UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
DEPARTAMENTO DE MORFOLOGIA E FISIOLOGIA ANIMAL
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL TROPICAL

ESTUDO DE ASSOCIAÇÃO DE POLIMORFISMOS NOS GENES CCR2 e CCR5 COM O DESENVOLVIMENTO DE LESÕES CERVICAIS INDUZIDAS PELO HPV

ERINALDO UBIRAJARA DAMASCENO DOS SANTOS

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Dissertação apresentada para o Programa de Pós-graduação em Ciência Animal Tropical da Universidade Federal Rural de Pernambuco (PGCAT-UFRPE), como requisito para obtenção do título de Mestre em Ciência Animal Tropical.

Orientador: Profº. Drº. Paulo Roberto Eleutério De Souza

Co-orientadora: Profª. Drª Maria de Mascena Diniz Maia

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Sumário

Lista de Figuras

Lista de Tabelas

Artigo I

Lista de Abreviaturas, Siglas e Símbolos

CC	Câncer cervical
CCL2	Quimiocina Ligante 2
CCL5	Quimiocina Ligante 5
CCR2	Receptor de quimiocina 2
CCR2-64I	Variante polimórfica do receptor de quimiocinas 2
CCR5	Receptor de quimiocina 5
CCR5Δ32	Variante polimórfica do receptor de quimiocinas 5
DNA	Ácido Desoxirribonucleico
E	Proteína Precoce do *papillomavírus humano*
E1	Proteína Precoce 1 do *papillomavírus humano*
E2	Proteína Precoce 2 do *papillomavírus humano*
E3	Proteína Precoce 3 do *papillomavírus humano*
E4	Proteína Precoce 4 do *papillomavírus humano*
E5	Proteína Precoce 5 do *papillomavírus humano*
E6	Proteína Precoce 6 do *papillomavírus humano*
E7	Proteína Precoce 7 do *papillomavírus humano*
E8	Proteína Precoce 8 do *papillomavírus humano*
HPV	*Papillomavírus humano*
HR-HPV	HPV de alto risco oncogênico (do inglês, high-risk hpv)
HL-HPV	HPV de baixo risco oncogênico (do inglês, low-risk hpv)

IARC	Agência de Pesquina no Câncer
L	Proteína Tardia Presente no *papillomavírus humano*
L1	Proteína Tardia 1 presente no *papillomavírus humano*
L2	Proteína Tardia 2 presente no *papillomavírus humano*
LCR	Longa Região de Controle presente no *papillomavírus humano*
NIC	Neoplasias Intraepiteliais Cervicais
pb	Pares de Base
PCR	Reação em Cadeia da Polimerase
pRb	Proteina do Retinoblastoma
PV	*Papillomavírus*
RFLP	Polimorfismo de Comprimento de Fragmento de Restrição
SNP	Polimorfismos de Nucleotideo Único

Resumo

O câncer cervical (CC) afeta cerca de meio milhão de mulheres a cada ano em todo o mundo. O principal agente etiológico que pode levar ao desenvolvimento do CC é a infecção por Papillomavírus humano (HPV). Porém, nem todas as mulheres infectadas pelo HPV terão uma progressão para o câncer, visto que, o desenvolvimento neoplásico envolve fatores imunológicos, genéticos e ambientais. Os receptores de quimiocinas desempenham um importante papel na resposta imunológica e progressão de neoplasia intraepitelial cervical (NIC) para o CC. Variações genéticas relacionados com os genes destes receptores podem levar a formação de neoplasia cervical. O presente estudo teve como objetivo associar polimorfismos nos genes receptores das quimiocinas *CCR2-64I* (rs1799864) and *CCR5-Δ32* (rs333) com a susceptibilidade para o desenvolvimento de lesão cervical (NIC ou CC) em mulheres residentes no Estado Pernambuco-Brasil. A população de estudo consistiu de 139 mulheres com lesões cervicais (pacientes) e 151 mulheres saudáveis (controles). Os polimorfismos *CCR2-64I* e *CCR5-Δ32* foram analisados pela técnica da PCR-RFLP. A detecção do HPV foi realizada através da técnica de PCR convencional. Um efeito protetor foi observado para indivíduos carreadores de um dos genótipos mutantes (GA ou AA) em relação aos indivíduos com lesão cervical para o polimorfismo no gene *CCR2-64I* (OR = 0,37, p= 0,0008). O mesmo foi observado para o alelo A (OR = 0,39, P = 0,0002). Contrariamente, nenhuma associação para o polimorfismo no gene *CCR5-Δ32* foi observada (p> 0,05). A prevalência dos tipos de HPV mostrou que 38,8% dos pacientes estavam infectados pelo HPV16; 22,3% HPV 18; 2,9% HPV31; 3,6% HPV 33; e 14,4% por outros tipos de HPV. Em relação a infecção múltipla, 18% dos pacientes foram co-infectados pelos tipos 16 e 18. Quando analisada a associação do tipo de HPV com o polimorfismo no gene *CCR2-64I*, entre os indivíduos do grupo de pacientes, observou-se um efeito protetor da infecção para o HPV 16 (OR = 0,35, p = 0,0184). Além disso, quando os pacientes foram estratificados de acordo com a gravidade das lesões cervicais, 28,78% (40/139) apresentaram NIC I (lesão de baixo grau), 62,58% (87/139) tinham NIC II ou III (lesão de alto grau) e 8,63% (12/139) tiveram CC. Em resumo, nosso estudo mostrou um efeito protetor do polimorfismo *CCR2-64I* tanto para susceptibilidade para a infecção pelo HPV 16 como para o desenvolvimento de lesões cervicais (CIN e CC).

Abstract

Cervical cancer (CC) affects about half a million women each year worldwide. The main etiological agent that can lead to the development of the CC is the human papillomavirus (HPV). However, not all women infected with HPV will have a progression to cancer, since the neoplastic development involves immune, genetic and environmental factors. Chemokine receptors play an important role in immune response, and progression of cervical intraepithelial neoplasia (CIN) for CC. Genetic variations related to the genes of these receptors may lead to the formation of cervical neoplasia. This study aimed to associate polymorphisms in genes of CCR2-64I chemokine receptor (rs1799864) and CCR5-Δ32 (rs333) with susceptibility to the development of cervical lesions (CIN or CC) in women from the State of Pernambuco, Brazil. The study population consisted of 139 women with cervical lesions (patients) and 151 healthy women (controls). The CCR2-64I and CCR5-Δ32 polymorphisms were analyzed by the technique of PCR-RFLP. The HPV detection was performed using the standard PCR technique. A protective effect for individuals carriers of a mutant genotypes (GA or AA) for individuals with cervical injury to the polymorphism in CCR2-64I gene (OR = 0.37, p = 0.0008). The same was observed for the A allele (OR = 0.39, P = 0.0002). In contrast, no association to the polymorphism in the CCR5-Δ32 gene was observed (p> 0.05). The prevalence of HPV types showed that 38.8% of patients were infected with HPV16; 22.3% HPV 18; HPV31 2.9%; 3.6% HPV 33; and 14.4% for other types of HPV. For multiple infection 18% of patients were co-infected with types 16 and 18. When we analyzed the association of HPV type with CCR2-64I polymorphism in the gene between individuals of the group of patients there is an effect protector of infection for HPV 16 (OR = 0.35, p = 0.0184). Moreover, when patients were stratified according to the severity of cervical lesions, 28.78% (40/139) had CIN I (low grade lesion), 62.58% (87/139) had CIN II or III (high-grade lesions) and 8.63% (12/139) had CC. In summary, our study showed CCR2-64I polymorphism protective effect of both susceptibility to infection with HPV 16 and for the development of cervical lesions (CIN and CC).

1. Introdução

O Câncer Cervical (CC), também conhecido como câncer do colo do útero, é uma das principais neoplasias que afetam mulheres em todo o mundo. Cerca de meio milhão de mulheres são afetadas por esse tipo de câncer a cada ano, segundo a *Agency for Research on Cancer* (IARC) (De Casadevante et al., 2015). Dentre os principais fatores de risco para o desenvolvimento do CC, destaca-se a infecção pelo Paillomavírus Humano (HPV) encontrado em 99% dos casos de CC (de Oliveira et al., 2013).

O papilomavírus (PV) faz parte de um grupo diversificado de vírus que infectam mamíferos, aves e répteis (Freitas et al., 2011). O HPV é um vírus pequeno não envelopado de simetria icosaédrica, com genoma de DNA dupla fita circular, com cerca de 8.000 pares de bases (García-Espinosa et al., 2009). Mostrando um tropismo específico para as células humanas epiteliais da pele e de membranas mucosas (Bernard et al., 2010). São classificados de acordo com a propensão das células infectadas à transformação neoplásica, em alto ou baixo risco oncogênico (de Villiers et al., 2004). Os tipos de HPV considerados de baixo risco oncogênico (LR-HPV) são representados principalmente pelos tipos 6, 11, 40, 42, 43, 54, 61, 70, 72 e 81 e se destacam por formar verrugas. Por outro lado, os HPVs de alto risco oncogênico (HR-HPV) frequentemente são associados com o desenvolvimento de neoplasias intraepiteliais cervicais (NIC) sendo representado principalmente pelos tipos 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 e 82 (Muñoz et al., 2003). Existem aproximadamente 120 tipos de HPVs que têm sido identificados até o momento. Destes, 18 são classificados como sendo HR-HPV (Bouvard et al. 2009; Bernard et al. 2010).

A maioria das mulheres infectadas com um dos tipos de HR-HPV não desenvolverá CC, uma vez que, o processo carcinogênico depende de outros fatores como imunológicos, genéticos e ambientais (Zheng et al., 2006). Dentre os produtos génicos envolvidos na mucosa cervical, quimiocinas e seus receptores têm demonstrado um papel chave na imunidade contra tumores cervicais (Ghaderi et al., 2000, Ohta et al., 2002). As quimiocinas são proteínas quimioatrativas de baixo peso molecular que promovem a aderência das células alvo, além disso, estão envolvidas em processos de

angiogênese, direcionamento de fagócitos, linfócitos e de órgãos linfóides secundários (Rossi & Zlotnik, 2000, Kleine-Lowinski et al., 2003, Zheng et al., 2006).

Os receptores para as quimiocinas são expressos principalmente em células do sistema imunológico e ajudam na diferenciação e na migração destas células para os tecidos inflamados (Bromley et al., 2005).

O receptor de quimiocina 5 (*CCR5*) é o principal receptor de quimiocina e seus ligantes são o MIP-1α / CCL3, MIP-1β / CCL4, e RANTES / CCL5 (Lehner 2002, Ahmadabadi et al. 2012). Variações polimórficas deste gene, em particular a mutação Δ32 (uma deleção de 32 pares de base do gene CCR5) leva à diminuição da expressão e disfunção do receptor CCR5 (Ahmadabadi et al. 2012, Nahon et al., 2008). Os estudos relatam que os indivíduos homozigotos para o polimorfismo no gene *CCR5-Δ32* (rs 333) reduziram o risco para a asma e enfarte do miocárdio, atenuação da severidade na artrite reumatoide e lenta progressão para AIDS (Berger et al., 1999, Hall et al., 1999, Zapico et al., 2000, Gonzalez et al., 2001). Além disso, em conjunto com o *CCR2*, funcionam como co-receptores para o HIV-1 (Zheng et al., 2006).

O receptor de quimiocina 2 *(CCR2*) é o receptor para quimiocina ligante 2 (CCL2), também conhecido como quimioatrativa de monócitos da proteína-1 (MCP-1), sendo associada a carcinogênese e angiogênese (Charo et al., 1994, Naohiko et al., 2004, Huang et al. 2013). O polimorfismo de nucleotídeo único (SNP) no códon 64 (CCR2-64I, rs1799864) do gene CCR2 que codifica a isoleucina (ATC) em vez de valina (GTC) tem sido amplamente estudado, e há relatos de associação entre este polimorfismo e o efeito protetor na progressão de doenças inflamatórias tais como esclerose múltipla (Miyagishi et al., 2003), aterosclerose da carótida (Nyquist et al., 2009) e no desenvolvimento de cancro da mama (Zafiropoulos et al., 2009). No entanto, resultados contraditórios acerca do papel do polimorfismo do CCR5 e CCR2 no desenvolvimento de CC têm sido relatados até agora (Zheng et al 2006, Coelhoet al., 2005, Ivansson et al., 2007, Chatterjee et al., 2010).

Diante disto, o objetivo do presente estudo foi analisar a associação de ambos os polimorfismos nos genes *CCR2-64I* e *CCR5-Δ32* com o desenvolvimento de lesão cervical (NIC ou CC) em mulheres infectadas por HPV do Estado de Pernambuco.

2. Fundamentação teórica

2.1. Distribuição mundial do câncer do colo uterino

Com uma incidência em todo o mundo de cerca de meio milhão de casos por ano principalmente em países em desenvolvimento o câncer cervical (CC) permanece como um dos mais importantes e danosos cânceres da mulher (Neto, 1991; IARC, 2013).

As últimas estatísticas publicadas pela *Agency for Research on Cancer* (IARC) a agência especializada em câncer da Organização Mundial da Saúde, mostra que o câncer do colo do útero ocupa a quarta posição na lista dos cânceres mais comuns que afetam mulheres de todo o mundo, precedidos pelo câncer de mama, colorretal, e de pulmão (De Casadevante et al., 2015). Mais especificamente, a incidência estimada de câncer do colo do útero foi de 527.624 novos casos em 2012. No mesmo ano, está neoplasia foi responsável por 265.653 mortes no mundo, que constituiu a quarta causa mais comum de morte por câncer em todo o mundo (De Casadevante et al., 2015).

O CC é frequentemente definido como uma doença de disparidade, porque afeta diferentemente os países pobres e ricos, pelo menos 80% das mortes por câncer do colo do útero ocorrem nos países em desenvolvimento (Ferlay et al., 2012). A mortalidade varia de 18 vezes entre as diferentes regiões do mundo, com taxas que variam de menos de 2 casos por 100.000 habitantes na Ásia Ocidental, Europa Ocidental, Austrália e Nova Zelândia para mais de 20 por 100.000 nas regiões da Melanésia, Médio (20,6), Oriental (22,2) e África (27,6) (Ferlay et al., 2012) (Figura 1).

No Brasil, bem como em outros países em desenvolvimento, o câncer do colo do útero corresponde ao terceiro tipo de câncer feminino mais frequente, com estimativa de 16.340 casos novos para 2016 e risco de 17 casos para cada 100.000 mulheres, perdendo em termos de frequência, para o câncer de mama, com 57.960 casos novos para o ano de 2016 e para o câncer colorretal com 42.280 novos casos do para o ano de 2016 (INCA, 2016).

