão destas lesões até um quadro de câncer cervical (WANG et al., 2013).

Níveis elevados de IL-10 foram observados na cérvice uterina de pacientes apresentando LIEAG ou LIEBG (MINDIOLA et al., 2008), assim como em pacientes com câncer cervical, sendo este aumento, em geral, associado ao grau de lesão observado. Dentre os casos de câncer cervical este padrão tende a ser mantido, com pacientes em estádios mais avançados tendo níveis mais elevados de IL-10 (BERMÚDEZ-MORALES et al., 2008; WANG et al., 2013)

No soro, em geral, os níveis de IL-10 também se encontram aumentados em pacientes com LIEBG ou LIEAG (WANG et al., 2013), apesar de alguns estudos não detectarem diferença significativa entre os níveis de IL-10 destes grupos quando comparados a mulheres sem lesão cervical (ALI; ALI; JUBRAEL, 2012). No entanto, nos casos de câncer cervical, a grande maioria das pacientes

apresenta níveis de IL-10 aumentados sistemicamente. E da mesma forma que na secreção cervical, a concentração sérica de IL-10 parece aumentar progressivamente, a medida que o grau de lesão aumenta (SHARMA et al., 2007). Dentre as principais células produtoras de IL-10, responsáveis pela produção desta na cérvice uterina, em diferentes fases da infecção, destacam-se algumas subpopulações de DC, células Treg, além dos próprios queratinócitos transformados (ALCOCER-GONZÁLEZ et al., 2006; BOLPETTI et al., 2010; GOPAL, 2015; KOBAYASHI et al., 2008).

O papel da IL-10 no câncer tem sido amplamente discutido, e resultados controversos vêm sendo apresentados na literatura. Todavia, no câncer cervical, o papel imunossupressor desta citocina parece prevalecer, exercendo efeito pró-tumoral, favorecendo a evasão da resposta imune e contribuindo para a progressão do tumor (TORRES-POVEDA et al., 2014; WANG et al., 2013). A IL-10 e o HPV compartilham alguns efeitos imunossupressores como, por exemplo, a regulação negativa da expressão de moléculas de MHC (I e II) (RITEAU et al., 2001), além de exercerem individualmente efeitos imunossupressores que se somam, comprometendo uma resposta imune efetiva (WANG et al., 2013). Sendo assim, a manutenção de níveis aumentados de IL-10, no microambiente cervical, pode favorecer um estado de imunotolerância, permitindo a progressão de lesões pré-malignas a lesões cancerosas. E adicionalmente a outros fatores imunossupressores, especialmente ao HPV, esta citocina parece promover a evasão imune de HPV-AR e, consequentemente, a progressão de lesões cervicais (SYRJÄNEN et al., 2009).

3.3 POLIMORFISMO rs1800872 (C.-592 C>A) DE *IL-10*

Diversos polimorfismos no gene que codifica a IL-10 têm sido associados a maior susceptibilidade a infecções e ao desenvolvimento de doenças, exercendo efeitos sobre o padrão de expressão e produção desta citocina. A região promotora do gene da IL-10 é polimórfica, contendo três importantes SNPs, entre eles o rs1800872 (c.-592C>A) (ESKDALE et al., 1998).

Até o presente momento não há estudos que avaliem a associação do polimorfismo c.-592C>A com a susceptibilidade à infecção por HPV e com a persistência viral. Todavia, alguns trabalhos investigaram a associação entre este e

o desenvolvimento de LIE, apresentando resultados divergentes entre si, dependendo da população estudada. Enquanto alguns trabalhos demonstraram que a presença do alelo A (TORRES-POVEDA et al., 2012; ZOODSMA et al., 2005) está associada a maior susceptibilidade de desenvolver estas lesões, outros não encontraram tal associação (FERNANDES et al., 2008; MARANGON et al., 2013). Da mesma forma, a associação entre este polimorfismo e maior susceptibilidade ao desenvolvimento de câncer em geral (DING et al., 2013; ZHANG et al., 2012) e câncer cervical (IVANSSON et al., 2007; SHEKARI et al., 2012; TORRES-POVEDA et al., 2016; ZIDI et al., 2015) é igualmente controversa, variando de acordo com a população avaliada.

Em relação a influência deste polimorfismo sobre a produção de IL-10, foi demonstrado que pacientes portadores do alelo A apresentam níveis elevados desta citocina no soro (TORRES-POVEDA et al., 2012), porém, neste estudo tais pacientes apresentavam LIEBG ou LIEAG, sendo que a influência sobre os níveis de IL-10 séricos pode estar associada à presença de lesão e não ao genótipo, uma vez que esta não foi considerada. Contudo, em relação à influência deste polimorfismo de *IL-10* sobre os níveis cervicais desta citocina, não há estudos avaliando tal associação.

OBJETIVOS

4 OBJETIVOS

4.1 Objetivo Geral

Avaliar a influência do polimorfismo rs1800872 (c.-592C>A) do gene *IL-10* na infecção por HPV, assim como sobre os níveis plasmáticos e cervicais desta citocina, em mulheres infectadas pelo HPV e controles, atendidas pelo Programa de Prevenção ao Câncer de Colo do Útero do Sistema Único de Saúde (SUS), na região norte do Paraná.

4.2 Objetivos Específicos

- Revisar o papel da IL-10 na infecção por HPV, no desenvolvimento de LIE e na progressão para o câncer cervical, produzindo artigo de revisão sobre o tema;
- Realizar a detecção do HPV em amostras de pacientes atendidas em diferentes Unidades Básicas de Saúde (UBS) e no Consórcio Intermunicipal de Saúde do Médio Paranapanema (CISMEPAR);
- Realizar a genotipagem viral nas amostras que apresentaram diagnóstico molecular positivo para o HPV;
- Identificar a presença, em heterozigose ou em homozigose, do polimorfismo c.-592C>A nas amostras de pacientes infectadas pelo HPV e controles;
- Dosar os níveis plasmáticos e cervicais de IL-10 de amostras provenientes de pacientes infectadas pelo HPV e controles;
- Avaliar a associação do perfil sócio-demográfico, clínico e de comportamento sexual com a presença do HPV em pacientes infectadas pelo HPV e controles;
- Comparar a frequência do polimorfismo c.-592C>A de *IL-10* nas pacientes infectadas pelo HPV e controles;
- Associar o polimorfismo c.-592C>A de *IL-10* com os níveis plasmáticos e cervicais de IL-10 em mulheres infectadas pelo HPV e controles.

PRODUÇÃO CIENTÍFICA

5 PRODUÇÃO CIENTÍFICA

5.1 ARTIGO 1

IL-10 ROLE IN HPV INFECTION, DEVELOPMENT OF SQUAMOUS INTRAEPITHELIAL LESIONS AND PROGRESSION TO CERVICAL CANCER

ABSTRACT

Although Human Papillomavirus (HPV) exerts a vital influence on cervical carcinogenesis, other factors influence the development of a squamous intraepithelial lesion (SIL) that may or not progress to cervical cancer. Among several cytokines, Interleukin 10 (IL-10) stands out as an important anti-inflammatory factor, leading to immune system evasion through an immunosuppressive state. In the cervical microenvironment, during different stages of HPV infection, IL-10 production can be induced and maintained by different cell sources, including infected keratinocytes, some subsets of dendritic cells (DC), tumor associated macrophages (TAM), T regulatory cells (Treg) and tumor cells. Further, a wide range of effects can be exerted by IL-10 on different cell populations, such as inhibiting proinflammatory cytokine production, DCs differentiation, antigen presenting function and T-helper 1 (Th1) polarization. IL-10 is one of several cytokines involved in cancer development and sustenance, although its role in cancer is still controversial and poorly understood. However, cervical IL-10 levels tend to increase in parallel to SIL development and are even higher within cervical tumors. Accumulating data have shown that after HPV infection, IL-10 levels are enhanced as a result of HPV E2, E6 and E7 proteins action over IL-10 gene transcription, while IL-10 stimulates HPV E6 and E7 expression. Therefore, this interplay between HPV and IL-10 creates a vicious cycle that could favor an immunosuppressive microenvironment in the cervix, facilitating the progression of a simple HPV infection to SIL or cervical cancer.

Keywords: Human Papillomavirus; Interleukin 10; Immune modulation; Squamous intraepithelial lesions; Cervical carcinoma.

INTRODUCTION

Several viruses are capable of transforming infected cells into benign or malignant tumor cells, stimulating cell growth and survival by a wide range of mechanisms. Different oncogenic DNA viruses present this ability, including Human Papillomavirus (HPV) (MINAROVITS et al., 2016). HPVs show strict tropism for epithelial cells at different anatomic locations, infecting both mucosal and cutaneous epithelium and represent a heterogeneous group of viruses that are associated with a spectrum of manifestations ranging from unapparent infections to malignant neoplasias (GALLOWAY; LAIMINS, 2015).

To date, more than 200 types of HPV have been identified and approximately 150 HPV types have been sequenced (NGUYEN; RAMÍREZ-FORT; RADY, 2014). The viral genome contains approximately 8000 base pairs, typically compounding a non-coding segment named long control region (LCR), involved in transcription and replication control, and eight open reading frames (ORF) divided in two regions, the early (E) and the late (L) regions. The E region encodes the viral regulatory proteins (E1, E2, E4, E5, E6 and E7) which are required for viral DNA replication and are expressed in both productively (i.e., cells from the superficial layer, with intense virions release) and nonproductively (i.e., cells from the basal layer, with no virions release) infected cells. Together, E1, E2 and E4 proteins are responsible for viral amplification and viral release, while E5, E6 and E7 proteins show tumor-promoting activities, with E6 and E7 corresponding to the primary transforming viral proteins while E5 is mostly responsible for enhancing cell proliferation. E6 and E7 proteins interact with tumor suppressor proteins such as tumor suppressor p53 (p53) and retinoblastoma protein (pRb), respectively, allowing cell cycle disruption and cell immortalization (DOORBAR et al., 2015; GALLOWAY; LAIMINS, 2015; NGUYEN; RAMÍREZ-FORT; RADY, 2014). The L region encodes the viral structural proteins, L1 and L2, both expressed only in productively infected cells. L1 is the major capsid protein and L2 the minor component of the capsid, recruiting L1 protein for virus assembly. HPV L1 and L2 late gene expression is required for virus production which normally occurs in terminally differentiated cells at the top of the epithelium and plays an important role in virus infectivity (BRAVO; FÉLEZ-SÁNCHEZ, 2015; DOORBAR, 2006).

According to their carcinogenic potential, HPVs are classified as low risk (LR-HPV), causing benign or low-grade cervical cell abnormalities, genital warts and laryngeal papillomas (MUÑOZ et al., 2003; SCHIFFMAN et al., 2007), high risk (HR-HPV), acting as carcinogens in the development of cervical cancer and other anogenital cancers and as undetermined risk (UR-HPV). Among the HR-HPV group, HPV16 and HPV18 are related with several cancers, both accounting for approximately 70% of cervical cancer cases (SMITH et al., 2007; WOODMAN; COLLINS; YOUNG, 2007).

Generally, up to half of infections are asymptomatic and clear within 6-8 months. Among the other half, about 90% clear within a few years (i.e., 1 - 3 years) after acquisition, while 10% persist (WINER et al., 2011). Some HPV types can establish chronic infection due to viral persistency, allowing the development of squamous intraepithelial lesions (SIL), such as low squamous intraepithelial lesions (LSIL) and high squamous intraepithelial lesions (HSIL) that may result in progression to cervical cancer. LSIL is associated with a productive HPV infection, intense viral replication and only mild alterations in the growing keratinocytes, uncommonly progressing to HSIL. HSIL is characterized by a progressive deregulation of the cell cycle resulting in an increased cell proliferation, decrease in viral replication and blockage of epithelial maturation, which may lead to a transformed malignant phenotype, establishing the cervical carcinoma (KAJITANI, SCHWARTZ, 2015; SCHIFFMAN, M. et al., 2007).

In spite of its importance to cervical carcinogenesis, factors other than HPV influence SIL progression. In the cervical microenvironment, several immune components play important roles in establishing HPV infection as well as on SIL regression or progression to cervical cancer. One of these components is Interleukin 10 (IL-10), an immunoregulatory cytokine, produced by many cell types. After HPV infection, certain HPV proteins seem to influence IL-10 expression, while IL-10 induces some HPV proteins expression, leading to an amplified state of immunosuppression, allowing SIL development and, eventually, progression to cervical cancer (BHAIRAVABHOTLA et al., 2007; SARAIVA; O'GARRA, 2010; SYRJÄNEN et al., 2009).

