NÁDIA CALVO MARTINS OKUYAMA

INTERAÇÃO DAS VARIANTES DE NUCLEOTÍDEO ÚNICO DE CXCL12 rs1801157 e CXCR4 rs2228014 E EXPRESSÃO TECIDUAL DE CXCR4 NA INFECÇÃO PELO PAPILOMAVÍRUS HUMANO, NO DESENVOLVIMENTO DE LESÃO INTRAEPITELIAL ESCAMOSA E DE CÂNCER CERVICAL

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Orientador: Profª. Drª. Karen Brajão de Oliveira.

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Londrina, 20 de maio de 2021.

Aos meus pais, com todo carinho.

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OKUYAMA, N.C.M. **Interação das variantes de nucleotídeo único de *CXCL12* rs1801157 e *CXCR4* rs2228014 e da expressão tecidual de CXCR4 na infecção pelo papilomavírus humano, no desenvolvimento de lesão intraepitelial escamosa e do câncer cervical**. 2021. 75 f. Tese (Doutorado em Patologia Experimental) - Universidade Estadual de Londrina, Londrina, 2021.

RESUMO

O papilomavírus humano (HPV) está associado ao desenvolvimento de verrugas genitais bem como ao desenvolvimento de lesões no colo do útero e ao câncer cervical. No entanto, para o desenvolvimento de lesões precursoras e do câncer são necessárias também condições intrínsecas ao indivíduo como o sistema imunológico. Neste contexto, destaca-se o eixo CXCL12/CXCR4 e suas variantes genéticas, uma vez que este eixo tem papel fundamental na regulação do tráfego de células imunológicas e tumorais. O gene *CXCL12* pode conter um uma variante de nucleotídeo único (do inglês, *single nucleotide variant* (SNV)) na região 3' não traduzida (3'UTR) também conhecido como rs1801157. Em relação ao gene do receptor *CXCR4,* o SNV é identificado como rs2228014. Até o presente momento, não existem estudos avaliando a interação entre o SNV rs1801157 de *CXCL12* e o SNV rs2228014 de *CXCR4* no contexto da infecção pelo HPV e o desenvolvimento de câncer de colo de útero. Amostras de muco cervical e sangue periférico foram coletadas de 424 mulheres submetidas ao exame citológico, e amostras de tecido tumoral embebidas em parafina foram provenientes do Hospital do Câncer de Londrina. As participantes foram estratificadas com base na presença ou ausência de DNA do HPV, testada pela reação em cadeia da polimerase (PCR), e de acordo com o a presença de lesões conforme determinado por análise citológica ou biópsia cervical. Dados clínicos e histopatológicos de pacientes com câncer e dados sociodemográficos de todas as participantes também foram coletados. As análises das variantes genéticas mostraram uma associação significativa para *CXCR4* rs2228014 em relação a infecção por HPV em diferentes modelos genéticos: codominante CT (ORadj=2,005, 95%IC (1,03-3,87) p=0,038) e dominante CT+TT (ORadj=2,254, 95%IC (2,25-4,20) p=0,011), e ao desenvolvimento de câncer cervical: codominante CT (CC: ORadj=4,755, 95%IC (1,92-11,84) p=0,001), dominante CT+TT (CC: ORadj=4,755, 95%IC (2,09-10,81) p<0,001). *CXCL12* rs1801157 encontrou-se associado a lesão intraepitelial escamosa (LIE) e ao câncer cervical: CXCL12 codominante AA (LIE: ORadj=8,857, 95%IC (3,24-24,20) p<0,001; CC: ORadj =5,031, 95%IC (1,48-17,00) p=0,009) e AA (LIE: ORadj=9,425, 95%CI (3,26-23,14) p<0,0001; CC: ORadj=5,962, 95%IC (1,89-18,72) p=0,005). A análise da interação entre ambas variantes genéticas mostrou que as variantes combinadas se encontram independentemente associadas à infecção por HPV: CXCL12 GA + CXCR4 CT (codominante) (ORadj=7,345, 95%IC (2,124-25,400) p=0,002); CXCL12 GA+AA + CXCR4 CT+TT (ORadj=10,138, 95%IC (3,466-29,652) p<0,001). Em relação a LIE as interações significativas foram: CXCL12 GA+AA + CXCR4 CC+CT (ORadj=2,068, 95%IC (1,123 – 3,811) p=0,002). Para o câncer cervical: CXCL12 AA + CXCR4 CT (ORadj=7,634, 95%IC (2,580-22,571) p=0,015), CXCL12 GA+AA + CXCR4 CT+TT (ORadj=4,207, 95%IC (1,641-10,753) p=0,006), CXCL12 GA+AA + CXCR4 CT (ORadj=3,021, 95%IC (1,382-10,570) p=0,011). A marcação tecidual do receptor CXCR4 por imunohistoquímica, não se mostrou associada aos parâmetros

clinicopatológicos [tipo de tumor (p=0,310); grau histológico (p=0,959) e estadiamento (p=0,776)] bem como as variantes de nucleotídeo único rs2228014 [codominante (p=0,372); dominante (p=0,198); recessivo (p=0,639) e overdominante (p=0,200)] e rs1801157 [codominante (p=0,116); dominante (p=0,163); recessivo (p=0,050) e overdominante (p=0,545)]. Embora o modelo recessivo do SNV rs1801157 de CXCL12 tenha apresentado uma forte marcação para o CXCR4 (p=0.050). Os SNVs também não foram associados aos parâmetros clinicopatológicos. Esta é a primeira vez que a análise de interação dos SNVs rs1801157 de *CXCL12* e rs2228014 de *CXCR4* são analisados em relação a infecção pelo vírus HPV, lesões intraepiteliais escamosas e ao câncer cervical. Com base nos resultados apresentados, ambas as variantes genéticas podem ser consideradas interessantes candidatas a suscetibilidade para essa a progressão do câncer de colo de útero uma vez que a presença dos alelos variantes foi associada a uma maior susceptibilidade tanto à infecção pelo HPV como ao desenvolvimento de LIE e do câncer cervical.

Palavras-chave: câncer de colo de útero; SDF-1; polimorfismo genético, rs1801157; rs2228014.

OKUYAMA, N. C. M. **Interaction analysis of single nucleotide variants of *CXCL12* rs1801157 and *CXCR4* rs2228014 and CXCR4 tissue expression in human papillomavirus infection, development of squamous intraepithelial lesions and cervical cancer**. 2021. 75 f. Thesis (Doctoral in Experimental Pathology) – State University of Londrina, Londrina 2021.

ABSTRACT

Human papillomavirus (HPV) associated with the development of genital warts is associated with the development of cervical lesions and cervical cancer. However, for the development of precursor lesions and cancer, conditions intrinsic to the individual, such as the immune system, are also necessary. In this context, it is important to highlight the role of the CXCL12 / CXCR4 axis and its genetic variants. This axis has a fundamental role in regulating the traffic of immune and tumor cells. The *CXCL12* gene may contain a single nucleotide variant (from the English, single nucleotide variant (SNV)) in the 3 'untranslated region (3'UTR) also known as rs1801157. Regarding the *CXCR4* SNV receptor gene, it is identified as rs2228014. To date, there are no studies evaluating the interaction between rs1801157 of *CXCL12* and rs2228014 of *CXCR4* in the context of HPV infection and the development of cervical cancer. Samples of cervical mucus and peripheral blood were collected from 424 women who underwent cytological examination and samples of tumor tissue embedded in paraffin came from the Londrina Cancer Hospital. Participants were stratified based on the presence or absence of HPV DNA, tested by polymerase chain reaction (PCR), and according to the presence of lesions as determined by cytological analysis or cervical biopsy. Clinical and histopathological data from cancer patients and sociodemographic data from all participants were also collected. The analysis of the genetic variants showed a significant association for CXCR4 rs2228014 in relation to HPV infection: codominant CT (ORadj = 2.005, 95% CI (1.03-3.87) p = 0.038) and dominant CT + TT (ORadj = 2,254, 95% CI (2.25-4.20) p = 0.011), and the development of cervical cancer (CC): codominant CT (CC: ORadj = 4.755, 95% CI (1.92-11.84) p = 0.001), dominant CT + TT (CC: ORadj = 4,755, 95% CI (2.09-10.81) p <0.001) and overdomimant CT (CC: ORadj = 4.292, 95% CI (1.97-10.43) p = 0.001). CXCL12 rs1801157 was found to be associated with squamous intraepithelial lesion (SIL) and cervical cancer: CXCL12 codominant AA (SIL: ORadj = 8,857, 95% CI (3.24-24.20) p <0.001; CC: ORadj = 5.031, 95% CI (1.48-17.00) p = 0.009) and AA (SIL: ORadj = 9.425, 95% CI (3.26-23.14) p <0.0001; CC: ORadj = 5,962, 95% CI (1.89-18.72) p = 0.005). The analysis of the interaction between both genetic variants was independently associated with HPV infection: CXCL12 GA + CXCR4 CT (codominant) (ORadj = 7.345, 95% CI (2.124-25,400) p = 0.002); CXCL12 GA + AA + CXCR4 CT + TT (ORadj = 10,138, 95% CI (3.466-29.652) p <0.001). In relation to SIL, the significant interactions were: CXCL12 GA + AA + CXCR4 CC + CT (ORadj = 2.068, 95% CI (1.123 – 3.811) p = 0.002). For CC: CXCL12 AA + CXCR4 CT (ORadj = 7.634, 95% CI (2.580-22.571) p = 0.015), CXCL12 GA + AA + CXCR4 CT + TT (ORadj = 4.207, 95% CI (1.641-10.753) p = 0.006), CXCL12 GA + AA + CXCR4 CT (ORadj = 3.021, 95% CI (1.382-10.570) p = 0.011). Tissue marking of the CXCR4 receptor by immunohistochemistry was not associated with clinicopathological parameters [tumor type (p = 0.310); histological grade (p = 0.959) and staging (p = 0.776)] as well as the single nucleotide variants

rs2228014 [codominant (p = 0.372); dominant (p = 0.198); recessive (p = 0.639) and overdominant (p = 0.200)] and s1801157 [codominant (p= 0.1160); dominant (p = 0.163); recessive (p = 0.050) and overdominant (p = 0.545)]. Nor are SNVs associated with clinicopathological parameters, this is the first time that the interaction analysis of SNXs rs1801157 from CXCL12 and rs2228014 fromCXCR4 has been analyzed in relation to HPV virus infection, scaly intraepithelial lesions and cervical cancer. in the results presented, both genetic variants can be considered interesting candidates for susceptibility to the progression of cervical cancer once the presence of the variant alleles was associated with a greater susceptibility to both HPV infection and the development of SIL and cancer.

Keywords: cervical cancer; rs1801157; rs2228014.

LISTA DE ILUSTRAÇÕES

LISTA DE QUADROS

LISTA DE ABREVIATURAS

3'UTR	*Untranslated Region*
AIDS	*Acquired immunodeficiency syndrome*
AIP4	*Atrophin interacting protein 4*
AKT	*Protein kinase B*
BAD	*Death-promoting protein*
BC	*Breast cancer*
Bcl-2	*B cell lymphoma 2*
CC	*Cervical cancer*
CISMEPAR	Consórcio Intermunicipal de Saúde do Médio Paranapanema
CEP-UEL	Comitê de Ética em Pesquisa – Universidade Estadual de Londrina
CI	*Confidencial interval*
CRC	*Colorectal cancer*
CXCL12	*Chemokine ligand (family CXC) 12*
CXCR4	*Chemokine receptor (family CXC) 4*
CXCR7	*Chemokine receptor (family CXC) 7*
DNA	*Desoxyribonucleic acid*
dNTP	*Deoxynucleotide triphosphate*
E	*Early*
E2F	*E2 promoter-binding factor*
E6AP	*E6-associated protein*
EDTA	*Ethylenediaminetetraacetic acid*
ER	*Estrogen receptor*
ERK1/2	*Extracellular signal-regulated kinases ½*
FFPE	*Formalin-fixed-parafin-embedded*
GATA 2	*GATA binding protein 2*
GDP	*Guanosine diphosphate*
GTP	*Guanosine triphosphate*
GPCR	*G protein coupled receptor*
HCC	*Hepatocellular carcinoma*
HIV	*Human Immunodeficiency Virus*
HSIL	*High-grade intraepithelial lesion*
HPV	*Human Papillomavirus*
HPV-AR	HPV de alto risco
HPV-BR	HPV de baixo risco
HPV-RI	HPV de risco indeterminado
HSIL	*High-grade squamous intraepithelial lesion*
HR	*High risk*
INCA	Instituto Nacional do Câncer
IP3	*Inositol 3-trisphosphate*

JAK	*Janus kinase*
L	*Late*
LCR	*Long control region*
LIEAG	Lesão intraepitelial de alto grau
LIEBG	Lesão Intraepitelial de baixo grau
LSIL	*Low-grade squamous intraepithelial lesion*
MAPK	*Mitogen activated protein kinase*
MDR	*Multifactor dimensionality reduction*
mRNA	*Messenger RNA*
NIC	*Neoplasia Intraepitelial Cervical*
NILM	*Negative for intraepithelial lesion*
NK	*Natural Killer*
NF-κB	*Nuclear Factor Kappa B*
ORF	*Open reading frames*
OR	*Odds ratio*
OSCC	*Oral squamous cell carcinoma*
P16INK4a	*Cyclin-dependent kinase inhibitor*
PCR	*Polymerase chain reaction*
PI3K	*Phosphoinositide-3 kinase*
PKC	*Protein kinase C*
SIL	*Squamous intraepithelial lesion*
SNV	*Single nucleotide variant*
VEGF	*Vascular endothelial growth factor*

SUMÁRIO

1. INTRODUÇÃO

O papilomavírus humano (HPV) foi identificado por Harald zur Hausen associado ao desenvolvimento de verrugas genitais (condilomas acuminados), desenvolvimento de lesões no colo do útero e ao câncer cervical. No entanto, essas lesões podem regredir espontaneamente devido ao *clearance* pelo sistema imunológico, ou então, se a infecção persistir, estas podem progredir dando origem ao carcinoma de colo uterino (WOODMAN *et al*., 2007). Existem mais de 200 tipos de HPV (BZHALAVA; EKLUND; DILLNER, 2015) que podem ser classificados, de acordo com seu potencial carcinogênico, em alto risco (HPV-AR), risco indeterminado (HPV-RI) e baixo risco (HPV-BR), estes últimos, responsáveis pelo condiloma acuminado (WOODMAN *et al*., 2007). A grande maioria dos casos de câncer de colo de útero está associada aos tipo virais de alto risco HPV-AR 16 e HPV-AR 18 (SMITH *et al*., 2007).

O câncer cervical é considerado um grande problema de saúde pública mundial, sendo o terceiro tipo que câncer mais frequente entre as mulheres brasileiras e o quarto entre mulheres em todo o mundo (INCA, 2020; SUNG et al., 2020). Esta patologia está fortemente associada ao HPV, que está presente em 99% dos casos de cânceres (WALBOOMER, *et a*l., 1999). O vírus HPV pode ser responsável também, por outros tipos de câncer como o de vulva (LEE *et al*., 2016), vagina (LEVOVITZ *et al*., 2014), anus (MAI *et al*., 2015) e orofaringe (EGAWA et al., 2015) bem como por patologias benignas como verrugas genitais e papilomatose respiratória (ARBYN *et al*., 2012).