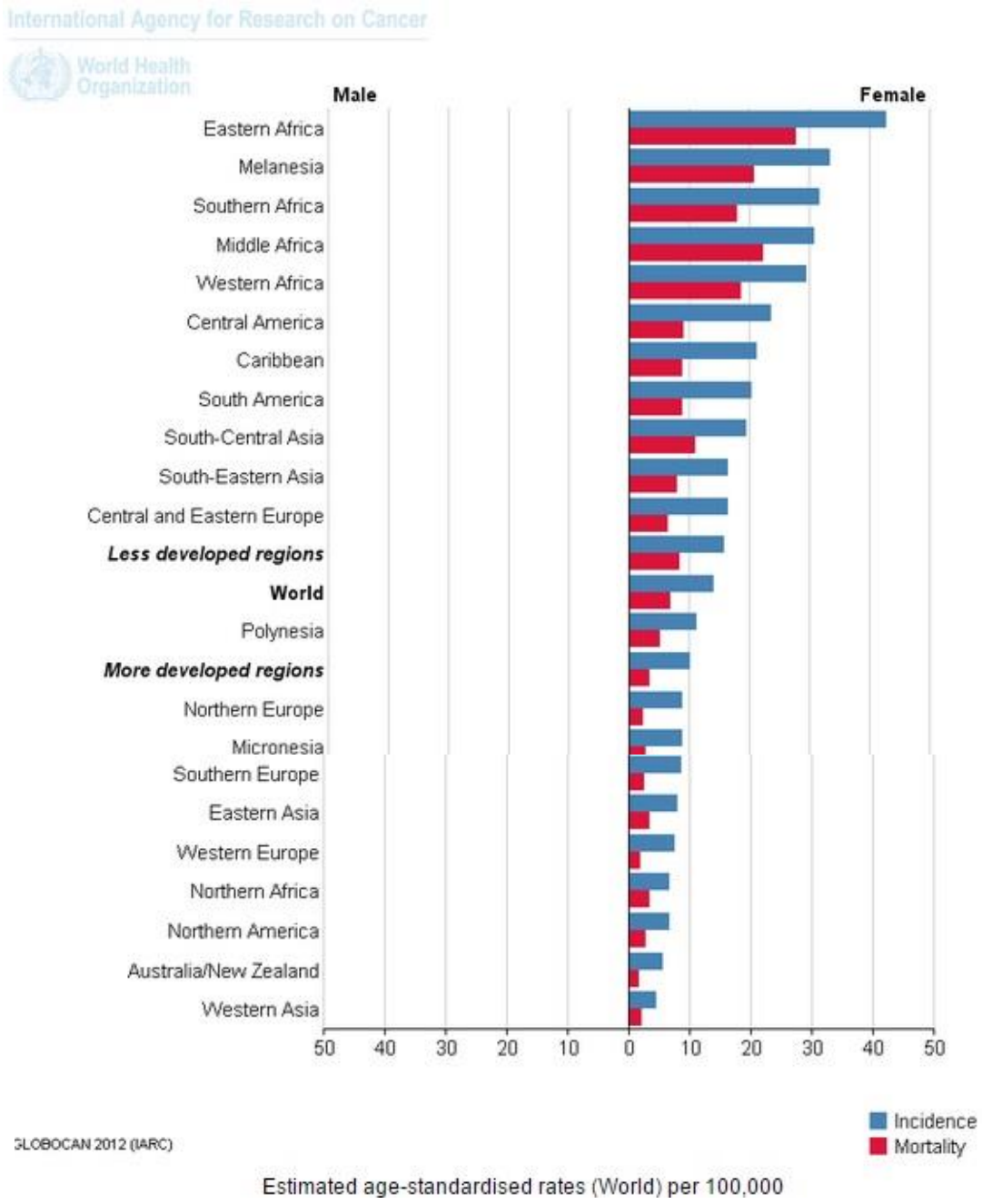

Figura 1: Incidência e mortalidade do Câncer de colo de útero. (IARC, 2012).

A região Nordeste do Brasil apresenta a maior incidência de CC, com uma incidência de 5.370 novos casos, as regiões Sudeste e sul correspondem a 4.370 e 2.320 novos casos, respectivamente. A região norte apresenta incidência de 1.890 casos e, o Centro-Oeste com 1.640 casos para o ano de 2016 (INCA, 2016). Evidenciando-se assim que a disparidade do câncer do colo do útero varia consideravelmente dentro de cada região (Kesic et al., 2012).

Pernambuco, para o ano de 2014, tem estimativa de CC de 970 casos novos para cada 100.000 habitantes. No município do Recife, também para o mesmo período, a estimativa é de 180 novos casos para cada 100.000 habitantes (INCA, 2014).

2.2. Histologia do colo do útero

Conforme descrito por Neto em 1991 e aceito até os dias de hoje, existem dois tipos celulares básicos que formam o revestimento do colo uterino, as células epiteliais escamosas estratificadas e células epiteliais glandulares. Onde o encontro destes dois tipos celulares forma a junção escamo-colunar (INCA, 2012). Nesse local se iniciam as alterações celulares da cérvice, ocorrendo as metaplasias que são transformações de um epitélio maduro em outro epitélio maduro, isto é, no colo, o epitélio glandular se transforma em epitélio escamoso. Deste modo, essa região é onde se inicia a oncogênese no colo uterino (INCA, 2012).

O CC habitualmente inicia-se como neoplasia intraepitelial cervical (NIC), uma condição pré-invasiva limitada ao epitélio cervical, conforme a classificação histológica, ou como lesão intraepitelial escamosa, de acordo com o diagnóstico citológico (Rama et al., 2008). A NIC também pode ser classificada em graus de acordo com a espessura epitelial acometida: no grau 1 (NIC 1), a alteração celular acomete as camadas mais baixas do epitélio, equivalendo a um terço desse; no grau 2 (NIC 2), o desarranjo celular ocorre em dois terços do epitélio; e, no grau 3 (NIC 3), toda a espessura epitelial é acometida, respeitando a membrana basal, sem invasão do tecido conjuntivo subjacente (Rama et al., 2008; INCA, 2012).

2.3. Rastreamento e diagnóstico das lesões pré-neoplásicas e neoplásicas

Historicamente, o rastreamento para o câncer cervical é baseado no exame citológico do esfregaço cervical (Papanicolaou), utilizado há mais de 50 anos (Forbes et al., 2002). Consiste no esfoliamento das células do colo uterino para estudo ao microscópio, com o objetivo de identificação de lesões pré-neoplásicas e neoplásicas em fases iniciais, antes mesmo do aparecimento dos sintomas (INCA, 2011). Visto que, o diagnóstico das lesões glandulares da cérvice é realizado pela associação da citologia, colposcopia e estudo histopatológico (McIntyre, 2005; IARC, 2012).

A colposcopia baseia-se na visualização do colo através do colposcópio, cuja iluminação e lentes permitem a magnificação da visão (McIntyre, 2005). Além disso, orienta a melhor topografia para realização de biópsias e cirurgias de alta frequência (CAF), obtendo-se material para o estudo histopatológico (McIntyre, 2005; IARC, 2012).

Nos países onde há eficientes programas de rastreio é possível comparar as taxas de cobertura às curvas de sobrevida para o câncer cervical, pois a identificação de lesões pré-malignas reduz a incidência e previne o câncer em estágios mais agressivos (Forbes et al., 2002).

Apesar do sucesso na prevenção do câncer cervical, alguns estudos relatam que as lesões glandulares são pouco expressivas na colposcopia e na citologia (Dalrymple et al., 2008). Apenas 51,9% das lesões glandulares são diagnosticadas pela citologia, tornando o estudo histopatológico após conização do colo uterino o método mais acurado (Van et al., 2004; Dalrymple et al., 2008). Cerca de 30% dos diagnósticos histológicos de NICs grau 2 e 3 são negativos nos esfregaços citológicos (Ferreccio et al., 2003). Cerca de 10% das lesões histologicamente classificadas como NIC 1 podem evoluir para NIC 2 ou 3; estima-se que 22% dos casos não tratados de NIC 2, por sua vez, possam evoluir para NIC 3. As mulheres com NIC 3, que inclui o carcinoma in situ, apresentam risco substancial para o câncer cervical invasivo (Pagliusi & Teresa Agudo, 2004).

2.4. Fatores associado ao desenvolvimento de lesão cervical

O fator de risco mais importante para o desenvolvimento de lesão cervical (CIN ou CC) é a infecção pelo *Papillomavírus Humano* (HPV). Vários outros fatores foram encontrados para aumentar o risco de CC, possivelmente através da sua relação com o risco de infecção pelo HPV: número de parceiros sexuais, atividade sexual precoce, paridade, o uso a longo prazo de contraceptivos orais, tabagismo, Vírus da Imunodeficiência Humana (HIV) e *Chlamydia trachomatis* (CT) (Plummer et al., 2012; Franceschi et al., 2006; Kjellberg et al., 2000; Applebey et al., 2007a, 2007b; Denny et al., 2012; De Vuyst et al., 2012; Tavares et al., 2014).

Além desses fatores, se destacam também os fatores genéticos do paciente e características virais como tipo, variante, carga viral, integração do vírus (Wang & Hildesheim, 2003). Uma vez que, a possibilidade de predisposição genética é reforçada pela observação de um risco de duas vezes mais de desenvolver câncer cervical em parentes de primeiro grau biológico (Magnusson et al., 1999).

2.5. *Papillomavírus Humano* (HPV)

Os Papilomavírus (PV) são um grupo diversificado de vírus que infectam mamíferos, aves e repteis (Freitas et al., 2011). Eles são caracterizados por serem espécie-específicos e não infectarem outro hospedeiro que não seja o seu natural mesmo quando submetidos a condições experimentais (Campo, 2006).

Inicialmente, os papilomavírus eram classificados na subfamília Papilomavirinae, dentro da família Papovaviridade que incluíam os Polyomavírus (Bernard, 2005). Posteriormente, os PV foram re-classificados e formam a grande família Papilomaviridae, que compreende 29 gêneros (Alphapapillomavirus a Dyoiotapapillomavirus) e mais de 200 tipos virais (de Villiers et al., 2004; Bernard et al., 2010).

O Papilomavírus humano (HPV) é um vírus pequeno não envelopado de simetria icosaédrica, com capsídeo composto por 72 capsômeros e um genoma de DNA dupla fita circular, com cerca de 8.000 pares de bases (García-Espinosa et al., 2009). Mostrando um tropismo específico para as células humanas epiteliais da pele e membranas mucosas (Bernard et al., 2010).

Até agora, 120 tipos de HPV foram totalmente caracterizados, compreendendo tanto o cutâneo que são os HPV que causam manifestações clínicas benignas conhecidas como verrugas de pele (papiloma) e o tipo de HPV que infectam as mucosas induzindo a papiloma benigna, câncer invasivo e neoplásias intraepiteliais na mucosa anogenital, bem como no trato respiratório (sinonasal, laringe, traquéia, brônquios) e trato digestivo superior (mucosa oral, orofaringe, esófago) (Syrjänen & Syrjänen, 2000).

Embora, existam mais de 120 tipos de HPV identificados, cerca de 40 destes infectam o trato genital feminino (García-Espinosa et al., 2009). Os tipos de HPV são classificados entre vírus de alto ou baixo risco oncogênico, de acordo com a propensão

das células infectadas à transformação neoplásica (de Villiers et al., 2004). Os tipos de HPV considerados de baixo risco oncogênico (LR-HPV) são representados principalmente pelos tipos 6, 11, 40, 42, 43, 54, 61, 70, 72 e 81. Aqueles considerados, HPV alto risco oncogênico (HR-HPV), por estarem frequentemente associados com as lesões cervicais de NICs 2 e 3 e às neoplasias invasoras, são representados principalmente pelos tipos 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 e 82 (Muñoz et al., 2003).

O risco oncogênico do vírus está diretamente relacionado ao comportamento de seu genoma no núcleo da célula hospedeira. HPVs de baixo risco oncogênico tendem a manter o seu DNA íntegro, circular e episssomal, diferente dos HR-HPVs, cujas fitas de DNA circular se abrem, sofrem deleções e se integram ao genoma da célula hospedeira (Scheurer et al.,2005; Muñoz et al., 2006).

O genoma do HPV possui oito regiões conhecidas como fases de leitura aberta (*Open Reading Frames*) e uma região não-codificadora. As fases de leitura aberta são organizadas em três regiões: a região precoce (E, early), região tardia (L, *late*) e longa região de controle (LCR, *Longe control region*) (Muñoz et al., 2003). A região precoce (composta pelos genes E1, E2, E4, E5, E6, E7), a região tardia (composta pelos genes L1 e L2), e a região controladora (URR) (Muñoz et al., 2003) (Figura 2). Essas três regiões dos HPVs são separadas por dois sítios de poliadenilação um na região precoce (AE) e outro na região tardia (AL) (Zheng & Baker, 2006).

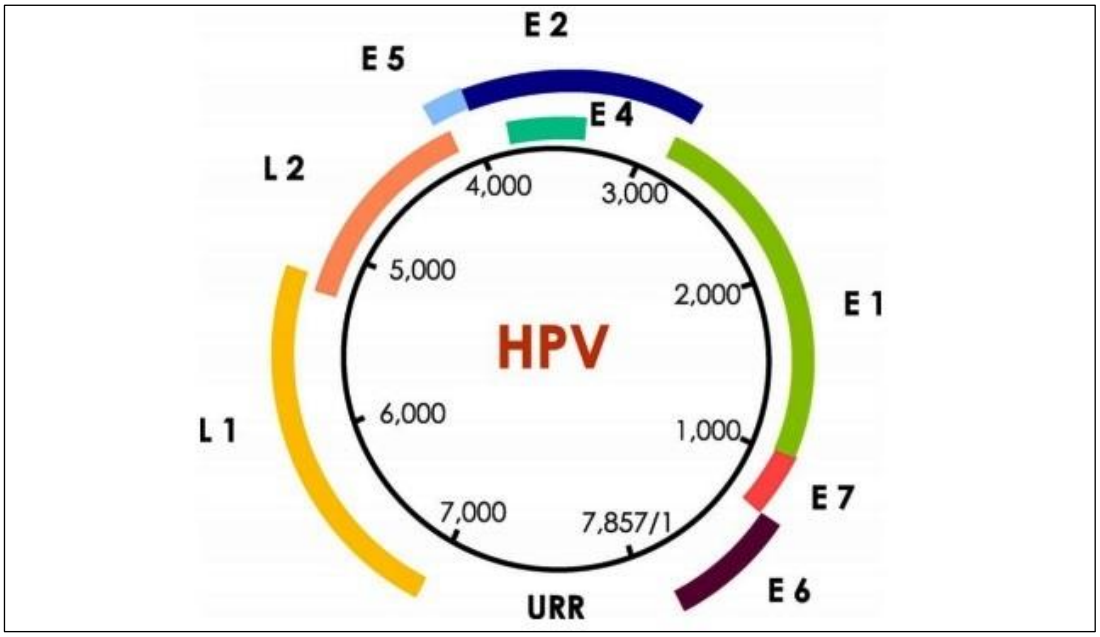

Figura 2: Representação esquemática do genoma de HPV que mostra o arranjo dos genes precoces E ou não estruturais, os genes do capsídeo (L1 e L2) e a região reguladora (URR) (Muñoz et al., 2003).

Resumidamente, os genes E1 e E2 codificam proteínas que são vitais para a replicação do DNA viral e controle da transcrição gênica do vírus. A proteína E4 é expressa nos estágios tardios da infecção e tem um papel importante na alteração da matriz intracelular, maturação e liberação das novas partículas virais (Muñoz et al., 2006). As proteínas E6 e E7 são importantes para a amplificação do genoma viral. As regiões tardias L1 e L2 codificam as proteínas virais dos capsídeos durante os últimos estágios da replicação dos vírus (Scheurer et al.,2005; Muñoz et al., 2006).

O ciclo infeccioso dos HPVs está ligado a diferenciação epitelial, visto que, estes vírus se replicam no epitélio escamoso estratificado de peles e mucosas. As células infectadas se dividem e se espalham lateralmente (Frazer, 2004) (Figura 03). Outras células se deslocam para as camadas suprabasais e se diferenciam. Neste processo, alguns genes virais são ativados viabilizando a formação do capsídeo viral (Frazer, 2004).