HPV AND IMMUNE RESPONSE

After HPV infection and viral proliferation, there is an integrated immune response that plays an important role in clearing most of these infections. Although most HPV infection cases regress, occasionally the virus cannot be eliminated and the infection persists for several years. In the early stages of an HPV infection, the innate immune responses involving macrophages, dendritic cells (DC), Langerhans cells (LC), natural killer (NK) and natural killer T (NKT) cells, as well as keratinocytes, are the first line of defense against HPV infection. This type of response acts in a non-specific manner and is important for eliminating the virus. As the second line of defense, the adaptive immunity appears to eliminate infected cells and prevent reinfection, producing a strong specific cytotoxic T cell response, with cytotoxic T lymphocytes (CTL) targeting HPV proteins, such as E2 and E6 (AMADOR-MOLINA et al., 2013; SASAGAWA; TAKAGI; MAKINODA, 2012).

First, infected keratinocytes seem to be important during the initial HPV infection immune response, acting as immune sentinels. Despite their limitation, they are able to act as non-professional antigen presenting cells, inducing the expression of T-helper 1 (Th1) and T-helper 2 (Th2) type cytokines in CD4+ T cells, as well as a cytotoxic response in CD8+ T cells (BLACK et al., 2007). They express several Toll-like receptors (TLR), such as TLR-3 and TLR-9, which are endosomal TLR involved in the recognition of viral nucleic acids and in the innate immune response against viral infections, promoting the production of several cytokines, and creating a powerful pro-inflammatory environment (NASU; NARAHARA, 2010; ZHOU; ZHU; CHENG, 2013).

Despite keratinocyte cooperation, other cells such as macrophages and DC are responsible for the effective innate immunity response. Macrophages are activated by binding to viral components, also through TLR, releasing several inflammatory cytokines, chemokines, and interferons (IFNs), leading to HPV-infected cell elimination via tumor necrosis factor alpha (TNF-α) secretion or antibody-dependent cytotoxicity. Generally, normal keratinocytes release the monocyte chemotactic protein-1 (MCP-1) in the presence of TNF-α, attracting macrophages into the site of viral infection (HACKE et al., 2010; ROUTES et al., 2005; SASAGAWA; TAKAGI; MAKINODA, 2012). DC act as professional antigen presenting cells, linking both innate and adaptive immune response, orchestrating a

T-cell-inducing response against HPV. In human skin, several DC phenotypes can be found, comprising LC, in the epidermis, and different subsets in the dermis, characterized by the expression of CD1a+, CD14+, or CD141+ (VALLADEAU; SAELAND, 2005). DC, macrophages, and B-lymphocytes located in the dermis appear to have no access to HPV proteins (STANLEY, 2012), but eventually, due to microinjury induced by bacterial infection or sexual contact, HPV proteins are exposed and recognized by LC in the epidermis. Once HPV proteins are recognized and presented, the adaptive immunity response initiates, producing different effects, including local inflammation and activation of T CD8+ cells, which differentiate into CTL. In the presence of Th1 cytokines, such as Interleukin 2 (IL-2), Interleukin 12 (IL-12), and interferon-γ (IFN-γ), CTL cells are activated and become effector T cells able to kill preneoplastic cervical cells or cancer cells expressing HPV antigens (SASAGAWA; TAKAGI; MAKINODA, 2012). In addition, NK and NKT cells also participate in the immune response against HPV infection and NK cells can directly eliminate HPV infected cells (RENOUX et al., 2011). Nevertheless, the role of NKT cells in the spontaneous regression of HPV lesions is still uncertain, and even controversial (CERUNDOLO; BARRAL; BATISTA, 2010; HU et al., 2015; VAN KAER; PAREKH; WU, 2011).

However, even with several immune defense mechanisms, HPV is able to evade the immune response. First, because immune response to HPV is generally low-level, due to virus localization during initial stages (i.e., confined to basal epithelial cells) and also because HPV proteins expression tends to be limited during this initial phase. Further, HPV infection presents a non-lytic characteristic, avoiding part of the inflammation response (FELLER et al., 2010; TORRES-POVEDA et al., 2014). In addition, HPV oncoproteins also downregulate the expression of different molecules in a wide range of cells, including: HPV16 E6 and E7 downregulates type-1 IFNs expression in host cells, leading to a lack of co-stimulatory signals by inflammatory cytokines; HPV16 E6 downregulates MCP-1 secretion by normal keratinocytes in the presence of TNF-α, limiting macrophage translocation to the HPV infection site; and HPV16 E5 protein downregulates the expression of the major histocompatibility complex (MHC) class I, facilitating the evasion of CTL attack. Therefore, different escape mechanisms induced by HPV may promote a state of immune tolerance, allowing HPV infection persistence (HACKE et al., 2010; SASAGAWA; TAKAGI; MAKINODA, 2012; STANLEY, 2012).

In this context we highlight some immunosuppressive cytokines involved in different physiological and pathological processes, especially IL-10, an important anti-inflammatory factor, with a crucial role leading to HPV immune system evasion through an immunosuppressive state in the local microenvironment (SARAIVA; O'GARRA, 2010).

IL-10 AND GENERAL FEATURES

IL-10 was isolated and characterized in the late ´80s (FIORENTINO; BOND; MOSMANN, 1989), and it was originally named as cytokine synthesis inhibitory factor (CSIF). IL-10 is the main member of the IL-10 superfamily, along with Interleukin 19 (IL-19), Interleukin 20 (IL-20), Interleukin 22 (IL-22), Interleukin 24 (IL-24), Interleukin 26 (IL-26) and, type III IFN-γ subfamily. Human IL-10 has 178 aminoacids (aa) in total, with a 160 aa mature segment and an 18 aa signal sequence. IL-10 is a V-shaped homodimeric cytokine with a molecular weight of 37 kDa (MANNINO et al., 2015; PESTKA et al., 2004), and is encoded by a gene located on chromosome 1 (1q31-1q32), which is composed of 5 exons and 4 introns. The *IL-10* gene is highly polymorphic, with at least 40 identified single nucleotide polymorphisms (SNP), including three SNP in the promoter region (c.−1082A>G; c.−819C>T; and c.−592A>C), that all show effects on IL-10 expression, as demonstrated by different data, in several cancer types, including cervical cancer (DING et al., 2013; NI et al., 2013). Thus, IL-10 levels seem to be significantly influenced by genetic factors (FERNANDES et al., 2015; NI et al., 2013).

The regulation of IL-10 production by immune cells seems complex and regulated at transcription (e.g., epigenetic influence, such as changes in the structure of the chromatin) (SARAIVA; O'GARRA, 2010) and post-transcription level (STOECKLIN et al., 2008). Some authors believe that immune cells may constitutively transcribe the *IL-10* gene and regulate its expression at post-transcriptional level in order to shorten signal response time, since IL-10 is an important immunomodulatory cytokine that needs to act promptly (MANNINO et al., 2015; POWELL et al., 2000).

A wide range of cell populations produces human IL-10, including Th2, T regulatory cells (Treg), type 1 T regulatory (Tr1), T-helper 17 (Th17), and

cytotoxic CD8+ T cells. Also, some subsets of DC, macrophages, granulocytes, B cells, NK cells, mast cells, fibroblasts, keratinocytes, and epithelial cells can produce IL-10. Additionally, various tumor cells are able to produce IL-10, such as multiple myeloma, melanoma and cervical squamous carcinoma cells (KOBAYASHI et al., 2008; MOORE et al., 2001; O'GARRA; VIEIRA, 2004; RONCAROLO et al., 2006; SARAIVA; O'GARRA, 2010).

The produced IL-10 homodimer acts on different cell types, through IL-10 receptor (IL-10R), a tetrameric transmembrane cytokine receptor composed of two ligand-binding subunits (IL-10R1) and two accessory signaling subunits (IL-10R2) (PESTKA et al., 2004). Distinct IL-10 activated intracellular pathways have been described in different cell types, and the JAK1/Tyk2/STAT3 (Janus Kinase-1/ Tyrosine Kinase-2/ Signal transducer and activator of transcription 3) pathway is the most commonly activated in myeloid cells. STAT3 is primarily involved in the negative regulation of macrophage activation, leading to a down-modulating in the expression of important inflammatory cytokines such as TNF-α, Interleukin 6 (IL-6) and Interleukin 1 beta (IL-1β) (HU et al., 2007; MOORE et al., 2001).

The JAK1/Tyk2/STAT3 pathway is initiated when IL-10 homodimer binds to the extracellular domains of both IL-10R1 subunits, activating the phosphorylation of several receptor-associated kinases, including JAK 1, constitutively associated to IL-10R1 subunits and TyK2, constitutively associated to IL-10R2 subunits. Once phosphorylated, these kinases phosphorylate specific tyrosine residues, located in the IL-10R1 intracellular domain (Y446 and Y496), which act as transitory anchorage sites for the latent transcription factor STAT3, a key translocation nuclear factor that induces activation of specific gene encoding for anti-inflammatory cytokines. After binding to these anchorage sites, JAK1 and Tyk2 phosphorylate STAT3, which is homodimerized and translocates to the nucleus. There, STAT3 binds to STAT-Binding-Elements (SBE) motifs, which are located in the promoter region of different IL-10 responsive genes, including *SOCS3*, which encodes the suppressor of cytokine signaling 3 (SOCS3) protein (WILLIAMS et al., 2004).

SOCS3 elicit different intracellular effects including JAK1 inhibition that leads to a negative feedback of the JAK1/Tyk2/STAT3 pathway (WILLIAMS et al., 2004). Further, it appears to inhibit endotoxin-inducible expression of many

inflammatory cytokines, by inhibiting the Mitogen-Activated Protein Kinase (MAP-kinase) pathway, affecting the nuclear translocation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), and consequently inhibiting IL-6, TNF-α and IL-1β synthesis (BERLATO et al., 2002; MURRAY, 2006; WILLIAMS et al., 2004). STAT3 also stimulates anti-apoptotic gene transcription, such as *B-cell lymphoma-extra large* (*Bcl-XL*) and *B-cell lymphoma 2* (*Bcl-2*), and also of genes involved in cell-cycle progression (DONNELLY; DICKENSHEETS; FINBLOOM, 1999). Besides STAT3 phosphorylation, IL-10/IL-10R binding may also result in signal transduction and activation of transcription 1 (STAT1) and 5 (STAT5) phosphorylation in monocytes and T cells (FINBLOOM; WINESTOCK, 1995; WEHINGER et al., 1996), although their effects in IL-10 intracellular signaling transduction are uncertain and still unclear (WILLIAMS et al., 2004).

Since its discovery, IL-10 has been considered the prototype of anti-inflammatory cytokines, modulating cytokine synthesis, and exerting effects on resident and circulating immune cells. IL-10 was first described as a Th2 anti-inflammatory cytokine that participates in immune response regulation at several levels and can have pleiotropic effects on cell mediators of adaptive and innate immunity. This cytokine is predominantly known for its effect on regulating the Th1/Th2 balance and it can inhibit both the Th1-type and Th2-type responses, with a predominant effect on the Th1 subpopulation (FIORANELLI; GRAZIA, 2014). Nowadays, IL-10 has also been associated with tolerant or regulatory cell responses, expressed by Th2 or Treg cells, among others (SARAIVA; O'GARRA, 2010; O'GARRA; VIEIRA, 2004).

In different microenvironments, including the uterine cervix, IL-10 exerts a wide range of effects on several cell types (Figure 1), which respond to its anti-inflammatory, immunosuppressive and antiproliferative role, including inhibition of proinflammatory cytokines production normally synthesized by activated macrophages and mononuclear cells, such as IL-2, IL-12 and IFN-γ (inhibiting Th1 polarization and also NK cells activation), and TNF-α, IL-1β, IL-6 (decreasing cell infiltration and inflammation) (BOLPETTI et al., 2010; TORRES-POVEDA et al., 2014).