No entanto, a infecção pelo vírus HPV, o desenvolvimento de lesões precursoras e câncer dependem não somente do vírus, mas também de condições intrínsecas ao indivíduo como o sistema imunológico. Neste contexto, é importante ressaltar o papel do eixo formados pelos genes CXCL12 (*chemokine ligand (CXC family) 12*) e CXCR4 (*chemokine receptor (CXC family) 4*) e de suas variantes genéticas. Este eixo tem papel fundamental na regulação do tráfego de células imunológicas e tumorais (SUN *et al*., 2010), na angiogênese (RATAJCZAK *et al*., 2006), no desenvolvimento e progressão de diversos tipos câncer, como mama, próstata, gástrico e ainda pode ser alvo de tratamento antitumoral através da inibição da ação do receptor CXCR4 (DOMANSKA *et al*., 2013).

Deste modo, este trabalho avaliou a interação das variantes de nucleotídeo único (SNV) rs1801157 do gene *CXCL12* e rs2228014 do gene *CXCR4,* na infecção pelo HPV e progressão para o câncer de colo de útero.

2. PAPILOMAVÍRUS HUMANO (HPV)

O papilomavírus humano é um vírus pequeno, não envelopado e seu material genético é composto por uma dupla fita de DNA (ácido desoxirribonucleico) circular. Seu genoma contém cerca de 8000 pares de bases que se encontram organizados em 8 regiões de leitura abertas, também conhecidas como *open reading frame* (ORF) e uma região não-codificante conhecida como *long control region* (LCR). As ORFs são denominadas *early* (regiões de expressão precoce) que são responsáveis pela replicação viral e duas denominadas *late* (regiões de expressão tardia) que têm a função de codificar o capsídeo proteico (DOOBAR, 2007) (Figura 1).

O vírus HPV pode permanecer na forma episomal ou pode se integrar ao genoma da célula hospedeira, em fases mais avançadas da infecção. No estado episomal, a proteína E2 regula a expressão das proteínas E6 e E7, suprimindo sua expressão. A integração do DNA viral ao genoma da célula hospedeira ocorre normalmente com a ruptura da região E2, o que interrompe a expressão da proteína E2, causando uma desregulação dos genes de expressão precoce, incluindo E6 e E7, bem como um aumento da capacidade proliferativa, uma etapa crucial na progressão para o câncer (MOODY; LAIMINS, 2010)

A proteína E4 participa dar replicação do genoma viral bem como da proteína L1 responsável pela formação do capsídeo proteico. E4 tem papel importante na amplificação do genoma viral, ativação da via MAPK e pode interagir e estabilizar a oncoproteína E2 (DAVY, MCINTOSH, JACKSON et al. 2009; EGAWA, WANG, GRIFFIN *et al*., 2017). O gene da proteína E5 parece ser conservado em muitos tipos do vírus HPV. Este gene tem um papel importante no ciclo de vida do vírus e está envolvido com os oncogenes E6 e E7 (DOOBAR, 2013; DIMAIO; PETTI, 2013). As proteínas E6 e E7 estão fortemente expressas. A proteína E7 liga-se a proteína do retinoblastoma (pRb), responsável pela regulação do ciclo celular. Uma vez hipofosforilada, a pRb libera o fator de transcrição E2 (*transcriptor family E2F*) no núcleo, permitindo a entrada da célula na fase S do ciclo celular. Enquanto que a proteína E6 interage com a ubiquitina-ligase E6AP e direciona sua atividade do

supressor tumoral PT53 (*tumor supressor protein 53*), importante molécula de controle da apoptose, levando a sua degradação pelo proteassoma (BEAUDENON; HUIBREGTSE, 2008)

Além disso, E6AP pode atuar como coativador para o receptor de estrógeno (ER), o qual fosforilado liga-se a E6AP formando um complexo que é recrutado para ativar a transcrição de um subgrupo de genes promotores do receptor de estrógeno (ZHOU; SLINGERALND, 2014). Existe forte evidência que o estrógeno contribui para o desenvolvimento do câncer de colo de útero através de seu receptor α. Este eixo hormônio-receptor é recrutado para a gênese do câncer cervical e sua persistência, uma vez que o estrógeno parece aumentar a expressão dos oncogenes E6 e E7 (SCHIFFMAN; WENTZENSEN, 2013; zur HAUSEN, 2009). Neste sentido, a participação de E6AP como coativador do receptor de estrógeno na proteólise de p53, sugere que a sinalização via receptor de estrógeno poderia participar dos mecanismos da carcinogênese desencadeada pelo HPV devido ao E6AP no desenvolvimento do câncer de colo de útero (ZHOU; SLINGERALND, 2014).

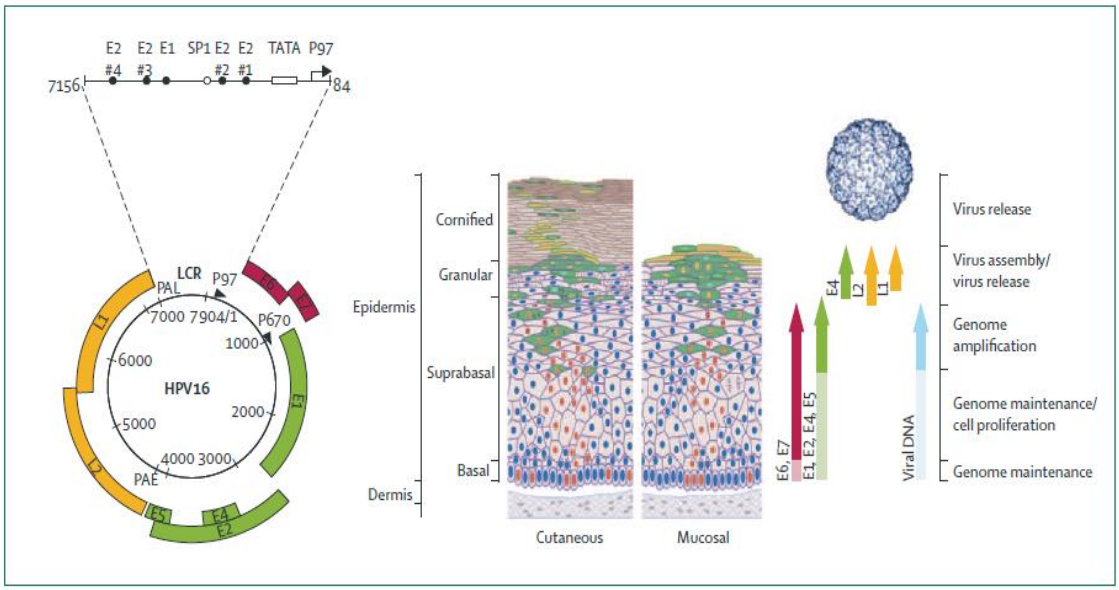

Figura 1**. Genoma do vírus HPV e sua expressão no epitélio**. O genoma do HPV contém 8000 pares de bases contidos em um DNA circular. Existem oito estruturas de leitura abertas e uma região reguladora. Os genes de HPV são designados como E ou L de acordo com sua expressão no estágio de diferenciação que pode ser inicial ou tardia: E1, E2, E5, E6 e E7 são expressos no início da diferenciação, E4 é expressa a maior parte do tempo e L1 e L2 são expressas durante os estágios finais de diferenciação. O genoma viral é mantido na camada basal do epitélio, onde a infecção por HPV é estabelecida. As proteínas iniciais são expressas em níveis baixos para manutenção do genoma (aumentando a possibilidade de um estado latente) e proliferação celular. À medida que as células epiteliais basais se diferenciam, o ciclo de vida viral passa por estágios de amplificação do genoma, montagem e liberação do vírus, com uma mudança concomitante nos padrões de expressão de genes precoces para genes tardios, incluindo L1 e L2, que montam o capsídeo viral. L1 é a principal proteína do capsídeo enquanto L2 serve como ligação para o DNA do plasmídeo. Fonte: SCHIFFMAN et al., 2007.

2.1 DESENVOLVIMENTO DE LESÕES E DO CÂNCER CERVICAL

O vírus HPV é transmitido pelo contato pele-pele, mucosa-mucosa e pele-mucosa. Diferentes tipos de HPV podem ser transmitidos ao mesmo tempo devido à via comum de transmissão (zur HAUSEN, 2009). Fatores de risco como idade da primeira relação sexual, número de parceiros sexuais, uso de preservativo e contraceptivo oral, múltiplos partos e tabagismo são importantes para o desenvolvimento de lesões relacionadas ao HPV, porém o motivo pelo qual eles funcionam como cofatores não está bem estabelecido (SCHIFFMAN *et al., 2007)*.

A infecção pelo HPV ocorre através de micro lesões na camada basal do epitélio do colo do útero e o vírus infecta a célula através de receptores ainda não estabelecidos. As células basais infectadas migram para as camadas superficiais do epitélio onde começam a se diferenciar. O DNA viral é então empacotado e novos vírus são liberados das células. A minoria das mulheres apresenta infecção persistente pelo HPV, que pode evoluir de lesão intraepitelial de baixo grau, para lesão intraepitelial de alto grau que podem regredir ou então, posteriormente, progredir para o desenvolvimento câncer de colo de útero (BRUNI *et al*., 2010).

As lesões cervicais pré-malignas são classificadas de acordo com o grau de severidade e são correspondentes a alterações citológicas ou histológicas anormais. A classificação histológica de Richart baseia-se na proporção de espessura do epitélio escamoso, o qual é constituído por células maduras e diferenciadas. Nesta classificação, pode-se encontrar a seguinte organização acerca do nível de lesão: neoplasia intraepitelial cervical grau 1, considerada displasia leve; grau 2, considerada displasia moderada e grau 3, displasia severa/carcinoma *in situ*. Para a classificação do exame citológico, utiliza-se a classificação do sistema de Bethesda: lesões intraepiteliais escamosas de baixo e alto grau (LIEBG e LIEAG) e adenocarcinoma *in situ* (INCA, 2016) (Quadro 1).

O subtipo mais comum de câncer cervical é o carcinoma de células escamosas representando cerca de 80% dos casos, seguido pelo adenocarcinoma cervical (15%) e por carcinomas mais raros, os adenoescamosos e neuroendócrinos (5%). O câncer de células escamosas pode ser visualizado como projeções do epitélio escamoso maligno, que pode ser queratinizado ou não e invade o estroma subjacente. Os adenocarcinomas, por sua vez, são caracterizados pela proliferação do epitélio glandular composto por células endocervicais malignas que apresentam núcleos

grandes e hipercromáticos, citoplasma depletado de mucina. O câncer cervical avançado pode invadir tecidos paracervicais, bexiga, ureter, reto e vagina. Pode ocorrer comprometimento de linfonodos locais e distantes bem como metástases no fígado, pulmões, medula óssea, ente outros (KUMAR; ABBAS; ASTER, 2015).

Contudo, somente a infecção por vírus HPV de alto risco não é suficiente para promover a imortalização celular e sua malignidade. Modificações genéticas como a presença de variantes polimórficas no hospedeiro, mutações químicas e físicas podem contribuir para o processo (zur HAUSEN, 2009). Fatores endógenos e exógenos como o uso de tabaco, paridade, uso de contraceptivo oral (CASTELSAGUÉ; MUÑOZ, 2003), sistema imunológico deficiente e interações imunológicas no sítio da infecção (PATEL; CHIPLUNKAR, 2009) podem influenciar a progressão da infecção pelo HPV para lesões relacionadas.

Neste contexto, podemos evidenciar as quimiocinas, uma vez que estudos sugerem que estas moléculas são reguladores importantes no desenvolvimento de infecções virais (MBEUNKUI; JOHANN, 2010) e também são responsáveis por controlarem a migração celular, particularmente de leucócitos durante e inflamação, a qual se prolongada pode facilitar a carcinogênese por promover um microambiente ideal para o desenvolvimento e crescimento da célula tumoral (VANDERCAPELLEN *et al*., 2008).

Classificação citológica de Papanicolaou (1941)	**Classificação histológica da OMS (1952)**	**Classificação histológica de Richart (1967)**	**Sistema Bethesda (2001)**	**Classificação Citológica Brasileira (2006)**
Classe I	-	-	-	-
Classe II	-	-	Alterações benignas	Alterações benignas
-	-	-	Atipias de significado indeterminado	Atipias de significado indeterminado
Classe III	Displasia leve Displasia moderada e acentuada	NIC I NIC II e NICIII	LSIL HSIL	LSIL HSIL
Classe IV	Carcinoma *in situ*	NIC III	HSIL Adenocarcinoma *in situ* (AIS)	HSIL AIS
Classe V	Carcinoma invasor	Carcinoma invasor	Carcinoma invasor	Carcinoma invasor

Quadro 1. **Nomenclatura citopatológica e histológica para lesões cervicais.** NIC 1, 2 e 3, neoplasia intraepitelial cervical grau 1, 2 e 3**;** LSIL, lesão intraepitelial escamosa de baixo grau; HSIL, lesão intraepitelial escamosa de alto grau; AIS, adenocarcinoma *in situ***.** Fonte: INCA 2016, p. 26.

3. QUIMIOCINAS E O MICROAMBIENTE TUMORAL

O termo quimiocinas foi elaborado em 1992 no encontro internacional de imunologia em Budapeste (BAGGIOLINI, 2001). Quimiocinas são proteínas pequenas do sistema imunológico que possuem atividade quimiotática que estimula a migração de diferentes tipos celulares como linfócitos, monócitos, neutrófilos, células endoteliais, células tronco mesenquimais e células epiteliais malignas. Constituem uma grande família de citocinas com aproximadamente 50 quimiocinas endógenas em humanos e camundongos. As quimiocinas são divididas em 4 subfamílias de acordo com a posição dos primeiros resíduos de cisteína formando as subfamílias CC, CXC, CX3C e XC (BAGGIOLINI, 2001).

Os receptores de quimiocinas constituem uma grande subfamília tipo rodopsina que formam um receptor composto por 7 domínios transmembranas. Os receptores são expressos em todos os leucócitos e podem ser divididos em dois grupos: receptores de quimiocinas acoplados a proteína G (GPCR), e receptores atípicos de quimiocinas, que parecem modular o gradiente de quimiocinas diminuindo a inflamação através da limpeza de quimiocinas de maneira independente da proteína G. Existem aproximadamente 20 receptores de quimiocinas sinalizantes e 5 não-sinalizantes (BAGGIOLINI, 2001; GRIFFTHI *et al*., 2014).

Tecidos normais cuidadosamente controlam a produção e liberação de sinais promotores de crescimento que induzem a progressão do crescimento celular e do ciclo celular mantendo a homeostasia no número de células e na manutenção da arquitetura tecidual e sua função (POZZOBON *et al*., 2016). Diversos estudos têm demonstrado que as quimiocinas e seus receptores estão envolvidos no crescimento e progressão tumoral (WANI *et al*., 2014).

Outro mecanismo mediado por quimiocinas que contribui para a tumorigênese é a angiogênese, necessária para permitir a propagação e progressão do tumor e indução da vascularização tumoral (BERGERS; BENJAMIN, 2003). Como os tecidos normais, o tecido tumoral também necessita de um suprimento de oxigênio adequado, metabólitos e uma forma eficiente de remover moléculas que não são necessárias. Dentre os diferentes tipos de tumor, estas necessidades podem variar e mudar de acordo com a progressão do tumor. Foi demonstrado que quimiocinas e seus receptores são mediadores do processo de formação vascular (BERGERS; BENJAMIM, 2003).

A progressão do tumor em direção a metástase é descrita como um processo de múltiplos estágios no qual células malignas saem do tumor original e vão colonizar órgãos distantes. A sequência de eventos que podem resultar no processo metastático pode ser definida em invasão local, sobrevivência na circulação, extravasamento e colonização, o que tem ajudado a racionalizar o complexo conjunto de propriedades biológicas que devem ser adquiridas para um tumor maligno se tornar uma doença metastática (NGUYEN *et al*., 2009). As células tumorais podem circular por todo o organismo e o fazem sob a influência de sinais que determinam o comportamento migratório. Para o sucesso da metástase, são necessários dois estágios: primeiro, as células devem responder a sinais quimiotáticos que as encaminham a um local propício para a sua instalação; e segundo, elas devem sobreviver e se estabelecer no novo tecido (ZLOTNIK; YOSHIE, 2012). Evidências que apontam para o papel das quimiocinas na formação de metástases são provenientes da observação que a expressão de receptores de quimiocinas por células tumorais não é aleatória, e sim, que as células tumorais expressam receptores de quimiocinas selecionados (ZLOTNIK; YOSHIE, 2012).

