Universidade Federal de Pernambuco
Centro de Biociências
Programa de Pós-Graduação em Genética

RITA DE CÁSSIA PEREIRA DE LIMA

IDENTIFICAÇÃO, CARACTERIZAÇÃO E AVALIAÇÃO FUNCIONAL DE VARIANTES DO ONCOGENE E7 DO PAPILOMAVÍRUS HUMANO 16

Recife
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Tese apresentada ao Programa de Pós-Graduação em Genética da Universidade Federal de Pernambuco como parte dos requisitos exigidos para obtenção do título de Doutor em Genética.

Orientador: Dr. Antonio Carlos de Freitas
Coorientadores: Dra. Bárbara Simas Chagas
Dr. Marcelo Nazário Cordeiro

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Aprovado em 08/03/2017

Banca Examinadora:

_____

Dr. Antonio Carlos de Freitas

Universidade Federal de Pernambuco

_____

Dr. Lindomar José Pena

Fundação Osvaldo Cruz/Centro de Pesquisa Aggeu Magalhães

_____

Dra. Neide Santos

Universidade Federal de Pernambuco

_____

Dra. Heidi Lacerda Alves da Cruz

Universidade Federal de Pernambuco

_____

Dr. Jacinto da Costa Silva Neto

Universidade Federal de Pernambuco

Recife

2017

Dedico a realização deste trabalho ao meu Deus e salvador todo poderoso que sempre esteve presente em cada momento da minha vida.

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"Que os vossos esforços desafiem as impossibilidades, lembrai-vos de que as grandes coisas do homem foram consquistadas do que parecia impossível."
(Charles Chaplin)

RESUMO

Cervical e um subconjunto de cânceres de cabeça e pescoço são causados pela infecção persistente por papilomavírus humano (HPV) de alto risco. A oncogenicidade do HPV está fortemente associada a fatores ambientais, a exemplo dos tipos virais e suas variantes, e também tem sido associado a presença de polimorfismos em oncogenes virais, os quais podem estar envolvidos no risco de progressão maligna. O conhecimento sobre como a variância genética é bem distribuído entre diferentes populações e sua relação com lesões de alto grau pode ser crucial para o diagnóstico e estratégias terapêuticas contra doenças relacionadas ao HPV. Os polimorfismos podem levar à alteração da função biológica com conseqüências clínicas. Além de prejudicar o controle do ciclo celular mediado por pRB, as atividades de E7 incluem perturbação da via NF-κB afetando uma importante via crítica celular anti-viral. Em pacientes do Nordeste do Brasil, nosso estudo descreveu a presença de tipos de HPV e variantes de amostras cervicais por técnica de PCR e sequenciamento e realizou uma das primeiras análises funcionais baseadas na atividade NF-κB dos polimorfismos E7 de HPV-16. Adicionalmente, para se ter acesso aos níveis de expressão de mRNA de E7 a partir de culturas de células. O HPV-16 foi encontrado como o tipo mais prevalente, seguido por HPV-31 e HPV-58. Entre as variantes do HPV-16, as linhagens C e D foram detectadas principalmente em lesões cervicais de alto grau. Além disso, identificamos quatro polimorfismos do gene E7 do HPV-16. Um polimorfismo não-sinônimo foi localizado em epítopos preditos de células T e B, que estão sob seleção positiva. Este polimorfismo também está localizado em uma região de loop próximo ao domínio da hélice LXCXE, que é responsável pela interação da proteína E7 e pRb, passo crucial no desenvolvimento do câncer. Novas mutações adaptativas podem levar o vírus a evadir o sistema imunológico do hospedeiro e aumentar a patogenicidade. Sobre os resultados da via NF-kB, estes mostraram que as variantes do gene E7 do HPV-16 nas quais os três polimorfismos estão presentes podem exercer um forte efeito supressor nos pacientes infectados com HPV com implicações na persistência da infecção e na sua progressão. No entanto, as alterações resultantes dos polimorfismos podem não estar necessariamente relacionadas com os níveis de expressão diferencial das variantes. Estes resultados adicionam dados importantes para os estudos sobre a variabilidade de E7, o que poderia contribuir para uma melhor compreensão da diversidade e infecção do HPV.