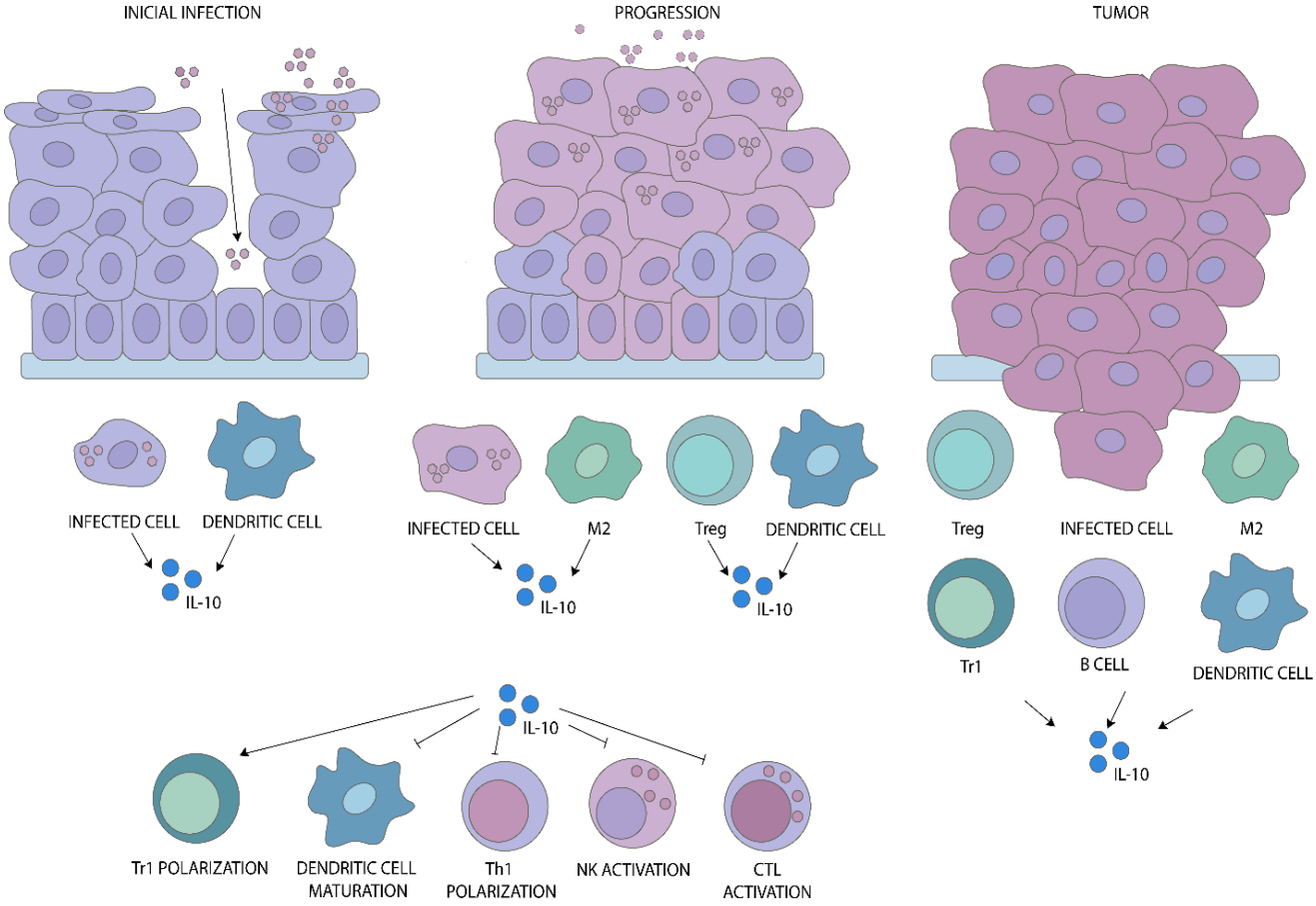

Figure 1 Major IL-10 sources during different stages of HPV infection and IL-10 actions on different cell types. During initial HPV infection, infected queratinocytes and DC are responsible for an increase in IL-10 cervical levels. As infection progresses, other cells increase and sustain IL-10 levels, such as M2-machrophages and Treg cells, as well as tumor cells within cervical cancer. IL-10 exerts several effects over different cell populations, including expansion of regulatory Tr1 cells, an inducible subset of regulatory T cells that play a pivotal role in promoting and maintaining tolerance; DC maturation inhibition (i.e., impairing antigen presentation); Th1 polarization inhibition (i.e., through decreased proinflammatory cytokines production) and suppression of NK and CTL cell activation.

Further, IL-10 is responsible for a down-regulation of MHC class I (MHC I) and MHC class II (MHC II) molecules, co-stimulatory molecules (e.g., CD80 and CD86), and intercellular adhesion molecules (e.g., intercellular adhesion molecule 1 - ICAM-1), and also interferes with monocyte maturation to DC. Thus, IL-10 has an inhibitory effect on DC differentiation and impairs their antigen presenting properties (TORRES-POVEDA et al., 2014; SABAT et al., 2010). DC seem to be the major IL-10 target (GRÜTZ, 2005), and it was proposed that its immunosuppressive effect on T cells is mainly indirect, being mediated by DC and Treg cells (DENNIS et al., 2013; SABAT et al., 2010).

Additionally, IL-10 appears to mediate the immunosuppressive activity of Tregs (ASKENASY; KAMINITZ; YARKONI, 2008; RONCAROLO et al., 2006) and can act as a negative regulator in the crosstalk between innate and adaptive immunity, and may regulate angiogenesis (GOPAL, 2015). Furthermore, a few studies have shown that IL-10 is able to activate mast cells, enhances CD8+ T cell, NK cell and B cell function and, induces T and B cell proliferation (O'GARRA et al., 2008; MUMM; OFT, 2013). However, a role for IL-10 as an immunostimulating factor is still controversial and needs to be fully understood before IL-10 status as an omnipotent anti-inflammatory cytokine changes.

IL-10-MEDIATED MODULATION IN HPV INFECTION

Although it seems unlikely that IL-10 levels have a direct impact in acquiring an HPV infection, it has been demonstrated that they do influence HPV persistence (BIJJIGA; MARTINO, 2013; BROOKS et al., 2006), which is strongly associated with progression to SIL and cervical cancer (MOSCICKI et al., 2012; SCHIFFMAN; SOLOMON, 2013). Several reports have shown that IL-10 is a common target for many viruses in their attempt to subvert the human immune system (WANG et al., 2013; BERMÚDEZ-MORALES et al., 2011). Furthermore, there is an interplay between IL-10 and HPV, with IL-10 inducing some early HPV proteins expression (e.g., E6 and E7), while certain HPV proteins (e.g., E2, E6 and E7) induce IL-10 expression, creating a vicious cycle (TORRES-POVEDA et al., 2014).

It has been reported that IL-10 exerts an impact on HPV transcription, inducing transcription of the HPV16 early promoter through the 5'-sequence of the upstream regulatory region (URR). Treatment of HPV 16-positive cervical carcinoma cells with IL-10 increased HPV16 E7 mRNA levels moderately, but significantly, and a similar increase in E7 mRNA levels in other HPV16-positive cell lines was also observed. Furthermore, protein levels of HPV16 E7 were increased after IL-10 treatment in all HPV16-positive cells. Moreover, the functional analysis of the HPV16 URR was performed in order to identify the possible IL-10 responsive region. The HPV16 URR contains three distinct regions: the 5´-, the central and the 3´-segments, respectively, with these segments binding different sets of transcription factors and thus affecting transcription from the epithelium specific

early promoter 97 (p97). Employing mutant deletion, the authors determined that this induction is mapped in the 5´-segment of the URR, which is responsive to IL-10, and that IL-10 effect on the URR was dose-dependent (ARANY; GRATTENDICK; TYRING, 2002).

Furthermore, Arany, Grattendick and Tyring (2002) also demonstrated that IL-10 regulates HPV16 E7 transcription through STAT3, increasing phosphorylation of STAT3 but not STAT1. In addition, transient transfection of an antisense-STAT3-expression vector completely abolished IL-10-induced reporter activity as well as HPV 16 E7 expression, suggesting that STAT3 either directly binds to the URR and stimulates transcription or affects expression and/or binding of transcription factors that bind to the 5´-region, playing a role in regulating transcription from the p97 early promoter. Therefore, based on these findings, it was proposed that in addition to its immunosuppressive effects, IL-10 might enhance HPV-related lesion persistence and progression under certain conditions (e.g., dysplastic progression) when the cytokine expression in the cervical microenvironment changes. Thus, the effects of IL-10 are amplified, with this cytokine acting not only as an immune modulator, but also as a transcription regulator on HPV transcription (ARANY; GRATTENDICK; TYRING, 2002; WANG et al., 2013).

Despite IL-10 effects over HPV proteins expression, a counter effect may occur. Some HPV proteins present the ability to bind to the IL-10 gene promoter region, leading to an increase in IL-10 levels. In a physiological state, the transformation zone of the cervix already tends to show a discrete increase in several cytokines levels, including Interleukin 4 (IL-4), IL-6, Transforming growth factor beta 1 (TGF-β1) and IL-10 levels (TORRES-POVEDA et al., 2014). Additionally, after acquisition of an HPV infection, especially with a HR-HPV, the levels of these cytokines, notably IL-10 and TGF-β1, appear to increase even more and are highly expressed locally within SIL and cervical tumors (DANIILIDIS et al., 2016; NYGUEN, RAMÍREZ-FORT, RADY, 2014; TORRES-POVEDA et al., 2014).

IL-10 expression is directly associated with cervical lesion degree and correlates with the presence of HPV infection. This suggests that IL-10 is produced by cervical epithelial cells, which have the potential to influence inflammation and cell immunity in the cervical mucosa, and also that HPV proteins exert an important impact on IL-10 expression (BERMÚDEZ-MORALES et al., 2008; BHAIRAVABHOTLA et al., 2007). Besides altering immune response by a wide

variety of cell mechanisms, HPV may induce soluble immunosuppressive factors, such as IL-10. It has been demonstrated that specific HPV proteins regulate several human heterologous promoters and accumulating data support this hypothesis, showing that the expression of certain cytokines may be induced by HPV, as a mechanism of escape from the immune response (BERMÚDEZ-MORALES et al., 2011). Among HPV proteins, E2, E6 and E7 are known to be involved in HPV-infected cell malignant transformation. Additionally, these viral proteins appear to play a role in the immunosuppression state HPV causes, leading to an increase in IL-10 expression (TORRES-POVEDA et al., 2014; BERMÚDEZ-MORALES et al., 2011).

Besides regulating the transcription and replication of the HPV genome, HPV E2 protein also has the ability to transactivate IL-10 gene expression. Band shifting analysis demonstrated that the HPV E2 protein binds to the regulatory region of the human IL-10 gene (-2054 nt), inducing high promoter activity in epithelial cells. While human IL-10 mRNA expression was detected in cells transfected with the HPV16 E2 gene, no expression of the IL-10 gene was found in cells not transfected with this particular gene. Similarly, cervical cancer cells transfected to express the HPV E2 protein induced elevated IL-10 mRNA levels in HPV-infected cells, supporting the hypothesis that the IL-10 gene is regulated by HPV16 E2 protein activity. Even though the function of the DNA-binding domain of HPV E2 protein within the IL-10 gene regulatory region is still uncertain, it is believed that the effect of HPV E2 protein on the transactivation of the IL-10 gene regulatory region is mediated through the physical and functional interaction of HPV E2 protein with the IL-10 gene regulatory region (BERMÚDEZ-MORALES et al., 2011). Therefore, induction of IL-10 gene expression by HPV E2 protein is an important event during HPV infection that may influence cervical cancer development, representing an additional escape mechanism of the antitumor immune response (BERMÚDEZ-MORALES et al., 2011; TORRES-POVEDA et al., 2014).

However, HPV E2 influence on IL-10 expression may be limited to early HPV infection stages. HPV persistence often causes viral integration in the genome of the host cell, leading to viral E1/E2 region disruption, releasing E6/E7 viral promoters and increasing expression of these oncogenes. *E1/E2* rupture may lead to E1 and E2 protein replication loss (TAN; ANKATHIL, 2015). Thus, in an advanced SIL stage and, more often, at the cervical cancer stage, E2 actions on IL-10 expression might be suppressed, although the upregulated E6 and E7 expression

pattern still contributes to IL-10 production. In addition, high IL-10 levels, along with other immunosuppressive cytokines, might be sustained by different cell sources in the microenvironment, including tumor cells, Tregs and M2-macrophages.

In addition to the HPV E2 effects, HPV E6 and E7 may also have a direct impact on IL-10 levels. Some authors believe that as HPV E6 and E7 proteins induce activation of TGF-β1 promoter through the specificity protein 1 transcription factor (Sp1) recognition sequence, they may induce IL-10 expression. Such a belief is based on the fact that regulatory regions of the human IL-10 gene present Sp1 regulatory elements, which suggests that HPV E6 and E7 proteins may bind to the IL-10 regulatory region to activate IL-10 gene transcription, leading to an increase in IL-10 expression in HPV-transformed cervical cells (PERALTA-ZARAGOZA et al., 2006; TORRES-POVEDA et al., 2014). Moreover, IL-10 and TGF-β1 show an interesting interplay, with IL-10 enhancing TGF-β1 expression and TGF-β1 enhancing IL-10 expression (COTTREZ; GROUX, 2001). And considering that IL-10 has the ability to increase E6 and E7 proteins, which are responsible for an increase in TGF-β1 expression, an amplified immunosuppressive state seems to be sustained (TORRES-POVEDA et al., 2014).