As quimiocinas e seus receptores são mediadores chaves não somente no crescimento do tumor, formação de vasos sanguíneos e metástases, mas também no recrutamento de diferentes tipos celulares para o microambiente tumoral. Isto inclui células infiltradas como macrófagos, neutrófilos e fibroblastos associados a tumores, linfócitos, células tronco mesenquimais e células endoteliais (BALKWILL, 2004).

Dentre as quimiocinas, podemos destacar a CXCL12 e seu receptor CXCR4. Este eixo apresenta função quimioatraente para linfócitos, monócitos e células hematopoiéticas, sendo constitutivamente expressa por diversos órgãos como fígado, pulmão, linfonodos, cérebro e pode ter papel importante na imunovigilância (BLEUL *et al*., 1996).

3.1 VIAS DE SINALIZAÇÃO DO EIXO CXCL12/CXCR4

CXCL12 se liga ao CXCR4 para ativar uma série de cascatas de sinalização mediadas principalmente por meio de proteínas G heterotriméricas e β-arrestinas. As proteínas G são compostas por três subunidades: Gα, Gβ e Gγ. A ligação de CXCL12 ao receptor CXCR4, leva a substituição de uma molécula de GDP por GTP na subunidade Gα, resultando na ativação e dissociação do heterotrímero em duas

subunidades distintas: Gα e um dímero Gβγ. Proteínas G ativadas sinalizam através da *phosphatidylinositol 3-kinase* (PI3K) e da *Akt serine/threonine kinase family* (Akt), *Inositol 1,4,5-trisphosphate* (IP3), *mitogen activated protein kinases* (MAPK) e *protein kinase C* (PKC) para convergir nas vias de promoção do crescimento ou para promover a migração através da mobilização de cálcio intracelular. Além de sinalizar por meio de proteínas G, receptores ativados recrutam β-arrestina, o que pode levar a ativação da proteína G independente de Akt e ERK1/2 (*extracellular signal-regulated kinases ½)* (COJOC, et al., 2013).

A duração da sinalização, CXCR4 é regulada em dois níveis. Primeiro, a ligação receptor-ligante leva à fosforilação de CXCR4 por PKC e GRKs (*G protein-coupled receptor kinase*). Receptores fosforilados são rapidamente internalizados pela β-arrestina e ubiquitinados pela E3 ligase da família *itchy E3 ubiquitin protein ligase*, que os direciona para a degradação lisossomal. Portanto, a sinalização CXCL12 reduz os níveis do receptor CXCR4 em tecidos (MUELLER *et al*., 2013; DONÀ *et al*., 2014). Em segundo lugar, os reguladores de sinalização da proteína G (RGS – *regulators of G protein singnaling*) aumentam a atividade GTPase da subunidade Gα, promovendo a hidrólise de GTP. Gα se reassocia com o dímero Gβγ para retornar a um estado inativo. Ambos os mecanismos levam ao encerramento de sinalização (COJOC, *et al*., 2013).

Além de se ligar ao receptor CXCR4, a quimiocina CXCL12 se liga ao receptor CXCR7 não-canônico. Dependendo do contexto, a ligação CXCL12-CXCR7 pode resultar em duas diferentes respostas: internalização do ligante seguida por sua degradação (depuração de quimiocina) e sinalização independente de proteína G, mediada por β-arrestina. Várias observações apoiam um papel para CXCR7 na depuração de quimiocinas. Por exemplo, CXCR7 não se associa com proteínas G heterotriméricas, e a ligação de CXCL12 não promove o influxo de cálcio. Além disso, CXCR7 liga CXCL12 com dez vezes mais afinidade quando comparado a ligação com CXCR4, e o receptor é rapidamente reciclado de volta para a membrana plasmática após internalização induzida pelo ligante (Figura 2) (COJOC *et al*., 2013).

Durante o desenvolvimento embrionário, CXCR4 é expresso em células progenitoras, permitindo a migração de seu local de origem para seu destino onde eles se diferenciarão em órgãos e tecidos. Camundongos deficientes em CXCR4/CXCL12 mostram um fenótipo letal, confirmando a importância crítica do eixo no desenvolvimento embrionário (CHENG *et al*., 2014). Células do sistema

imunológico inato, como neutrófilos e macrófagos, também expressam CXCR4, o que permite que essas células migrem ao longo de um gradiente de CXCL12 presente em o local da inflamação (POZZOBON *et al*., 2016).

No câncer, CXCL12 atua principalmente por meio de mecanismos: (a) por efeitos autócrinos diretos que promovem o crescimento celular de células tumorais, metástase e angiogênese e (b) por efeitos, incluindo o recrutamento de células tumorais que expressam CXCR4 para órgãos que expressem CXCL12 ou células estromais CXCR4-positivas para locais de tumor (GUO *et al*, 2016). CXCL12 pode ainda suprimir a apoptose através da ativação de NF-κB, bem como ativar o gene anti-apoptótico *Bcl-2 (B-cell lymphoma 2*) pela inibição direta da proteína BAD (proteína promotora de morte associada a *Bcl-xl/Bcl-2*) (GANJU *et al*., 1998; WANG, KNAUT, 2014).

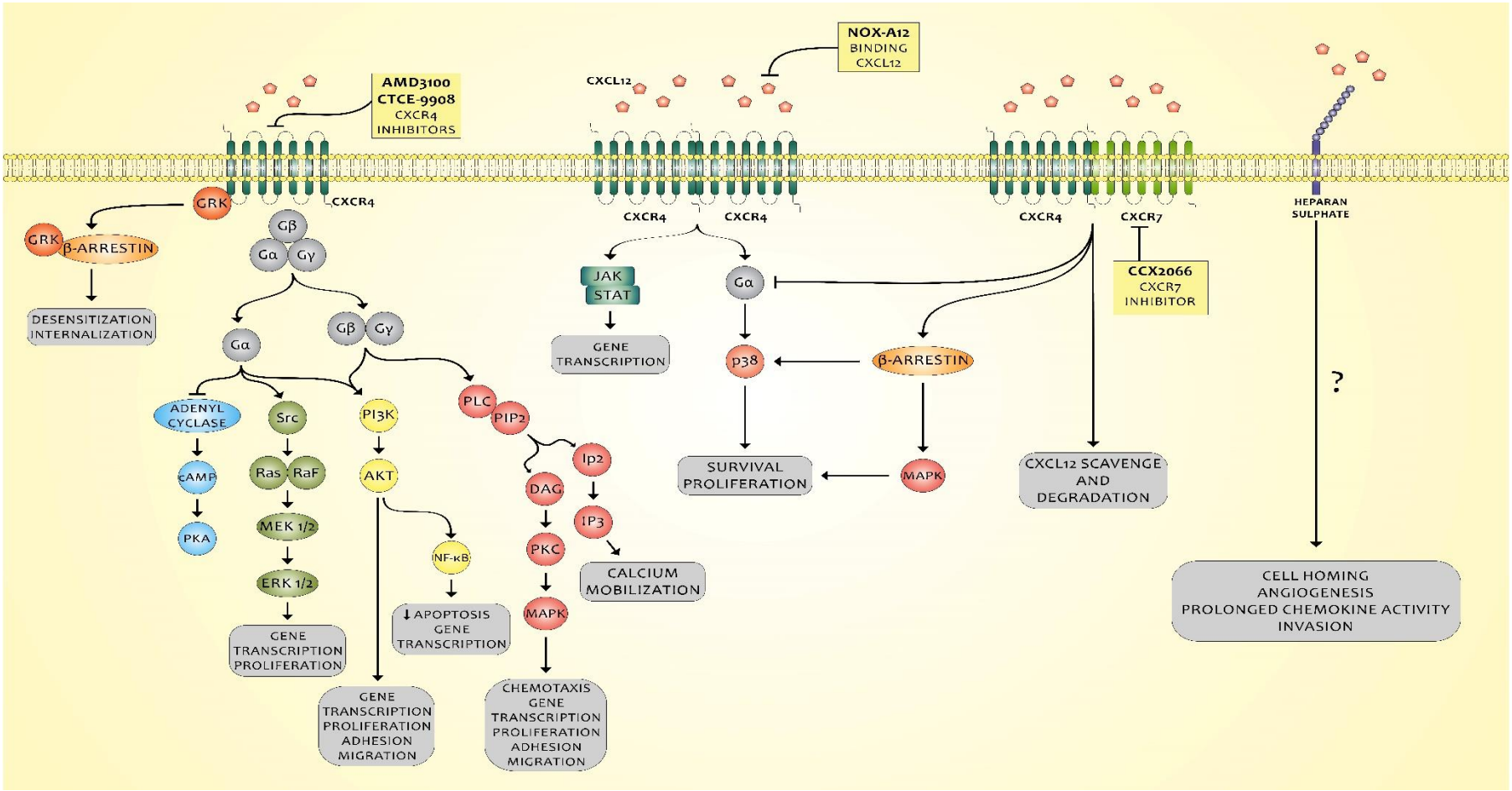

Figura 2: Vias de sinalização CXCL12/CXCR4/CXCR7. A ligação do CXCL12 ao CXCR4, desencadeia a sinalização acoplada à proteína G e subsequente ativação das vias PI3K/AKT, PLC/IP3, ERK1/2, resultando na transcrição do gene, adesão celular, migração, proliferação e sobrevivência celular. A via da β-arrestina pode ser ativada por meio de GRK, necessária para a internalização do CXCR4. A oligomerização CXCR4 também pode ativar a via JAK/STAT (a qual é independente da proteína G), induzindo a transcrição do gene; p38 também pode ser ativado modulando a sobrevivência e a proliferação. A ligação de CXCL12 a heterodímeros CXCR4-CXCR7 pode inibir a sinalização de Gα e potencializar a sinalização a jusante dependente de β-arrestina, ativando p38 e MAPK para aumentar a sobrevivência celular. No último caso, o CXCR7 altera a conformação dos complexos CXCR4/proteína-G e anula a sinalização. Além disso, a ativação da via β-arrestina pode levar à eliminação e degradação de CXCL12. A ligação de CXCL12 ao sulfato de heparano presente na superfície celular e na matriz extracelular impede sua proteólise e medeia eventos como migração, angiogênese e invasão de células tumorais. A figura mostra, também, drogas testadas em estudos clínicos e pré-clínicos capazes de bloquear a via CXCL12 por meio da inibição do receptor ou ligação ao ligante. Adaptado de: OKUYAMA *et al*., 2016.

3.2 VARIANTES DE NUCLEOTÍDEO ÚNICO (SNV) DE *CXCL12* rs1801157 E *CXCR4* rs2228014 E O DESENVOLVIMENTO TUMORAL

Diversos SNVs já foram associados como fatores de risco ou proteção para o desenvolvimento de algumas doenças uma vez que, dependendo de sua localização, podem gerar diferentes consequências. Se o SNV se apresenta em regiões codificantes, podem ocorrer alterações na estrutura proteica, alterando função e/ou atividade. Quando presentes em regiões não codificantes, podem ocorrer alterações na regulação da expressão gênica (FAREED; AFZAL., 2013).

O gene responsável por produzir a quimiocina CXCL12, está localizado a posição 10q11.1 e foi mapeado por hibridização *in situ* (SHIROZU *et al*., 1995). Este gene foi clonado pela primeira vez de uma linhagem celular derivada da medula óssea e posteriormente identificado como fator estimulante de crescimento de células pré-B. O gene *CXCL12* pode conter um uma variante de nucleotídeo único (do inglês, *single nucleotide variant* (SNV)) na região 3' não traduzida (3'UTR) também conhecido como rs1801157, no qual ocorre a troca de uma guanina por uma adenina, descrito pela primeira vez por Cheryl Winkler em um estudo com pacientes com AIDS (WINKLER *et al*., 1998).

O SNV rs1801157 de *CXCL12* está também associado a doenças coronarianas (FENG *et al*., 2014), possível efeito protetor nos estágios tardios no HIV na população brasileira (REICHE *et a*l., 2006) e a um elevado risco no desenvolvimento de diversos tipos de câncer incluindo mama, próstata, colo retal (DE OLIVEIRA *et al*., 2009; HIRATA et al., 2007; CHANG et al., 2009).

O papel biológico do SNV de *CXCL12* no prognóstico de diferentes tipos de câncer é controverso. Uma prevalência do genótipo AA deste polimorfismo foi observada em pacientes com carcinoma de células renais (CCR) (OR=3,07, IC 95% 1,98-5.46, p= $6.1x10^{-6}$) e a taxa de sobrevivência foi menor quando comparada a pacientes que apresentavam os genótipos GG e GA (CAI *et al*., 2013). Pacientes portadores dos genótipos CT e TT de CXCR4 apresentaram um risco elevado para CCR (OR = 1.77, IC 95%, 1.28–2.71, p = 0.0003; OR = 4.01, IC 95%, 1.87–9.12, p= 7.8×10^{-4}, respectivamente) (CAI *et al*., 2013). De acordo com Schimanski *et al*. (2011), pacientes portadores dos genótipos AA e GA são mais suscetíveis ao desenvolvimento de metástases distantes no câncer gastroesofágico (p=0.026).

De acordo com Razmkhah et al. (2005), esta variante está associada ao aumento na susceptibilidade para o desenvolvimento de câncer de mama. A combinação entre baixos níveis plasmáticos e seu polimorfismo pode identificar pacientes com susceptibilidade a um pior prognóstico (HASSAN *et al*., 2008). Deste modo, os níveis de CXCL12 pode ser um importante biomarcador no prognóstico do câncer. Phillips et al. (2003), utilizando um modelo animal de câncer de pulmão de células não pequenas, observaram que os níveis de CXCL12 estão significativamente mais elevados nas glândulas adrenais, nos pulmões, fígado e medula óssea, órgãos altamente susceptíveis a metástase neste tipo de câncer. Além disso, os níveis plasmáticos de CXCL12 podem se encontrar elevados em pacientes com câncer de próstata quando comparados aos pacientes com a forma benigna da doença e aos controles (DEHGHANI *et al*., 2014), assim como esse aumento foi relatado em células tumorais da medula óssea no mieloma múltiplo (BEIDER *et al*., 2014).

Sei et al (2001) demonstraram que *CXCL12* pode estar superregulado devido a uma variação no gene localizado na região 3'UTR, sugerindo que o polimorfismo poderia influenciar os níveis da quimiocina. Entretanto, Oliveira et al. (2011) observaram que pacientes com câncer de mama portadoras do alelo A, apresentaram expressão de RNAm de CXCL12 2,1 vezes menor do que pacientes com o genótipo GG, sugerindo que o alelo A está associado a baixa expressão de *CXCL12* no sangue periférico em pacientes com câncer de mama receptor de estrógeno positivo.