Palavras-chave: análise funcional, polimorfismos, NF-kB

ABSTRACT

Cervical and a subset of head and neck cancers are caused by high-risk human papillomavirus (HPV) persistent infection. HPV oncogenicity is strongly associated with environmental factors, viral types, and their variants, where even polymorphisms in viral oncogenes may be involved in malignant progression risk. Knowledge about how genetic variance is distributed among populations and its relationship with high-grade lesions may be critical to diagnosis and therapeutic strategies against HPV-related diseases. Polymorphisms may lead to altered biological function with clinical consequences. Besides impairing pRB-mediated cell cycle control, E7 activities include NF-κB disturbing, which affects an anti-viral cellular critical pathway. In Brazilian Northeastern patients, our study described the presence of HPV types and variants from cervical cells by PCR technique and sequencing and performed one of the first NF-κB activity-based functional analysis of HPV-16 E7 polymorphisms. In addition, in order to access E7 mRNA expression levels from cell cultures. HPV-16 was found as the most prevalent type, followed by HPV-31 and HPV-58. Among HPV-16 variants, C and D lineages were detected mostly from high-grade cervical lesions. Furthermore, we have identified four HPV-16 E7 gene polymorphisms. A non-synonymous polymorphism was located into predicted T and B-cell epitopes, which are under positive selection. This polymorphism is also located in a loop region next to LXCXE helix domain, which is responsible for the interaction of E7 protein and pRb, critical step in cancer development. Novel adaptive mutations could lead the virus to evade host immunological system and increase pathogenicity. About the NF-kB pathway, results have shown that HPV-16 E7 gene variants in which the three polymorphisms are present may perform a strong suppressive effect on the in HPV-infected patients with implications for infection persistence and its progression. However, the changes resulting from polymorphisms may not necessarily be related to the differential expression levels of the variants. These results add important data on E7 variability studies, which could contribute to a better understanding of HPV diversity and infection.

Key words: functional evaluation, polymorphisms, NF-κB

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Capítulo I

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

Item	Definição
ASC-US	células escamosas atípicas de significado indeterminado
ASC-H	células escamosas atípicas HSIL
Bp	Pares de base
BPV	Papilomavírus bovino
CDK	Quinase dependente de ciclina
CIN	Neoplasia Intrepitelial Cervical
CK	Caseína Quinase
CKI	Inibidores de quinases dependente de ciclina
COPV	Papilomavírus Oral Canino
CRPV	Papilomavírus de Coelho
DNA	Ácido Desoxirribonucleico
DMEM	Meio Dulbecco's Modified Eagle's
E2F	Fator Transcricional E2F
E6	Oncogene E6
E6AP	Proteína de associação a E6
E7	Oncogene E7
EV	Epidermodisplasia verruciforme
F	Aminoácido Fenilalanina
HEK-293	Rim Embrionário Humano -293
HIV	Vírus da Imunodeficiência Humana
HLA	Antígeno leucocitário humano
HSIL	Lesão intraepitelial escamosa de alto grau

HSPG	Heparan sulfato proteoglicano
HPV	Papilomavírus humano
HR	Papilomavírus humano de alto risco oncogênico
HSIL	Lesão intraepitelial de alto grau
I	Aminoácido Isoleucina
IL	Interleucina
INCA	Instituto Nacional do Câncer
Kb	Kilobase
LCR	Longa região de controle
LSIL	Lesão intraepitelial de baixo grau
Luc	Luciferase
MHC	Complexo principal de histocompatibilidade
N	Aminoácido Asparigina
NF-κB	Fator Nuclear -kappaB
NIC	Neoplasia intra-epitelial cervical
ORF	Open reading frame
pAE	Sinal de poliAdenilação precose
PBS	Tampão fosfato salino
PCR	Reação de cadeia de polymerase
pRb	Proteína Retinoblastoma
p53	Proteína 53
PV	Papillomavirus
RNA	Ácido Ribonucleíco
qPCR	PCR quantitativa
S	Aminoácido Serina