Taken together, it is possible to assume that HPV E2, E6 and E7 proteins enhance the IL-10 expression (i.e., and E6 and E7 of TGF-β1), while IL-10 stimulates HPV E6 and E7 expression, not forgetting the interplay between IL-10 and TGF-β1 (Figure 2). Thus, this seems to be only a part of the molecular mechanism by which HPV influences IL-10 gene expression and IL-10 induces HPV proteins expression, representing a vicious cycle that could favor an immunosuppressive microenvironment in the cervix, facilitating the progression of a simple HPV infection to SIL or cervical cancer.

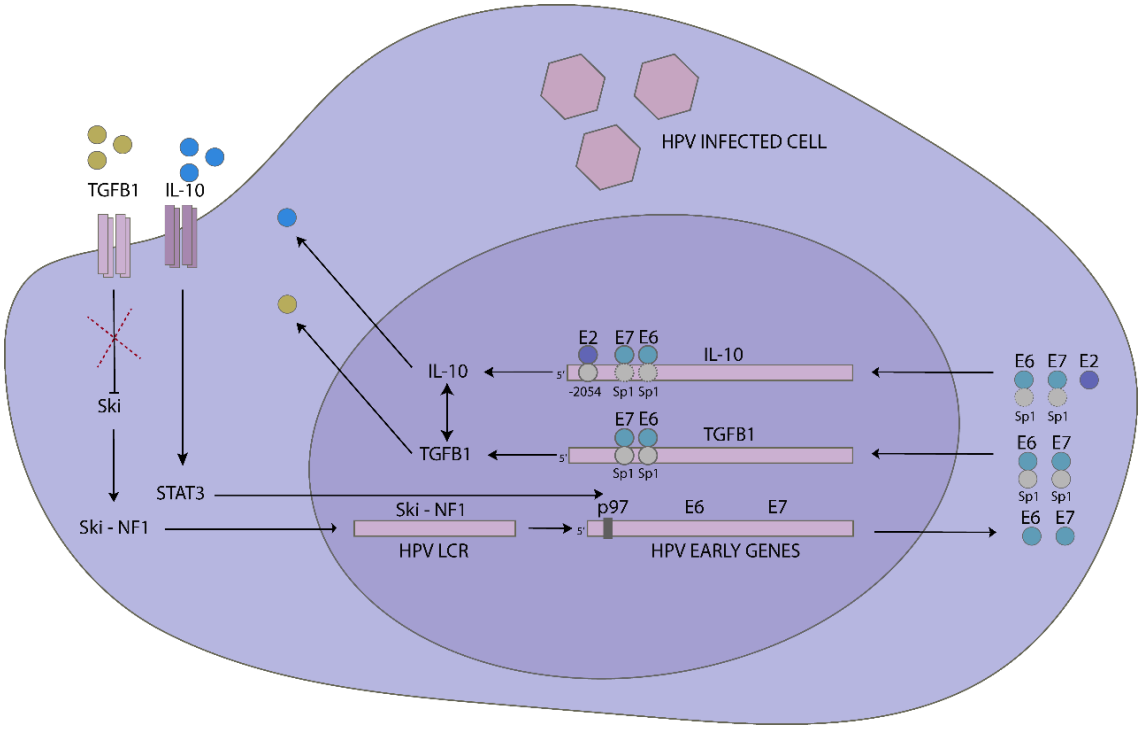

Figure 2 Interplay between HPV and IL-10. After HPV infection, IL-10 level tends to be increased as a result of HPV E2, E6 and E7 protein actions on the *IL-10* gene. HPV E2 protein binds to the regulatory region of the human IL-10 gene (-2054 nt), inducing high promoter activity in epithelial cells. Additionally, HPV E6 and E7 may also bind to the *IL-10* gene through Sp1 recognition sequence, as it does to the *TGF-β1* gene, increasing IL-10 expression. Besides HPV oncoprotein effects on IL-10 expression, IL-10 also exerts an impact on HPV transcription, affecting the transcription of the HPV early promoter p97, regulating E6 and E7 transcription in a STAT3-dependent manner. In addition, IL-10 levels are also increased by TGF-β1 action and TGF-β1 levels are also increased by IL-10. TGF-β1 exerts a particular regulatory effect on HPV early gene expression, but only after initial infection. As infection persists, this regulatory ability is lost and E6 and E7 expression increases, also increasing TGF-β1 and IL-10 expression.

IL-10-MEDIATED MODULATION IN SIL DEVELOPMENT AND PROGRESSION TO CERVICAL CANCER

Following an HPV infection, cervical IL-10 levels tend to increase as a result of HPV proteins actions over the IL-10 gene, as mentioned above. The maintenance of an elevated IL-10 expression pattern, along with other cytokines and all the other immunosuppressive effects that HPV causes, may contribute to the start of SIL because it allows HPV to subvert the innate immunological surveillance, enabling HPV-persistence. Consequently, as SILs persist, viral integration into the

epithelial cells genome, cell transformation and immortalization may also occur, leading to cervical cancer progression (WANG et al., 2013).

In the cervix of patients with SILan increased number of IL-10-positive cells was detected, which were associated with different grades of cervical dysplasia. Using indirect immunofluorescence, the expression of IL-10, IL-2 and IL-2 receptor (IL-2R) was evaluated in different grades of cervical intraepithelial neoplasia (CIN) of the exocervix. An increased number of IL-2, IL-10 or IL-2R positive cells were found in tissues from patients with CIN when compared to adjacent normal tissues and normal controls. Such increment was present in the different grades of CIN and the number of cytokine-positive cells was associated to the severity of the cervical lesion. In addition, an increased number of cytokine-positive cells in normal tissues adjacent to the lesions were found, suggesting that the cytokine expression is not limited to the lesion. The progressive increased expression of IL-10 seems to participate in the immunoescape of preneoplastic cervical keratinocytes (MINDIOLA et al., 2008).

In SIL and within cervical tumors, IL-10 cervical levels are very elevated when compared to healthy women with no lesion and absence of the HPV genome. In general, this increase is positively associated with cervical lesion stage and patients with cervical cancer and HSIL present the highest IL-10 concentration (WANG et al., 2013). It has been demonstrated that cervical IL-10 expression in patients with different grades of SIL (i.e., LSIL and HSIL) and cervical cancer presents a clear tendency to increase with advancing cervical cancer stage (BERMÚDEZ-MORALES et al., 2008). Such elevated cervical IL-10 expression in patients with SIL and cervical cancer also translates the local state of cervical immunosuppression they present (WANG et al., 2013).

A recent study evaluated the differences in several cytokine levels among various intraepithelial cervical lesion stages, including patients with LSIL and HSIL, as well as women with normal smear. Lower levels of the several cytokines studied, including IL-10, were detectable in the LSIL group when compared to tissue obtained from the HSIL group. Additionally, within the control group IL-10 levels remained undetectable, along with IL-2, IL-6, IL-12 and TGF-β1 levels. Thus, the levels of the regulatory lymphocyte factors families and immune inflammation regulatory factors were low in the control group, high in the LSIL group and even higher in subjects diagnosed with HSIL, suggesting that significant differences in

tissue cytokine levels exist among different SIL stages, and also that the underlying cause for the IL-10 production, as well as other regulatory factors, was HPV infection (DANIILIDIS et al., 2016).

Such findings are in accordance with previous reports, in which increased levels of IL-2, IL-12 and TNF-α in LSIL lesions and elevated levels of IL-4, IL-6 and IL-10 in HSIL lesions were observed. For LSIL, higher levels of Th1 cytokines were detected, while HSIL cases were associated with a Th2 cytokine profile. Further, cervical cancer tissues were associated with the strongest expression of a Treg cytokine profile and the highest IL-10 and TGF-β1 levels were observed at this stage, suggesting that tumor progression is dependent on cell immunity suppression. In this study IL-10 was associated with a Th2 cytokine profile, as well as a Treg one (PEGHINI et al., 2012). In addition, another recent study evaluated the cervical cytokine profile at various stages of cervical cancer (i.e., as well as its association with cervical microbiome), and determined cervical expression levels of IL-4, IL-6, IL-10, TGF-β1, TNF-α and IFN-γ mRNA. Even though discrete differences could be observed between cervical expression levels of IL-4, IL-6, TGF-β1, TNF-α and IFN-γ mRNA, only the median levels of IL-10 mRNA were significantly higher in SIL cases when compared to women without cervical lesions (AUDIRAC-CHALIFOUR et al., 2016).

In addition, the correlation between the presence of HPV and different cytokines (IFN-γ, IL-4, IL-10 and IL-6) expression was also investigated in cervical carcinomas. Results demonstrated a significant increase in type-2 cytokines mRNA in cervical tumor biopsies, including IL-10 mRNA, while none of the biopsies showed expression for IFN-γ. In addition, a positive correlation was observed between the presence of HPV16 and IL-10 mRNA in cervical tumor biopsies which demonstrated a pronounced shift form type-1 to type-2 cytokine production that are correlated to HPV infection (BHAIRAVABHOTLA et al., 2007). Previous studies also reported such a tendency in cervical cancer, which might reflect the reduced protective cell-mediated immunity found in these patients (CLERICI et al., 1997; EL-SHERIF et al., 2001; SALAZAR-ONFRAY; LÓPEZ; MENDOZA-NARANJO, 2007; TJONG et al., 2001). Further, an increased percentage of circulating Tregs was observed in patients with cervical cancer, reinforcing the immunosuppression state of cervical microenvironment (BHAIRAVABHOTLA et al., 2007).

Furthermore, IL-10 serum levels are increased in patients with SIL (WANG et al., 2013), although some authors have shown no detectable differences between normal controls and patients presenting SIL (ALI; ALI; JUBRAEL, 2012). On the other hand, circulating IL-10 levels are highly increased in the great majority of patients with cervical cancer. Cytokine production patterns of peripheral blood mononuclear cells (PBMCs), including IL-10 levels, were evaluated in patients with LSIL and HSIL in whom HPV infection was limited to the cervix or involved other sites of the lower genital tract. Results showed an increased IL-10 production in PBMCs from patients with cervical lesions when compared to women with normal smear and IL-10 levels increased significantly with dysplasia severity, suggesting that HPV-associated LSIL/HSIL are also characterized by secretion of high levels of IL-10 (CLERICI et al., 1997).

Cytokines and growth factors levels were assayed in the serum of women in various stages of cervical cancer, as well as in the serum of healthy controls. The results indicated that the IL-10 serum levels were significantly higher in later cancer stages, confirming that cervical cancer progression is associated with increased serum levels of several type-2 cytokines, including IL-10, angiogenin and granulocyte macrophage colony-stimulating factor (GM-CSF) (CHOPRA; DINH; HANNIGAN, 1998). Therefore, all this evidence shows that IL-10 levels tend to increase both in serum and in cervical tissues of women presenting SIL and cervical cancer, in a progressive and HPV-dependent manner, thus confirming IL-10 mediated modulation in this scenario.

Moreover, several cell sources are involved in maintaining such IL-10 levels, as discussed before, with different biophenotypes of immune cells predominantly producing IL-10 in human normal cervix, SIL and cervical cancer (Figure 1). It was previously reported that an increased number of cells expressing tolerogenic factors, including IL-10 and TGF-β1, is observed during HSIL compared with normal cervix, suggesting the presence of a dynamic immune equilibrium in precancerous lesions (KOBAYASHI et al., 2004). Another study has characterized the biochemical evolution of the local immune microenvironment in HPV-negative normal cervix, as well as in HSIL and invasive cervical cancer, with regard to the phenotypes of stromal DC, macrophages, and T cells. IL-10 expression and cell localization, along with other factors, were examined by immunofluorescence and immunohistochemistry, and the results showed that the mean cell densities of IL-10-

positive cells (i.e., and other immune cell biophenotypes) significantly increased from normal cervix to cancer. In addition, a unique subset of morphologically immature stromal DC expressing IL-10 and indoleamine 2,3-dioxygenase (IDO) was more numerous in cancer than in normal cervix and SIL, as significant trends in increasing density of IDO+ and IL-10+ cells were observed in HSIL compared with normal cervix (KOBAYASHI et al., 2008).