Em relação a quimiocina CXCL12 e seu SNV associados ao vírus HPV, ao desenvolvimento de lesões cervicais bem como do câncer de colo de útero, os dados existentes na literatura são escassos e controversos. Para Tee *et al*. (2012), o polimorfismo rs1801157 de *CXCL12* não está associado ao risco de desenvolvimento de lesões neoplásicas cervicais. Em uma análise de 5 SNVs do gene *CXCL12,* Maley *et al*. (2010) investigaram mulheres com carcinoma escamoso invasivo e adenocarcinoma *in situ*, e concluíram que o SNV rs1801157 não está relacionado ao desenvolvimento de câncer de colo de útero. De acordo com Roszak *et al*. (2015), a presença deste polimorfismo pode ser um fator de risco para o desenvolvimento de neoplasia de colo uterino em pacientes tabagistas. Estes trabalhos não apresentaram dados relacionados à associação desta variante a infecção pelo HPV e ao desenvolvimento de lesões cervicais de baixo grau (LIEBG) e de alto grau (LIEAG) e do câncer cervical.

O gene do receptor CXCR4 está localizado na posição 2q2 onde um polimorfismo de nucleotídeo único, rs2228014 (C/T), foi encontrado no códon 138 (FEDERSPPIEL *et al*., 1993). Este códon traduz o aminoácido isoleucina (Ile), e como este SNV é silencioso, não há alteração na formação do aminoácido. Embora seja um SNV que não provoca mudança de aminoácido e, consequentemente, não há alteração da estrutura da proteína, o estudo desta variante genética é necessária, pois, de acordo com o ENCODE Project Consortium/Haploreg v4.1, nesta região existe a ligação da RNA polimerase II e alteração dos sítios de ligação para fatores de transcrição da família SOX, RXRA e NRSF (WARD; KELLIS, 2012). O fator de transcrição SOX 18 está relacionada ao desenvolvimento do câncer cervical e a desregulação desses fatores tem sido recentemente associada a uma variedade de doenças (DENG *et al*., 2018; HALSTEAD *et al*., 2017; KUMAR; MISTRI., 2019).

Este SNV está associado a estágios III e IV bem como metástase em linfonodos no câncer oral (TENG *et al*., 2009). De acordo com Cao et al. (2019) o genótipo CT do SNV rs2228014 de CXCR4 parece estar correlacionado ao risco de desenvolvimento de leucemia mielóide aguda.

Em câncer cólon e reto, pacientes portadores do genótipo CC apresentam sobrevida maior quando comparados aos pacientes portadores do alelo T, durante tratamento quimioterápico com bevacizumab. Esta variante genética também pode afetar o estabelecimento de tumor do endotélio, independente da inibição de VEGF (fator de crescimento endotelial vascular) e a expressão de CXCR4 em células endoteliais (MATSUSAKA *et al*., 2016). No entanto, para Cacina *et al*. (2012) este polimorfismo não apresenta correlação com a suscetibilidade ao desenvolvimento do câncer de endométrio. Aumento na expressão de RNAm de CXCR4 e da proteína foram encontrados em tumores de câncer de mama, porém, este aumento não foi influenciado pelo SNV rs2228014 (DE OLIVEIRA *et al*., 2011).

Até o presente momento, não existem estudos avaliando a interação entre o SNV rs1801157 de *CXCL12* e o SNV rs2228014 de *CXCR4* dentro do contexto da infecção pelo HPV e o desenvolvimento de câncer de colo de útero. Portanto, o desenvolvimento de estudos onde se possa fazer a análise destas variantes genéticas em conjunto, é necessário para a melhor compreensão do papel destes polimorfismos na imunopatogênese do câncer cervical.

4. OBJETIVOS

4.1 OBJETIVOS GERAIS

O objetivo geral deste estudo foi avaliar a influência do eixo CXCL12/CXCR4 na infecção por HPV e no desenvolvimento das lesões intraepiteliais escamosas de baixo e alto grau (LIEBG e LIEAG) e do câncer cervical.

4.1 OBJETIVOS ESPECÍFICOS

- Avaliar o perfil sociodemográfico das mulheres atendidas pelos programas de prevenção ao cânder de colo do útero do Sistema Único de Saúde (SUS) na região norte do Paraná;

- Correlacionar a infecção pelo HPV e a presença das lesões provocadas por ele com dados sociodemográficos das mulheres atendidas, como faixa etária, estado civil, grau de escolaridade, renda familiar e hábito tabagista;

- Correlacionar a infecção por HPV e a presença das lesões provocadas por ele com as variáveis sexuais e reprodutivas, como menarca, idade da primeira relação sexual, uso de métodos contraceptivos, número de parceiros sexuais ao longo da vida e nos últimos seis meses, número de partos e tipo(s) de parto(s) realizado(s);

- Avaliar a frequência dos alelos e genótipos do polimorfismo rs1801157 de *CXCL12* e rs2228014 de *CXCR4* em mulheres atendidas pelos programas de prevenção ao câncer de colo de útero;

- Avaliar a suscetibilidade à infecção por HPV e desenvolvimento de LIE e câncer cervical associado aos alelos e genótipos dos polimorfismos rs1801157 de *CXCL12* e rs2228014 de *CXCR4* em mulheres atendidas pelos programas de prevenção ao câncer de colo de útero;

- Avaliar a interação dos SNVs de CXCL12 rs1801157 e CXCR4 rs2228014, na infecção por HPV, desenvolvimento de lesões intraepiteliais escamosas e no câncer de colo de útero.

-Avaliar a correlação das variantes genéticas de CXCL12 e CXCR4 com parametros de prognóstico das pacientes com cancer cervical.

5. PRODUÇÃO CIENTÍFICA

5.1 Artigo 1

Genotypes and interaction analysis of *CXCL12* rs1801157 and *CXCR4* rs2228014 single nucleotide variants and the susceptibility of HPV infection, squamous intraepithelial lesions and cervical cancer

ABSTRACT

Every year, more than half a million women are diagnosed with cervical cancer (CC). High risk HPV is the main cause of cervical cancer and may be classified in squamous cell carcinoma and adenocarcinoma, corresponding to 70% and 25% of CC, respectively. Individual factors may contribute to the cervical cancer development, such as immunogenetic variation. *CXCL12* and *CXCR4* regulate pathways involved in tumor progression and aggressiveness. In the present study, we aimed to investigate a possible association between two single nucleotide variants (*CXCL12* rs1801157 and *CXCR4* rs2228014) with HPV infection and cervical cancer development. PCR technique was used to test HPV positivity in 424 women, in which the allelic frequency of *CXCL12* rs1801157 and *CXCR4* rs2228014 was also assessed by PCR-RFLP. *CXCL12* rs1801157 was significantly associated with HPV infection in the allelic distribution as well in the codominant, dominant, and recessive genetic models; regarding its association with SIL/CC it was verified in the codominant and dominant models. *CXCR4* rs2228014 was associated to HPV infection in the codominant model and allelic distribution; as well with SIL/CC in the codominant, dominant, and allelic models. In the adjusted analysis, *CXCL12* AA genotype was independently associated to HPV infection, SIL and CC development. *CXCR4* allele T carriers were independently associated to HPV infection and CC. The variants interaction analysis demonstrated that the allelic variants presence for both polymorphisms simultaneously increases the susceptibility of HPV infection in 10.1 times, SIL (2 times) and CC development in 4.2 times. Therefore, this is the first time that the interaction of CXCL12 and CXCR4 variants contributes to the increased susceptibility of HPV infection, squamous intraepithelial lesions and cervical cancer development.

Keywords: cervical cancer, rs1801157, rs2228014

1. INTRODUCTION

Every year more than half a million women are diagnosed with cervical cancer (CC) and from these, 300 000 women die due to the disease (COHEN *et al*., 2019). Most of the deaths, 85%, occur in less developed countries (BRAY *et al*., 2018). High risk human papillomavirus (HR-HPV) is the main cause of cervical cancer, between the different histological subtypes the squamous cell carcinoma and adenocarcinoma are the most common representing 70% and 25%, respectively (COHEN *et al*., 2019). Even though HPV is necessary to the cervical cancer development, the virus alone is not sufficient to transform the cervix cells, which lead us to investigate individual intrinsic factors, such as genetic variation in immunological genes that act as important agent to susceptibility, progression and disease outcome (TORRES-POVEDA *et al*., 2016).

CXCL12 gene is located at 10q11.1 and there are three GC boxes and one CAAT box on promoter region of the gene that are binding sites for the transcription factors SP1 and CTF, respectively (SHIROZU *et al*., 1995). CXCL12 binds to specific G protein-coupled seven transmembrane receptor CXCR4, which activate a biological axis responsible for cell trafficking, activation and differentiation. *CXCR4* gene is located at 2q21 and is expressed on multiple cell types, including lymphocytes, hematopoietic stem cells, endothelial cells, epithelial cells, cancer cells and stromal fibroblasts (GUYON, 2014). The *CXCL12 rs1801157* and *CXCR4* rs2228014 single nucleotide variants (SNV) are associated to glioma (CHANG *et al*., 2015), breast cancer (GUEMBAROVSKI *et al*., 2018), hepatocellular carcinoma (QUIN *et al*., 2018) and lymphocytic leukemia (BUTRYM *et al*., 2016).

Regarding the HPV infection, the CXCR4 upregulation in cervical cancer, promoted by the virus, was significantly associated with the histologic grade of cervical carcinoma (MORTAZAEE, 2020). Cervical cancer cells that do not express *CXCL12* may initiate its migration and invasion towards a CXCL12 gradient (ZANOTTA, *et al*., 2016) and the CXCL12/CXCR4 axis may affect tumor malignant progression and clinical aggressiveness (LECAVALIER-BARSOUM *et al*., 2018). Studies involving *CXCL12* and *CXCR4* SNVs in cervical cancer are scarce. *CXCL12* rs1801157 was previously associated to HPV infection (OKUYAMA *et al*., 2018) but not associated to the development of squamous intraepithelial lesion (SIL) (TEE *et al*., 2012). No data

about *CXCR4* rs2228014 and infection, lesions and cervical cancer may be found in the literature.

In this context, in the present study we investigated the possible association of *CXCL12* rs1801157 and *CXCR4* rs2228014 with HPV, squamous intraepithelial lesions and cervical cancer susceptibility.

2. MATERIALS AND METHODS

2.1 ETHICAL APPROVAL, PATIENTS AND SAMPLES

This study was approved by the Institutional Ethics Committee Involving Humans at State University of Londrina, Londrina – PR, Brazil (CEP/UEL 133/2012; CAAE 05505912.0.0000.5231). The study purpose and procedures were explained to all patients and written informed consent was obtained prior samples collection.

Between 2013 and 2018, 424 women were enrolled. They were recruited in public health services in Londrina- PR, Brazil. Biological materials (cervical secretion, blood samples, or formalin-fixed-paraffin-embedded (FFPE) tumor tissues), were collected from participants who attended cervical cancer prevention programs at an ambulatory colposcopy facility of Intermunicipal Consortium of Health of the Middle Paranapanema, at University Hospital and Clinic Center of the State University of Londrina, at Basic Healthcare Units in Londrina—PR, Brazil, and at Cancer Hospital of Londrina.

After sample collection, cytobrushes were stored in 2 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) at -10 C° until analysis. Peripheral blood was drawn into sterile syringes containing EDTA as anticoagulant and stored at -10°C until analysis. The cervical tumor tissue samples embedded in paraffin were provided by Londrina Cancer Hospital. A structured questionnaire was applied to all the patients to collect socio-demographic and sexual behavioral data. Participants were stratified based on presence or absence of HPV DNA, as tested by PCR and based on SIL and CC diagnosis, as determined by cervical cytology. Clinical and pathological data of cervical cancer patients were available from Londrina Cancer Hospital. Clinical staging was determined according to the International Federation of Gynecology and Obstetrics (FIGO) criteria. Pathological features analyzed included: histological classification and

histopathological grade, according to World Health Organization (WHO) histological classification of tumors of the uterine cervix.

2.2 DNA EXTRACTION

Genomic DNA was obtained from: a) cervical cytobrushes using DNAzol (Invitrogen™ Inc., Carlsbad, CA, USA), b) peripheral blood using Biopur Mini Spin Plus Kit (Biometrix®, Curitiba – PR, Brazil), c) FFPE tumor tissues using PureLink™ Genomic DNA Mini Kit (InvitrogenTM, Carlsbad, CA, EUA), all extraction were performed according to the manufacturer's instructions, and stored at -10 °C until use. DNA concentration was measured at 260 nm on a NanoDrop 2000c™ Spectrophotometer (Thermo Fisher Scientific, USA), and purity was assessed by A260/A280 ratio.

2.3 HPV DETECTION

HPV was detected by PCR using the primers MY09 (5'-CGTCCMAARGGAWACTGATC-3') and MY11 (5'-GCMCAGGGWCATAAYAATGG-3'), which are designed to amplify a conserved region of approximately 450bp in the HPV L1 gene (GenBank Accession number: AJ236888). 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 (Invitrogen™), with an annealing temperature of 55ºC. β-globin gene amplification(268 bp) was performed as an internal control, using primers GH20 (5'-GAAGAGCCAAGGACAGGTAC-3') and PC04 (5'-CAACTTCATCCACGTTCACC-3') (DA SILVA et al., 2012) under the same conditions of HPV PCR. Reactions without template DNA were used as negative control to test for contamination, and DNA from HeLa cells, which are stably integrated with HPV18, was used as positive control. PCR products were electrophoresed on 10% polyacrylamide gel and stained with silver nitrate.

2.4 *CXCL12* rs1801157 AND *CXCR4* rs2228014 POLYMORPHISMS DETECTION AND GENOTYPING

Genomic DNA from peripheral blood and FFPE tumor tissue samples was used to amplify regions of the *CXCL12* and *CXCR4* genes. Primers used for the amplification of the *CXCL12* gene were designed according to the nucleotide sequence deposited in GenBank which code is L36033. The primers forward 5'CAGTCAACCTGGGCAAGCC3' and reverse :5' CCTGAGAGTCCTTTTCGCGG3' were utilized to amplify the 3'UTR of *CXCL12*. For the amplification of the *CXCR4* gene the following primers were used: 5'AACTTCCTATGCAAGGCAGT3' (forward) and 5'TATCTGTCATCTCTCACT3' (reverse). PCR reactions were conducted using 100nM of dNTPs, 250μM of each primer, 1.5mM of $MgCl_2$, 1X of Buffer and 1U of Taq polymerase for CXCL12 polymorphism. For CXCR4 was used 0.75 mM de $MgCl_2$; 100 nM de dNTP, 0.2 μM of each primer, 1U of Taq polymerase and approximately 100ng of DNA in both cases (Invitrogen™).

The CXCL12 and CXCR4 products amplification correspond to a 293bp and 236bp fragments, respectively. The enzymatic restriction was performed using PCR products in the presence of the restriction enzyme *MspI* (at 37°C for 1 hour) for CXCL12 and *BccI* (at 37°C, for 1 hour) for CXCR4 (New England Biolabs, Ipswich, MA, USA). For CXCL12, *MspI* cleaves the amplified fragment of DNA in the presence of an adenine, producing fragments of 100bp and 193bp and in the presence of a guanine, the fragment of 293bp remains intact. For CXCR4, *BccI* cleaves in the presence of a thymine, generating fragments of 103 and 133 pb and in the presence of a cytosine, remains the fragment of 236 bp.