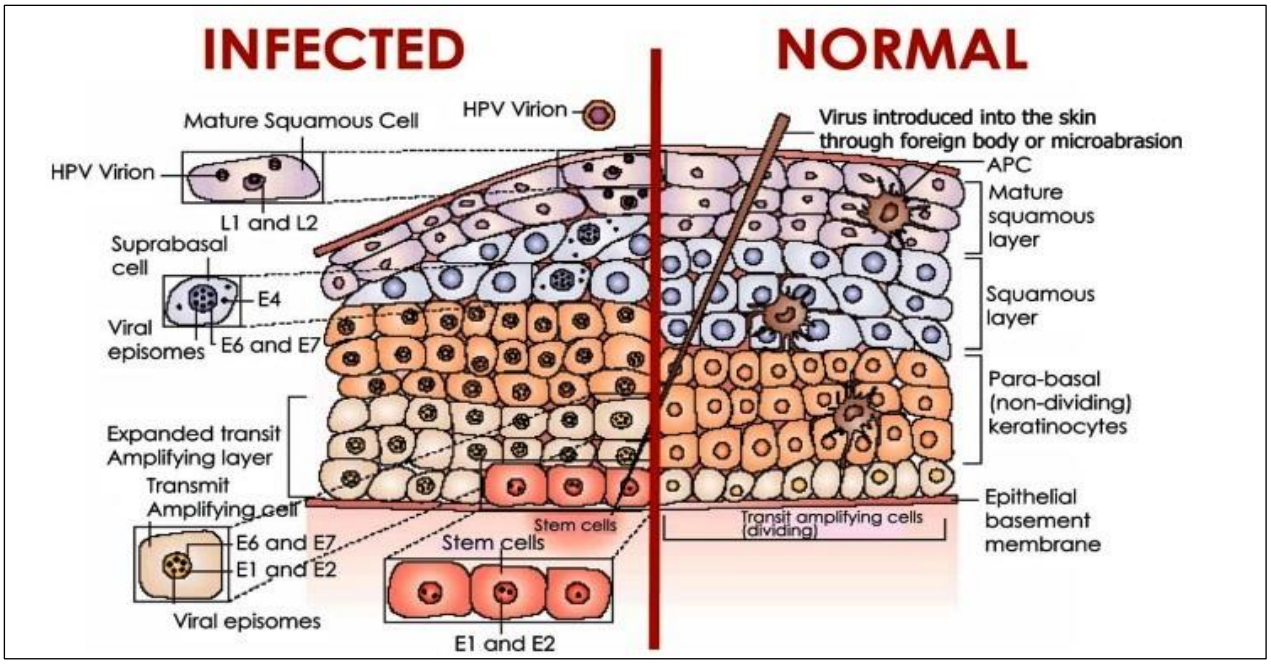

Figura 3: A localização no epitélio escamoso das principais fases do ciclo de vida do vírus do papiloma. Epitélio escamoso estratificado do colo do útero de células epiteliais e a expressão de proteínas do HPV após a infecção. Células filhas de células-tronco epiteliais se dividem ao longo da membrana basal e, em seguida, amadurecem verticalmente através do epitélio sem mais divisão (lado direito). Após a introdução do HPV para dentro das células estaminais na camada basal do epitélio, expressão de proteínas não estruturais virais ocorre. Também ocorre a regulação destas proteínas, a população de célula em divisão expande verticalmente e diferenciação de células epiteliais é retardada e é menos completa. As proteínas virais são expressas, sequencialmente, com a diferenciação, como mostrado, e os viríons maduros são produzidos apenas nas camadas superficiais do epitélio. As células apresentadoras de antigénio (APCs) intraepitelial estão esgotadas no epitélio infectadas com o HPV (Frazen, 2004).

Não foi ainda completamente elucidado. Como os fatores carcinogênicos e agentes promotores estão envolvidos no desenvolvimento de papilomas e carcinomas, porém já foram descobertos dois estágios no mecanismo de proteínas carcinogênese, a iniciação e a promoção, que possui componentes independentes (zur Hausen, 1996).

Entretanto, a ação oncogênica viral envolve a expressão de genes que codificam as proteínas precoces (E6 e E7) na célula hospedeira. Essas oncoproteínas interferem no controle do ciclo celular através da interação com proteínas específicas, tais como a proteína 53 (p53) e a proteína do retinoblastoma (pRB) (Campo, 2003).

Tendo em conta a diversidade de eventos que o HPV pode desencadear. Tipos de HPV induzindo infecções assintomáticas ou transientes podem utilizar estratégias distintas para a transmissão e propagação dentro do epitélio, e também para as suas interações com o sistema imunitário (Doorbar et al., 2012).

2.6. Sistema imunológico

A função imunológica tem sido conceitualmente dividida em imunidade inata e imunidade adaptativa (Medzhitov & Janeway, 2000). A imunidade inata representa uma resposta rápida e estereotipada a um número grande, mas limitado, de estímulos (Leibowitz & Schiffrin, 2011). É representada por barreiras físicas, químicas e biológicas, células especializadas e moléculas solúveis, presentes em todos os indivíduos, independentemente de contato prévio com imunógenos ou agentes agressores, e não se altera qualitativa ou quantitativamente após o contato (Medzhitov & Janeway, 2000). As principais células efetoras da imunidade inata são: macrófagos, neutrófilos, células dendríticas e células Natural Killer (Leibowitz & Schiffrin, 2011). Fagocitose, liberação de mediadores inflamatórios, ativação de proteínas mediadoras, bem como síntese de proteínas de fase aguda, citocinas e quimiocinas são os principais mecanismos na imunidade inata (Leibowitz & Schiffrin, 2011).

Em contraposição à resposta inata, a resposta imune adaptativa depende da ativação de células especializadas, os linfócitos, e das moléculas solúveis por eles produzidas (Delves & Roitt, 2000). As principais características da resposta adquirida são: especificidade e diversidade de reconhecimento, memória, especialização de resposta, autolimitação e tolerância a componentes do próprio organismo (Leibowitz &

Schiffrin, 2011). Embora as principais células envolvidas na resposta imune adquirida sejam os linfócitos, as células apresentadoras de antígenos (APCs) desempenham papel fundamental em sua ativação, apresentando antígenos associados a moléculas do complexo de histocompatibilidade principal (MHC, *major histocompatibility complex*) para os linfócitos T (LT) (Delves & Roitt, 2000).

2.7. Quimiocinas e seus receptores

As quimiocinas constituem um grupo de proteínas de baixo peso molecular (8–15 kDa) que se ligam às proteínas G acopladas aos receptores de quimiocinas (Raman et al., 2011). Elas desempenham um importante papel na migração celular, no desenvolvimento, a vigilância imunitária, inflamação, bem como em muitas condições patológicas (Raman et al., 2011).

As quimiocinas e os seus receptores são divididos em quatro famílias com base no padrão de resíduos de cisteína: CXC, CC, C e CX3C, em que C representa a cisteína e o X representa os aminoácidos (Zlotnik & Yoshie, 2000; Murphy et al., 2000). Cerca de 20 receptores de quimiocinas e 50 quimiocinas foram identificados em humanos (Murphy et al., 2000). Essas proteínas, também podem ser divididas em dois grupos com base na sua função: quimiocinas inflamatórias e quimiocinas homeostáticas. Como os nomes sugerem, quimiocinas inflamatórias são induzidas por inflamação enquanto que as quimiocinas homeostáticos são constitutivamente expressas e são envolvidos na regulação homeostática imune (Sarvaiya et al., 2013).

Essas moléculas desempenham um papel fundamental na regulação da resposta imune e a inflamação por seu envolvimento na regulação do tráfico e posicionamento de leucócitos. A ligação das quimiocinas aos seus receptores leva a uma alteração conformacional, que ativa as vias de sinalização e promove a migração, quimiotaxia dos leucócitos (Raman et al., 2011). Além disso, algumas quimiocinas são importantes para outros processos fisiológicos tais como a hematopoese, a angiogênese, organogênese e embriogênese (Krieg & Boyman, 2009). No entanto, também tem sido relatado, que a migração de células de tumor e o crescimento são dependentes de sinais de quimiocinas, direto para as células tumorais (Krieg & Boyman, 2009). As células tumorais segregam e respondem as quimiocinas, que facilitam o crescimento que é viabilizado por aumento do recrutamento de células endoteliais, subversão de vigilância imunológica e manobra

do perfil de leucócitos tumoral para que a libertação de quimiocina permita o crescimento de tumores e metástases para locais distantes (Raman et al., 2007). Assim, as quimiocinas são vitais para a progressão de tumores (Raman et al., 2007).

Os receptores de quimiocinas abrangem sete proteínas transmembranares que estão acoplados a proteína G (GPCRs), estes receptores são nomeados de acordo com os ligantes de quimiocinas aos quais eles se ligam (Zlotnik & Yoshie, 2000; Murphy et al., 2000). Os receptores de CXC (CXCR1, 2, 3, 4 e 5) se ligam os receptores de quimiocinas CXC, CC (CCR1, 2, 3, 4, 5, 6, 7, 8, 9) se ligam as quimiocinas CC; CX3C receptor se liga as quimiocinas CX3C e, por último, o receptor XC se liga a quimiocina C (Sarvaiya et al., 2013). Apesar do fato de que os receptores de quimiocinas se ligam aos seus sub-grupos específicos, algumas quimiocinas podem se ligar a mais de um único receptor (Sarvaiya et al., 2013). Além disso, as respostas a alguns receptores de quimiocinas podem ser provocados por no máximo 10 ligantes (Schall & Proudfoot, 2011).

Muitas modificações pós-traducionais podem afetar a sinalização do receptor de quimiocina, especificidade do receptor, bem como propriedades quimiotáticas das quimiocinas e, assim, afetar as suas funções biológicas. Algumas das modificações pós-traducionais nas quimiocinas incluem glicosilação, citrulinação, e processamento proteolítico no N e C terminal (Proost et al., 2006; Loss et al., 2008; Struyf et al., 2009; Loss et al., 2009). Além disso, variações genéticas podem modificar as respostas desses receptores e alterar o poder de ligação que ocorre entre essas moléculas que acaba por desencadear em processos neoplásicos (Zeng, et al., 2006; Raman et al., 2007).

2.8. *Chemokine receptor* 2 (CCR2)

O receptor de quimiocina *CCR2* é codificado por um gene denominado CCR2 que está localizado no cromossomo 3p21 (Murphy et al., 2000). É um receptor de quimiocinas CC com afinidade pelas quimiocinas CCL2, CCL7, CCL8 e CCL13 (Yoshie et al., 2001; Narter et al., 2010). A maior afinidade desse receptor é pela quimiocina ligante CCL2 também conhecido como proteína quimioatrativa de monócitos-1 (MCP-1) sendo expresso em basófilos, monócitos, células dendríticas (DC), células T e células NK (Charo et al., 1994; Polentarutti et al., 1997; Sanders et al., 2000). Consistente com seu importante papel no tráfego de monócitos, o CCR2 parece

dirigir a inflamação em alguns modelos animais de doenças. Estas incluem desordens imunológicas (como artrite reumatóide, doença de Crohn, rejeição a transplantes), bem como doenças cardiovasculares, incluindo aterosclerose e hiperplasia intimal (Zhao, 2010).

O alelo variante do gene CCR2 que sofreu uma substituição nucleotídica de G para A (SNP – *Single Nucleotide Polymorphism*, rs1799864) na posição 190, substitui o resíduo de aminoácido valina na posição 64 por uma isoleucina (CCR2-64I) (Smith et al., 1997), uma mudança conservativa de aminoácidos no primeiro domínio transmembrana do receptor CCR2 (Nakayama et al., 2004), como pode ser observado na figura 4.

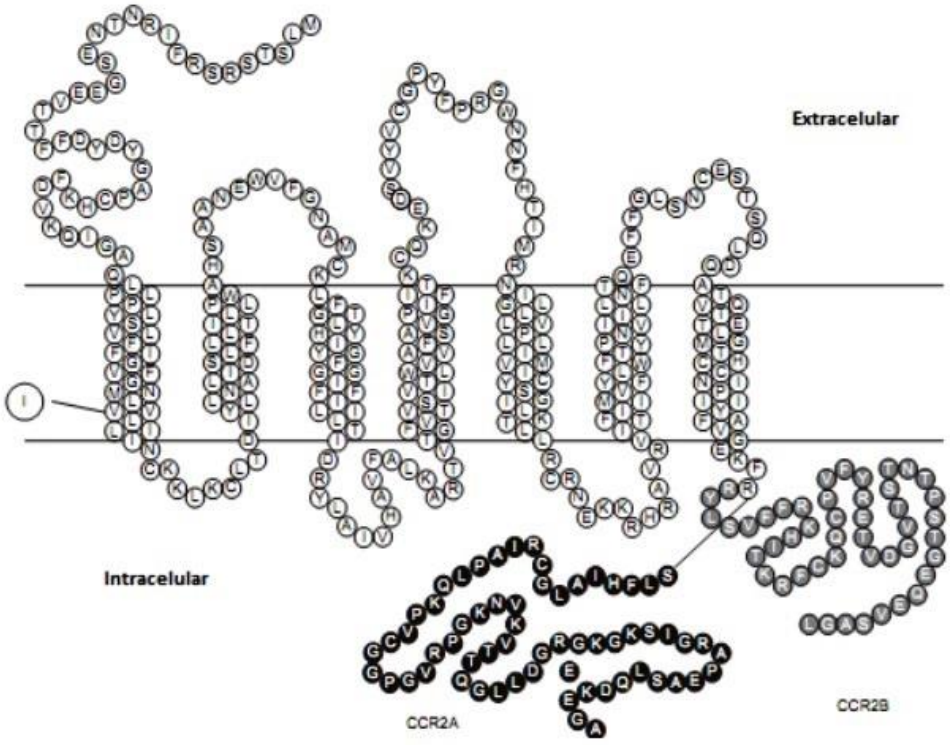

Figura 4: Estrutura da molécula CCR2 (isoforma CCR2A e CCR2B). A letra "I" na esfera maior denota a substituição na posição 64, de uma valina (V) por uma isoleucina (I), presente na variante polimórfica CCR2-64I. As esferas em cor preta correspondem aos resíduos de aminoácidos presentes apenas na isoforma CCR2A, enquanto as esferas em cinza correspondem aos presentes apenas na isoforma CCR2B. As duas linhas paralelas horizontais representam a membrana plasmática [modificado de (Nakayama et al., 2004)].

CCR2 tem duas isoformas, CCR2A e CCR2B, que são os produtos de gene CCR2 como resultado de um *splicing* alternativo. Esta mudança faz com que o CCR2A fique mais estável, aumenta a sua meia-vida, mas não afeta de qualquer forma a estabilidade da isoforma CCR2B (Nakayama et al., 2004). A alteração CCR2-64I parece ser comum a todos os grupos étnicos. Sua frequência alélica é de 0,098 em Caucasianos; 0,151 em Afro-americanos; 0,172 em Hispânicos; e 0,250 em Asiáticos (Smith et al., 1997).

Estudos iniciais também demonstraram que esse polimorfismo, embora não exerça nenhuma influência sobre a incidência da infecção pelo HIV-1, está associado a um retardo na progressão da AIDS (Síndrome da Imunodeficiência Adquirida) (Smith et al., 1997). Vários estudos tentam desvendar uma possível diferença funcional de CCR2-64I com relação ao CCR2 e tentando entender de que modo o alelo variante poderia influenciar no desenvolvimento de várias doenças e cânceres. Assim, alguns estudos têm sido realizados com o intuito de elucidar o mecanismo molecular referente ao efeito desse alelo (Mummidi et al., 1998; Bartoli et al., 2001; Nakayama et al., 2004; Navratilova, 2006; Deshmane et al., 2009; Narter et al., 2010).