SIL	Lesão Intraepitelial Escamosa
SNP	Polimorfismos de base única
T	Aminoácido Treonina
TNF	Fator de Necrose Tumoral
UV	Ultravioleta
WHO	Organização Mundial da Saúde

SUMÁRIO

1. INTRODUÇÃO

O câncer cervical é considerado o quarto tipo de câncer a ocasionar mortes entre as mulheres no mundo, com cerca de 529.800 casos diagnosticados a cada ano, representando cerca de 7.5% de todas as mortes por câncer e ocasionando cerca de 270.000 mortes por ano. No Brasil, o câncer cervical é o terceiro tumor mais frequente em mulheres, com estimativa de 16.340 casos para o ano de 2016, sendo considerado a quarta causa de morte entre mulheres portadoras de câncer com 5.430 casos registrados no ano de 2013.

A infecção persistente pelo Papilomavírus Humano (HPV – *Human Papillomavirus*) é o principal fator de risco para o desenvolvimento de câncer cervical. Os prognósticos mais graves na patogênese cervical estão relacionados com o tipo de HPV e o período de replicação do vírus nas células epiteliais da zona de transformação do colo do útero. A relação entre tipos de HPV e câncer cervical foi estabelecida a partir de estudos epidemiológicos, onde o vírus foi detectado em mais de 99,7% do carcinoma de células escamosas e em 94-100% do carcinoma cervical adenoescamoso.

O genoma do HPV pode ser dividido em três regiões: a longa região de controle (LCR – *Long Control Region*), a região precoce (E – *Early*) e a região tardia (L – *Late*). A porção reguladora LCR compreende aproximadamente 1kb e está localizada entre as *Open Reading Frames* (ORFs) L1 e E6. Nessa região, existem sequências acentuadoras e repressoras da transcrição viral, além da sequência de origem de replicação. Com alguma variação a depender do tipo de HPV, a região E é formada pelos genes: *E1*, *E2*, *E4*, *E5*, *E6* e *E7*, os quais são expressos imediatamente após a infecção da célula hospedeira pelo vírus e cujos produtos participam da replicação do genoma viral, controle de expressão viral, desestabilização da rede de queratina, imunoevasão e pertubação do ciclo celular. A região L é formada pelos genes *L1* e *L2*, os quais são expressos tardiamente e respondem por proteínas estruturais do vírus.

A atividade transformante dos HPVs de alto risco é promovida pelas oncoproteínas E6 e E7, as quais inativam os produtos dos supressores tumorais, *TP53* e *pRb*, respectivamente. Ambas oncoproteínas induzem a proliferação, imortalização e transformação maligna das células infectadas pelo vírus.

Regiões polimórficas nos oncogenes E6 e E7 podem responder por expressão diferencial ou mesmo propriedades funcionais das proteínas que devem ser consideradas para o diagnóstico, prevenção e terapia empregados no controle de infecções persistentes. As proteínas E6 e E7 do HPV são importantes para várias atividades virais, onde variações na sequência do gene de uma ou ambas as proteínas podem conduzir a função biológica alterada e, portanto, afetar o resultado clínico.

O presente projeto tem como finalidade identificar as linhagens e as variantes no oncogene E7 do HPV-16 presentes na região Nordeste do Brasil e avaliar a função biológica destas variantes identificados por meio de ensaios de atividade da luciferase e qPCR. Através da realização deste estudo foi possível iniciar uma fundamentação para o potencial dessas variantes e a importância clínica de seus polimorfismos quanto à oncogenicidade em mulheres da região Nordeste do Brasil.