2.5 STATISTICAL ANALYSIS

HPV infection's frequency reported in this paper was compared to the overall prevalence of cervical HPV in Brazil through the one-sample general z-test (ALTMAN, 1991), and the exact Clopper-Pearson confidence interval for the observed proportion was calculated (CLOPPER; PEARSON, 1934). Pearson's Chi-square test of independence (χ^2) followed by Bonferroni correction was employed in the analysis of contingency tables to identify differences in baseline features between HPV-infected and non-infected women, and those bearing SIL and CC. Allele frequency was calculated as [1(h + 2H)]/2 N, where h represents the heterozygous genotype, H is the

homozygous genotype, and N is the sample size for each population. χ^2 test followed by Bonferroni correction was employed in SNVs CXCL12 rs1801157 and CXCR4 rs2228014 distribution testing. Adjusted odds ratio (OR) with 95% confidence interval (95%CI) were estimated by a binary or multiple logistic regression in the forced entry method to test the association between genetic models of inheritance of the SNVs rs1801157 and rs2228014 and HPV infection, SIL and CC, as appropriate, adjusting for baseline factors that were associated with HPV infection, SIL and CC diagnosis with a significance level lower than 0.10 found in the χ2 test. These variables were included in a forward stepwise variable selection method. The gene-gene association analysis between the SNVs were also determined by logistic regression models. All tests were two-tailed, and data were analyzed in SPSS Statistics 22.0 software (SPSS Inc., Chicago, Illinois, USA), considering a significance level alpha set at 0.05**.**

3 RESULTS

3.1 Patient baseline characteristics

In this study, 424 women were included and categorized as HPV uninfected (187/56.2%), HPV infected (146/43.8%), NILM (negative for intraepithelial lesion and malignancy, (187/56.2%), SIL (squamous intraepithelial lesion, 63/14.8%) and CC (cervical cancer 92/21.7%). We previously reported data from HPV infection and CXCL12 rs1801157 association analysis for a smaller group of patients (OKUYAMA et al., 2018). Sociodemographic, sexual lifestyle and gynecological and obstetric data for NILM, SIL and CC are summarized in Tables 1 and 2. In order to provide an epidemiological perspective, we compared the frequency of cervical HPV infection found in our cohort (43.8%) (*i.e.*, composed by women from the north of the Paraná state (BRA)) with the overall cervical HPV prevalence in Brazil (25.41%), recently reported in a meta-analysis conducted by Colpani et al. (2020), and our results significantly differ from national data (z-statistic: 7.70; $p<0.0001$; 95%CI of observed proportion: 38.40% - 49.31%).

A higher frequency of SIL and CC was observed in women with no knowledge about HPV and its ways of transmission ($p= 0.03$ and $p=0.008$, respectively), 55 years old or more ($p <0.0001$), receiving <1 minimum wage ($p=0.004$), smokers ($p=0.005$),

women with incomplete elementary education (p<0.0001) and single (p=0.001) (Table 1). Regarding the sexual lifestyle and gynecological and obstetric data, a higher frequency of SIL and CC was found in women who use hormonal contraceptive (p =0.009), who have had sex with or under 17 years old (p=0.011), had 4 or more sexual partners during lifetime (p=0.005), who had 5 or more number of pregnancies and women without prior cervical exam (p=0.001) (Table 2).

3.2 *CXCL12* and *CXCR4* SNVs distribution among diagnostic groups

We compared the allelic frequency of *CXCL12* rs1801157 and *CXCR4* rs2228014 with genomic big data from the Trans-Omics for Precision Medicine (TOPMed) Program, which used Whole-Genome Sequencing (WGS) to sequence approximately 155.000 genomes of participants from >80 different studies with varying designs, and data is publicly available at the National Center for Biotechnology Information site (https://www.ncbi.nlm.nih.gov/). Applying a general z-test for proportion comparisons, we found that the allele distribution of *CXCL12* rs1801157 and *CXCR4* rs2228014 was consistent with that reported by the TOPMed Program (Figure 1). *CXCL12* rs1801157 and *CXCR4* rs2228014 allelic and genotype distributions and p-values for the χ^2 test are shown in Table 3. Significant association was found for the following models for *CXCL12* rs1801157 HPV infected: codominant, dominant, recessive, models and allele; SIL/CC: codominant and dominant models. For *CXCR4* rs2228014, the following models were HPV infected: codominant model and allele; SIL/CC: codominant, dominant and allele.

3.3 Multivariate logistic models of association between *CXCL12* and *CXCR4* SNVs and SIL/CC diagnosis

When the models were adjusted by age and partners during lifetime in the binary logistic regression employing *CXCL12* rs1801157 genetic models as explanatory variables, the following models were independently associated with HPV infection: codominant GA (OR_{adj}=3.006, 95%CI (1.77-5.08) p<0.001); AA ($OR_{adj=}$18.027, 95%CI (4.87-66.71) p<0.001); dominant GA+AA (OR_{adj} =3.841, 95%CI (2.32-6.35) p<0.001); recessive AA (OR_{adj}=12.801, 95%CI, (3.33-4382),

p<0.0001). When the effects of *CXCR4* rs2228014 were evaluated, the following significant associations were found: codominant CT (OR_{adj}=2.005, 95%CI (1.03-3.87) *p*=0.038) and dominant model CT+TT (OR_{adj}=2.254, 95%CI (2.25-4.20) *p*=0.011) (Table 4).

Case-control analysis of association between *CXCL12* rs1801157 and SIL/CC adjusting by age, monthly income and partners during lifetime in the multiple logistic regression, the following genetic models were independently associated: *CXCL12* codominant AA (SIL: OR_{adj}=8.857, 95%CI (3.24-24.20) *p*<0.001; CC: OR_{adj} =5.031, 95%CI (1.48-17.00) *p*=0.009) and recessive AA (SIL: OR_{adj}=9.425, 95%CI (3.26-23.14) *p*<0.0001; CC: OR_{adj}=5.962, 95%CI (1.89-18.72) *p*=0.005). For *CXCR4* codominant CT (CC: OR_{adj}=4.755, 95%CI (1.92-11.84) *p*=0.001), dominant CT+TT (CC: OR_{adj}=4.755, 95%CI (2.09-10.81) *p*<0.001) presented themselves independently associated to CC (Table 4).

3.4 Impact of interaction between *CXCL12* and *CXCR4* SNVs on HPV infection, SIL and CC

Through binary logistic regression adjusted for age and sexual partners during lifetime, significant interactions for HPV infection were found for the following cases: CXCL12 GA + CXCR4 CT$^{\#}$ (codominant model) (OR_{adj}=7.345, 95%CI (2.124-25.400) p=0.002); CXCL12 GA+AA + CXCR4 CT+TT (OR_{adj}=10.138, 95%CI (3.466-29.652) p<0.001). For SIL and CC, multiple logistic regression adjusted for age, monthly income and sexual partners during lifetime was performed. Significant susceptibility for SIL was evidence by the interactions CXCL12 GA+AA + CXCR4 CC+CT (OR_{adj}=2.068, 95%CI (1.123 – 3.811) p=0.002). For CC diagnosis, the significant interactions were CXCL12 AA + CXCR4 CT (OR_{adj}=7.634, 95%CI (2.580-22.571) p=0.015), CXCL12 GA+AA + CXCR4 CT+TT (OR_{adj}=4.207, 95%CI (1.641-10.753) p=0.006), CXCL12 GA+AA + CXCR4 CT (OR_{adj}=3.021, 95%CI (1.382-10.570) p=0.011) (Table 5).

4. DISCUSSION

In this study, we provided novel information about the effect of single nucleotide genetic variants rs1801157 of *CXCL12* and rs2228014 of *CXCR4* on the HPV infection, squamous intraepithelial lesion (SIL) and cervical cancer (CC) susceptibility.

Tumors are dynamic tissue masses, so requiring continuous exposure to the host cells, nurturing them into pave a path for tumor growth and metastasis. CXCL12/CXCR4 is the key signaling for such aim. Gathering knowledge about the activity within this axis would deepen insight into the utmost importance this signaling taken to attract and cross-connect multiple cells within the tumor microenvironment. A large number of studies showed that individual genetic factors can affect the incidence and progression of cancer. Among genetic factors, single nucleotide variants (SNV) located in the promoter region or coding region of gene expression regulation have important functions (DENG; *et al*. 2017; MORTAZAEE *et al*., 2020).

Synonymous SNVs can also accelerate or decelerate the speed at which the ribosome moves along the mRNA, thus changing the dynamics of translation, and the subsequent protein structure and function. They may also result in different mRNA secondary structures and protein secondary structures such as α helix β folding (DENG, *et al*., 2016). Mutations in the 3′UTR are involved in many diseases because they affect gene progression. As a regulatory region, the 3′UTR is indispensable for normal gene expression. Therefore, polymorphisms in the 3′UTR can alter miRNA binding sites and affect mRNA degradation and protein translation (SETHUPATHY; COLLINS, 2008).

In the present study, our data indicates that *CXCL12* rs1801157 AA genotype is associated with the susceptibility of developing SIL and CC. A previous study from our group had already demonstrated that the CXCL12 genotype AA and allele A were associated to HPV infection susceptibility (OKUYAMA, *et al*., 2018). On the other hand, previous studies did not analyze the possible association with HPV infection but verified that this SNV is not associated with CC. According to Rozask *et al.* (2015) *CXCL12* rs1801157 is a risk factor for cervical cancer development in women with a positive history of tobacco. For Maley *et al*. (2009), this single nucleotide variant is not a risk for CC which corroborates with studies from Tee *et al*. (2012) who also did no find association between rs1801157 and susceptibility to cervical neoplasia.

For CXCR4 rs2228014 we demonstrated for the first time in the literature that women who were allele T carriers presented a significant increased risk of HPV infection and development of CC. Regarding other types of cancers, the CXCR4 T allele was more frequent in patients compared with controls and individuals who had TT genotype presented an elevated risk for endometrial carcinoma and for CXCL12 rs1801157 AA genotype was higher in patients as well (CACINA *et al*., 2012).

SNVs interaction analysis were performed to evaluate possible significant combination between *CXCL12* rs1801157 and *CXCR4* rs2228014. In this work, several significant results were found for HPV infection, SIL and CC. It was observed that among the SNV interactions, models with the presence of genotypes AA and CT from *CXCL12* and *CXCR4* SNV, respectively, strongly indicated a greater risk for the patients in developing squamous intraepithelial lesion and cervical cancer. There are no previous studies evaluating the interaction between both SNVs and HPV, SIL and CC. Data so far has shown a significant interaction for oral squamous cell carcinoma (OSCC) in the genotypic combination of CXCL12 and CXCR4 SNVs. Patients with genetic polymorphisms of the genotype combination CXCL12/CXCR4 had a higher risk of OSCC (p=0.033). The effects of CXCR4 genetic variants on susceptibility to OSCC in patients with different risk habits of tobacco smoking and alcohol consumption, and revealed that CT+TT genotypes exerted an increased risk only in patients with one or two risk habits (HUANG *et al*., 2019). The axis CXCL12/CXCR4 is extensively studied in breast cancer (BC). Combination of both SNVs were analyzed by Lin *et al*. (2009) in BC and evidenced a genetic interaction between CXCL12 and CXCR4 SNVs in which wild type homozygote for both reduced BC susceptibility, but failed in finding any association of both SNVs in isolation. This data is in accordance with Guembarovski et al. (2018) which also observed no SNV association in isolation but showed a significant increased susceptibility in the interaction between heterozygous genotypes for both single nucleotide variants. On the contrary, using an analysis called multifactor dimensionality reduction (MDR), a bioinformatic technique to provide a non-linear model associated with disease, Fu et al. (2016) evidenced an interaction between CXCL12 rs1801157 and CXCR4 rs2228014 for the BC susceptibility.

For Chang *et al*. (2009), expression of CXCL12 rs1801157 mRNA in GA/AA fibroblasts was three times that in GG fibroblasts. GA/AA (but not GG) fibroblasts harvested from patients enhanced colon cancer cell line (HCT116) proliferation and migration. The authors concluded that this SNV could work as a predictive marker of

lymph node metastasis in CRC. In prostate cancer patients, CXCL12 rs1801157 presented protein expression in AA + GA genotype carriers was significantly higher than that in GG genotype carriers. Among the CXCL12 allele A carriers the protein expression was also significantly higher compared to those with the GG genotype (HIRATA *et al*., 2007). CXCR4 mRNA relative expression did not differ regarding the presence or absence of T allele and even though the expression was higher in BC patients, there was no correlation with patient clinicopathological features (OKUYAMA *et al*., 2015). CXCL12 mRNA expression in BC demonstrated that allele A carrying patients presented smaller expression compared to GG patients and the ones with positive estrogen receptor with allele A showed a significantly lower expression of CXCL12 in peripheral blood than GG hormone positive patients (DE OLIVEIRA *et al*., 2011). Further analysis regarding *CXCL12* rs1801157 and *CXCR4* rs2228014 are required in order to evaluate the role of both SNVs in mRNA expression and protein in the development of SIL and CC progression.

A possible role for CXCL12/CXCR4 axis in HPV infection is that keratinocytes immortalized by oncogenic HPV16 or HPV18 upregulate CXCL12 and CXCR4 in a manner dependent upon expression of the viral proteins E6 and E7. Autocrine signaling activated by CXCL12-engagement of its receptor could control responses as motility and survival of the infected cells. Chemokine produced by dermal fibroblasts could increase proliferation and migration of adjacent keratinocytes. The paracrine activation would enhance cell permissiveness to viral genome replication and production of the E6 and E7 proteins. As a consequence, E6 and E7 expressed in the suprabasal layers would then upregulate levels of CXCL12 and CXCR4, which further enhance cell proliferation and viral DNA replication. This effect might happen for low- or high-risk HPV infection and could possibly explain the basis of HPV associated oncogenesis (CHOW *et al*., 2010). Furthermore, *CXCL12* proximal promoter in its 5'flanking and 5'UTR region contain six Sp1 binding sites, and Sp1 transcription factor seems to be the major positive regulator of *CXCL12* expression. After HPV infection of basal epithelial cervical cells, E6 and E7 oncoproteins are expressed, and may bind specifically to protein 1 transcription factor (Sp1). The E6-Sp1 and E7-Sp1 complex can migrate into the nucleus and probably induce the CXCL12 gene expression (GARCÍA-MORUJA *et al*., 2005). In case of CXCR4 SNV, the site of the mutation is a target of RNA polymerase II and may alter motifs for SOX, RXRA and NRSF transcription factors. The deregulation of these transcription factors has been

associated to a plethora of cancers (DENG *et al*., 2018; HALSTEAD *et al*., 2017; KUMAR; MISTRI., 2019).

Leukocytes bearing the CXCR4 receptor are attracted to the malignant sites and are themselves stimulated to produce more chemokine, cytokine and stimuli an inflammatory environment (LAZENNEC, 2010). Autocrine and paracrine networks signalization attract more leukocytes into the site of infection, especially T-helper 2 (TH2) lymphocytes, type-2 macrophages (M2) and pre-dendritic cells (PDC). The inflammatory chemokines such as CXCL12 produced by these leukocytes contribute to tumor growth and progression inducing the production of proteases, angiogenic factors, growth factors and immunosuppressive cytokines by others cells in the tumor microenvironment (TME). Within the TME, CXCL12 ligand secretion is often altered compared to healthy tissue. This facilitates recruitment of pro-tumorigenic immune cells such as myeloid-derived suppressor cells (MDSCs), tumor-associated neutrophils (TAN), tumor-associated macrophages (TAM), and regulatory T cells (Treg). These cells expand during tumor progression, suppress effector lymphocytes, and are associated with worse prognosis in patients with solid malignancies (SUSEK, 2018). These factors may amplify the inflammatory response by recruiting additional inflammatory cells. Research into the cancer chemokine network is revealing parallels between the pathology of inflammation and malignancy, parallels that enhance our understanding of both types of disease and indicate new approaches for treatment (BAKLWILL, 2004).

The small number of low-grade squamous intraepithelial lesion (LSIL) patients difficulted an analysis stratified in lesions levels: LSIL and HSIL (high-grade squamous intraepithelial lesion). Also, further analyses are necessary to confirm the influence of *CXCL12* rs1801157 and *CXCR4* rs2228014 SNVs on gene expression and protein levels in cervical cancer development. To the best of our knowledge, this is the first time that *CXCR4* rs2228014 is associated to the HPV infection susceptibility and CC. In this work, we also demonstrated that *CXCL12* rs1801157 is strongly associated to SIL and CC. An expressive number of patients and a strong interaction analysis between *CXCL12* rs1801157 and *CXCR4* rs2228014 showed that patients carriers of allele A of *CXCL12* and allele T of *CXCR4* simultaneously, are more susceptible to HPV infection, squamous intraepithelial lesions and cervical cancer which may be considered the highlight in the present study.