A principal quimiocina ligante do CCR2, a CCL2, é amplamente expressa em muitos carcinomas humanos e sua produção corresponde ao recrutamento de macrófagos (Balkwill, 2004). CCL2 é secretada também por macrófagos associados ao tumor (TAMs) (Ueno et al., 2000). Até o momento, nas neoplasias de mama e esôfago, o aumento da expressão de CCL2 tem sido associado ao aumento do influxo de Macrófagos associados a tumores (TAMs) e correlacionado com um fenótipo invasivo, metástases de linfonodos e pobre prognóstico (Hembruff & Cheng, 2009; Raman et al., 2007). O efeito de CCL2 sobre as células neoplásicas parece variar de acordo com sua quantidade no microambiente tumoral. No melanoma, baixa expressão de CCL2 com modesta infiltração macrofágica parecem promover o crescimento tumoral (Nesbit et al., 2001). De modo semelhante, no câncer de colo do útero a perda da expressão de CCL2 tem sido associada à diminuição dos níveis de macrófagos, e correlacionada com pobre prognóstico (Kleine- Lowinski et al., 1999; Narter et al., 2010). No entanto, a perda de expressão de CCL2 no câncer de ovário (Arnold et al., 2005), assim como a reduzida expressão de CCR2 nos pacientes com mieloma (Van de Broek et al., 2006)

indicam um possível papel de supressão tumoral para a sinalização de quimiocinas (Hembruff & Cheng, 2009).

A variante polimórfica CCR2-64I tem sido relatada como protetora no desenvolvimento e progressão de doenças inflamatórias, como esclerose múltipla, arteriosclerose e no desenvolvimento do câncer de mama (Miyagishi et al.,2003 Nyquistet al., 2009; Zafiropoulos et al., 2004). Porém, contrariamente, parece ser um fator de risco no desenvolvimento de câncer de bexiga (Narter et al., 2010).

Além disso, CCR2-64I tem sido relatado com efeito protetor para o desenvolvimento de lesões cervicais (Coelho et al., 2005), contrariamente observado na população africana e sueca onde essa variante esteve associada com o aumento do risco para o desenvolvimento de CC (Chatterjeeet al., 2010; Ivansson et al., 2007). Resultados conflitantes na literatura quanto ao papel do CCR2-64I ainda são observadas quanto ao desenvolvimento de cânceres, fazendo-se necessária a realização de mais estudos sobre essa variante.

2.9. *Chemokine receptor* 5 CCR5

O CCR5 é um receptor de quimiocinas pertencente à classe CC, responsável por mediar as funções das quimiocinas CCL3 (MIP-1α) (Nibbs et al., 1999), CCL4 (MIP-1β), CCL8 e CCL5 (RANTES) (Combadiere et al., 1996; Raport et al., 1996; Samson et al., 1996a; Gong et al., 1998). Este receptor está presente principalmente em células do sistema imunitário, tais como macrófagos e linfócitos T, que desempenham um importante papel na migração destas células para sítios inflamatórios (Vargas et al., 2006). Ele também é expresso por células dendríticas, células endoteliais e epiteliais, músculo liso vascular, fibroblastos, neurônios, astrócitos e timócitos (Murphy et al., 2000; Yoshie et al., 2001). O gene que codifica CCR5 (CKR5) está localizado na região p21.3 do cromossoma humano 3 (Vargas et al., 2006), formando um *cluster* com outros genes dos receptores de quimiocinas, incluindo o CCR2, distante apenas cerca de 10 kb dele (Smith et al., 1997). Alguns estudos relatam que as variantes alélicas CCR2-64I e CCR5-Δ32 (rs333) estão em forte desequilíbrio de ligação em algumas populações, como na população norte-americana (Smith et al., 1997; Vargas et al., 2006).

O gene CCR5 está sujeito a várias mutações que afetam sua expressão. Foi identificado um alelo mutante cuja deleção de 32 pares de bases (pb) encontra-se dentro da região codificante do CCR5, resultando em uma mudança no quadro de leitura que gera um receptor não funcional, denominado CCR5-Δ32 (Liu et al., 1996), como demonstrado na figura 5. Assim, a proteína truncada resultante não é expressa na superfície celular, permanecendo no retículo endoplasmático (Zafiropoulos et al., 2004). Essa variação alélica ganhou grande destaque na literatura científica quando se descobriu que indivíduos homozigotos eram resistentes à infecção pelo HIV-1 (Ahlenstiel et al., 2004). Além disso, o CCR5-Δ32 tem sido relatado para reduzir o risco de asma (Hall et al., 1999), infarto do miocárdio precoce (Gonzalez et al., 2001), diminuição da gravidade da artrite reumatoide (Zapico et al., 2000). O CCR5 tem sido relatado para atuar como co-receptor na infecção para o HIV-1 (Zheng et al., 2006).

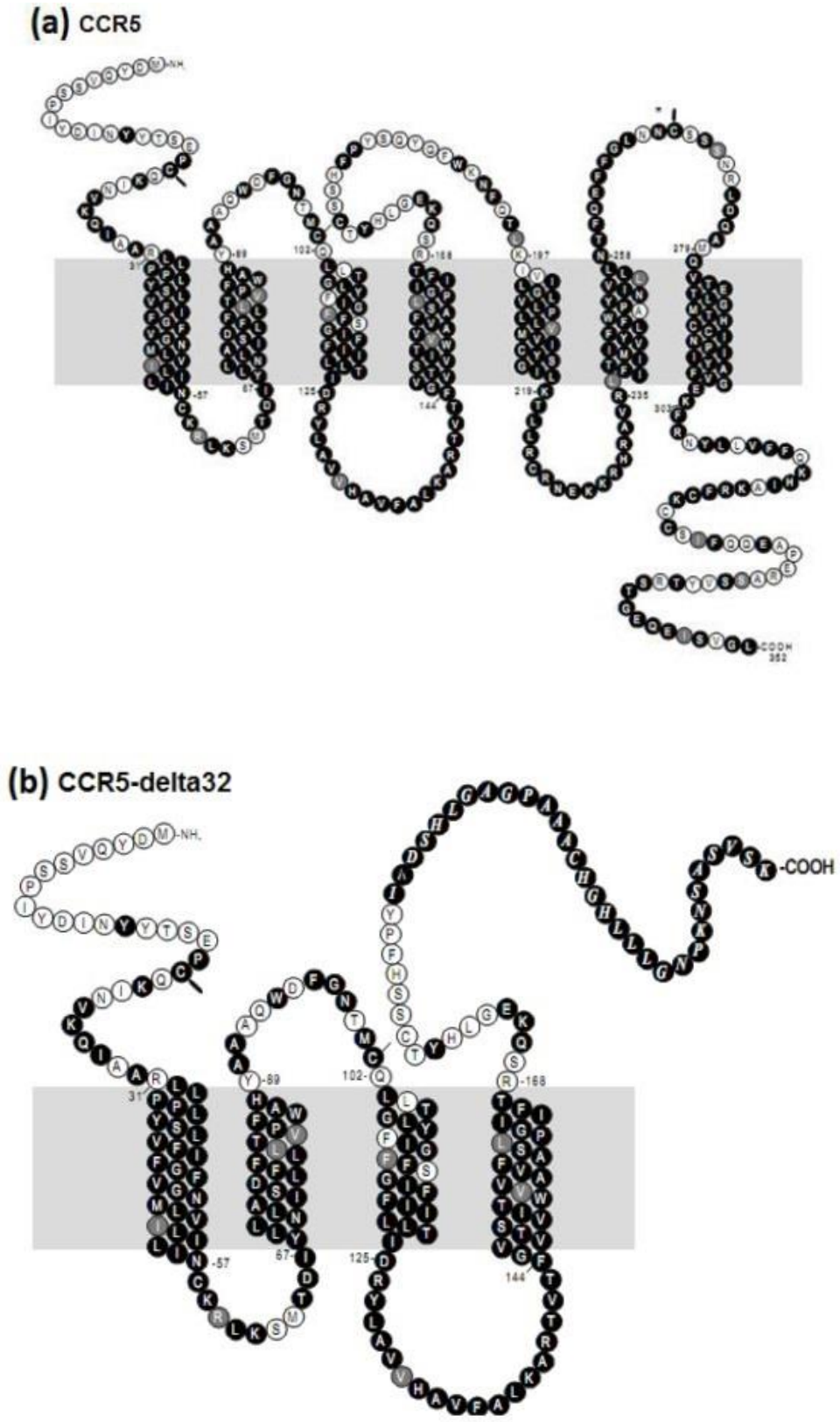

Figura 5: Estrutura prevista das moléculas CCR5 e CCR5-Δ32. (a) Estrutura prevista da molécula do CCR5 e sua sequência de aminoácidos. A estrutura típica de serpentina está representada com três loops (alças) extracelulares (superiores), três loops intracelulares (inferiores) e sete domínios transmembrana. Os aminoácidos são representados por uma única letra. (b) Estrutura prevista da forma mutante humana CCR5-Δ32 e sua sequência de aminoácidos. Na proteína mutante falta os últimos 3 segmentos transmembrana do CCR5, bem como as regiões envolvidas no acoplamento à proteína-G. A faixa horizontal sombreada representa a membrana de organelas intraceluares [modificado de (McNicholl et al., 1997)

Posteriormente, verificou-se que os indivíduos heterozigotos não eram resistentes à infecção, mas apresentavam menor carga viral e progressão mais lenta da doença. Além disso, dentre os indivíduos soropositivos, os heterozigotos apresentavam-se em menor frequência (Ahlenstiel et al., 2004). Embora os indivíduos homozigotos CCR5-Δ32 pareçam saudáveis e não apresente qualquer fenótipo aparente, a expressão alterada devido à mutação deve afetar as vias mediadas pelo CCR5 nas respostas imunológicas (Ahlenstiel et al., 2004).

A frequência do CCR5-Δ32 varia de zero, em populações africanas, a aproximadamente 14%, em populações europeias. Cerca de 1% da população caucasiana é homozigota para o polimorfismo (Mañes et al., 2003). É possível que as frequências elevadas do CCR5-Δ32 em europeus sejam atribuídas a uma forte pressão seletiva exercida por patógenos como *Yersinia pestis* (agente da peste bubônica), *Shigella*, *Salmonella* e *Mycobacterium tuberculosis*, que são alvos de macrófagos, ou por outras doenças infecciosas como sífilis, varíola e influenza (Vargas et al., 2006). Nas populações brasileiras em geral, a frequência do CCR5-Δ32 varia de 4,2% na região Norte a 6,4% na Sul. Quando estratificadas por origem étnica, as frequências são baixas entre afro-descendentes, variando de 0,7% a 2,6% e entre euro-descendentes (Vargas et al., 2006).

O conjuto ligante do receptor de quimiocina CCR5/ CCL5 tem sido estudado no câncer e relacionado com o crescimento e metástase de muitos tipos de carcinomas, como os de mama e mieloma múltiplo (Narter et al., 2010). Também se tem o conhecimento que os receptores de quimiocinas desempenham um papel crucial na imunidade antitumoral e que estão envolvidos na inflamação e patogênese das neoplasias (Srivastava et al., 2008). Acredita-se que exista uma relação entre CCR5 e a proteína supressora de tumor p53 representando mais um mecanismo de controle da progressão tumoral em humanos (Mañes et al., 2003).

Esses trabalhos mostram que o papel dos receptores de quimiocinas e de seus ligantes na progressão tumoral é complexo e pouco entendido (Mañes et al., 2003). Também fornecem indícios que CCR5/CCL5 e CCR2 representam uma via de sinalização por quimiocinas potencialmente significativa no desenvolvimento tumoral

(Hembruff & Cheng, 2009; Coelhoet al., 2005, Ivansson et al., 2007, Chatterjee et al.2010).

3. Objetivos

3.1. Objetivo Geral

- ✓ Relacionar os polimorfismos nos genes CCR2 e CCR5 com o desenvolvimento de lesões cervicais induzidas pelo HPV.

3.2. Objetivos Específicos

- ✓ Analisar a prevalência dos tipos de HPV em mulheres com lesão cervical;
- ✓ Relacionar o polimorfismo no gene CCR2 com a susceptibilidade ao desenvolvimento de lesão cervical;
- ✓ Relacionar o polimorfismo no gene CCR5 com a susceptibilidade ao desenvolvimento de lesão cervical;
- ✓ Associar os polimorfismos nos genes CCR2 e CCR5 com a infecção do HPV 16.

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CCR2 and *CCR5* genes polymorphisms in women with cervical lesions from Pernambuco, Northeast Region of Brazil: a case-control study

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Polymorphisms in chemokine receptors play an important role in the progression of cervical intraepithelial neoplasia (CIN) to cervical cancer (CC). Our study examined the association of CCR2-64I *(rs1799864) and* CCR5-Δ32 *(rs333) polymorphisms with susceptibility to develop cervical lesion (CIN and CC) in a Brazilian population. The genotyping of 139 women with cervical lesions and 151 women without cervical lesions for the* CCR2-64I *and* CCR5-Δ32 *polymorphisms were performed using polymerase chain reaction-restriction fragment length polymorphism. The individuals carrying heterozygous or homozygous genotypes (GA+AA) for* CCR2-64I *polymorphisms seem to be at lower risk for cervical lesion [odds ratio (OR) = 0.37, p = 0.0008)]. The same was observed for the A allele (OR = 0.39, p = 0.0002), while no association was detected (p > 0.05) with* CCR5-Δ32 *polymorphism. Regarding the human papillomavirus (HPV) type, patients carrying the* CCR2-64I *polymorphism were protected against infection by HPV type 16 (OR = 0.35, p = 0.0184). In summary, our study showed a protective effect of* CCR2-64I *rs1799864 polymorphism against the development of cervical lesions (CIN and CC) and in the susceptibility of HPV 16 infection.*

Key words: chemokine receptors - cervical intraepithelial neoplasia - cervical cancer - single nucleotide polymorphism

Infections by oncogenic types of human papillomavirus (HPV) are found in 99% of women with cervical cancer (CC) (de Oliveira et al. 2013). Therefore, this virus is classified as the most important carcinogenic risk factor according to the criteria of the International Agency for Research on Cancer (Bonanni et al. 2015). Among more than 120 types of HPV have been identified, 18 of these are classified as high-risk oncogenic (Bouvard et al. 2009, Bernard et al. 2010). However, the majority of women infected with HPV will not develop cervical carcinoma, since the carcinogenic process depends on several other genetic, environmental, and immune factors (Zheng et al. 2006).

The loss of cell cycle control mechanism, infiltration of leukocyte, and of other immunocompetent cells, as well as the altered expression of immune response genes, have been considered critical in neoplasms cervical pathogenesis and progression for CC (Evans et al. 1997, Ghaderi et al. 2000, O'Brien et al. 2001).

Among the gene products present into cervical mucosa, chemokines and their receptor have shown a key role in immunity against cervical tumours (Ghaderi et al. 2000, Ohta et al. 2002). Chemokines are chemoattractant proteins of low molecular weight that promote adhesiveness of target cells; then the angiogenesis process drive homing of phagocytes and lymphocytes into secondary lymphoid organs (Rossi & Zlotnik 2000, Kleine-Lowinski et al. 2003, Zheng et al. 2006). The receptors for chemokines are mainly expressed on immune cells and assist in the differentiation and migration of these cells to inflamed tissues (Bromley et al. 2005).