1.1 Problematização

Em 2013 foi publicada uma portaria (N° 874, de 16 de Maio de 2013) do Ministério da Saúde do Brasil, que institui a Política Nacional para a Prevenção e Controle do Câncer na Rede de Atenção à Saúde das Pessoas com Doenças Crônicas no Âmbito do Sistema Único de Saúde. A Política Nacional para a Prevenção e Controle do Câncer tem como objetivo reduzir a mortalidade e a incapacidade e diminuir a incidência de alguns tipos de câncer, bem como contribuir para a melhoria da qualidade de vida dos usuários com câncer, por meio de ações de promoção, prevenção, detecção precoce, tratamento oportuno e cuidados paliativos. Além disso, essa portaria também preconiza a promoção do intercâmbio de experiências que visem estimular o desenvolvimento de estudos e pesquisas que busquem o aperfeiçoamento, a inovação de tecnologias e a disseminação de conhecimentos voltados à promoção da saúde, à prevenção e ao cuidado das pessoas com câncer.

De acordo com a alta incidência do HPV 16, principalmente entre países em desenvolvimento (WHO, 2015), evidencia-se a necessidade da realização de estudos funcionais sobre estes tipos virais de alto risco oncogênico na população

brasileira, e em especial no Nordeste. Contudo, apesar do HPV-16 ser considerado o tipo viral mais prevalente na região do Nordeste do Brasil e em alguns países, até o presente momento existem poucos dados referenciando a funcionalidade de variantes dos oncogenes E6 e E7 e dos HPVs 16.

1.2. OBJETIVOS

1.2.1 **Objetivo Geral**

Descrever a presença dos tipos de HPVs e variantes do HPV-16 circulantes no Brasil, em particular na região Nordeste e verificar a função biológica dos polimorfismos do oncogene E7 do HPV-16 na via NF-κB.

1.2.2 **Objetivos Específicos**

a) Identificar a presença e os tipos de HPVs presentes em amostras cervicais de pacientes da região Nordeste do Brasil;
b) Realizar a análise filogenética de variantes do E7 HPV-16 na população estudada;
c) Identificar os polimorfismos no oncogene E7 do HPV-16 e verificar a ocorrência de alteração conformacional resultantes destes;
d) Avaliar a função biológica destes polimorfismos na via NF-kB, e efeitos na expressão gênica do E7.

2. REVISÃO DA LITERATURA

2.1 Câncer cervical e a infecção pelo HPV

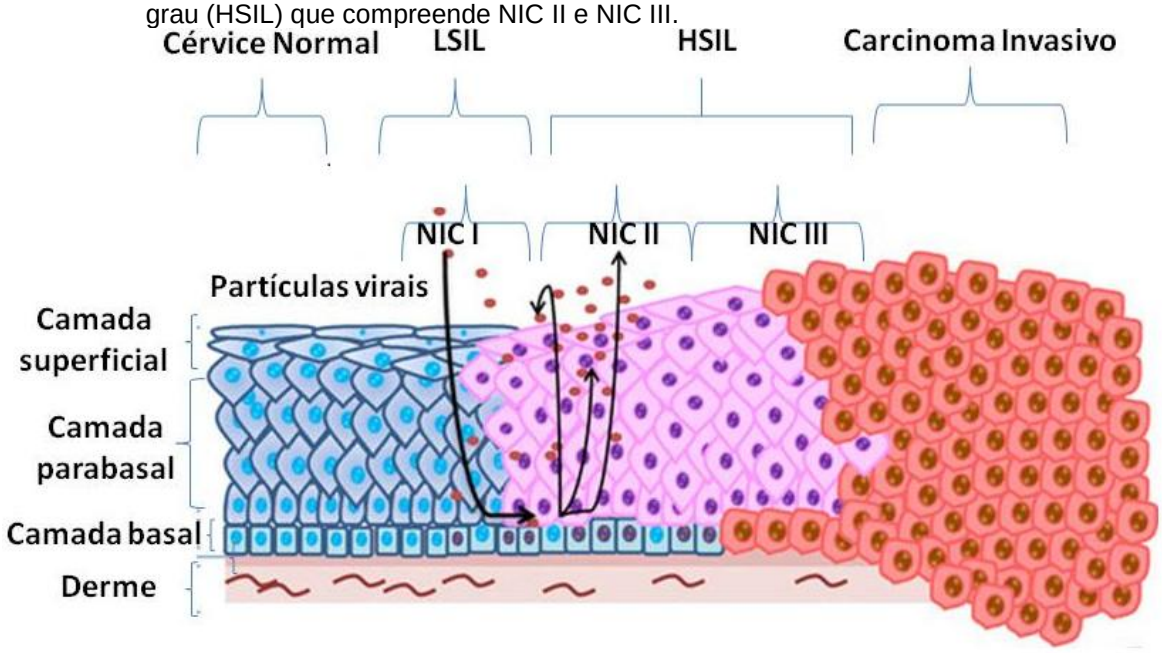
O câncer cervical é considerado o quarto tipo de câncer a ocasionar mortes entre a população feminina no mundo, com diagnóstico de 530.00 casos por ano, representando cerca de 7.5% de todas as mortes por câncer e ocasionando cerca de 270.000 mortes por ano (WHO, 2015).