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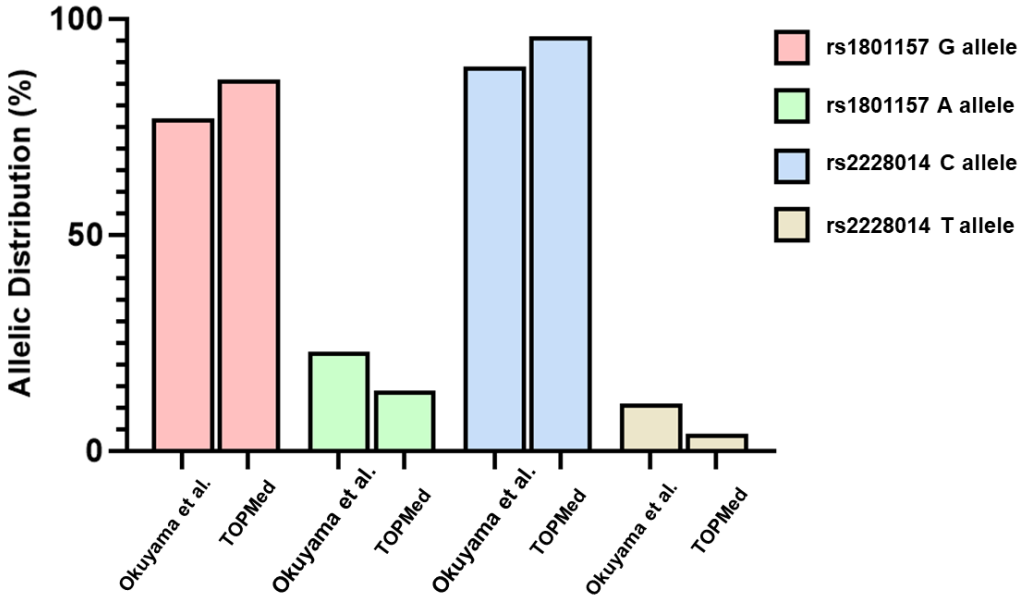

Figure 1. **Allelic distribution of the *CXCL12* rs1801157 and *CXCR4* rs2228014 SNVs in the present study compared to genomic big data from Trans-Omics for Precision Medicine (TOPMed) Program.** *CXCL12* rs1801157 allelic frequency of Okuyama et al. vs. TOPMed: z-statistic = 1.639; p = 0.101. *CXCR4* rs2228014 allelic frequency of Okuyama et al. vs. TOPMed: z-statistic = 1.879; p = 0.060.

Table 1 Sociodemographic characteristics of patients and controls

Variable	NILM		SIL		CC		*p*-value*
	n	(%)	n	(%)	n	(%)	
Knowledge about HPV							**0.003**
No	35	(18.7)	20	(31.3)	16*	(41.1)	
Have ever heard	104	(55.6)	30	(46.9)	16	(41.0)	
Yes	48	(25.7)	14	(21.8)	7	(17.9)	
Knowledge about ways of HPV transmission							**0.008**
No	88	(47.1)	34	(53.1)	29*	(74.4)	
Yes	99	(52.9)	30	(46.9)	10	(25.6)	
Age (years)							**<0.001**
≤ 24	10	(5.3)	13	(20.3)	1	(1.1)	
25 – 34	43	(23.0)	20	(31.3)	8	(8.7)	
35 – 44	46	(24.6)	14	(21.8)	27	(29.7)	
45 – 54	55	(29.4)	11	(17.2)	21	(23.1)	
≥ 55	33	(17.7)	6	(9.4)	34*	(37.4)	
Monthly income[a]							**0.004**
<1 minimum wage	83	(44.9)	36	(57.1)	26*	(72.2)	
1 – 3 minimum wages	95	(51.4)	27	(42.9)	8	(22.2)	
>3 minimum wages	7	(3.8)	0	(0.0)	2	(5.6)	
Smoking status							**0.003**
No	142	(75.9)	36	(56.3)	56	(62.2)	
Yes	29	(15.5)	24*	(37.4)	23	(25.6)	
Former smoker	16	(8.6)	4	(6.3)	11	(12.2)	
Educational level[b]							**<0.0001**
Incomplete elementary	58	(31.4)	26	(22.6)	62*	(45.1)	
Complete elementary	23	(12.4)	6	(14.5)	11	(19.1)	
Incomplete secondary	26	(14.1)	10	(20.9)	4	(9.4)	
Complete secondary	59	(31.9)	17	(31.0)	9	(13.9)	
Incomplete higher education	6	(3.2)	3	(7.4)	0	(0.0)	
Complete higher education	13	(7.0)	1	(3.6)	5	(12.5)	
Marital status							**0.001**
Single	135	(72.2)	41*	(21.8)	6	(6.6)	
Married / Civil partner	16	(8.6)	38	(59.4)	54	(60.0)	
Divorced	25	(13.4)	9	(14.1)	15	(16.7)	
Widowed	11	(5.8)	3	(4.7)	15*	(16.7)	

[a]Based on Brazilian minimum wage (approximately U$ 206.00). [b]Based on Brazilian educational system. *Analysis by two-sided Chi-square (X^2) test and $p<0.05$ set as significance level (SPSS Inc., Chicago, Illinois, USA). NILM negative for intraepithelial malignancy; SIL squamous intraepithelial lesion; CC cervical cancer. Some categories did not complete the total of patients due to lack of data.

Table 2 Sexual behavioral and reproductive characteristics of patients and control

Variable	NILM		SIL		CC		*p* value*
	n	(%)	n	(%)	n	(%)	
Contraceptive method: Condon							0.215
Yes	21	(11.3)	9	(14.1)	17	(19.4)	
No	165	(88.7)	55	(85.9)	72	(80.6)	
Contraceptive method: Hormonal							**0.009**
Yes	54	(29.0)	26	(40.6)	42*	(47.2)	
No	132	(71.0)	38	(59.4)	47	(52.8)	
Number of pregnancies							**0.001**
0	21	(11.2)	10	(15.6)	4	(4.4)	
1	30	(16.0)	8	(12.5)	12	(13.6)	
2	57	(30.5)	15	(23.4)	20	(22.0)	
3	41	(21.9)	16	(25.1)	17	(18.7)	
4	18	(9.6)	10	(15.6)	9	(9.4)	
≥ 5	20	(10.8)	5	(7.8)	29*	(31.9)	
Abortion							0.477
No	136	(79.1)	40	(72.7)	67	(73.6)	
Yes	36	(20.9)	15	(27.3)	24	(26.4)	
Age at first sexual intercourse (years)							**0.011**
≤17	92	(49.5)	44*	(69.8)	52	(61.2)	
≥18	94	(50.5)	19	(30.2)	33	(38.8)	
Age at menarche							0.511
≤11	42	(22.7)	18	(28.6)	9	(23.1)	
12	42	(22.7)	17	(27.0)	10	(25.6)	
13	46	(24.9)	16	(25.4)	6	(15.6)	
≥14	55	(29.7)	12	(19.0)	14	(35.9)	
Sexual partners during the lifetime							**0.002**
1	74	(39.6)	8	(12.5)	14	(36.8)	
2 – 3	62	(33.2)	27	(42.2)	11	(28.9)	
≥4	51	(27.3)	29*	(45.3)	13	(34.3)	
Prior Exam							**<0.001**
No	7	(3.8)	0	(0.0)	27*	(42.0)	
Yes	179	(96.2)	64	(100.0)	51	(58.0)	

*Analysis by two-sided Chi-square (X^2) test and $p<0.05$ set as significance level (SPSS Inc., Chicago, Illinois, USA). NILM negative for intraepithelial malignancy; SIL squamous intraepithelial lesion; CC cervical cancer. Some categories did not complete the total of patients due to lack of data.

Table 3 Genotype and allele distribution considering HPV infection status, SIL diagnosis, cancer and inheritance models testing

SNVs	HPV uninfected	HPV infected	p-value	NILM	SIL	CC	p-value
CXCL12							
Codominant model			**<0.001**				**<0.001**
GG	139 (74.3)	69 (47.3)		139 (74.3)	32 (50.0)	58 (63.7)	
GA	45 (23.6)	58*(39.7)		45 (24.1)	18 (28.1)	23 (25.3)	
AA	3 (2.1)	19*(13.0)		3 (1.6)	14* (21.9)	10* (11.0)	
Dominant model			**<0.001**				**0.001**
GG	139 (74.3)	69 (47.3)		139 (74.3)	32 (50.0)	58 (63.7)	
GA + AA	48 (25.7)	77* (52.7)		48 (25.7)	32 (50.0)	33 (36.3)	
Recessive model			**<0.001**				**<0.001**
AA	3 (1.6)	19* (13.0)		3 (1.6)	14* (21.9)	10* (11.0)	
GG + GA	184 (98.4)	127 (87.0)		184 (98.4)	50 (78.1)	81 (89.0)	
Alleles			**<0.001**				0.490
Allele G	323 (86.3)	196 (67.1)		323 (86.3)	82 (64.06)	119 (73.46)	
Allele A	51 (16.7)	96* (32.9)		51 (16.7)	46 (35.94)	43 (26.54)	
CXCR4							
Codominant model			0.064				**0.010**
CC	163 (87.2)	114 (78.1)		225 (83.6)	52 (81.3)	64 (70.3)	
CT	22 (11.7)	27 (18.5)		37 (13.8)	12 (18.7)	24* (26.4)	
TT	2 (1.1)	5 (3.4)		7 (2.6)	0 (0.0)	3 (3.3)	
Dominant model			**0.028**				**0.003**
CC	163 (86.9)	14 (78.1)		225 (83.6)	52 (81.2)	64 (70.3)	
CT+TT	24 (13.1)	32* (21.9)		44 (16.4)	12 (18.8)	27* (29.7)	
Recessive model			0.137				0.194
TT	2 (1.1)	5 (3.4)		7 (2.6)	0 (0.0)	3 (3.3)	
CC+CT	185 (98.9)	141 (96.6)		262 (97.4)	64 (100.0)	88 (96.7)	
Alleles			**0.012**				**<0.001**
Allele C	348 (93.0)	225 (87.3)		348 (93.0)	116* (90.6)	152 (83.5)	
Allele T	26 (7.0)	37* (12.7)		26 (7.0)	12* (9.4)	30* (16.5)	

Data presented as absolutely number and percentage. Twi-sided χ^2 test, with p<0.05 considered significant> SNV, single nucleotide variant; NILM, negative for intraepithelial lesion and malignancy; SIL squamous intraepithelial lesion; CC, cervical cancer.

Table 4 Case-control multivariate analysis considering HPV infection and SIL/CC inheritance models.

Models	Case groups [OR (CI 95%)]		
	HPV infection[a]	**SIL[b]**	**CC[b]**
CXCL12			
Codominant model	1.0	1.0	1.0
GG			
GA	**3.006**** (1.77-5.08)	1.054 (0.53-2.07)	0.739 (0.28-1.89)
AA	**18.027**** (4.87-66.71)	**8.857*** (3.24-24.20)	**5.031*** (1.48-17.00)
Dominant model			
GG	1.0	1.0	1.0
GA + A/A	**3.841*** (2.32-6.35)	1.960 (1.10-3.47)	1.225 (0.56-2.83)
Recessive model			
GG + GA	1.0	1.0	1.0
AA	**12.081*** (3.33-43.82)	**9.425*** (3.26-23.14)	**5.962*** (1.89-18.72)
CXCR4			
Codominant model			
CC	1.0	1.0	1.0
CT	**2.005*** (1.03-3.87)	0.28 (0.01 – 5.09)[c]	**4.755*** (2.09-10.81)
TT	5.287 (0.93-2.93)	1.340 (0.62-2.85)	2.853 (0.50-26.21)
Dominant model			
CC	1.0	1.0	1.0
CT + TT	**2.254*** (2.25-4.20)	1.228 (0.57-2.63)	**4.544**** (1.96-11.11)
Recessive model			
CC + CT	1.0	1.0	1.0
TT	4.716 (0.83-26.56)	0.27 (0.02 – 4.71)[C]	2.645 (0.44-15.76)

*p<0.05.**p<0.01; SIL squamous intraepithelial lesion; CC cervical cancer.

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

[b] OR (odds ratio) and CI (confidence interval) 95% estimated by multinomial logistic regression with "NILM group" as reference and adjusted by age and sexual partners during lifetime.

[c] Crude OR with 95% CI was calculated using Haldane's modification, which adds 0.5 in all cells to accommodate possible zero counts

Table 5 *CXCL12* rs1801157 and *CXCR4* rs2228014 single nucleotide variants interaction models for HPV and SIL/CC

Models	**Case groups [OR (CI 95%)]**		
	HPV infected [a]	SIL [d]	CC [d]
CXCL12 GA + CXCR4 CT	7.345* (2.12-25.40)	-	-
CXCL12 AA + CXCR4 CT	-	-	7.634* (2.580 - 22.571)
CXCL12 GA+AA + CXCR4 CT+TT	10.138** (3.46-29.65)	2.068* (1.123-3.811)	4.207* (1.641 - 10.753)
CXCL12 GA+AA + CXCR4 CT	-	-	3.021* (1.382 -10.570)

*p<0.05 **p<0.001; SIL squamous intraepithelial lesion; CC cervical cancer.

[a]Odds ratio (OR) and 95% confidence interval (95% CI) obtained through binary logistic regression with major alleles as reference and adjusted by age, monthly income, sexual partners during lifetime. [b]Note that interaction analyses between only homozygous genotypes were not performed because none of the patients co-inherited the two SNVs in homozygosity. [c]An exploratory analysis of heterozygous co-inheritance was carried out, in which all patients bearing any of the heterozygous genotypes for both SNVs were grouped.[d] OR (odds ratio) and CI (confidence interval) 95% estimated by multinomial logistic regression with “SIL group” as reference and adjusted by age, monthly income and sexual partners during lifetime.

ARTIGO 2

CXCL12 and CXCR4 genetic variants do not affect the tissue expression of CXCR4 in cervical cancer

ABSTRACT

Cervical cancer is the third most common cancer in women worldwide and inflammation is a crucial component of tumor progression, but other cofactors must be present for the development of a malignancy such as individual genetic factors. In this context, *CXCL12* and *CXCR4* genes may have a single nucleotide variant (SNV) rs1801157 and rs2228014, respectively, which are involved in survival, angiogenesis and invasion of malignant cells. The present work objective was to verify a possible association between SNVs of CXCL12 and CXCR4 and its influence in CXCR4 expression in cervical tumor tissue, analyze association between CXCL12 and CXCR4 single nucleotide variants with clinicopathological features (tumor, histology grade and stage) and CXCR4 tumor tissue expression. CXCL12 and CXCR4 were assessed by PCR followed by restriction fragment length polymorphism for 90 patients, and immunohistochemistry was performed in 35 formalin-fixed-paraffin-embedded cervical tumor tissues. No difference in the genotype distribution between groups was found for the assessed variables. Neither the main effect of SNVs nor the interaction term (GA + AA by CT + TT) were associated with the evaluated clinicopathological characteristics. Regarding CXCR4 staining, no significant data was related with the evaluated clinicopathological features. Evaluation of CXCL12 rs1801157 and CXCR4 rs2228014 and immunostaining showed no significant relationship between the degrees of CXCR4 immunostaining and each model of *CXCL12* and *CXCR4* SNVs. Although a strong CXCR4 immunostaining was observed in patients presenting the CXCL12 AA genotype (p=0.05). This is the first time that CXCL12 and CXCR4 SNVs were analyzed in order to verify possible association with CXCR4 immunostaining and clinicopathological features in cervical cancer.