Chemokine receptor (CCR)5 is the major receptor for the chemokine and their ligands are the macrophage inflammatory protein (MIP)-1α/chemokine ligand (CCL)3, MIP-1β/CCL4, and regulated on activation, normal T cell expressed and secreted/CCL5 (Lehner 2002, Al-Abdulhadi & Al-Rabia 2010, Ahmadabadi et al. 2012). Polymorphic variations in this gene, in particular the Δ32 mutation (a 32 bp deletion in the *CCR5* gene) leads to decreased expression and dysfunction of CCR5 receptor (Nahon et al. 2008, Ahmadabadi et al. 2012). Studies report that individuals homozygotes for *CCR5-Δ32* (rs333) gene have reduced risk for asthma and early-onset myocardial infarction, attenuation of severity in rheumatoid arthritis, and slower acquired immune deficiency syndrome (AIDS) progression (Berger et al. 1999, Hall et al. 1999, Zapico et al. 2000, González et al. 2001). In addition CCR5, together with CCR2, act as co-receptors for human immunodeficiency virus-1 (Zheng et al. 2006).

CCR 2 is the receptor for CCL 2, also known as monocyte chemoattractant protein (MCP)-1, being associated with carcinogenesis and angiogenesis (Charo et al. 1994, Zhang et al. 2003, Koide et al. 2004, Huang

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et al. 2013). The single nucleotide polymorphism at codon 64 (*CCR2-64I*, rs1799864) of *CCR2* gene that encodes isoleucine (ATC) instead of valine (GTC) has been widely studied, and there are reports of association between this polymorphism and the protective effect in the progression of inflammatory diseases such as multiple sclerosis (Miyagishi et al. 2003), carotid atherosclerosis (Nyquist et al. 2009), and in development of breast cancer (Zafiropoulos et al. 2004). However, conflicting results on the role of the *CCR5* and *CCR2* polymorphisms in the development of the CC have been reported so far (Coelho et al. 2005, Zheng et al. 2006, Ivansson et al. 2007, Chatterjee et al. 2010).

Therefore, the aim of this study was to analyse the association for *CCR2-64I* and *CCR5-Δ32* polymorphisms with development of cervical intraepithelial neoplasia (CIN) or CC in women infected by HPV from Northeast Region of Brazil.

SUBJECTS, MATERIALS AND METHODS

Population - The present study was a hospital-based cross-sectional prospective one carried out in the outpatient clinics of the Lower Genital Tract Pathology Clinic at the Women's Healthcare Center of the Prof Fernando Figueira Institute of Integrated Medicine, Recife, state of Pernambuco, Brazil. Patients were selected by spontaneous demand from January 2009 until 2011 and the study population consisted of 290 sexually active women ranging between 16-75 years old. Information was collected from all women pertaining to their age, smoking, alcohol consumption, number of offspring, number of sexual partners, and age at first coitus. The inclusion criteria was as follows: women with oncotic cytology submitted to Papanicolaou test (cytological) according to Bethesda System terminology (Solomon et al. 2002) performed on the state accredited networks, presenting diagnostic of CIN of low-grade and high-grade or CC, and confirmed by histological analysis. Subjects were evaluated for clinical features of other sexually transmitted infections on history and examination. Patients that were previously submitted to radiotherapy or chemotherapy to invasive CC were excluded. The Institutional Ethical Committee approved this study (protocol 355/08). Informed written consent was taken from the women informing them about the background of the study, risks and benefits, and voluntary nature of participation. After histological analysis, patients were stratified according to the presence or absence of cervical lesion (CIN or CC) as case and control groups, respectively.

Clinical samples - Cervical smears were obtained using Cytobrushes. Each Cytobrush was packed in a Tris-ethylenediamine tetraacetic acid (EDTA) buffer solution (Tris-HCl 10 mM and EDTA 1 mM pH 8.0) and conserved at -20°C until analysis.

DNA extraction - Genomic DNA extraction was performed from 300 µL of vaginal fluid from each study subject, following the manufacturer's instructions of the kit Wizard® Genomic DNA Purification (Promega, USA). The analyses samples were executed the Laboratory of Genetics, Biochemistry, and DNA Sequencing at Rural Federal University of Pernambuco.

HPV detection and typing - Amplification of human β-globin gene segment was used as an internal control for DNA quality and samples negative for this assay were excluded from analysis. Then, our samples were tested for HPV presence using MY09/11, GP05+ and GP06+ consensus primers by polymerase chain reaction (PCR) (Tavares et al. 2014). The typing of high-risk HPV (HR-HPV) 16, 18, 31, and 33 was performed using specific primers (da Silva et al. 2009, Tavares et al. 2015).

Analysis of the CCR2-64I polymorphism - The *CCR2-64I* polymorphism was analysed through PCR followed by restriction fragment length polymorphism (Coelho et al. 2005). DNA was amplified using primers sense 5'-TTGTGGGCAACATGATGG-3' and antisense 5'-GCATTCCCAAAGACCCACTC-3'. The PCR products of 163 bp length were then digested with *BsaBI* restriction enzyme. The fragments originated after of the use the restriction enzyme, 163 bp for G allele, and 145 and 18 bp for A allele were revealed using 3% agarose gel stained with gel red (UNISCIENCE).

Analysis of the CCR5-Δ32 polymorphism - The *CCR5-Δ32* polymorphism was analysed through PCR (Kristiansen et al. 2001) using primers sense 5'-CTTCATCATCCTC-CTGACAATCG-3' and antisense 5'-GACCAGCCCCAAGTTGACTATC-3'. PCR products of 262 bp for CCR5 wild-type allele and 230 bp for *CCR5-Δ32* allele were detected with 3% agarose gel stained with gel red (UNISCIENCE).

Sequencing - A total of 20% of the all samples (randomly chosen) was submitted to bidirectional sequencing (MegaBACE 1000 DNA sequencer; GE Healthcare, USA) in order to double-check the genotyping results for each polymorphism (Tavares et al. 2015).

Statistical analyses - Univariate statistical analysis was performed using the BioEstat 5.0 software. The study was cross-sectional with independent samples consisting of nominal data (genotypes). The influence of each polymorphism on the risk for development of (pre) neoplastic cervical disease was estimated by odds ratio (OR) and a 95% confidence interval (CI). Allele frequencies were estimated by direct counting. Comparison between genotypic frequencies of patients and control groups was performed by chi-square test and Fisher's exact test was used to compare the allele frequencies in contingency tables.

For identification of relevant risk factors, a logistic regression analysis was carried out (comparing HPV-positive women with history of lesions with HPV-positive women with no history of lesions or CC). This modelled the influence of genetic polymorphisms, HPV 16 single infection or multiple HPV strains co-infection, smoking and alcohol consumption on the risk of developing high-grade squamous intraepithelial lesions. The OR and their respective 95% CI were determined. The R software v.3.0.2 (R-project.org/) was used to perform the regression analysis. Power analysis was performed through G*Power software v.3.1.9.2 (Faul et al. 2007). All p-values ≤ 0.05 were considered statistically significant.

RESULTS

Within the 290 HPV positive enrolled women, 139 had cervical lesions (CIN or CC) (HPV+L) while the 151 had no cervical lesions (HPV+). When considering the prevalence of type-specific HR-HPV infection, we observed that 38.8% of the patients had HPV 16, 22.3% HPV 18, 2.9% HPV 31, 3.6% HPV 33, and 14.4% other HPV types. Furthermore, the presence of co-infection by HPV types 16/18 was found in 18% of the patients (Table I). Moreover, when the patients were stratified according to

TABLE I

Human papillomavirus (HPV) genotypes prevalence and histologic diagnosis

Types	CIN I (n = 40) n (%)	CIN II/III (n = 87) n (%)	CC (n = 12) n (%)	Total (n = 139) n (%)
HPV 16	10 (25)	38 (43.7)	6 (50)	54 (38.8)
HPV 18	9 (22.5)	20 (22.9)	2 (16.7)	31 (22.3)
HPV 31	2 (5)	2 (2.3)	0 (0)	4 (2.9)
HPV 33	0 (0)	4 (4.6)	1 (8.3)	5 (3.6)
Others HPV	14 (35)	5 (5.8)	1(8.3)	20 (14.4)
Co-infection (16/18)	5 (12.5)	18 (20.7)	2 (16.7)	25 (18)

CC: cervical cancer; CIN: cervical intraepithelial neoplasia.

TABLE II

Genotypic distribution of the *CCR2-64I* and *CCR5-Δ32* gene polymorphisms in human papillomavirus (HPV) positive patients with cervical intraepithelial neoplasia (CIN) and cervical cancer (CC) (HPV+L) or without cervical lesions (HPV+)

SNP	HPV+ (n = 151) n (%)	HPV+L (n = 139) n (%)	$\chi 2$ (p)	OR (95% CI)	p[a]
CCR2 64I genotypes					
GG	99 (65.6)	116 (83.4)		1	
GA	43 (28.4)	21 (15.1)	8.820 (0.0047)	**0.42 (0.23-0.75)**	**0.0047**
AA	9 (6)	2 (1.5)	5.367 (0.0447)	**0.19 (0.04-0.89)**	**0.0447**
AA + GA x GG	52/99 (52.5)	23/116 (19.8)	12.082 (0.0005)	**0.37 (0.21-0.66)**	**0.0008**
Allele					
G	241 (79.8)	253 (91)	14.393 (0.0001)	1	
A	61 (20.2)	25 (9)		**0.39 (0.23-0.64)**	**0.0002**
CCR5-Δ32 genotypes					
WT/WT	141 (93.3)	125 (89.9)		1	
WT/Δ32	10 (6.7)	14 (10.1)	1.134 (0.2868)	1.57 (0.67-3.68)	0.3943
Δ32/Δ32	0 (0)	0 (0)	ND	ND	ND
WT/Δ32 + Δ32/Δ32 x WT/WT	10/141 (7)	14/125 (11.2)	1.134 (0.2868)	1.57 (0.67-3.68)	0.3943
Allele					
WT	292 (96.7)	264 (94.9)		1	
Δ32	10 (3.3)	14 (5.1)	1.085 (0.2975)	1.54 (0.67-3.54)	0.4047

a: value of the odds ratio (OR); CI: confidence interval; ND: not determined; SNP: single nucleotide polymorphism; WT: wild-type; χ^2: chi-square test. Bolded values mean significant values.

the severity of cervical lesions, 28.78% (40/139) exhibited CIN I (low-degree of lesion), 62.58% (87/139) had CIN II or III (high-degree of lesion), and 8.63% (12/139) had CC.

Among the 139 HPV+L, five risk factors for cervical lesions were analysed: smoking, alcohol consumption, number of offspring, number of sexual partners, and age at first coitus. With the 139 HPV+L, 32.37% (45/139) reported smoking, 60.03% (89/139) alcohol consumption, 28.78% (40/139) number of offspring > 3, 20.17% (24/119) had number of sexual partners > 4, and 59.71% (83/139) had first coitus with ≤ 16 years old.

The distribution of *CCR2-64I* and *CCR5-Δ32* polymorphisms genotypes in 139 women with cervical lesions (CIN or CC) and 151 HPV+ were according to the Hardy-Weinberg equilibrium. A significant difference in the distribution of *CCR2-64I* polymorphism between HPV+ L and HPV+ was observed using a dominant genetic model (OR = 0.37; p = 0.0005), being the variant carrier (GA+AA) associated with protection to cervical lesions (Table II). When considering the *CCR5-Δ32* polymorphism, no statistical difference between HPV+-L and HPV+ was observed (p = 0.3943) (Table II).

Statistical significant association was found between *CCR2-64I* polymorphism and susceptibility to HPV 16 infection (OR = 0.35; p = 0.0184) (Table III); the *CCR5-Δ32* variant did not show any association with HPV types studied.

Patients clinical features are shown in Table IV. There were no significant statistical differences between *CCR2-64I* and *CCR5-Δ32* polymorphisms with age, smoking, alcohol consumption, number of offspring, number of sexual partners, or age at first coitus (p > 0.05).

DISCUSSION

In this study, we examined the possible association between *CCR2-64I* and *CCR5-Δ32* polymorphisms and the presence of cervical lesions (CIN or CC) in HPV infected women from Northeast Region of Brazil.

The CCRs genes *CCR5* and *CCR2* have been associated with carcinogenesis and angiogenesis (Zheng et al. 2006), inflammatory disorders, and autoimmune diseases (Rossi & Zlotnik 2000). Immunological studies, regarding to immune response genes, showed a significant decrease in intraepithelial macrophages (Tay et al. 1987), Langerhans cells (Spinillo et al. 1993), and cytotoxic T lymphocytes in CIN advanced (Evans et al. 1997).

The *CCR2-64I* polymorphism has been reported to influence several diseases as multiple sclerosis (Miyag-

TABLE III

Genotypic distribution of the *CCR2-64I* and *CCR5-Δ32* polymorphisms in human papillomavirus (HPV)-16 positive patients and in patients with HPV genotypes other than type 16

SNP	Other HPV (n = 85) n (%)	HPV 16 (n = 54) n (%)	$\chi 2$ (p)	OR (95% CI)	p[a]
CCR2 64I genotypes					
GG	69 (81.1)	47 (87.1)		1	
GA	1 (1.1)	6 (11.1)	13.76 (0.001)	8.80 (1.02-75.56)	0.0509
AA	15 (17.8)	1 (1.8)		**0.09 (0.01-0.77)**	**0.0167**
GG/GA + AA	69/16	47/7		0.64 (0.24-1.68)	0.5015
Allele					
G	139 (81.7)	100 (92.6)	6.93 (0.031)	1	
A	31 (18.3)	8 (7.4)		**0.35 (0.15-0.81)**	**0.0184**
CCR5-Δ32 genotypes					
WT/WT	80 (94.1)	46 (85.2)		1	
WT/Δ32	5 (5.9)	8 (14.8)	3.01 (0.221)	2.78 (0.86-9.01)	0.1432
Δ32/Δ32	0	0		ND	
WT/WT x WT/Δ32 + Δ32/32	80/5	46/8			
Alelle					
WT	165/170 (97.1)	100/108 (92.6)		1	
Δ32	5/170 (2.9)	8/108 (7.4)	2.86 (0.239)	2.64 (0.84-8.29)	0.1534

a: value of the odds ratio (OR); CI: confidence interval; ND: not determined; SNP: single nucleotide polymorphism; WT: wild-type; χ^2: chi-square test. Bolded values mean significant values.