Na população brasileira, o câncer cervical é o terceiro tumor mais frequente em mulheres, com estimativa de 16.340 casos para o ano de 2016, precedido apenas pelo câncer de mama e colorretal. Em relação à mortalidade por cânceres em mulheres este representa o quarto mais associado com 5.430 mortes registradas no ano de 2013 (INCA, 2016).

O câncer cervical é precedido por lesões pré-malignas não invasivas, denominadas neoplasias intraepiteliais cervicais-NIC (*cervical intraepithelial neoplasia-CIN*) ou lesão intraepitelial escamosa (*squamous intraepithelial lesions-SIL*). Histologicamente as neoplasias intraepiteliais cervicais são classificadas com base na morfologia e estágio progressivo das células epiteliais: em NIC I - displasia leve; NIC II - displasia moderada e NIC III - displasia severa, podendo esta progredir para câncer invasivo (carcinoma *in situ*) (WOODMAN et al., 2007).

Citologicamente as lesões intraepiteliais são classificadas em lesão intraepitelial escamosa de baixo grau (*low-grade* SIL - LSIL), que compreende NIC I, e lesão intraepitelial escamosa de alto grau (*high-grade* SIL - HSIL), que compreende NIC II e III (Figura 1). Ainda é possível encontrar células escamosas atípicas de significado indeterminado (ASC-US), e células escamosas atípicas HSIL (ASC-H) quando não é possível diagnosticar NICII e NICIII (STEENBERGEN et al., 2005). De acordo com o estágio dessas lesões, é possível a regressão ou progressão, sendo de extrema relevância o diagnóstico precoce e exato (STEENBERGEN et al., 2005).

Figura 1: Progressão do câncer cervical mediada pela infecção do Papilomavírus Humano (HPV). O vírus atinge a camada basal através de micro-abrasões no epitélio cervical. Nas camadas superiores do epitélio ocorre a replicação do genoma viral e formação de novas partículas virais que são liberados podendo iniciar uma nova infecção e, posteriormente poderá ocorrer a progressão para o câncer invasivo. As lesões intraepiteliais podem ser classificadas escamosa de baixo grau (LSIL) que compreende a neoplasia intraepitelial cervical I (NIC I) ou lesão intraepitelial de alto grau (HSIL) que compreende NIC II e NIC III.



Fonte: Adaptado de Saavedra et al., (2012).

O teste de rastreamento mais utilizado na prevenção do câncer cervical é a colpocitologia, também conhecido como exame Papanicolao ou preventivo, utilizado na identificação de lesões pré-neoplásicas, que se forem diagnosticadas precocemente e tratadas podem evitar a progressão para o câncer invasivo (PEIRSON et al., 2013).