Keywords: cervical cancer, rs1801157, rs2228014

1. INTRODUCTION

Cervical cancer is the third most common cancer in women worldwide and recent studies have expanded the concept that inflammation is a crucial component of tumor progression though the mechanism triggering the transformation from inflammation to malignancy is still unknown. Many women, for example, are infected with high-risk HPV, but only a subset of infected women will ever develop cervical cancer, suggesting that other cofactors must be present for the development of a malignancy (HUANG *et al*., 2013). CXCR4 (CXC chemokine receptor 4) is a transmembrane receptor that belongs to the CXC chemokine receptor family and was firstly associated to leukocyte homing to the CXCL12 tissue producers (WALENKAMP *et al*., 2017). This receptor extends to most human neoplastic cells and was found to be altered dramatically in neoplastic tissue, particularly at the leading edge of transformation, survival, angiogenesis and invasion (FURUSATO *et al*., 2010).

The CXCR4 gene may have a single nucleotide variant (SNV), rs2228014, which has been associated to breast cancer, endometrial cancer, among others (OKUYAMA *et al*., 2015; CACINA *et al*., 2012). CXCR4 is abundantly expressed on cells from several metastatic cancers, including ovarian, prostate and bladder cancer (PORCILE *et al*., 2005; TAICHMAN *et al*., 2002; RETZ *et al*., 2005). CXCR4 in cervical cancer has been associated to lymph node metastasis in squamous cell carcinoma and adenocarcinoma (KODAMA *et al*., 2006; YANG *et al*., 2007). Furthermore, CXCR4 level in breast, ovarian, pancreatic tumor tissue has been associated with clinical outcomes (BALKWILL, 2012).

In this context we aimed to investigate the influence of *CXCL12* rs1801157 and *CXCR4* rs2228014 single nucleotide variants in the clinicopathological features and on its protein expression in cervical cancer samples.

2. MATERIALS AND METHODS

2.1 ETHICAL APPROVAL, PATIENTS AND SAMPLES

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

05505912.0.0000.5231). The study purpose and procedures were explained to all patients and written informed consent was obtained prior samples collection.

Between 2017 and 2019, 90 women were enrolled. Biological materials (blood samples or formalin-fixed-paraffin-embedded (FFPE) tumor tissues), were collected from participants. From Cancer Hospital of Londrina Londrina - PR, Brazil, 35 patients accepted the invitation to participate and from Erasto Gaertner Hospital, Curitiba – Paraná, Brazil, were 55 patients.

2.2 DNA EXTRACTION

Genomic DNA was obtained from: a) peripheral blood using Biopur Mini Spin Plus Kit (Biometrix®, Curitiba – PR, Brazil), b) FFPE tumor tissues using PureLink™ Genomic DNA Mini Kit (Invitrogen™, Carlsbad, CA, EUA), all extraction were performed according to the manufacturer's instructions, and stored at -10 °C until use. DNA concentration was measured at 260 nm on a NanoDrop 2000c™ Spectrophotometer (Thermo Fisher Scientific, USA), and purity was assessed by A260/A280 ratio.

2.3 *CXCL12* rs1801157 AND *CXCR4* rs2228014 POLYMORPHISMS DETECTION AND GENOTYPING

Genomic DNA from peripheral blood and FFPE tumor tissue samples were used to amplify regions of the *CXCL12* and *CXCR4* genes. Primers used for the amplification of the *CXCL12* gene were designed according to the nucleotide sequence deposited in GenBank which code is L36033. The primers forward 5'CAGTCAACCTGGGCAAGCC3' and reverse :5' CCTGAGAGTCCTTTTCGCGG3' were utilized to amplify the 3'UTR of *CXCL12*. The amplification of the *CXCR4* gene used the following primers: 5'AACTTCCTATGCAAGGCAGT3' (forward) and 5'TATCTGTCATCTCTCACT3' (reverse). PCR reactions were conducted using 0.1 mM of dNTPs, 0.2μM of each primer, 1.5mM of $MgCl_2$, 1X of Buffer and 1U of Taq polymerase for CXCL12 polymorphism. For CXCR4 was used 0.75 mM de $MgCl_2$; 0.1 mM de dNTP, 0.2 μM of each primer, 1U of Taq polymerase and approximately 100ng of DNA in both cases (Invitrogen™).

The CXCL12 and CXCR4 products amplification correspond to a 293bp and 236bp fragments, respectively. Genotyping was performed through an enzymatic restriction, using PCR product in the presence of the restriction enzyme *MspI* (2 units of enzyme, at 37°C for 1 hour) for CXCL12 and *BccI* (2 units of enzyme, at 37°C, for 1 hour) for CXCR4 (New England Biolabs, Ipswich, MA, USA). For CXCL12, *MspI* cleaves the amplified fragment of DNA in the presence of an adenine, producing fragments of 100bp and 193bp and in the presence of a guanine, the fragment of 293bp remains intact. For CXCR4, *BccI* cleaves in the presence of a thymine, generating fragments of 103 and 133 pb and in the presence of a cytosine, remains the fragment of 236 bp.

2.4 CXCR4 IMMUNOHISTOCHEMISTRY

Briefly, formalin-fixed paraffin-embedded (FFPE) sections of cervical tumor tissue samples were obtained from 35 patients. Additionally, normal adjacent tissue sections could also be obtained from 9 of these patients. Sections of 5 µ*l*m thickness were obtained from several representative areas of each tumor specimen and were mounted on to glass slides for immunostaining (Starfrost, Knittel, Germany). Briefly, after the slides were dewaxed in xylene and rehydrated in an alcohol series, antigen retrieval was carried out in a pressured chamber (Easypath diagnostics, code ep31-20292, series number 31112007mp, Brazil), in Tris-EDTA buffer, pH 9, for 1 hour. Peroxidase blockage was performed with Easypath diagnostic kit (ref ep-11-20522, 090420/1, Brazil). Anti-human CXCR4 monoclonal antibody (TermoFisher, Scientific, Waltham, Massachusetts, USA) was used as primary antibody, at a dilution of 1:50, overnight at 8ºC. The secondary antibody (Mouse/Rabbit Immuno detector HRP/DAB, Bio SB INC, Santa Barbara, CA, USA) was added and the slides were counterstained with hematoxylin-eosin and fixed by Canada balsam. CXCR4 staining was evaluated in tumor and normal adjacent tissues by an experienced pathologist. Staining scores were considered as follows: 0 = no staining, + = weak staining, ++ = moderate staining and +++ = strong staining.

2.5 STATISTICAL ANALYSIS

Differences in genotype distribution in cervical clinicopathological data were tested by Chi-square test. Association between *CXCL12* rs1801157 or *CXCR4* rs2228014 SNVs and tumor's clinicopathological characteristics was tested through age adjusted logistic regression. CXCR4 immunohistochemistry vs clinicopathological features and CXCR4 immunohistochemistry and SNVs were analyzed through Kendall's Tau rank. Data were analyzed in IBM SPSS Statistics 22.0 software (SPSS Inc., Chicago, Illinois, USA), considering a significance level alpha set at 0.05.

3. RESULTS

3.1 Clinicopathological characteristics and median age description of patients

In this study, patients were characterized by age (years) and clinicopathological data according to the variables analyzed such as "type of tumor", "histology grade" and "stage". Groups did not present significant difference between median age and the variables: type of tumor p=0.113; histology grade p=0.066 and stage p=0.859 Table 1.

3.2 Analysis of CXCL12, CXCR4 SNVs and patients' clinicopathological features

Genotyping of *CXCL12* (rs18011157) and *CXCR4* (rs2228014) SNVs was performed for the 90 patients. In Table 2, the genotypes of both SNVs were distributed according to the grouping variables "type of tumor", "histology grade" and "stage". There was no difference in the genotype distribution between groups of each variable.

Taking into account the small number of AA (*CXCL12*) and TT (*CXCR4*) patients, the combination of these categories with their respective heterozygotes (GA + AA and CT + TT) was used during the age-adjusted logistic regression analysis, as shown in the Table 3. Neither the main effect of SNVs nor the interaction term (GA + AA by CT + TT) were associated with the evaluated clinicopathological characteristics, when compared with the GG and CC genotypes. Then, according to present data, *CXCL12* and *CXCR4*

SNVs do not seem to influence the type of tumor, histology grade and stage of cervical cancer.

3.3 Correlation between CXCR4 immunohistochemistry and clinicopathological features

FFPE sections of cervical tissue containing the tumor from 35 of the 90 patients were immunostained for CXCR4. Adjacent non-tumor tissues (in 9 patients) and tumor tissues were evaluated, with leukocyte positivity being used as adequate internal positive control for each case. Only the tumor cells but not the normal epithelial cells in all patients were found to be stained, mainly involving cytoplasm compared to membrane (Fig. 1). The CXCR4 expression of cancer cells in each case was homogenously and diffusely stained to show different degrees of positivity as follow: + weak staining in 10 (28.6 %) patients, ++ moderate staining in 16 (45.7 %), and +++ strong staining in 9 (25.7 %). However, in the present study, such degrees of CXCR4 staining were not significant f related with the evaluated clinicopathological features (Table 4).

3.4 Correlation between CXCR4 immunohistochemistry and CXCL12 rs1801157 and CXCR4 rs2228014 SNVs

Finally, the relationship between *CXCL12* and *CXCR4* SNVs with CXCR4 immunostaining was assessed. For this, inheritance models were created for each SNV: genotypic model (minor allele homozygotes, heterozygotes, and major allele homozygotes), dominant model (major allele homozygotes, and heterozygotes plus minor allele homozygotes), and recessive model (heterozygotes plus major allele homozygotes, and minor allele homozygotes).

There was no significant relationship between the degrees of CXCR4 immunostaining and each model of *CXCL12* and *CXCR4* SNVs, even though *CXCL12* genotype AA almost reached a significant correlation with CXCR4 immunostaining as seen in Table 5 ($p=0.05$).

4. DISCUSSION

In the present work, data comparing CXCL12 rs1801157, CXCR4 rs2228014 and clinicopathological features (type of tumor, histology grade and stage) showed no significant association. Rozask *et al*. (2015) and Tee *et al*. (2012), authors who previous studied cervical cancer and CXCL12 rs1801157 SNV did not evaluated possible association between both SNVs and clinicopathological features in cervical cancer, therefore data analyzing these variables are nonexistent. In the health system in Brazil the type of tumor (squamous cell carcinoma or adenocarcinoma) and stage are clinicopathological parameters frequently analyzed. Stage shows the localization of the tumor, infiltration depth and invasion in adjacent structures, bladder and rectum. Clinicopathological features are important to define the treatment for each patient individually (INCA, 2016).

Association between rs1801157 and rs2228014 SNVs and clinical parameters have been studied and analyzed in other types of cancers. A work involving hepatocellular carcinoma (HCC), *CXCL12* rs1801157 allele A carriers had a higher risk to develop a status of stage III or IV disease, while no significant association was found between *CXCR4* rs2228014 variant and either HCC risk or pathological status (CHANG *et al*., 2009).

The biological effect of both SNVs vary according to the disease, type and subtype of tumor, therefore, there is not a consensus among the data published so far. For Amara *et al*. (2015) CXCL12 protein was higher expressed in colorectal cancer (CRC) Tunisian patients with genotypes AA and GA and with TNM stage II and III. The expression was significantly increased from normal mucosa to primary tumor suggesting that CXCL12 overexpression was an early event during tumorigenesis. In esophagogastric cancer, CXCL12 mRNA expression in rs1801157 allele A carriers was significantly associated with distant metastasis but had no correlation with the tumor infiltration depth, lymphatic metastasis and grading (SCHIMANSKI *et al*., 2011).

The CXCR4 tissue expression was more evidenced in cytoplasm in cervical carcinoma cells which is in accordance with previous studies (HUANG *et al*., 2013; YANG *et al*., 2007). Different localization patterns of chemokine receptors, i.e. nuclear versus

cytoplasmic, appear to have a different biological significance for the metastatic potential of cancer cells (CABIOGLU *et al*., 2005). This might represent a functional status of the receptor because binding to a specific ligand CXCL12 induces receptor internalization (KODAMA *et al*., 2006). Endosomes are gaining considerable attention as scaffolds for signaling complexes. The assembly of signaling complexes on intracellular endosomal membranes indicates that the intracellular trafficking itinerary of chemokine receptors may have important implications for signaling (NEEL et al., 2005). Receptor/ligand internalization induces activation of many transduction signaling pathways which are responsible for apoptosis, such as Bcl-2 (*B-cell lymphoma 2)*, proliferation, angiogenesis and metastasis through PI3K (*phosphatidylinositol 3-kinase),* MAPK (*mitogen activated protein kinases)*, Akt (*serine/threonine kinase Family)* and ERK1/2 (*extracellular signal-regulated kinases 1/2)* (GUO *et al*., 2015). High cytoplasmatic expression of CXCR4 appears to be more commonly in breast cancer, melanoma and colon cancer with distant lymphatic metastasis. Unfavorable prognosis decreases in median disease-free survival and high risk of recurrence (CABIOLGU et al., 2005; SCALA *et al*., 2005; KIM *et al*., 2005).

Moreover, enhanced signaling in infected keratinocytes could promote the proliferation and migration of nearby keratinocytes, facilitating virus entry into adjacent cells, promoting cell proliferation and viral gene expression. The enhanced metastatic activity conferred by E6 and E7 expression in HPV-positive tumor cells could aid in virus dissemination and maintenance. The implications to the host, as increased expression and activity of the CXCR4-CXCL12 axis could promote the proliferation and survival of transformed HPV positive cells and increase the metastatic potential of HPV-positive cervical cancers (ARNOLDS and SPENCER 2014).

In HPV16/18-infected cells, E6 and E7 were demonstrated to enhance CXCR4 and CXCL12 expression and activity, leading to increased CXCR4-mediated migration, survival, and proliferation (AMINE et al., 2009; CHOW et al., 2010). In the present work, were also analyzed for the first time a possible association between the SNVs rs1801157 of *CXCL12* and rs2228014 of CXCR4 genes and CXCR4 tissue expression, since the CXCL12 variant may alter protein expression and CXCR4 variant may alter the linkage site of some transcription factors as the belonging to the SOX, RXRA and NRSF families (WARD; KELLIS, 2012).

Dai *et al.* (2017) observed that CXCR4 expression was significantly elevated in patients with squamous cell carcinomas and lymph node metastasis, however there was no significant association between CXCR4 expression and FIGO stage or differentiation, which is in accordance with our findings. For Huang et al. (2013) the expression of CXCR4 was associated with histology grade of cervical cancer, and upregulation of CXCR4 expression was positively correlated with the expression of its ligand CXCL12 in both malignant and premalignant epithelia. This suggests a direct link between CXCL12 and CXCR4 in cervical tumorigenesis. CXCL12 and CXCR4 interaction may induce migration of HeLa cells (ZHANG *et al*., 2007). CXCL12 induced the direct migration of HeLa cells with a concentration-dependent model, which was inhibited by CXCR4 monoclonal antibody. Then, the CXCL12/CXCR4 axis probably participates in the metastasis toward lymph nodes in cervical cancer (UCHIDA *et al*., 2003).

Although the role of chemokines and their receptors in human cancers is complex, the chemokine receptors CXCR4 may have a critical role in determining lymph node metastasis in solid tumors (KODAMA, *et al*., 2006). HeLa cells expressing CXCR4 had the chemokine receptor downregulated after treatment with CXCL12 but reappeared on the cell surface after 18 hours which indicates the recycle from the internalized pool of receptors (YANG, 2007).