TABLE IV

Genotypic distribution of the *CCR2-64I* and *CCR5-Δ32* polymorphisms in patients with cervical lesions (cervical intraepithelial neoplasia and cervical cancer and clinical features

			CCR2-64I				
Clinical features	A/P	(n = 139)	G/G	G/A + A/A	χ^2 (p)	OR (95% CI)	p[a]
Smoking	P	45	36	9	0.4484	1.42	0.6072
	A	94	80	10		(0.56-3.60)	
Alcohol consumption	P	89	77	12	0.1947	0.55	0.2896
	A	50	39	11		(0.41-4.36)	
Number of offspring	> 3	40	33	7	0.7452	1.18	0.9521
	≤ 3	79	67	12		(0.42-3.28)	
Number of sexual partners	> 4	24	21	3	0.6038[a]	0.70	0.8360[a]
	≤ 4	95	79	16		(0.18-2.65)[a]	
Age at first coitus	≤ 16	83	67	16	0.2916	1.67	0.4111
	≤ 16	56	49	7		(0.63-4.37)	
			CCR5-Δ32				
Clinical features	A/P	(n = 139)	Wt/Wt	Wt/Δ32 + Δ32/Δ32	χ^2 (p)	OR (95% CI)	p[a]
Smoking	P	45	40	5	0.6222	1.34	0.8561
	A	94	86	8		(0.41-4.36)	
Alcohol consumption	P	89	79	10	0.0529	6.20	0.1077
	A	50	49	1		(0.77-49.96)	
Number of offspring	> 3	40	36	4	0.9827	0.76	0.7637
	≤ 3	79	71	8		(0.27-3.49)	
Number of sexual partners	> 4	24	22	2	0.7499[a]	0.77	0.9517
	≤ 4	95	85	10		(0.15 -3.78)[a]	
Age at first coitus	≤ 16	83	76	7	0.6506	0.76	0.8761
	> 16	56	50	6		(0.24-2.41)	

a: were used a total of 119 patients; A: absence of the characteristic; CI: confidence interval; OR: odds ratio of A x G alleles; P: presence of the characteristic; WT: wild-type; χ2: chi-square test.

ishi et al. 2003), carotid atherosclerosis (Nyquist et al. 2009), breast cancer (Zafiropoulos et al. 2004), AIDS progression (Smith et al. 1997, Mulherin et al. 2003), and CIN or CC (Coelho et al. 2005, Ivansson et al. 2007, Chatterjee et al. 2010). Our results showed a protective effect of *CCR2-64I* polymorphic variant against the development of cervical lesions (OR = 0.37); Coelho et al. (2005) found the same outcome in a Portuguese population. Nevertheless, Chatterjee et al. (2010) and Ivansson et al. (2007) reported that the A allele conferred risk to development of CC in African and Swedish women. The contradictory results on the A allele in relation to the development of CIN or CC (Coelho et al. 2005, Zheng et al. 2006, Ivansson et al. 2007) might be due to its conflicting role in the macrophages recruitment reported by some authors. Wallin et al. (1999) suggested that mutant allele *CCR2-64I* can be linked with decreased macrophages recruitment in the process of tumour angiogenesis, which could be a key during progression of cervical neoplasia to CC. However, Chatterje et al. (2010) proposed that the raised attraction of the macrophages through the increased expression of MCP-1 could be auxiliary in the process of destruction or progression of tumour. These discordant findings reinforce the possibility of several factors associated with the multifactorial neoplastic development, including the difference in ethnic origin of the populations studied, sample sizes, and the low percentage of the mutant allele (*CCR2-64I*).

Studies reported that *CCR5-Δ32* is involved in slower AIDS progression (Berger et al. 1999), in decreasing the severity of rheumatoid arthritis (Zapico et al. 2000), and in the reduced risk to asthma (Hall et al. 1999). To our knowledge until now, only one study conducted by Zheng et al. (2006) in a Swedish population related the *CCR5-Δ32* polymorphism with CC. The authors observed that individual carriers of the allele Δ32 had 4.58 fold-increased risk to HPV infection, but they did not found association in relation to progression of cervical

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Apêndice I

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ORIGINAL ARTICLE

Association between p21 Ser31Arg polymorphism and the development of cervical lesion in women infected with high risk HPV

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Abstract Infection by high-risk human papillomavirus (HR-HPV) and single nucleotide polymorphism (SNP) in genes involved in cell cycle control, as *p21* and *p27*, are important factors in the development of different types of human cancers. This study aims at investigating whether both the *p21* Ser31Arg and *p27* V109G polymorphisms are associated with susceptibility to the development of cervical lesions in women HR-HPV positive. We analyzed 132 women HPV positive and with cervical lesions or CC and 154 healthy control (HPV negative and without cervical lesions). *p21* Ser31Arg and *p27* V109G polymorphisms were analyzed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method and sequencing. The *p21* 31Arg allele was associated with susceptibility for the development of cervical lesions ($P^* = 0.0009$), while *p27* V109G polymorphism showed no significant differences for this association ($P^* = 0.89$). However, the combined effect of the polymorphisms showed that the presence of the CC genotype (SNP *p21* Ser31Arg) conferred protection for the development of cervical lesions (OR = 0.39). *p21* Ser31Arg and *p27* V109G polymorphisms were not associated with the grade of cervical lesions (CINI, CINII, and CINIII) or CC ($P^* > 0.05$). The HR-HPV more frequent in this study were of 16 (57.6 %) and 18 (37.1 %) types; however, no association was observed when both polymorphisms and risk factors analyzed were compared ($P^* > 0.05$). Our findings suggest a possible association between *p21* Ser31tabArg polymorphism and susceptibility to the development of cervical lesions in women from Pernambuco. Brazil.

The work was performed at the Rural Federal University of Pernambuco (UFRPE), Recife, PE, Brazil.

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Keywords SNPs · Cell cycle · Cervical cancer · HPV

Introduction

Cervical cancer (CC) represents the second most common cancer in Brazil and is the fourth leading cause of death in Brazilian women. In years 2014–2015, 15,590 new cases of CC are estimated, while in Pernambuco, 6.13 new cases per 100,000 women are expected [1]. The main etiological agent of CC is the human papillomaviruses (HPV) infection, which is found in more than 90 % of the cases [2, 3]. Among over 100 types of HPV, the 16, 18, 31, and 33 are the most frequent high-risk HPV (HR-HPVs) types found in both cervical intraepithelial neoplasia (CIN) and cervical cancer [3]. Moreover, several other factors appear to increase susceptibility to develop cervical cancer, including genetic and environmental factors (tobacco use, multiple sexual partners, alcohol consumption, and number of children) [4, 5].

The cell cycle is divided into four phases, G1, S, G2, and M, and its progression is regulated by cyclins and cyclin-depent kinases (CDK). This complex operates in the phosphorylation of proteins, such as pRb, involved in cell cycle progression [6, 7]. The pRB protein inhibits the activity of the transcription factor E2F blocking the G1 to S phase transition, when phosphorylated by the cyclin CDK complex becomes inactive, liberating the E2F factor and thus enabling the continuity of the cell cycle [8]. DNA damage in normal cells causes the activation of the cyclin-dependent kinase inhibitors (CKIs) that inhibit the cyclin-CDK complex aimed at preventing the unregulated proliferation of cells [9].

The CKIs are classified into subclasses: Kip/Cip, which include P21 and P27, and INK4 [10]. The P21, upregulated by wild-type tumor suppressor protein P53, inhibits cyclin-CDK2 or cyclin-CDK4 complexes and hinders the transition from the G1 to the S phase [7, 8]. Also, high levels of P27 inhibit cyclin E/CDK2 complex in the G1 phase and block the advance of the cell cycle [11].

The development of cancers results from the uncontrolled proliferation of cells mainly caused by interference in the cell cycle checkpoints. The HR-HPV infection increases the expression of oncoproteins E6 and E7 that inhibit the activity P21 and P27 proteins, facilitating malignant transformation [7, 12].

Single nucleotide polymorphism (SNP) of the *p21* gene (C>A) at codon 31 (*p21* Ser31Arg; rs1801270) produces an amino acid substitution of arginine for serine, resulting in altered levels of protein [13]. Genetic studies have found that SNPs of the *p21* gene could influence on the development of several pathology, as cervical, lung, and gastric cancer [14–19]. The SNP (T>G) at codon 109 of *p27* gene (*p27* V109G; rs2066827), which promotes a valine-to-glycine substitution, is associated with altered activity of the protein [20]. Mutations in this tumor suppressor gene has been reported to be involved in human tumor progression, as in a colon cancer, breast cancer, oral squamous cell carcinoma, and endometriosis [21–25]. Besides, some studies suggest that decreased levels of *p27* may be important in the development of cervical carcinoma, since that both a diminished expression in women with lesions and high levels of *p27* in normal cervix are found [26–28]

Prior studies have related these polymorphisms with cervical cancer, but the results are conflicting and even at present moment, no study has been conducted relating these polymorphisms with the development of cervical lesion [22–25, 28]. Therefore, further studies to assess these possible associations are needed. Thus, the aim of the present work was to evaluate the possible association of the single nucleotide polymorphisms in the *p21* and *p27* genes with the development of cervical lesions in women with HR-HPV infection.

Methods

Study population

The study group consisted of 132 sexually active women from Recife metropolitan region (Pernambuco, Northeast Brazil), with ages between 16 and 75 years and mean age 33.9 ± 10.3 years, presenting HR-HPV infection and different grade of cervical intraepithelial neoplasia (low grade or CIN I, and high grade or CIN II, III) or cervical cancer, which were confirmed by cytological and histological analysis. All patients were initially assessed by colposcopy, and subsequently, cervical smears were collected. Histological diagnosis was made according to Solomon et al. [29] and Associacao Brasileira de Ginetoscopia [30]. Patients were also stratified according to smoking, alcohol consumption, multiple sexual partners, and number of children.

One hundred fifty-four unrelated women volunteers without HPV and cervical lesions from Recife metropolitan region (healthy controls group), with ages between 14 and 70 years (mean age 37.7 ± 10 years), with no history of lesions or neoplastic disease as evaluated by the physician were enrolled as controls, and written informed consent was obtained.

The study population was recruited between January 2009 and December 2009 at the Lower Genital Tract Pathology Clinic at the Women's Healthcare Center of the Prof. Fernando Figueira Institute of Integrated Medicine, Pernambuco, Brazil. All women survey participants answered a questionnaire including social and demographic features, including age, level of instruction, age of the first sexualintercourse, number of partners, sexual behavior, smoking, and alcohol consumption. The institutional ethics and research committees (no. 355/08) approved this study.

DNA extraction

All the analyses were realized in the Laboratory of Genetic, Biochemistry and DNA Sequencing at Rural Federal University of Pernambuco. DNA was extracted from 300 μL vaginal fluid following the manufacturer's instructions using the Wizard® Genomic DNA Purification Kit Protocol – Promega.

HPV detection and typing

HPV DNA was detected in all samples using MY09/11, GP05+, and GP06+ consensus primers following PCR protocols published elsewhere [31, 32]. Four types of HR-HPV (such as 16, 18, 31, and 33), which infect the region anogenital, were genotyped by use of specific primers and following protocols published elsewhere [33].

Analysis of the polymorphism in the *p21* gene codon 31

The genotyping of the *p21* Ser31Arg polymorphism was performed as described by Li et al. [34], using polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). The following primers were used for PCR amplification: sense (5′-GTCAGAACCGGCTGGGGATG-3′) and antisense (5′-CTCCTCCCAACTCATCCCGG-3′). The amplification produced a PCR product of 272 bp that was digested by BlpI restriction enzyme at 37 °C for 4 h, generating two fragments of 183 and 89 bp when in presence of the C allele, and only one fragment of 272 bp in presence of allele A.

Analysis of the polymorphism in the p27gene codon 109

The genotyping was also performed using PCR-RFLP. The sense primer (5-TGCAGACCCGGGAGAAAG-3′) and antisense primer (5′-CTAACCCCGTCTGGC-3), as described by Li et al. [24], were used. When the product of the PCR amplification was digested with the BglI enzyme restriction for 4 h at 37 °C, three fragments of 262, 116, and 76 bp were generated when in presence of the T allele and two fragments of 377 and 76 bp when in presence of G allele.

Sequencing

To double-check PCR-RLFP genotyping analysis of the *p21* Ser31Arg and *p27* V109G polymorphisms, 20 % of the samples were sequenced using the MegaBACE 1000 DNA sequencer (GE Healthcare, USA). Data were collected following the parameters (Dye Set "Z") set by the Data Collection program v1.0.1. The obtained sequence were compared with sequences available in GenBank database (www.ncbi.nlm.nih.gov) using BLAST tool and analyzed in the program BioEdit 5.0.

Statistical analysis

The associations between the *p21* and *p27* polymorphisms and disease were tested using the chi-squared test. Hardy-Weinberg equilibrium (HWE) test was applied to both datasets. The frequencies of alleles and genotypes were obtained by direct counting. The possible association between SNPs and cervical lesions or CC with HR-HPVs (16, 18, 31, or 33) type or risk factors, such as smoking, alcohol consumption, multiple sexual partners, and number of children, were evaluated by the odds ratio (OR) with 95 % confidence interval. Furthermore, the same test was used to analyze the combined effect of these polymorphisms. All analyses were done using the BioStat 5.0 software program.

Results

Association of the polymorphisms in the p21 and p27 genes with development of cervical lesions

The distribution of genotypes and allele frequencies of *p21* Ser31Arg and *p27* V109G polymorphisms among patients and healthy controls were all in accordance with Hardy–Weinberg equilibrium. For the *p21* Ser31Arg polymorphism, the genotypic distribution was (76.6 % CC, 21.4 % CA, and 2.0 % AA) and (57.6 % CC, 37.1 % CA, 5.3 % AA) in cases and healthy controls, respectively. There was an increase of twofold risk for the development of cervical lesions in the patients group (OR = 2.41; $P^* = 0.0009$) when the AA + AC genotypes were used as reference. However, no significant difference in the distribution of genotypic frequencies between the control and patient groups (27 % TT, 86 % TG, and 41 % GG and 23 % TT, 70 % TG, and 39 % GG, respectively) was observed in relation to the *p27* V109G gene polymorphism ($P^* = 0.89$; Table 1). Also, we examined the combined effect of both polymorphisms in the development of cervical lesions or CC. This assay showed that the presence of the CC genotype (SNP *p21* Ser31Arg) conferred protection even when the polymorphic variants in the *p27* gene (OR = 0.39; $P < 0.05$) are present as reported in Table 2.

Table 3 presents the correlation between of the *p21* Ser31Arg and *p27* V109G polymorphisms with the grade of lesion (CIN I or CIN II, III), and in their progression to CC, no significant difference was observed ($P > 0.05$).

Association of the polymorphisms in the *p21* and *p27* genes with HR-HPV infection or with risk factors for CIN and CC

The analyses of the distribution of HR-HPV types in women with cervical lesions or CC showed elevated frequency of HPV 16 and 18 types (HPV 16 = 57.6 %, HPV 18 = 37.1 %,

Table 1 Genotypic distribution and allelic frequencies of *p21* Ser31Arg and p27 V109G polymorphisms in patients with and without development of cervical lesions from Recife-Pernambuco population

	Patients		Controls		*P*	OR (95 % CI)	*P**
	n = 132	%	*n* = 154	%			
p21 Ser31Arg							
CC	76	57.6	118	76.6			
CA	49	37.1	33	21.4			
AA	7	5.3	3	2.0			
AA + CA × CC	56/76		36/118		0.0006	2.41 (1.45–4.01)	0.0009*
C	201	76.1	269	87.3	0.0005	1	0.0007*
A	63	23.9	39	12.7		2.16 (1.39–3.35)	
p27 V109G							
TT	23	17.4	27	17.5			
TG	70	53.0	86	55.8			
GG	39	29.6	41	26.7			
GG + TG × TT	109/23		127/27		0.98	1.00 (0.54–1.85)	0.89
T	116	43.9	140	45.4	0.71	1.06 (0.76–1.48)	0.78
G	148	56.1	168	54.6		1	

P *P* value of χ^2 test, *OR* odds ratio, *CI* confidence interval, *P** *P* value of odds ratio

*significant value

HPV 31 = 3.0 %, HPV 33 = 1.5 %, and other HPVs = 15.2 %). Besides that, multiple infections by HPV16/18 types were present in 14.4 % of the patients. However, the genotypic frequencies of the *p21* Ser31Arg and *p27* V109G polymorphisms were not associated with infection by any kind of HR-HPV analyzed neither with coinfection with HPV16/18 types (Table 4). Furthermore, comparing the genotypic frequencies of both *p21* Ser31Arg and *p27* V109G polymorphisms with risk factors as smoking, alcohol consumption, multiple sexual partners, and number of children, no significant association was observed (Table 5).