Other cells produce relevant amounts of IL-10, including HPV-transformed cervical cells, as koilocytes and tumor cells (ALCOCER-GONZÁLEZ et al., 2006), Tregs (GOPAL, 2015; TORRES-POVEDA et al., 2014) and tumor associated macrophages (TAM) (BOLPETTI et al., 2010; KIM; BAE, 2016). TAMs play an important role in HPV16 tumor growth and several studies show that in women infected with HPV there is a significant correlation between lesion grade and number of infiltrating macrophages, as well as with higher IL-10 expression. A HPV16 associated tumor model in mice demonstrated that TAMs are predominantly activated M2-machophages and are responsible for inducing T cell regulatory phenotype. This TAMs subtype secretes several cytokines, including IL-10, favoring immune escape that facilitates tumor growth and points to a possible mechanism behind the epidemiologic data that correlates higher IL-10 expression with risk of cervical cancer development in HPV infected women (BOLPETTI et al., 2010).

Regardless of the IL-10 source, levels of this cytokine are high in almost all cervical cancer cases (TORRES-POVEDA et al., 2014; WANG et al., 2013). In spite of this and the consolidated belief of IL-10 as a classic inhibitory factor, accumulating data based on experimental models have shown that IL-10 has both immunosuppressive and immunostimulating effects in different immune cells, depending on the context of the stimulation and the concentration of the multiple factors involved in such/each context. In certain situations, IL-10 induces immunosuppression and tumor immune escape, thus promoting tumor growth while in others IL-10 seems to promote an antitumor cytotoxic response leading to tumor regression, and additionally exerts an antiangiogenic property (MANNINO et al., 2015; MUMM; OFT, 2013).

Nevertheless, even though it plays a controversial role in different cancer types, in cervical cancer IL-10 acts rather as an immunosuppressive factor. This cytokine ability to impair an anti-tumor immune response and contribute to tumor progression relies on its effects over a wide range of molecules and cells, including

down-regulation of MHC I and MHC II expression or up-regulation of Human Leukocyte Antigen-G (HLA-G) expression in human cells. HLA-G is known to inhibit T cells and NK cell cytotoxic activity (RITEAU et al., 2001; RODRÍGUEZ et al., 2012), and its expression was found to be associated with advanced clinical stage and disease progression and it was also documented as an unfavorable prognostic factor for many kinds of solid malignancies, including cervical cancer (LIN; YAN, 2015). IL-10 and HLA-G mRNAs were significantly increased, along with protein expression, in cervical cancer tissues (YOON et al., 2007; WANG et al., 2013) and similarly to IL-10 increased expression, HLA-G expression appears to correlate with cervical lesion stage, being higher in cervical cancer patients (DONG et al., 2010). This and all the other effects mentioned previously (i.e., including down-regulation of co-stimulatory molecules on DC, inhibition of DC and macrophages differentiation and antigen presentation, suppression of NK-mediated cytotoxicity) support IL-10-mediated immunosuppression in cervical cancer.

Even though an IL-10 immunostimulating role in other cancer types seems more plausible than in cervical cancer, some authors defend such effect. They believe that cytokines, including IL-10, have multiple biological roles, based on the fact that their receptors are expressed by diverse cell populations which may result in apparently opposing effects (BROWER, 2005; WANG et al., 2013). A few have reported that low IL-10 levels are associated with risk for distinct cancers, including cervical cancer (BROWER, 2005) and that higher IL-10 levels may prevent cervical neoplasia by assisting HPV elimination (FARZANEH et al., 2006). In addition, others also proposed the clinical use of IL-10 in combination with IL-2 in the treatment of cervical cancer, based on IL-10 ability to enhance the proliferation and expression of immunologically important surface molecules and to increase type-1 cytokine production and the cytotoxic potential of HPV-specific CD8+ cytotoxic T lymphocytes (SANTIN et al., 2000). However, evidence showing IL-10 as a potential antitumor agent in cervical cancer is scarce and based only on *in vitro* data. Moreover, blocking certain cytokines such as IL-10 does not appear to be simple, as they have multiple immunomodulatory effects, at different concentrations and in different environments. Joost Oppenheim called this the “ying-yang effect” of cytokines and Frances Balkwill complements saying, “It's a question of balance” (BROWER, 2005).

Therefore, the persistence of IL-10 in SIL for a relative time may lead to a state of immune tolerance, allowing the progression of a premalignant lesion to

cancer. Along with other immunosuppressive factors (i.e., especially HPV), IL-10 may play an important role in creating a microenvironment that favors immune evasion by HR-HPV and progressive cervical disease. Thus, IL-10 certainly makes a substantial contribution to the markedly immunosuppressive state presented by cervical cancer patients, thus supporting its mediated modulation in SIL and also in cervical cancer progression (BHAIRAVABHOTLA et al., 2007; SYRJÄNEN et al., 2009).

CONCLUSION

The role of HPV in SIL development and cervical cancer progression is beyond question, although factors other than HPV influence the establishment of these clinical outcomes. IL-10 is an important anti-inflammatory cytokine that *per se* promotes an immunosuppressive state. Additionally, IL-10 appears to interact with HPV proteins, further favoring the cervical microenvironment immunosuppression. HPV E2, E6 and E7 proteins exert a positive influence on IL-10 transcription, increasing its expression, while IL-10 promotes HPV E6 and E7 expression. As a result, IL-10 cervical levels tend to increase and facilitate HPV infection progression, thus demonstrating the important modulation exerted by IL-10 in SIL development and in cervical cancer progression.

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5.2 ARTIGO 2

IL-10 GENE POLYMORPHISM c.-592C>A INCREASES HPV INFECTION SUSCEPTIBILITY AND INFLUENCES IL-10 LEVELS IN HPV INFECTED WOMEN

ABSTRACT

Human Papillomavirus (HPV) is an important sexually transmitted virus, presenting a vital role in squamous intraepithelial lesion and cervical cancer development. These clinical outcomes result from several factors, besides HPV infection. Distinct cytokines influence HPV infection and viral persistence, including Interleukin 10 (IL-10), an important anti-inflammatory cytokine that favors cervical microenvironment immunosuppression. IL-10 expression and production may be influenced by HPV itself and by *IL-10* polymorphisms, including rs1800872 (c.-592C>A). Therefore, the aim of the present study was to evaluate the influence of *IL-10* c.-592C>A polymorphism in HPV infection and in IL-10 plasmatic and cervical levels in HPV infected and non-infected women. This study included 174 infected and 186 non-infected patients classified as controls. Cervical epithelial scrapings were obtained to determine HPV DNA presence by polymerase chain reaction (PCR). Peripheral blood samples were obtained to determine *IL-10* polymorphism by PCR followed by restriction fragment length polymorphism analysis (RFLP). IL-10 levels were assessed by enzyme-linked immunosorbent assay (ELISA). HPV was more prevalent among allele A carriers (p<0.001), with *IL-10* c.-592C>A polymorphism being associated with HPV infection, as demonstrated by binary logistic regression analysis, including several confounders. Heterozygotes [OR_{adj}=2.081 95% CI (1.222–3.544), p=0.007] and homozygotes [OR_{adj}=3.745 95% CI (1.695 – 8.271), p=0.001] showed approximately 2 and 4 time's greater odds, respectively, of presenting HPV when compared to CC patients. HPV infected patients carrying polymorphic allele A showed higher IL-10 cervical levels when compared to CC patients (p=0.039). Binary logistic regression analysis demonstrated that IL-10 cervical levels were not independently associated to CA+AA genotypes (p=0.162), neither to HPV´s presence (p=0.061), thus IL-10 cervical levels are possibly increased as a result of both HPV and allele A presence. Taken together, these findings suggest that in this population *IL-10* c.-592 C>A polymorphism is independently associated with HPV infection susceptibility, exerting influence on IL-10 cervical levels in HPV infected women.

Keywords: rs1800872; HPV infection; plasmatic IL-10; cervical IL-10.

INTRODUCTION

Human Papillomavirus (HPV) stands as the most important infectious agent sexually transmitted, infecting sexually active men and women, turning into a serious health concern, due to its involvement in cervical carcinogenesis. HPV is a well-established cause of previous squamous intraepithelial lesion (SIL), such as low squamous intraepithelial lesion (LSIL) and high squamous intraepithelial lesion (HSIL), as well as cervical carcinoma (KAJITANI, SCHWARTZ, 2015). Some HPV types, especially high-risk HPV (HR-HPV), act as carcinogens in cervical and other anogenital cancers development (SMITH et al., 2007; WOODMAN; COLLINS; YOUNG, 2007), as a result of several mechanisms underlying keratinocytes malignant transformation, exerted by HPV E5, E6 and E7 oncoproteins (NGUYEN; RAMÍREZ-FORT; RADY, 2014). Although important, HPV-mediated modulation in SIL development and, consequently, progression to cervical cancer is not sufficient, with other factors influencing such process (FERNANDES et al., 2015).

Different immune components modulate the immune response and determine the acquisition of HPV and its outcome, as viral persistence and SIL progression, including immunosuppressive cytokines, as Interleukin-10 (IL-10) (SARAIVA; O'GARRA, 2010; WANG et al., 2013). This cytokine plays an important role in HPV infection and SIL development, not only by its anti-inflammatory properties, but also by its interaction with HPV proteins, creating an amplified immunosuppressive response, that may influence HPV infection and/or viral persistence, contributing to SIL development (TORRES-POVEDA et al., 2014).

Several polymorphisms were identified in *IL-10* gene, which is highly polymorphic, presenting at least 40 single nucleotide polymorphisms (SNPs), including rs1800872 (c.-592C>A) located in the *IL-10* gene promoter region (ESKDALE et al., 1998; PESTKA et al., 2004), which has been associated with different clinical outcomes, as SIL development and cervical cancer progression (DING et al., 2013).

To date, no association between *IL-10* c.-592C>A polymorphism and HPV infection have been described. Nevertheless, the association between *IL-10* c.-592C>A polymorphism and SIL development was investigated in different populations, showing conflicting results. Some have demonstrated that allele A presence is associated with a higher risk of developing SIL (TORRES-POVEDA et

al., 2012; ZOODSMA et al., 2005), while others found no association (FERNANDES et al., 2008; MARANGON et al., 2013). Similarly, the association of this polymorphism and cervical cancer development remains uncertain (IVANSSON et al., 2007; ROH et al., 2002; TORRES-POVEDA et al., 2016; ZIDI et al., 2015). Despite conflicting results, *IL-10* c.-592C>A polymorphism seems to be involved in SIL and cervical cancer development, and may also be involved in HPV acquisition and/or viral persistence.

IL-10 polymorphisms are associated with different patterns of IL-10 expression and production (SARAIVA; O'GARRA, 2010), what may explain *IL-10* c.-592C>A polymorphism influence over HPV-related clinical outcomes. It is possible that IL-10 levels may influence viral acquisition, and also contribute to HPV persistence. It has been demonstrated that allele A carriers present higher IL-10 serum levels, which may explain its influence on the inflammatory microenvironment caused by HPV infection (TORRES-POVEDA et al., 2012).

Considering that, until the present moment, the association between *IL-10* c.-592C>A polymorphism and HPV infection has not been investigated and due to HPV´s epidemiological importance on cervical lesions pathogenesis, we aimed to assess the influence of *IL-10* c.-592C>A polymorphism on HPV infection and over IL-10 plasmatic and cervical levels in HPV infected and non-infected women.

MATERIALS AND METHODS

Study Design and Ethical Approval

This case control study included 360 women, recruited from several health services located in Londrina (Paraná, Southern Brazil), including the Intermunicipal Consortium of Health of the Middle Paranapanema (Cismepar), University Hospital and Clinic Center of the State University of Londrina, as well as from two Basic Health-care Units, between June 2014 and June 2016. All study subjects received clear instructions about the purpose of the current study, as well as about the procedures they were going to be submitted to (cervical and blood collection) prior to sample collection, and signed a formal consent. Afterwards each subject was interviewed for several socio-demographic and sexual behavioral characteristics. After HPV detection, women were classified as HPV non-infected

(n=186) or HPV infected (n=174), with the former being defined as the control group. The present study was approved by the Institutional Ethics Committee Involving Humans of the State University of Londrina (Londrina, PR, Brazil) (CEP/UEL 133/2012; CAAE 05505912.0.0000.5231).

SAMPLES COLLECTING

Cervical epithelial scrapings were obtained from women, who were submitted to clinical evaluation, in an outpatient appointment at the colposcopy ambulatory of the distinct health services mentioned above. After sample collection for cytology, the cytobrushes were stored in 2mL of TE solution (10mM Tris-HCl and 1mM EDTA), at -20°C until laboratorial analysis. Peripheral blood was collected by venipuncture, in vacutainer tubes containing EDTA and stored at −20°C until analysis. Cervical and peripheral blood samples were obtained from all 360 women included in this study. Blood samples were used for IL-10 polymorphism analysis and IL-10 plasma levels determination, while cervical samples were tested for HPV, as well as for IL-10 cervical levels.