In the present study a strong CXCR4 immunostaining was observed among cervical cancer patients presenting CXCL12 AA genotype in the recessive genetic models. Although there are no studies demonstrating the influence of CXCL12 rs1801157 variants in its levels during cervical carcinogenesis, CXCL12 binding to CXCR4 generates various signaling mechanisms affecting regulation of angiogenesis, activation of cell invasion, promotion of growth, and inhibition of apoptosis. Expression of CXCR4 receptor on cervical adenocarcinoma cells is associated with invasion of lymph nodes as well as cell proliferation and survival (YANG, 2007).

This study also has some limitations. Preliminarily, there was insufficient sample size of adenocarcinoma, and second, AA from *CXCL12* rs1801157 and TT from *CXCR4* rs2228014 genotypes patients were too low or inexistent in some clinicopathological parameters which difficulted our analysis but these was solved using the genetic models.

The total number of patients reached in this study population is indeed relatively big when biological samples need to be collected.

More investigations are still needed to elucidate the role of the localization and functions of CXCR4 receptor in cervical cancer, specially concerning the role of the genetic variants on the chemokines' levels, and their influence during carcinogenesis.

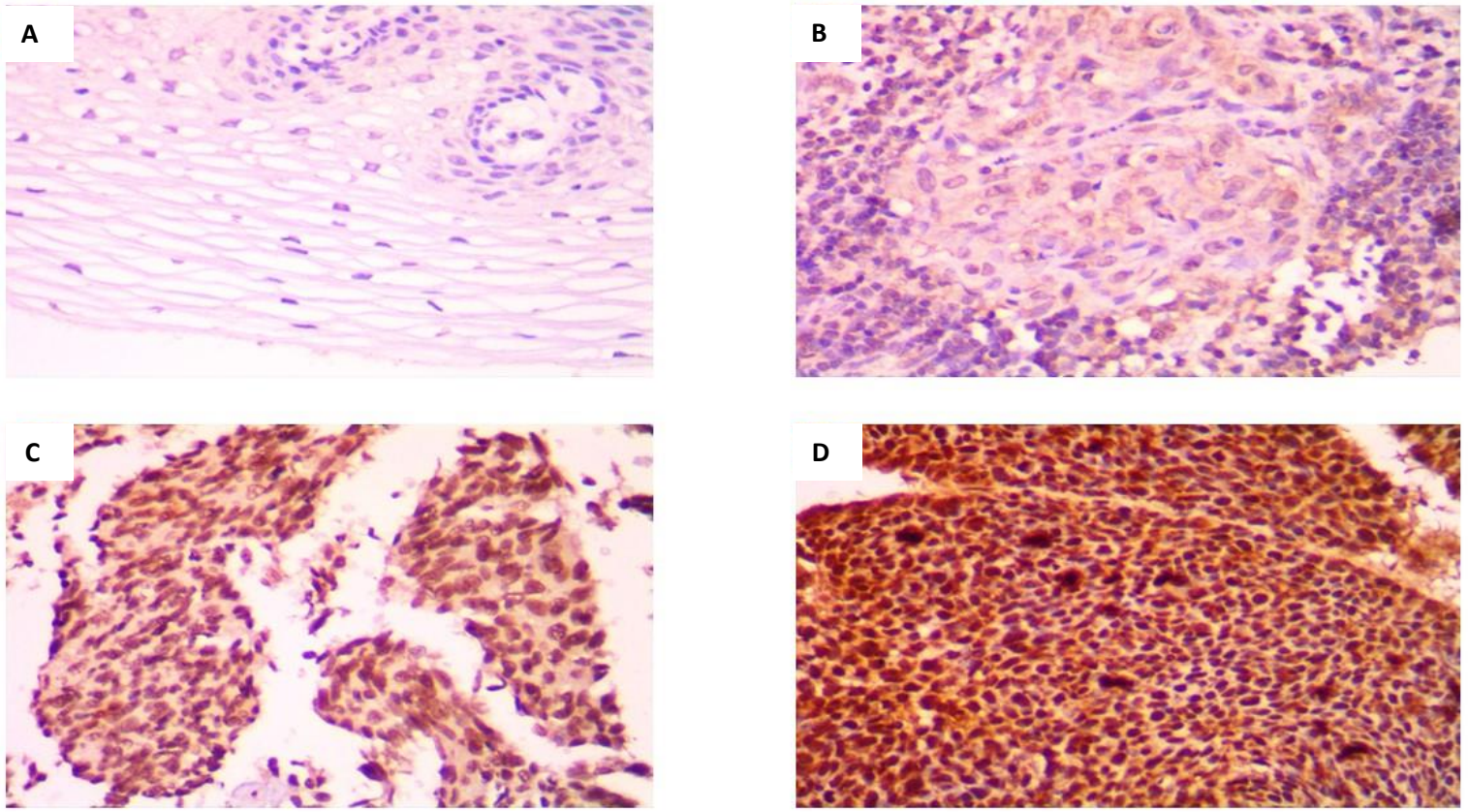

Figure 1. Representative section showing CXCR4 expression in cervical tissue from patients with cervical squamous cell carcinoma (CSCC). A) normal cervical tissue negative for CXCR4 (x400). B) CSCC: CXCR4 weak staining = 1+ (x400). C) CSCC: CXCR4 moderate staining = 2+ (x400). D) CSCC: CXCR4 strong staining = 3+ (x400).

Table 1 Clinicopathological data and median age of patients

	Type of tumor		Histology grade			Stage	
	SSC	AC	WD	MD	LD	I+II	III+IV
N (%)	78 (87.6)	11 (12.4)	12 (20.7)	33 (56.9)	13 (22.4)	51 (58.0)	37 (42.0)
Median age (IQR)	51 (25)	44 (13)	47 (22)	53 (24)	66 (32)	51 (21)	48 (29)
p	0.113			0.066		0.859	

Type of tumor and histology grade were analyzed by Kruskal-Wallis test and Stage were analyzed Mann-Whitney test. $p<0.05$ considered significant. IQR, interquartile range; SSC, squamous cell carcinoma; AC adenocarcinoma; WD, well-differentiated; MD, moderately differentiated; LD, little differentiated.

Table 2 Genotypic distribution of CXCL12 and CXCR4 SNVs and clinicopathological parameters

		Tumor Type		Histology grade			Stage	
SNV		SSC	AC	WD	MD	LD	I + II	III + IV
CXCL12	GG	51 (65.4)	7 (63.6)	8 (66.7)	24 (72.7)	8 (61.5)	35 (68.3)	22 (59.5)
	GA	19 (24.4)	4 (36.4)	3 (25.0)	7 (21.2)	3 (23.1)	4 (27.5)	9 (24.3)
	AA	8 (10.3)	0 (0.0)	1 (8.3)	2 (6.1)	2 (15.4)	2 (3.9)	6 (16.2)
	p	0.434			0.881		0.141	
	χ^2	1.670			1.182		3.924	
CXCR4	CC	54 (69.2)	9 (81.8)	8 (66.7)	22 (66.7)	8 (61.5)	39 (76.5)	23 (62.2)
	CT	21 (26.9)	2 (18.2)	3 (25.0)	9 (27.3)	5 (38.5)	11 (21.6)	12 (32.4)
	TT	3 (3.8)	0 (0.0)	1 (8.3)	2 (6.1)	0 (0.0)	1 (2.0)	2 (5.4)
	p	0.630			0.829		0.311	
	χ^2	0.924			1.489		2.338	

Data presented as absolute number and percentage. Two-sided χ^2 test, with $P<0.05$ considered significant. SSC, squamous cell carcinoma; AC adenocarcinoma; WD, well-differentiated; MD, moderately differentiated; LD, little differentiated.

Table 3 Logistic regression analysis between CXCL12 rs1801157, CXCR4 rs2228014 and clinicopathological parameters

	Odds Ratio (CI95%)			
	Type of Tumor[1]	Histology Grade[2]		Stage[3]
SNV	AC	MD	LD	III + IV
CXCL12				
GA + AA *vs* GG	0.647 (0.117 – 3.563)	1.512 (0.141 – 16.163)	3.222 (0.228 – 45.512)	1.114 (0.360 – 3.445)
CXCR4				
CT + TT *vs* CC	-	2.327 (0.235 – 22.993)	5.449 (0.400 – 74.187)	1.503 (0.430 – 5.258)
CXCL12 * CXCR4				
GA + AA by CT + TT				
vs	-	0.186 (0.006 – 6.104)	0.060 (0.001 – 3.192)	1.672 (0.243 – 11.495)
GG by CC				

[1]Binary logistic regression analysis adjusted by age using SSC (squamous cell carcinoma) as reference.
[2] Multinomial logistic regression analysis adjusted by age using WD (well-differentiated) as reference.
[3] Binary logistic regression analysis adjusted by age using I+II as reference.
* $p<0.05$ as significant; CI, Confidence Interval. SNV, single nucleotide variant

Table 4 Correlation analyses between CXCR4 staining and clinicopathological parameters

		Parameters						
		Type of tumor		Histology grade			Stage	
		Squamous N (%)	Glandular N (%)	Well differentiated N (%)	Moderately differentiated N (%)	Little differentiated N (%)	I + II N (%)	III + IV N (%)
CXCR4 Staining	+	10 (32.3)	0 (0.0)	1 (40.0)	5 (18.2)	1 (0.0)	6 (31.6)	4 (25.0)
	++	14 (45.2)	2 (50.0)	3 (40.0)	7 (54.5)	1 (50.0)	9 (47.3)	7 (43.8)
	+++	7 (22.5)	2 (50.0)	2 (20.0)	4 (27.3)	1 (50.0)	4 (21.1)	5 (31.2)
	τ (*p*-value)	0.100 (0.310)			0.630 (0.959)		0.501 (0.776)	

Kendall's Tau-b correlation coefficient; +: weak, ++: moderate, and +++: strong.

Table 5 Correlation between CXCR4 immunohistochemistry and CXCR4 and CXCL12 SNVs

		CXCR4 models								
		Codominant			Dominant		Recessive		Overdominant	
		CC	CT	TT	CC	CT + TT	CC + CT	TT	CC + TT	CT
		N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
CXCR4 Staining	+	4 (19.0)	5 (41.7)	1 (50.0)	4 (19.0)	6 (42.9)	9 (27.3)	1 (50.0)	5 (21.7)	5 (41.7)
	++	12 (57.1)	3 (25.0)	1 (50.0)	12 (57.1)	4 (28.6)	15 (45.5)	1 (50.0)	13 (56.5)	3 (25.0)
	+++	5 (23.8)	4 (33.3)	0 (0.0)	5 (23.8)	4 (28.6)	9 (27.3)	0 (0.0)	5 (21.7)	4 (33.3)
	τ (*p*-value)	0.356 (0.372)			0.469 (0.198)		0.315 (0.639)		0.759 (0.200)	
		CXCL12 models								
		Codominant			Dominant		Recessive		Overdominant	
		GG	GA	AA	GG	GA+AA	GG + GA	AA	GG + AA	GA
		N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
CXCR4 Staining	+	7 (29.2)	3 (42.9)	0 (0.0)	7 (29.2)	3 (27.3)	10 (32.3)	0 (0.0)	7 (25.0)	3 (42.9)
	++	13 (54.2)	2 (28.6)	1 (25.0)	13 (54.2)	3 (27.3)	15 (48.4)	1 (25.0)	14 (50.0)	2 (28.6)
	+++	4 (16.7)	2 (28.6)	3 (75.0)	4 (16.7)	5 (45.5)	6 (19.4)	3 (75.0)	7 (25.0)	2 (28.6)
	τ (*p*-value)	0.192 (0.116)			0.302 (0.163)		0.040 (0.050)		0.672 (0.545)	

Kendall's Tau-b correlation coefficient; +: weak, ++: moderate, and +++: strong.

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6. CONCLUSÃO

Artigo 1

No presente trabalho, verificamos uma alta frequência de LIE e câncer cervical em mulheres com 55 anos ou mais, fumantes, que recebiam 1 salário mínimo e não conheciam as formas de transmissão do vírus HPV. Em relação as características de comportamento sexual e reprodutivo, mulheres que iniciaram a vida sexual antes dos 17 anos de idade, que tiveram 4 ou mais parceiros sexuais e sem histórico de exame cervical prévio também apresentaram elevada frequência de LIE e câncer de colo de útero.

Em relação a análise dos aspectos imunogenéticos, as frequências alélicas das variantes de nucleotídeo único rs1801157 de *CXCL12* e rs2228014 de *CXCR4* encontradas neste trabalho, se mostrou consistente com a ferramenta Trans-Omics for Precision Medicine, um grande banco de dados utilizado para análises de Whole-Genome Sequencing. Pela primeira vez, foi demonstrado que o SNV rs2228014 de *CXCR4* encontra-se associado a infecção pelo vírus HPV e pacientes portadoras do alelo T desta variante estão mais suscetíveis ao desenvolvimento do câncer cervical. O SNV rs1801157 de *CXCL12*, mostrou-se associado ao desenvolvimento de lesão intraepitelial escamosa e cancer cervical. De forma inédita na literatura, o presente estudo mostrou com análise de interação entre os SNVs rs1801157 de *CXCL12* e rs2228014 de *CXCR4,* que os alelos variantes de ambos polimorfismos, quando presentes simultaneamente, estão associados a maior suscetibilidade à infecção pelo HPV, ao desenvolvimento de lesão intraepitelial escamosa cervical e ao câncer de colo de útero.

Artigo 2

Neste trabalho, a análise dos SNVs rs1801157 de *CXCL12* e rs2228014 de *CXCR4* em relação aos parâmetros clinicopatológicos como tipo de tumor, grau histológico e estadiamento, não mostrou associação entre as variantes genéticas e as variáveis analisadas. Quando realizada a análise de interação entre os SNVs, conclui-se também que a interação das variantes não parece influenciar nos parâmetros clinicopatológicos analisados.

Em relação a marcação da proteína CXCR4 e os dados clinicopatológicos não houve correlação entre os graus de marcação (+, fraca; ++, moderada; +++, forte) e as variáveis analisadas. No que diz respeito a análise da marcação de CXCR4 em relação as variantes rs1801157 de *CXCL12* e rs2228014 de *CXCR4*, também não se mostrou associação significante, embora o genótipo AA de CXCL12 tenha se aproximado da significância estatística.

7. CONSIDERAÇÃO FINAL

A infecção pelo vírus HPV tem papel importante no desenvolvimento do câncer cervical, porém, fatores intrínsecos ao indivíduo podem estar diretamente relacionados a progressão da doença. Na busca por um melhor entendimento, é de suma importância o conhecimento de mecanismos possivelmente associados a suscetibilidade e prognóstico para esclarecer o que leva apenas parte das mulheres infectadas a desenvolverem o câncer. Neste sentido, fatores imunogenéticos que podem relacionar-se a doença vem sendo intensamente investigados por este grupo de pesquisa. Este estudo mostra de forma inédita que a análise de interação entre as variantes de nucleotídeo único rs1801157 de *CXCL12* e rs2228014 de *CXCR4* está associada com a infecção por HPV bem como com o desenvolvimento de lesão intraepitelial escamosa cervical e com o câncer cervical. Neste estudo foi analisada também, pela primeira vez, a possível associação dos SNVs em relação imunomarcação de CXCR4 e em relação a mesma imunomarcação com os parâmetros clinicopatológicos como tipo de tumor, grau histológico e estadiamento no câncer cervical. Apesar dos achados inéditos demonstrados, estudos futuros são necessários para elucidar os mecanismos moleculares e validar os resultados encontrados no presente trabalho.

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APENDICE 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:_____

APENDICE 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 A

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