Ainda que o HPV seja muito frequente em população sexualmente ativa, o sistema imune do hospedeiro em geral combate a infecção eficientemente, ocasionando alterações citológicas ou histológicas cervicais menores transitórias ou até mesmo assintomáticas (BOSCH et al., 2002).Em alguns casos, as infecções ocasionadas por HPVs de alto risco carcinogênico, o organismo perde a capacidade de combatê-las, a qual torna-se persistente podendo ocasionar o desenvolvimento do câncer cervical (SNIJDERS et al., 2005).

O ciclo viral do HPV representa o principal fator de risco para o desenvolvimento de câncer cervical, estando relacionado com diversos fatores, como, por exemplo, o tipo de HPV (alto ou baixo risco) (VILLA et al., 2000;

SICHERO et al., 2007; WOODMAN et al., 2007) e o período de replicação do vírus nas células epiteliais da zona de transformação (HARMSEL et al.,1999; HOPMAN et al., 2000).

O HPV está associado com aproximadamente 98% dos tumores do colo do útero (ZUR HAUSEN, 1996; BASEMAN e KOUTSKY, 2005). A alta frequência do HPV é bem documentada, com detecção em mais de 90% das amostras do carcinoma cervical do tipo adenoescamoso (VAN MUYDEN et al., 1999; ZIELINSKI et al., 2003) e ainda sendo implicado em mais de 99% dos carcinomas de células escamosas (WALBOOMERS et al., 1999; MUNOZ et al., 2003).

Dados epidemiológicos também demonstram a associação do HPV em outros tipos de cânceres situados no pênis, vagina, vulva, ânus, orofaríngeo, câncer de pele não melanoma, e também em doenças benignas, a exemplo das verrugas anogenitais, psoríase e papilomas de laringe (PFISTER, 2003; HOORY et al., 2008; CHATURVEDI et al., 2011; CLEVELAND et al., 2011; LOWY e SHILLER, 2012; BRAVO et al., 2015).

A infecção pelo HPV é o principal fator de risco para a formação da neoplasia cervical (STANLEY, 2001; CLIFFORD et al., 2003), entretanto alguns cofatores ambientas podem influenciar o seu desenvolvimento entre eles o tabagismo, atuante no decréscimo da resposta imune e aumento nos danos genéticos (TRIMBLE et al., 2005; MACLAUGHLAN et al., 2011; MOKTAR et al., 2011; WARD et al., 2011). O uso de contraceptivos também pode estar relacionados, pois podem promover o aumento da expressão de oncogenes virais (EFIRD et al., 2011; GHANEM et al., 2011; AMARAL et al., 2014). Multiparidades decorrentes de mudanças hormonais induzidas pela gestação podem modular a resposta imune ao HPV (BOSCH et al., 2002).

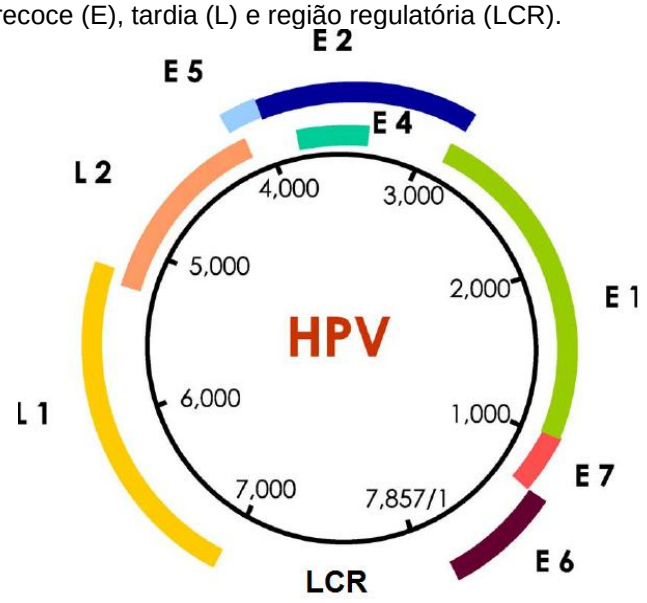
O próprio sistema imune do hospedeiro pode ser listado como um importante cofator que pode contribuir para o desenvolvimento do câncer cervical, pois foi relatado que determinados polimorfismos nos genes do HLA, relacionados com a apresentação de antígenos às células T CD4+, podem implicar na susceptibilidade genética à progressão maligna da neoplasia cervical (APPLE et al., 1994; MACIAG et al., 2002).