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. Genomic DNA from peripheral blood samples was extracted using the Biopur Mini Spin Plus Kit (Biometrix, Curitiba, PR, Brazil). DNA concentration was measured by Thermo Fisher Scientific NanoDrop 2000c® Spectrophotometer (USA) at 260nm and purity was assessed through 260/280 ratio.

HPV DETECTION BY PCR

HPV PCR was carried out using MY09 (5'-CGTCCMAARGGAWACTGATC-3') and MY11 (5'-GCMCAGGGWCATAAYAATGG-3') primers, according to GenBank Accession number: AJ236888. These primers amplify a conserved region of approximately 450 bp of L1 HPV gene (BAUER et al., 1991). Reaction conditions were 190nM of dNTPs, 500nM of each primer, 2mM of

$MgCl_2$, 1X of Buffer, approximately 80ng of DNA and 1,25U of Taq polymerase (InvitrogenTM, Carlsbad, CA, USA), with an annealing temperature of 55ºC. Co-amplification of the human b-globin gene (approximately 268 bp) was performed as an internal control, using primers GH20 (5'—GAAGAGCCAAGGACAGGTAC-3') and PC04 (5'-CAACTTCATCCACGTTCACC-3') under the same conditions as the HPV PCR. A negative control sample, with no DNA, was performed during all reaction sets in order to exclude possible contamination. Also, a positive control consisting of HeLa cell lineage DNA, which contains HPV18 genome integrated to cell genome, was adopted.

IL-10 POLYMORPHISM rs1800872 (C.-592C>A) GENOTYPING BY PCR-RFLP

Genomic DNA from peripheral blood samples was used to detect *IL-10* c.-592C>A polymorphisms, in wich a cytosine is replaced by an adenine. First, a set of primers, forward (5'-GTGGAAACATGTGCCTGAGA-3') and reverse (5'-ATGAGGGGGTGGGCTAAATA-3'), was used to amplify a region of 154 bp of IL-10 gene, containing the polymorphic region. PCR conditions were 100nM of dNTPs, 250uM of each primer, 1,5mM of $MgCl_2$, 1X of Buffer, approximately 100ng of DNA and 1U of Taq polymerase (InvitrogenTM, Carlsbad, CA, USA), with an annealing temperature of 58ºC. The amplified fragments of 154 bp were submitted to enzymatic restriction with 1U of *Afa I (Rsa*I) enzyme (Invitrogen, Life Technologies, Carlsbad, CA, USA) for polymorphism analysis, according to the manufacturer's instructions. This specific enzyme cleaves the amplified fragment when an adenine is present at loci -592, producing two fragments of 79 and 75 bp. In the presence of a cytosine, the 154 bp fragment remains intact (CAPASSO et al., 2007).

PLASMA AND CERVICAL IL-10 LEVELS DETERMINATION

IL-10 plasmatic levels of HPV infected (n=146) and non-infected (n=162) women, as well as IL-10 cervical levels of HPV infected (n=30) and non-infected (n=70) were determined using the Human IL-10 ELISA Ready-SET-Go!® (eBioscience, San Diego, CA, USA). Cervical samples collected by cytobrushes from endo- and ectocervices were placed into falcon tubes containing 2mL TE solution, which were subsequently centrifuged. The supernatants were separated and IL-10 protein levels were determined using the same Human IL-10 kit. The results were

expressed as picograms per milliliter (pg/mL). The reduced number of samples included in this analysis in comparison with the original study population is due to samples availability (i.e., adequate peripheral blood samples, TE availability).

Statistical Analyses

Categorical data were analyzed by χ^2 test and results expressed as absolute value and percentage. Continuous data were analyzed by parametric tests when normality was assumed (T Test) or by non-parametric among non-normal data (Mann-Whitney). The Kolmogorov-Smirnov or Shapiro-Wilk tests were used for testing for normality depending on sample size, while Levene's test was carried out for equality of variances when appropriate. Binary logistic regression analysis, with adjustment for several confounders was used to estimate the association between polymorphism genotypes and HPV infection status, as well as to estimate the association between polymorphism genotypes and IL-10 levels. All tests were two-tailed, with a *p* value <0.05 considered for statistical significance. The odds ratio (OR) was estimated for all analyses, adopting a 95% confidence interval (CI). All statistical analyses were carried out using the SPSS Statistics 22.0 software (SPSS Inc., Chicago, Illinois, USA).

RESULTS

In the present study, 360 women were included and categorized as HPV infected patients (174/ 48.3%) and HPV non-infected patients or controls (186/ 51.7%). HPV infected patients mean age was 36.30±12.71 years, while HPV non-infected women mean age was 41.84±11.60 years, being statistically different (p<0.001).

Socio-demographic and clinical characteristics, as well as reproductive sexual characteristics of HPV non-infected and HPV infected patients are presented in Tables 1 and 2, respectively. Comparing non-infected women to HPV infected, it was observed a higher frequency of HPV in women younger than 34 years old (p<0.001), who presented no knowledge about HPV (p=0.042) and among women receiving less than 1 minimum wage (p=0.013). Additionally, HPV infection was more common among single women (p=0.006), that had more than 4 sexual partners during lifetime (p=0.003) and among smokers (p<0.001).

IL-10 c.-592C>A polymorphism genotype distribution between HPV non-infected and HPV infected patients were in Hardy-Weinberg equilibrium (p>0.05). A significant difference in polymorphism genotypes distribution was observed between HPV non-infected and HPV infected patients, with a higher frequency of HPV being observed among allele A carriers (p<0.001), confirmed by codominant, dominant and recessive models (Table 3). In order to evaluate if *IL-10* c.-592C>A polymorphism is associated with HPV infection, considering other covariates that could also influence such infection, several models of binary logistic regression analysis were carried out, with different confounders included, as presented in Table 4. A significant association between allele A presence and HPV infection was confirmed in all five models, indicating that the polymorphism is independently associated to HPV infection, even when considering age, smoking status, knowledge about HPV, number of sexual partners during lifetime and marital status as confounders. As observed at model 5, heterozygotes [OR_{Adj}=2.081 $CI_{95\%}$ (1.122 – 3.544), p=0.007], as well as homozygotes [OR_{Adj} =3.745 $CI_{95\%}$ (1.695 – 8.271), p=0.001], presented an increased risk for HPV infection.

Adopting the dominant model due to its better distribution among genotype groups, and grouping HPV infected and non-infected women together, we found no association between IL-10 plasmatic levels and *IL-10* c.-592C>A

polymorphism (CC=3.74±0.19pg/mL; CA+AA=3.54±0.14pg/mL, *p*=0.377) (Supplementary Figure 1A), as well as between IL-10 cervical levels and *IL-10* c.-592C>A polymorphism (CC=3.89±0.40pg/mL; CA+AA=4.76±0.39pg/mL, *p*=0.166) (Supplementary Figure 1B). Additionally, IL-10 plasmatic levels did not vary among HPV non-infected and infected patients (3.85±0.18pg/mL; 3.37±0.14pg/mL, respectively, *p*=0.286) (Supplementary Figure 2A), however IL-10 cervical levels were higher among HPV infected patients (5.31±0.54pg/mL) when compared to HPV non-infected (4.00±0.32pg/mL) (*p*=0.026) (Supplementary Figure 2B).

Segregating patients in two groups, as HPV non-infected and infected patients, we found that IL-10 plasmatic (CC=3.65±0.26pg/mL; CA+AA=4.03±0.25pg/mL, *p*=0.416) and cervical (CC=3.89±0.49pg/mL; CA+AA=4.10±0.42pg/mL, *p*=0.712) levels did not vary according to the IL-10 genotype among HPV non-infected women (Figures 1A and 1C). However, among HPV infected women, IL-10 plasmatic levels were lower in allele A carriers (CC=3.91±0.28pg/mL; CA+AA=3.14±0.15pg/mL, *p*=0.017) (Figure 1B), while cervical levels showed to be higher among patients carrying the polymorphic allele A (CC=3.87±0.65pg/mL; CA+AA=6.15±0.70pg/mL, *p*=0.039), as shown in Figure 1D.

Binary logistic regression analysis showed that IL-10 plasmatic (*p*=0.647) and cervical (*p*=0.162) levels were not independently associated with *IL-10* c.-592C>A polymorphism, when HPV and age were included as confounders (Table 5). In addition, when *IL-10* c.-592C>A polymorphism and age were included as possible confounders, we observed that IL-10 plasmatic (*p*=0.055) and cervical (*p*=0.061) levels were not independently associated with HPV´s presence (Table 5).

DISCUSSION

To the best of our knowledge this is the first study that demonstrate a significant and independent association between *IL-10* c.-592C>A polymorphism and HPV infection, as well as its influence on IL-10 cervical levels in HPV infected women. Although *IL-10* gene polymorphism effect on SIL and cervical cancer development have been described in different populations, its association with HPV infection susceptibility and its influence over IL-10 levels in HPV infected women have not been previously reported.

HPV genome was detected in 48.3% of the study population. Assessing socio-demographical, clinical and sexual behavioral characteristics of HPV infected and HPV non-infected patients, a higher frequency of HPV was observed within patients <34 years old, and coincidentally the great majority of these patients were single and had more than 4 sexual partners during their lifetime. This is in accordance to a meta-analysis conducted by SanJosé et al. (2007), which has demonstrated that in all world regions, HPV prevalence was highest in women younger than 35 years old, decreasing in women of older age. Therefore, in this scenario, young age and marital status comes along with a sexual behavior pattern that appears to influence HPV acquisition. Young age is an established independent factor associated to HPV infection (COSER et al., 2016), due to a potential more intense sexual activity and also to cervical ectopy, a physiological change common at this age (HWANG et al., 2012).

Additionally, a higher frequency of HPV was observed within women who declared having no knowledge about HPV and who received less than 1 minimum wage, implying that lack of information about HPV and low family income, which may be associated to misinformation, are associated to an increased predisposition to contract HPV, as described previously (COSER et al., 2016). HPV infection was also correlated to smoking status, with a higher frequency of HPV among smokers. Such effect on HPV acquisition is still uncertain, although it may be related the immunosuppression caused by tobacco smoking (MOORE et al., 2001). In addition, some authors believe that smoking may be a proxy measure of sexual behaviors (BURCHELL et al., 2006).

Additionally, we investigated whether *IL-10* c.-592C>A polymorphism exert or not an impact on HPV infection susceptibility and observed a higher

frequency of HPV among allele A carriers, confirmed by different models (e.g., codominant, dominant and recessive models, as well as separating both alleles). Binary logistic regression analysis, including several confounders, showed a significant and independent association between allele A and HPV infection, with allele A carriers being at greater odds of presenting HPV as compared to patients with CC genotype. Model 5, for example, showed that heterozygotes and homozygotes are at approximately 2 and 4 time's greater odds, respectively, of presenting HPV as compared to patients with CC genotype. To our knowledge, this is the first study to demonstrate such association. Further studies are required in order to confirm if *IL-10* c.-592C>A polymorphism may be a plausible marker of HPV infection susceptibility. Moreover, *IL-10* c.-592C>A polymorphism may also influence HPV persistence although, as we have accessed only if HPV´s presence and we have not followed them up, it is not possible to determine with certainty if it also stands as a marker of HPV persistence.

In addition, we investigated if such association between *IL-10* c.-592C>A polymorphism and HPV infection was related to IL-10 produced levels. First, grouping all patients (HPV infected and non-infected) together and considering c.-592C>A polymorphism and IL-10 levels, we observed that IL-10 plasmatic and cervical levels were not significantly associated to the referred polymorphism. These results differs from another presented by Torres-Poveda et al. (2012) that have observed higher IL-10 plasmatic levels within allele A carriers, and may be explained by differences in the studied population. Furthermore, analyzing HPV presence and IL-10 levels, we observed that IL-10 plasmatic levels were not associated with HPV´s infection. However, IL-10 cervical levels were higher among HPV infected patients. Several studies have demonstrated HPV´s influence on IL-10 cervical levels, which tends to increase after HPV infection (TORRES-POVEDA et al., 2014).

Additionally, we evaluated the association between *IL-10* c.-592C>A polymorphism and IL-10 levels in HPV non-infected and infected patients, thus segregating patients in distinct groups. Among HPV non-infected women, no association was found between *IL-10* c.-592C>A polymorphism and IL-10 levels, althought within HPV infected women both IL-10 plasmatic and cervical levels varied due to the presence of allele A. Among HPV infected women IL-10 plasmatic levels were significantly lower in allele A carriers, although the exact mechanism for this reduction is unknown. Likewise, IL-10 cervical levels among CC and CA+AA HPV

infected patients varied, being higher among patients carrying polymorphic allele A. Thus, demonstrating that both *IL-10* c.-592C>A polymorphism and HPV are modulating IL-10 levels.