Discussion

In the present study, the association of the single nucleotide polymorphisms in the *p21* and *p27* genes with development of cervical lesions in women with HR-HPV infection was examined. Functional polymorphism in the *p21* gene, as *p21* Ser31Arg, promoting a nonsynonymous serine to arginine substitution and so modifying the expression and/or activity of this protein [13, 35], could increase the susceptibility to different types of cancer, including cancer cervical [14–16, 36].

In the present study, it was observed that the presence of A allele in the *p21* Ser31Arg polymorphism increased twofold the risk of cervical lesion. To our knowledge, until now, no other study reported this kind of association, this polymorphism, and the development of cervical lesion. However, studies as those performed by Harima et al. [14] and in Bhattacharya et al. [37] on the Japanese and Indian population, respectively, showed a risk of the A allelic variant to the development of CC. Contrarily, the work done by Ning et al. [38] reported that the A allele is more frequent in cancer-free controls, while Roh et al.[39], Tian et al. [40], and Jiang et al. [19] associated the presence of the C allele with increased risk for the development of CC. The

Table 2 Combined effect of the both *p21* Ser31Arg and *p27* V109G polymorphisms in the development of cervical lesions

Genotypes		Cases (*n* = 132)		Controls (*n* = 154)		OR (95 % CI)
p21 Ser31Arg	*p27* V109G	*n*	%	*n*	%	
CA + AA	TG + GG	47	35.6	29	18.8	1
CA + AA	TT	9	6.8	7	4.5	0.7933 (0.26–2.36)
CC	TG + GG	62	46.9	98	63.6	0.3904 (0.22–0.68)*
CC	TT	14	10.6	20	12.9	0.4319 (0.18–0.98)[a]

OR odds ratio, *CI* confidence interval

*$P < 0.05$

[a] Borderline effect

Table 3 Genotypic distribution of the *p21* Ser31Arg and *p27* V109G polymorphisms and their relationship with the level of lesion (CINI, CINII, and CINIII)

SNP	CIN II/III/CC		CIN I		*P*	OR (95 % CI)	*P**
	n = 94	%	*n* = 38	%			
p21 Ser31Arg							
CC	49	52.12	27	71.05			
CA	38	40.42	11	28.95			
AA	7	7.46	0				
AA + CA × CC	45/49		11/27		0.0464	2.25 (1.00–5.06)	0.0723
p27 V109G							
TT	15	15.95	8	21.05			
TG	49	52.12	21	55.26			
GG	30	31.93	9	23.69			
GG + TG × TT	79/15		30/8		0.4847	1.4044 (0.54–3.65)	0.6561

P *P* value of χ^2 test, *OR* odds ratio, *CI* confidence interval, *P** *P* value of odds ratio

different results for the association of the polymorphism *p21* Ser31Arg with cancer cervical may be due to the genetic heterogeneity of CC in different ethnicities and/or genotypic distribution and allelic frequencies between different populations [41].

Studies that investigated expression of the P27 protein suggest that downregulation of *p27* is fundamental for the development of cervical cancer, since high expression of P27 is present in quiescent cells and normal cervical squamous epithelium [42, 43]. The *p27* V109G polymorphism results in the substitution of glycine for valine, which causes an alteration in the expression, activation or degradation of P27, thereby contributing to tumorigenesis [20, 24]. However, the analyses showed no significant association between the genotypes for SNP in the *p27* gene (*p27* V109G; rs2066827) and the development of cervical lesions. This result can be explained considering that other genes could interact and contribute to the development of the cervical lesions, which is a multifactorial trait. Following this line of reasoning, a genetic analysis combining the SNPs studied was conducted to check if a joint effect of the two polymorphisms for the development of lesions in the cervix could occur. The results showed that the presence of the CC genotype (SNP *p21* Ser31Arg; rs1801270) conferred protection even when the polymorphic variants in the *p27* gene are present, suggesting a possible autonomous role of the *p21* gene.

The infection by HPV 16 and 18, the most common types within the Brazilian population, are a risk factor for the development of cancer [5, 44]. In this study, these types were also found as more frequent in patients with cervical lesions (57.6 and 37.1 %, respectively). Two studies conducted in Recife (capital of Pernambuco state, in Brazil) by Baldez et al. [45] and Tavares et al. [5], analyzing 213 and 142 women HPV infected, respectively, have observed that there was the prevalence of HPV 16 in more than 50 % of their samples. In contrast, a study realized at Recife by Lorenzato et al. [46] verified that among women infected by HPV, the largest

Table 4 Genotypic frequencies of the *p21* Ser31Arg and *p27* V109G polymorphisms and infection by any HR-HPV

HPV type	*p21*		*P*	OR (95 % CI)	*P**
	CC	CA + AA			
16+/16−	27/49	30/26	0.038	2.09 (1.03–4.23	0.06
18+/18−	19/56	10/46	0.30	0.64 (0.27–1.51)	0.41
31+/31−	3/73	1/55	0.47	0.44 (0.04–4.36)	0.84
33+/33−	1/75	1/55	0.82	1.32 (0.08–21.69	0.60
16+18+/16− and/or 18−	14/62	5/51	0.12	0.43 (0.14–1.28)	0.20
	p27				
	TT	TG + GG			
18+/18−	5/18	25/84	0.90	1.07 (0.36–3.17)	0.88
31+/31−	1/22	3/106	0.69	0.62 (0.06–6.26)	0.07
33+/33−	0/22	2/105	0.52	–	–
16+18+/16− and/or 18−	2/31	17/92	0.39	1.94 (0.41–9.04)	0.59

P *P* value of χ^2 test, *OR* odds ratio, *CI* confidence interval, *P** *P* value of odds ratio

Table 5 Genotypic frequencies of both *p21* Ser31Arg and *p27* V109G polymorphisms with risk factors for cervical lesions in patients from Recife-Pernambuco population

			p21 Ser31Arg				
Clinical features		$n=132$	*CC* ($n=76$)	*CA* + *AA* ($n=56$)	*P*	OR (95 % CI)	*P**
Smoking	Yes	44	29	15	0.1707	0.59 (0.27–1.2)	0.23
	No	88	47	41			
Alcohol consumption	Yes	79	42	37	0.2106	1.57 (0.77–3.22)	0.28
	No	53	34	19			
Number of children[a]	≤ 3	75	42	33	0.7279	0.86 (0.39–1.92	0.88
	>3	37	22	15			
Number of sexual partners	≤3	85	46	39	0.5637	0.80 (0.37–1.70)	0.70
	>3	47	25	17			
IPRS	≤18	104	64	40	0.0758	0.46 (0.20–1.09)	0.12
	>18	28	12	16			
			p27 V109G				
Clinical features		$N=132$	TT ($n=23$)	TG + GG ($n=109$)	P	OR (95 % CI)	P*
Smoking	Yes	44	9	35	0.5153	0002E74 (0.29–1.90)	0.69
	No	88	14	74			
Alcohol consumption	Yes	79	14	65	0.9125	0.95 (0.38–2.38)	0.90
	No	53	9	44			
Number of children[a]	≤3	75	17	58	0.0585	3.32 (0.90–12.17)	0.10
	>3	37	3	34			
Number of sexual partners	≤3	85	16	69	0.5687	1.32 (0.50–3.49)	0.74
	>3	47	7	40			
IPRS	≤18	104	17	87	–	–	–
	>18	28	6	22			

P value of χ^2, *OR* odds ratio, *CI* confidence interval, *P** *P* value of odds ratio

[a] Twenty patients were excluded

prevalence was of the viral type 31 (21.4 %). Different factors could explain these conflicting results, such as the fact that these studies had been done in different periods, the characteristics of the populations analyzed (in the present study, only women with cervical lesion were analyzed) and, also, the specificity of the technique used.

No significant difference was found when a possible association between the allelic variants *p21*Ser31Arg and *p27* V109G and susceptibility to infection by any HR-HPV was analyzed or when other risk factors to the development of cervical lesion were considered.

Conclusion

The presence of the HR-HPV infection together with the polymorphisms in *p21* Ser31Arg gene are associated with the susceptibility to development of cervical lesion in women in the state of Pernambuco, Brazil.

Compliance with ethical standards

Conflict of interest None.

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Apêndice II

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Polymorphisms in *TNF*-α and *IL-10* genes in women with cervical disease in Pernambuco, Brazil.

Abbreviated Title Genes *TNF*-α, *IL-10 and* cervical disease

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ABSTRACT

Susceptibility to infectious diseases is associated with the profile of genes involved in the immune response to infection. Here, we investigated whether the polymorphisms at promoters regions of *IL-10* -1082 (A> G, rs1800896) and *TNF*-α -308 (G>A, rs1800629) genes, were associated with susceptibility to HPV infection/progression to cervical dysplasia and adenocarcinoma. The study population consisted of 240 women infected with HPV (72 with adenocarcima and 168 with cervical intraepithelial lesion) and 169 healthy control women. *IL10* -1082 (A> G) and *TNF-α* -308 (G> A) polymorphisms were analyzed by PCR-SSP. There was significant increase in the frequency of *IL10* -1082G allele in both cervical dysplasia (OR = 1.39; P = 0.0372) and adenocarcinoma patients (OR = 2.19; P = 0.0002). The same was observed concerning individuals with GG genotype (OR = 2.17, P = 0.047 and OR = 3.8; P = 0.0029, respectively). For *TNF-α* -308 polymorphism, there was no association of the allele or genotype frequencies in relation to cervical disease (P> 0.05). On the other hand, there were susceptibility in relation to risk factors such as age> 35 years (OR = 2.57; p = 0.0057), age of first sexual intercourse 1st <18 years (OR = 6.6224, p <0.0001), smoking (OR = 3.80; P = 0.0003), African origin (OR = 5.18, p <0.0001) and co-infection by *Chlamydia trachomatis* (OR = 2.41; P = 0.0315). Our findings suggest that polymorphisms in the *IL-10* and *TNF-α* genes may play a role in susceptibility or severity of cervical disease in the study population.

Keywords: Tumor Necrosis Factor alpha, Interleukin 10, Glandular cervical lesions, Adenocarcinoma, HPV.

INTRODUCTION

Despite the infection by one of the types of human papillomavirus of high oncogenic risk (HR-HPV) to be the main cause of cervical cancer and its precursor lesions (Cervical Neoplasms Intraepitelais NIC) [1-5], the presence of other cofactors plays an important role in the viral etiology, such as: alcoholism, multiple partners, multiparous, use of oral contraceptives, age, co-infections with other types of pathogens [Human Immunodeficiency Virus (HIV), Herpes Simplex Virus (HSV), *Chlamydia trachomatis* (CT)] and also one should take into consideration the genetic factors of the host [2, 6-8]. The type and quality of the immune response are factors that favor an enabling environment for viral replication, and individuals with impaired cellular immune response have a high prevalence of cervical lesions induced by HPV [9-11]

Interleukin 10 (IL-10) and tumor necrosis factor alpha (TNF-α) are two multifunctional cytokine involved in the immune response of the host. IL-10 is an anti-inflammatory cytokine excreted by a variety of cells and has dual activity on immune response and suppresses the cellular immune response (Th-1), and stimulates the humoral immune response (Th-2) [12]. Patients with severe squamous intraepithelial lesions (SIL) has been found with the level of IL-10 expression increased [13-15]. The TNF-α is a pro-inflammatory cytokine secreted mainly by macrophages, and has a key role in inflammation, immune homeostasis and host defense. Furthermore, expression increased of adhesion molecules and activation of neutrophils, stimulates the production of cytokines, and acts as a T cell co-stimulatory activation and antibody production [16-18]. Additionally, TNF-α is involved in the defense against HPV infection, modulating viral replication [19].

Polymorphisms in genes related with the immune response, such as *IL-10* and *TNF*-α genes may be involved in the etiology of cervical disease [20]. Several polymorphisms have been described to *IL-10* gene, in particular three in the promoter region (-1082 A/G -819 T/C and - 592 A/C) may influence the transcriptional level of mRNA and protein expression *in vitro* and consequently they contributes to the development of cancer [21]. However, regardless of the position of the other polymorphic sites in relation to *IL-10* gene, individuals who had the -1082 AA, -1082

GA and -1082GG genotypes were associated with low, middle and high production of *IL-10* protein, respectively, *in vivo* and *in vitro* studies [21-22].

TNF-α also has several genic polymorphisms located in its promoter region: -1031 (T/C), -863 (C/A), -857 (C/T), -308 (G/A), -238 (G/A), -1196 (C/T), -1125 (G/C), -572 (A/C), -316 (G/A), -163 (G/A) and -70 (G/A). However, single nucleotide polymorphisms (SNP) located at position -308 (G> A) of *TNF*-α gene causes variation in the serum concentration of the protein encoded by this gene. The *TNF*-α -308 GG genotype was associated with low protein production, while -308 AA and -308 AG genotypes were associated with middle and high protein production, respectively [23].

In relation to cervical lesions, the presence of the *TNF*-α -308 GG genotype has been associated with the induction of cervical squamous cervical intraepithelial lesions (SCIL), whereas the presence of *TNF*-α -308AG and *TNF*-α -308AA genotypes were associated with the progression to cervical lesions and even in the formation of invasive cervical cancer (ICC), however there are conflicting results [23-28]

Despite of *IL-10* -1082 (A/G) and *TNF-α* -308 G/A polymorphisms have been studied with respect to their susceptibility to many infectious diseases, functional importance about these polymorphisms need to be clarified. Therefore, this research aimed to investigate the possible association among *IL-10* -1082 (rs1800896) and *TNF*-α -308 (rs1800629) gene polymorphisms and the susceptibility to cervical intraepithelial lesions as well as for adenocarcinoma in a population from Northeast of Brazil.

METHODOLOGY

Design and study site

A cross-sectional study was performed aimed at analyzing *IL-10* and *TNF-α* genes polymorphisms in women with squamous cervical intraepithelial lesion (SCIL) and cervical adenocarcinoma. Women were recruited at the Lower Genital Tract Pathology Clinic at Women´s Healthcare Center of the Prof. Fernando Figueira Institute of Integrated Medicine between January 2008 to April 2010.

The laboratorial analyses were conducted at the Laboratory of Genetics, Biochemistry and DNA sequencing Professor Tânia Falcão from Rural Federal

University of Pernambuco. The local Ethics Committee for Research (nº 3326/13) approved the study and all patients and controls agreed to participate, signing the Terms of Free and Informed Consent.

Patients and Controls

The study population consisted of 3 groups: group 1: 96 women with cervical lesions and HPV-positive; Group 2: 72 women with cervical adenocarcinoma and HPV-positive; Group 3: 169 healthy control women, HPV-negative. The samples of this study were selected from a DNA bank originated from cervical smears of women aged 16 to 75 years assisted by spontaneous demand in the Central Public Health Laboratory of Pernambuco (LACEN) and Integrative Medicine Institute Professor Fernando Figueira (IMIP), from January 2008 to April 2010. As regards the samples of Group 2, were included in this group women between 31 and 76 years of age with histopathological diagnosis of adenocarcinoma in situ and invasive cervical the uterus, identified in the file Cervical Pathology Service of the CAM-IMIP, in the period between 2001 and 2014. The study excluded women who had undergone treatment for the cervix or diagnosed with HIV and patients with adenocarcinoma not included paraffin blocks nail biopsy or surgical piece. For the healthy control group were excluded women who had the cytology diagnosed with atypical cells and colposcopy with abnormal colposcopic findings or suggestive of invasion and positive for HPV infection. For all groups, information was collected from medical records of patients in relation to biologic factors, sociodemographic, reproductive, and lifestyle habits.

***IL-10* and *TNF-α* genes polymorphisms analysis**

The presence of polymorphisms in the promoter regions of *IL-10* -1082 (G> A) and *TNF*-α -308 (G> A) genes were analyzed by sequence specific PCR technique (PCR-SSP). The analyzes of *IL-10* -1082 (rs1800896) polymorphism gene were performed as described by Crilly *et al*. [29] while *TNF*-α -308 (rs1800629) polymorphism gene was performed as described by Perrey *et al*. [30]. The sequences of the primers used for both regions are shown in Table 1.

The amplification reactions for analysis of both polymorphic sites were made to a final volume of 15 µl. The reaction mixture contained approximately 50 ng of DNA vaginal secretions, 1X Platinum Taq Buffer (Invitrogen Life Technologies), 200 µM dNTPs, 2.5 mM $MgCl_2$, 1U Taq DNA Platinum DNA polymerase (Invitrogen Life Technologies) and 1 µM of each primer (common and specific allele). The cycling conditions used were the same as previously described in the literature cited above.

Statistical analysis

Statistical analysis was performed using the BioEstat 5.0 software. The study was cross-sectional with independents samples consisting of nominal data (genotype). The influence of each polymorphism on the risk for development of (pre) neoplastic cervical disease was estimated by odds ratio (OR) using a confidence interval of 95% for the parameters.

Allele frequencies were estimated by direct gene counting. The prevalence of different genotypes in patients and controls was analyzed by the χ^2 test and by the Fisher exact test in contingency tables. P-value under or equal to 0.05 were considered statistically significant.

Results

Tables 2 and 3 show the distribution of allelic and genotypic frequencies of *IL-10* -1082 A> G and *TNF*-α -308 G> A genes polymorphisms, between cases and healthy control groups. Individuals with *IL-10* -1082GG genotype and *IL-10* -1082 G allele were significantly associated with risk to cervical lesions increased [OR = 2.17, p = 0.047; OR = 1.39, p = 0.037, respectively], as well as to development of adenocarcinoma [OR = 3.8; p = 0.0029; OR = 2.19, p = 0.0002]. However, no significant difference in the distribution of allelic and genotypic frequencies were observed relation to *TNF*-α -308 G>A gene polymorphism, when compared with SCIL (p> 0.05) and adenocarcinoma (p> 0.05) (Table 3).

In the Tables 3 and 4, we related clinical features, sociodemographic, reproductive and habits from patients selected with the two polymorphisms analyzed. Seven risk factors (age> 35anos, age at first sexual intercourse 1st <18 years in

pregnancies> 3, use of OCP, smoker, African origin, and coinfection with CT) were evaluated with relation to development for both cervical lesions and adenocarcinoma and they were correlated with *IL-10* -1082 A>G and *TNF*-α -308 G>A genes polymorphisms (Table 4 and 5, respectively). Of all the analyzed cofactors, only patients with aged over 35 years were associated with *IL-10* -1082 A> G gene polymorphism and the susceptibility to cervical lesions (Table 4). Regarding the *TNF*-α -308 G> A gene polymorphism, significant differences were observed with respect to age > 35 years (OR = 2.57, p = 0.0057), first sexual intercourse < 18 (OR = 6, 62; p <0.0001), smoking patients (OR = 3.80; p = 0.0003), use of OCP (OR = 14.17; p <0.0001), and coinfection *by Chlamydia trachomatis* (OR = 2.41; p = 0.0315) (Table 5),also related to cervical cancer. On the other hand, no significant difference was observed among the cofactors and the presence of adenocarcinoma for both the polymorphisms analyzed in this study (p> 0.05).

Discussion

In this study, we evaluated the correlation between the distribution of genotypic and allelic frequencies for both IL-10 (rs1800896) and TNF-α (rs1800629) polymorphisms and the susceptibility for cervical lesions and development of adenocarcinoma. Our findings suggest that the presence of variants GG and GA genotype as well as G allele in the promoter region of the *IL-10* (rs1800896) gene influence the developing cervical lesions and adenocarcinoma. Matsumoto *et al*. [31] studying a Japanese population, found association among high production genotypes (GA and GG) of IL-10 and the degree severity of cervical disease. These findings can be explained by the increase in the transcriptional nivel of IL-10 and, once IL-10 is a cytokine produced by Th-2 cells and possesses immunosuppressive and antiangiogenic activities, it may lead to an increased susceptibility for both, cervical lesions and adenocarcinoma. Concerning to the AA genotype, it was associated with decreased serum levels of protein in some studies involving different types of cancers (like as cervical, prostate and breast cancer) [32-33] and it has been considered as a biomarker for the development of cervical lesions as well as progression for cervical cancer [23, 34-37].

In relation to *TNF*-α -308 G/A gene polymorphism, it is known that the transcriptional level of mRNA of the TNF-α protein increases from 6 to 9 times in vitro in the presence of the transition from G to A, and may affect the susceptibility for various diseases [38-39]. However, literature data are still contradictory when related with the susceptibility for cervical lesions and progression to cervical cancer. In this study, no association was found among the *TNF*-α -308 G/A gene polymorphism and the susceptibility to cervical lesions or development of adenocarcinoma ($p > 0.05$). Souza *et al*. [40] studying a portuguese population also found no significant association with the development of pre-invasive cervical lesions. However, in this same study they found that individual with -308A allele and -308AA genotype had an increased risk for developing cervical cancer.

Kirkpatrick *et al*. [41] found a significant association between individuals carriers of GG genotype (low secretory) with the development of cervical intraepithelial lesion low grade, but they did not found association with respect to the development of cervical cancer. This last result was in agreement with other that also showed no association [24, 27, 42]. On the other hand, in others studies this polymorphism it was associated with the development of cervical cancer (26, 43-45]. Furthermore, a meta-analysis performed by Liu *et al.* [46] showed that individuals from African origin carrying the -308 AA genotype had been protected against developing cervical cancer, but no association in relation to Caucasian origin was observed. These discordant results may be explained by the difference in genetic background between the different populations analyzed.

Regarding the socio-behavioral factors our results showed that *IL-10* gene polymorphism was associated only with age above 35 years. On the other hand, when we analyzed the *TNF*-α -308 polymorphism, individuals carrying of the GA and AA genotypes were significantly associated with several risk factors, as age > 35 years, age at first intercourse <18 anos, prolonged use of oral contraceptives (ACO), smoking, and presence of co-infection with CT ($p < 0,05$). Duarte *et al*. [47] found similar results and suggested that these results could be explained due to HPV latency in the body, causing histological changes in women older age. Bezerra *et al*. [48] showed that the early age of first sexual intercourse considering the malformation of the female reproductive

system contributes directly to an increase in the chance of developing cervical lesions. A study performed by Rosa *et al*. [49] found that the use of ACO potentiates the onset of cervical lesions by about three times. In this same study, they found an increased risk for women that more parities. Furthermore, Simonetti *et al*. [50] suggested in their study that the co-infection by CT can cause inflammatory responses that damage the cervical mucosa, leading to lesions or even facilitate the HPV infection.

Conclusion

In our study, we found association of IL-10 (rs1800896) polymorphisms with the predisposition to develop of cervical lesions and adenocarcinoma. In addition, the clinical data of the patients presented significant differences for both IL-10 (rs1800896) and TNF-α (rs1800629) polymorphisms. Thus, our data suggest that *IL-10* and *TNF*-α gene can be used as molecular markers in patients predisposed to the screening cervical disease.

Conflicts of interest

The authors declare no conflicts of interest

Ethical approval

"All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards."

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Table 1. Sequence of primers used in the study

Primer	Sequence Sense	Antisense Sequence	Reference
IL-10 -1082 A	5´- ACTACTAAGGCTTCTTTGGGAA-3´	5´- CAGTGCCAACTGAGAATTTGG-3´	Crilly *et al.* (2003)
IL-10 -1082 G	5´- CTACTAAGGCTTCTTTGGGAG-3´		
TNF-α-308 G	5'-ATAGGTTTTGAGGGGCATGG-3'	5'-TCTCGGTTTCTTCTCCATCG-3'	Perrey *et al*. (1999)
TNF-α-308 A	5'-AATAGGTTTTGAGGGGCATGA-3'		

Table 2. genotypic and allelic distribution of the polymorphism of the promoter region of the IL10 (-1082 A / G) in patients with cervical lesion, patients with adenocarcinoma and healthy control.

IL10 Promoter -1082	Group 1 n=168 (%)	Group 2 n= 72 (%)	Group 3 n=169 (%)	χ^2 P (Value)	OR (95% CI)	*P**
Genotype						
AA	36 (21,5)	14 (19)	28 (16,57)		Reference	
GA	76 (45,2)	20 (28)	121 (71,6)	**6,158 (0,0131)[a]** **7,844 (0,0051)[b]**	**0,4885 (0,2760 - 0,0195)[a]** **0,3306 (0,1490 - 0,7336)[b]**	**0,0195[a]** **0,01[b]**
GG	56 (33,3)	38 (53)	20 (11,83)	**4,687(0,0304)[a]** **10,109 (0,0015)[b]**	**2,1778 (1,0703 - 4,4311)[a]** **3,8 (1,6413 - 8,7979)[b]**	**0,0470[a]** **0,0029[b]**
AA/GG+GA	36/132	14/58	28/141	1,294 (0,2554)[a] 0,290 (0,59)[b]	0,7281 (0,421 - 1,2595)[a] 0,8227 (0,4042 - 1,6745)[b]	0,3180[a] 0,7239[b]
Allele						
A	148 (44,0)	48 (33,3)	177 (52,37)		Reference	
G	188 (56,0)	96 (66,7)	161 (47,63)	**4,671 (0,0307)[a]** **14,698 (0,0001)[b]**	**1,3965 (1,0312 - 1,8912)[a]** **2,1988 (1,4637 - 3,3031)[b]**	**0,0372[a]** **0,0002[b]**

[a] Comparative results between the group of patients with cervical lesions and the control group
[b] Comparative results between the group of patients with adenocarcinoma and the control group
χ^2 –Chi squared test; P value de χ^2; OR – Odds ratio; CI –Confidence Interval; P* – P value de odds ratio
Characteristics in bold show significant values

Table 3. genotypic and allelic distribution of the polymorphism of TNF-α promoter region (-308 G / A) in patients with cervical lesions (Group 1), patients with adenocarcinoma (Group 2) and control patients

TNF-α Promoter -308	Group 1 n=168 (%)	Group 2 n= 72 (%)	Group 3 n=169 (%)	χ^2 P (Value)	OR (95% CI)	*P**
Genotype						
GG	103 (61,3)	44 (61,1)	95 (56,21)		Reference	
GA	58 (34,5)	26 (36,1)	74 (43,79)	2.07 (0.1502)[a] 0.898 (0.3433)[b]	0,7229 (0,4644 - 1,1254)[a] 0,7586 (0,4281 - 1,3444)[b]	0,1847[a] 0,4217[b]
AA	7 (4,2)	2 (2,8)	-		No analysis	
GG/GA+AA	103/65	44/28	95/74	0.903 (0.342)[a] 0.496 (0.4812)[b]	0,8102 (0,5247 - 1,2510)[a] 0.8170 (0.4653 - 1.4344)[b]	0.401[a] 0,5741[b]
Allele						
G	264 (78,6)	114(79,2)	264 (78,1)		Reference	
A	72 (21,4)	30 (20,8)	74 (21,9)	0.021 (0.8835)[a] 0.067 (0.7956)[b]	0.9730 (0.6744 - 1.4038)[a] 0.9388 (0.5823 - 1.5138)[b]	0.9577[a] 0.8902[b]

[a] Comparative results between the group of patients with cervical lesions and the control group
[b] Comparative results between the group of patients with adenocarcinoma and the control group
χ^2 –Chi squared test; P value de χ^2; OR – Odds ratio; CI –Confidence Interval; P* – P value de odds ratio
Characteristics in bold show significant values

Table 4. Distribution of genotype polymorphism of the promoter of the IL-10 gene region (-1082 A / G) in patients with cervical lesions and socio-behavioral characteristics.

socio-behavioral characteristics		n=168	Group 1		χ^2 (P Value)	OR (95%IC)	P*
			AA (n)	AG+GG (n)			
Age> 35 years	y	49	25	24	**35,979 (<0,0001)**	**0,0978 (0,0424-0,2255)**	**<0,0001**
	n	119	11	108			
Age of first sexual intercourse <18 years	y	116	26	90	0,543 (0,4616)	0,8242 (0,3644-1,8681)	0,3937
	n	52	10	42			
Above 3 pregnancies	y	85	19	66	0,087 (0,7676)	0,8947 (0,4278-1,8715)	0,9144
	n	83	17	66			
Using ACO[1]	y	83	19	64	0,209 (0,6479)	0,8421 (0,4026-1,765)	0,7882
	n	85	17	68			
Smoker	y	50	14	36	1,826 (0,1766)	0,5893 (0,2723-1,2751)	0,252
	n	118	22	96			
African derivation	y	58	8	50	3,067 (0,0799)	2,1341 (0,9023-5,0477)	0,1203
	n	110	28	82			
CT-positive	y	24	4	20	0,377 (0,5392)	1,4286 (0,4554-4,4810)	0,7298
	n	144	32	112			

[1] Oral contraceptive

χ^2 –Chi squared test; P value de χ^2; OR – Odds ratio; CI –Confidence Interval; P* – P value de odds ratio

Characteristics in bold show significant values

Table 4. Distribution of genotype polymorphism of the promoter region of the TNF-α gene (-308 G / A) in patients with cervical lesions and socio-behavioral characteristics

socio-behavioral characteristics		n = 168	Group 1		χ^2 (P Value)	OR (95%IC)	P*
			GG (n)	AG+AA (n)			
Age> 35 years	Y	93	49	44	**6,528 (0,0106)**	**2,5714 (1,3545-4,8815)**	**0,0057**
	N	75	54	21			
Age of first sexual intercourse <18 years	Y	99	44	55	**28,903 (<0,0001)**	**6,6224 (3,0571-14,3458)**	**<0,0001**
	N	69	59	10			
Above 3 pregnancies	Y	80	45	35	1,648 (0,1992)	1,5037 (0,8057-2,8066)	0,2605
	n	88	58	30			
Using ACO[1]	y	81	48	33	**0,277 (0,5986)**	**14,1797 (4,6013-43,6973)**	**<0,0001**
	n	87	55	32			
Smoker	y	47	18	29	**14,568 (0,0001)**	**3,8040 (1,8786-7,7030)**	**0,0003**
	n	121	85	36			
African derivation	y	60	22	38	**18,577 (<0,0001)**	**5,1818 (2,6194-10,2510)**	**<0,0001**
	n	108	81	27			
CT-positive	y	36	16	20	**5,494 (0,0191)**	**2,4167 (1,1422-5,1134)**	**0,0315**
	n	132	87	45			

[1] Oral contraceptive

χ^2 –Chi squared test; P value de χ^2; OR – Odds ratio; CI –Confidence Interval; P* – P value de odds ratio

Characteristics in bold show significant values