Finally, in order to investigate if such influence on IL-10 levels is independently associated with *IL-10* c.-592C>A polymorphism a binary logistic regression analysis, including HPV and age as confounders, was carried out. Results demonstrated that changes in IL-10 plasmatic and cervical levels were not independently associated to *IL-10* c.-592C>A polymorphism. And also, those levels were not independently associated to HPV when *IL-10* c.-592C>A polymorphism and age were included as confounders. Therefore, both are influencing IL-10 produced levels in a combined manner.

Peralta et al. (2006) have demonstrated that HPV E6 and E7 oncogenes trans-regulate many cell genes, and they analyzed the mechanism through which HPV16 E6 and E7 oncoproteins regulate the Transforming growth factor beta 1 (TGF-β1) gene promoter in cervical tumor cells. Demonstrating that E6 and E7 bind to Sp1 transcription factor, forming an E6-Sp1 and E7-Sp1 complex, which binds to a Sp1 recognition site (GGGGCGG) that is present within *TGF-β1* regulatory element site, inducing *TGF-β1* transcription. Similarly, the IL-10 regulatory region in human gene possesses the same Sp1 recognition site suggesting that the complexes E6-Sp1 and E7-Sp1 may bind to the IL-10 regulatory region, activating *IL-10* transcription (TORRES-POVEDA et al., 2014). Therefore, such interaction between E6 and E7 proteins with Sp1 and *IL-10* promoter region, may explain in part why these HPV infected women showed higher IL-10 cervical levels. Despite that, HPV infected patients with CC genotype presented lower IL-10 cervical levels as compared to allele A carriers. Therefore, besides HPV action at *IL-10* promoter region, allele A presence is somehow required for the observed IL-10 cervical increase.

Several promoter polymorphisms that influence transcriptional, phenotypic, and functional characteristics of a spectrum of genes have been previously described (HOWELL; ROSE-ZERILLI, 2007). For the human *IL-10* promoter, polymorphic changes at three well characterized sites, including −592 nt, are thought to contribute to dysregulated IL-10 production and to the onset and severity of several conditions, including infectious disorders (SHARMA et al., 2010). Steinke et al. (2004) have demonstrated that a specific binding site of the

transcription factors Sp1 to the IL-10 promoter is located immediately upstream of the -571 nt, which is quite near to -592 nt, where c.-592C>A polymorphism takes place. Further, Sharma et al. (2010) showed that −592 site was controlled by Sp1 transcription factor, with such interaction depending on distinct conditions, including if allele A or C is present on -592 nt position. Therefore, we hypothesize that in the presence of HPV, E6 and E7 proteins bind to Sp1 transcription factor, forming E6-Sp1 and E7-Sp1 complexes that may bind more easily to Sp1 recognition sequence when allele A is present at -592 nt site, explaining why HPV infected women carrying allele A showed increased IL-10 cervical levels. Despite being a plausible explanation, experimental studies are need in order to confirm the above hypothesis, as well as its underlying mechanisms.

Furthermore, previously our results showed that IL-10 plasmatic levels where lower within HPV infected women carrying A allele, which seems to contrast with the explanation presented above. Despite that, it is important to emphasize that HPV virions tend to remain restrict to infection site, probably not migrating systemically. Additionally, Sharma et al. (2010) demonstrated differential *IL-10* promoter transcriptional activation in specific cell types, showing that polymorphic changes may alter *IL-10* promoter activity in a cell-specific and environment-specific manner. Thus, a constitutively increased or diminished IL-10 production may be altered depending on cellular type and specific environment, as well as on Sp1 activity. Therefore, these data suggest a functional relevance for Sp1 binding differences at the -592 position, as well as a physical interaction and functional cooperation between viral oncoproteins and cellular regulatory elements of IL-10 gene promoter, and may explain the contribution of HPV and *IL-10* c.-592C>A polymorphism to IL-10 produced patterns.

Among limitations of our study, we could mention the lack of information about patient's body weight, which may also influence on cytokine levels. In addition, the reduced number of available cervical samples included in IL-10 cervical levels analysis may be another limitation. On the other hand, among strengths of our study stands the fact that our samples were obtained under the same conditions, from the same health service units, in the same location, being afterwards separated as HPV non-infected and infected patients. Moreover, we could mention the adjusted results obtained by binary logistic regression models,

considering several potential confounders, turning obtained data more real and substantial.

CONCLUSIONS

Taken together, our results have demonstrated for the first time an independent association between *IL-10* c.-592C>A polymorphism and HPV infection, with polymorphic allele A carriers showing an increased susceptibility to HPV infection. Additionally, *IL-10* c.-592C>A polymorphism along with HPV modulates IL-10 cervical pattern production, which may allow viral persistence and explain HPV infection maintenance in those patients.

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Table 1 Socio-demographic and clinical characteristics of HPV non-infected and infected patients

Variable		HPV non-infected		HPV infected		*p* value
		n*	(%)	n*	(%)	
Ethnicity						0.267
	Caucasian	94	(53.4)	62	(45.9)	
	Non-caucasian	82	(46.6)	73	(54.1)	
Age (years)						**< 0.001**
	≤ 34	58	(31.3)	85	(51.2)	
	35 – 44	49	(26.5)	38	(22.9)	
	45 – 54	52	(28.1)	25	(15.1)	
	≥ 55	26	(14.1)	18	(10.8)	
Educational level[a]						0.706
	Incomplete fundamental education	56	(31.8)	45	(33.1)	
	Complete fundamental education	17	(9.7)	18	(13.2)	
	Incomplete secondary education	26	(14.8)	19	(14.0)	
	Complete secondary education	60	(34.1)	44	(32.4)	
	Incomplete higher education	6	(3.4)	6	(4.4)	
	Complete higher education	11	(6.3)	4	(2.9)	
Monthly income[b]						**0.013**
	<1 minimum wage	40	(24.0)	48	(38.4)	
	1 – <3 minimum wages	112	(67.1)	66	(52.8)	
	3 – <5 minimum wages	11	(6.6)	11	(8.8)	
	≥ 5 minimum wages	4	(2.4)	0	(0.0)	
Marital status						**0.006**
	Single	18	(9.7)	38	(23.5)	
	Married / Civil partner	136	(73.5)	97	(59.9)	
	Divorced	22	(11.9)	19	(11.7)	
	Widowed	9	(4.9)	8	(4.9)	
Smoking status						**< 0.001**
	No	157	(85.8)	103	(69.6)	
	Yes	26	(14.2)	45	(30.4)	
Knowledge about HPV						**0.042**
	No	34	(19.2)	43	(31.4)	
	Have ever heard	100	(56.5)	68	(49.6)	
	Yes	43	(24.3)	26	(19.0)	

Analysis by two-sided Chi-square (χ^2) test and ($p<0.05$ as significance level). [a]Based on Brazilian educational system. [b]Based on Brazilian minimum wage (approximately U$ 220.00). SIL: squamous intraepithelial lesion; NSIL: non-squamous intraepithelial lesion; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion.

* For socio-demographic characteristics analysis between HPV non-infected and infected patients not all 360 patients were included, with variations depending on the characteristic analyzed.

Table 2 Sexual behavioral characteristics of HPV non-infected and infected patients

Variable	HPV non-infected		HPV infected		*p* value
	n*	(%)	n*	(%)	
Contraceptive method					0.307
No	108	(59.3)	87	(55.1)	
Yes, hormonal	60	(33.0)	54	(34.2)	
Yes, condon	13	(7.1)	12	(7.6)	
Yes, both	1	(0.5)	5	(3.2)	
Number of full-term pregnancies					0.070
0	15	(8.1)	24	(14.5)	
1	32	(17.3)	42	(25.5)	
2	58	(31.4)	38	(23.0)	
3	43	(23.2)	38	(23.0)	
4	21	(11.4)	12	(7.3)	
≥ 5	16	(8.6)	11	(6.7)	
Parturition					0.134
No	17	(9.2)	28	(17.0)	
Natural birth	75	(40.5)	68	(41.2)	
Cesarean birth	59	(31.9)	42	(25.5)	
Both	34	(18.4)	27	(16.4)	
Abortion					0.960
No	132	(79.5)	111	(79.3)	
Yes	34	(20.5)	29	(20.7)	
Age at first sexual intercourse (years)					0.150
≤17	95	(52.5)	95	(61.3)	
≥18	86	(47.5)	60	(38.7)	
Age at menarche (years)					0.718
≤11	41	(22.4)	39	(25.0)	
12	45	(24.6)	44	(28.2)	
13	44	(24.1)	32	(20.5)	
≥14	53	(29.0)	41	(26.3)	
Sexual partners during lifetime					**0.003**
1	74	(41.6)	32	(23.5)	
2 – 3	51	(28.7)	45	(33.1)	
≥4	53	(29.8)	59	(43.4)	
Sexual partners within the past 6 months					0.529
0	25	(14.0)	24	(17.4)	
1	150	(84.3)	110	(79,7)	
≥2	3	(1.7)	4	(2,9)	

Analysis by two-sided Chi-square (χ^2) test ($p<0.05$ as significance level).

* For sexual behavioral characteristics analysis between HPV non-infected and infected patients not all 360 patients were included, with variations depending on the characteristic analyzed.

Table 3 Association between *IL-10 c.-592C>A* polymorphism and HPV infection

***IL-10* - Genotypes** rs1800872 (c.-592C>A)	**HPV non-infected (n = 186)[a]**		**HPV infected (n = 174)[b]**		**OR**	**$CI_{95\%}$**	***p* value**
	n	**(%)**	**n**	**(%)**			
Codominant model							
CC	89	(47.8)	52	(29.9)	1.000	Reference	
CA	81	(43.5)	95	(54.6)	**2.010**	**1.281 – 3.160**	**0.002**
AA	16	(8.6)	27	(15.5)	**2.891**	**1.423 – 5.860**	**0.003**
Dominant Model							
CC	89	(47.8)	52	(29.9)	1.000	Reference	
CA+AA	97	(52.2)	122	(70.1)	**2.153**	**1.400 – 3.320**	**<0.001**
Recessive Model							
CC+CA	171	(91.4)	147	(84.5)	1.000	Reference	
AA	16	(8.6)	27	(15.5)	**2.094**	**1.073 – 4.086**	**0.028**
Alleles							
C	259	(69.92)	199	(57.18)	1.000	Reference	
A	113	(30.38)	149	(42.82)	**1.720**	**1.263 – 2.316**	**<0.001**

Analysis by two-sided Chi-square (χ^2) test (p<0.05 as significance level).

OR = Odds Ratio; CI = Confidence Interval. [a] χ^2 in HWE=0.092, p>0.05; [b] χ^2 in HWE=0.1833, p>0.05.

Table 4 *IL-10 c.-592C>A* polymorphism´s influence on HPV infection adjusted for several confounders

		Model 1	Model 2	Model 3	Model 4	Model 5
CC						
	OR	1.000	1.000	1.000	1.000	1.000
	$CI_{95\%}$	(Reference)	(Reference)	(Reference)	(Reference)	(Reference)
	p value					
CA						
	OR	**1.939**	**2.067**	**2.018**	**2.084**	**2.081**
	$CI_{95\%}$	(1.212 – 3.103)	(1.249 – 3.418)	(1.193 – 3.413)	(1.225 – 3.544)	(1.222 – 3.544)
	p value	**0.006**	**0.005**	**0.009**	**0.007**	**0.007**
AA						
	OR	**3.077**	**3.647**	**3.710**	**3.742**	**3.745**
	$CI_{95\%}$	(1.491 – 6.351)	(1.697 – 7.838)	(1.692 – 8.132)	(1.694 – 8.263)	(1.695 – 8.271)
	p value	**0.002**	**0.001**	**0.001**	**0.001**	**0.001**

Logistic regression analysis with HPV as dependent variable (reference group = non-infected women) and c.-592C>A polymorphism as explanatory variable, adjusted for several confounders according to the proposed models ($p<0.05$ as significance level).
Model 1: IL-10 polymorphism adjusted for age;
Model 2: IL-10 polymorphism adjusted for age and smoking status;
Model 3: IL-10 polymorphism adjusted for age, smoking status and knowledge about HPV;
Model 4: IL-10 polymorphism adjusted for age, smoking status, knowledge about HPV and number of sexual partners during lifetime;
Model 5: IL-10 polymorphism adjusted for age, smoking status, knowledge about HPV, number of sexual partners during lifetime and marital status.
OR = Odds Ratio; CI = Confidence Interval.

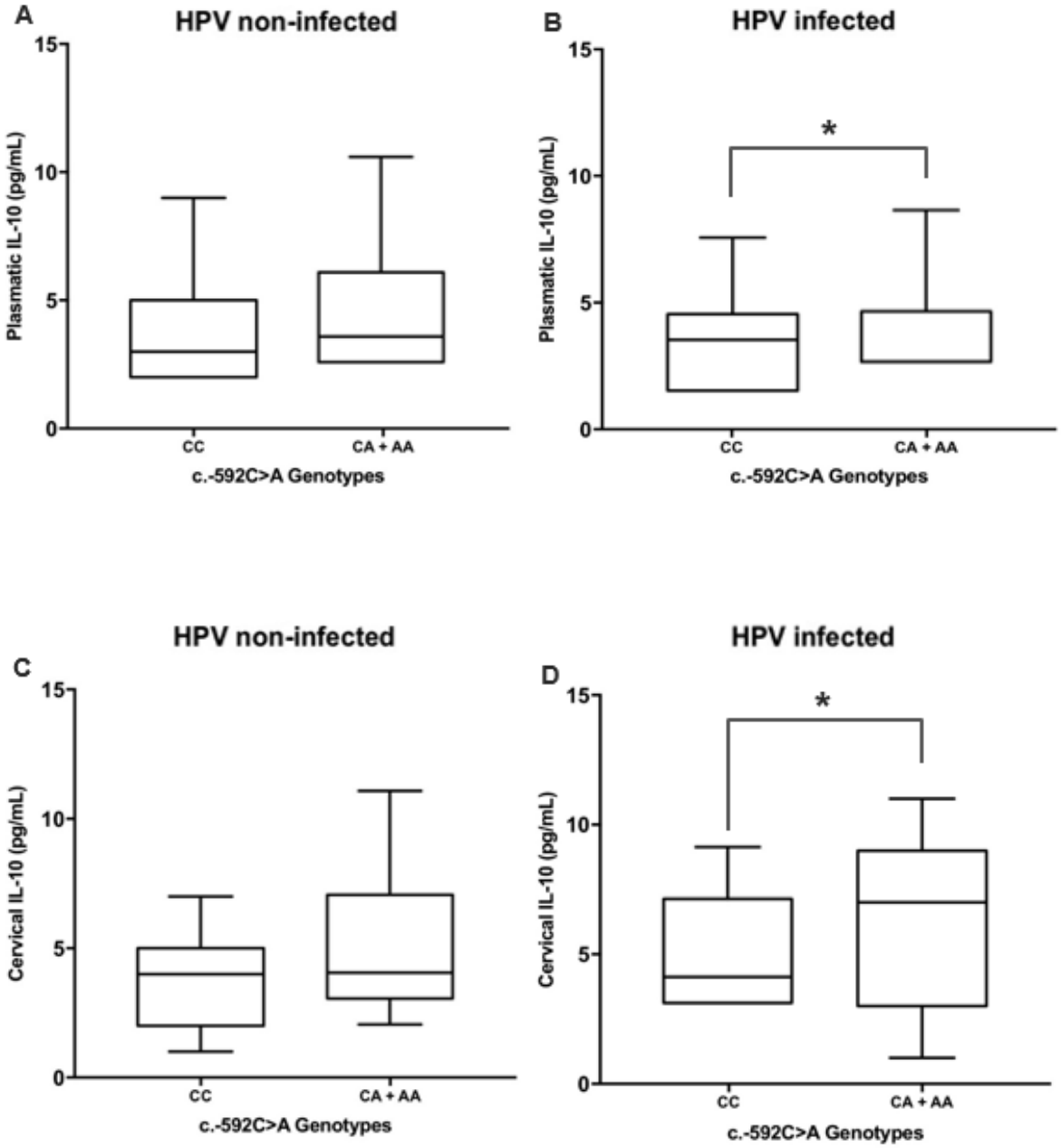


Figure 1 Plasmatic IL-10 levels association with CC or CA+AA genotype in HPV non-infected (A) and infected (B) patients. Cervical IL-10 levels association with CC or CA+AA genotype in HPV non-infected (C) and infected (D) patients. *The asterisk represents a statistically significant *p* value for Mann–Whitney test (*p*=0.017) (B) and for T test (*p*=0.039) (D).

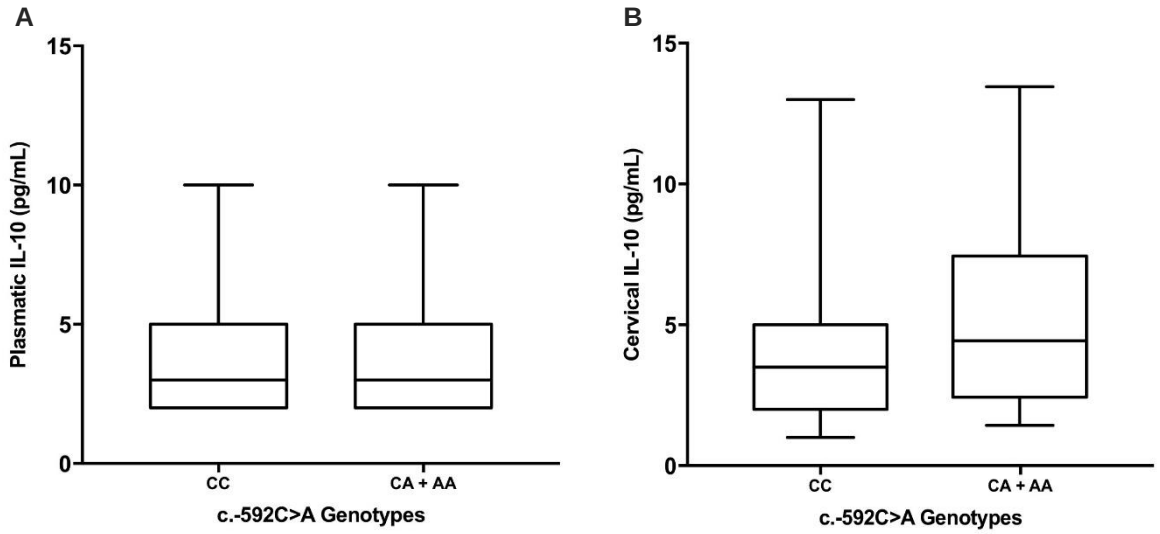
Table 5 Influence of *IL-10 c.-592C>A* polymorphism and HPV presence on IL-10 plasmatic and cervical levels

	Wald	df[a]	*p* value	OR	$CI_{95\%}$	
					Lower	Upper
Plasmatic IL-10 Levels						
IL-10 genotype[1]	0.210	1	0.647	0.974	0.870	1.091
HPV[2]	3.697	1	0.055	0.890	0.790	1.002
Cervical IL-10 Levels						
IL-10 genotype[1]	1.956	1	0.162	1.117	0.956	1.305
HPV[2]	3.516	1	0.061	1.160	0.993	1.354

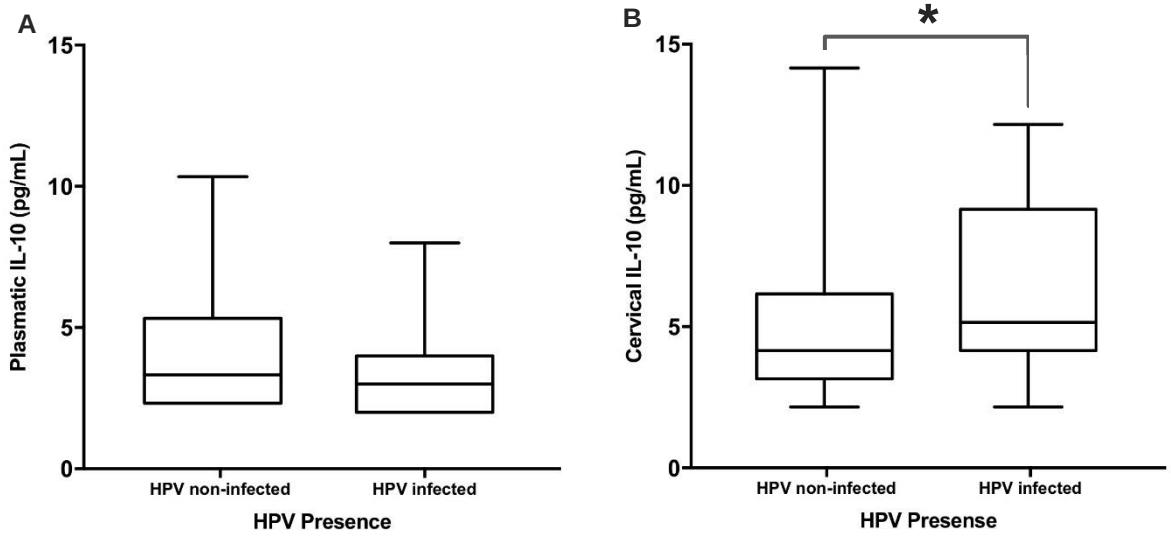
[1]Logistic regression analysis with *IL-10* c.-592C>A polymorphism genotypes (CC and CA+AA) as dependent variable (reference group = CC genotype) and IL-10 plasmatic or cervical levels as explanatory variables, adjusted for age and HPV presence.

[2]Logistic regression analysis with HPV as dependent variable (reference group = non-infected women) and IL-10 plasmatic or cervical levels as explanatory variables, adjusted for age and *IL-10* c.-592C>A polymorphism genotypes (CC and CA+AA).

OR = Odds Ratio; CI = Confidence Interval. [a] *df* = degrees of freedom



Supplementary Figure 1 (A) Plasmatic IL-10 levels in CC or CA+AA genotype patients (by Mann–Whitney test, *p*=0.377) and (B) Cervical IL-10 levels in CC or CA+AA genotype patients (by T test, *p*=0.166).



Supplementary Figure 2 (A) Plasmatic IL-10 levels in HPV infected and non-infected patients (by Mann–Whitney test, *p*=0.286) and (B) Cervical IL-10 levels in HPV infected and non-infected patients (by T test, *p*=0.026). *The asterisk represents a statistically significant *p* value.

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CONCLUSÕES

6 CONCLUSÕES

ARTIGO 1

- Após a infecção por HPV, os níveis de IL-10 tendem a se elevar no microambiente cervical, sendo resultado da interação entre proteínas do HPV e a IL-10;
- As proteínas E2, E6 e E7 estimulam a expressão, e consequente produção, de IL-10, enquanto esta citocina estimula a expressão, e consequente produção, de E6 e E7;
- A produção de IL-10 pode ser induzida e mantida por distintas populações celulares, incluindo queratinócitos infectados, exercendo diversos efeitos em diferentes tipos celulares;
- Os níveis de IL-10 tendem a ser proporcionais ao grau de lesão, sendo mais altos nos casos de câncer cervical;
- Juntamente com o HPV, a IL-10 promove um estado de imunossupressão na cérvice uterina, favorecendo o desenvolvimento de LIE e a progressão para o câncer cervical.

ARTIGO 2

- Mulheres mais jovens, solteiras, com multiplicidade de parceiros, sem conhecimento sobre o HPV, com renda inferior a 1 salário mínimo e fumantes mostraram-se mais susceptíveis à infecção por este vírus;
- O polimorfismo c.-592C>A de *IL-10* esteve independentemente associado à infecção por HPV, com portadores do alelo A apresentando maior frequência de infecção;
- Foi observada associação significativa entre o polimorfismo c.-592C>A de *IL-10* e os níveis plasmáticos de IL-10 em pacientes infectadas pelo HPV, portadoras do alelo A, sendo estes inferiores em relação aos níveis observados em controles;

- Foi observada associação significativa entre o polimorfismo c.-592C>A de *IL-10* e os níveis cervicais de IL-10 em pacientes infectadas pelo HPV portadoras do alelo A, sendo estes superiores em relação aos níveis observados em controles;
- Tanto o polimorfismo c.-592C>A de *IL-10* quanto a presença de HPV não estiveram independentemente associados aos níveis plasmáticos ou cervicais de IL-10, sendo as mudanças nos níveis desta citocina provavelmente resultantes da presença conjunta do HPV e do polimorfismo avaliado.

CONSIDERAÇÃO FINAL

7 CONSIDERAÇÃO FINAL

Na população estudada, foi demonstrada pela primeira vez a associação independente entre o polimorfismo rs1800872 (c.-592C>A) de *IL-10* e a infecção por HPV. Além disso, em mulheres infectadas pelo HPV este polimorfismo esteve associado, de maneira não independente, a níveis aumentados de IL-10 na cérvice uterina, fato que provavelmente está favorecendo a persistência viral nestas pacientes.

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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: _____

ANEXO

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