2.2 Estrutura viral e organização do genoma do HPV

Os papilomavírus são considerados pequenos entre os vírus analisados sob microscopia eletrônica. Seu capsídeo proteico, de configuração icosaédrica não ultrapassa os 55nm. Não há envelope recobrindo a partícula viral, em cujo interior encerra-se um genoma compreendido entre 6,8 a 8,0Kb de uma dupla-fita de ácido desoxirribonucleico (DNA) circular (ZHENG e BAKER, 2006). Os HPV restringem o tamanho de seu genoma a cerca de 7,8Kb como esboçado na Figura 2 com o esquema do genoma viral e suas principais regiões descriminadas.

O capsídeo dos papilomavírus é constituído por 72 capsômeros, cada qual composto pelo pentâmero da principal proteína estrutural do vírus, designada L1. A segunda proteína estrutural do vírus está presente em 12 cópias, sendo denominada L2, cuja extensão atravessa a estrutura da partícula viral para se associar ao DNA viral, sob a forma de um nucleossomo (BAKER et al., 1991; MODIS et al., 2002). O genoma dos papilomavírus encontra-se associado à histonas celulares, compondo um cromossomo rudimentar (TAN et al., 1998).

Figura 2: Esquema ilustrativo do genoma de HPV16 mostrando os genes de expressão precoce (E), tardia (L) e região regulatória (LCR).



Fonte: Modificado de Munoz et al., (2006).

O genoma dos papilomavírus pode conter entre 8 a 10 ORFs, cujas sequências são expressas de acordo com um padrão precoce e tardio conforme a diferenciação da célula hospedeira ao longo da infecção (GRAHAN, 2010). Os

genes designados como E (*early*) são comumente expressos desde os primeiros estágios da infecção e estão relacionados a funções regulatórias, como controle de transcrição e replicação virais, e transformação celular, enquanto a expressão dos genes L (*late*), somente é detectada nos estágios finais de infecção, envolvidos na composição do capsídeo viral. O DNA viral contém, entretanto, uma região não traduzida, denominada *Long Control Region* (LCR), na qual estão presentes sítios de interação *cis* com fatores transcricionais do vírus e da célula hospedeira, além da origem de replicação viral (DOLLARD et al., 1993; Berg e STENLUND, 1997).

A partícula viral do HPV segue o mesmo padrão morfológico dos demais papilomavírus, embora a organização do genoma possa variar em relação à quantidade, tamanho e sequência de ORFs, o que resulta em funções e padrão de expressão de algumas de suas proteínas. A região E do genoma codifica proteínas que atuam na fase precoce e tardia da infecção (E1, E2, E4, E5, E6 e E7), enquanto a região L contém as sequências codificantes de proteínas produzidas nos últimos estágios da infecção (L1 e L2) (SCHWARZ et al., 1985). Durante a infecção, logo na camada basal do epitélio, duas proteínas virais estão envolvidas na replicação viral, como a helicase E1, e no controle da transcrição viral em queratinócitos, como o regulador transcricional E2 (CHIANG et al., 1992). A progressão da infecção produtiva, vinculada ao atraso da diferenciação dos queratinócitos e avanço do ciclo celular, é desencadeada pela ação das proteínas E5, E6 e E7 (DIMAIO e PETTI, 2013; ROMANA e MUNGER, 2013; VANDE POL e KLINGELHUTZ, 2013).

Por suas capacidades de alterar o ciclo celular, como o comprometimento de vias celulares dependentes de proteínas supressoras tumorais, pRb e p53, além de conferir à célula hospedeira evasão do sistema imune, as proteínas E5, E6 e E7 são consideradas promotoras de oncogênese em queratinócitos primários. A matriz de queratina do epitélio infectado é desestabilizada nas camadas mais apicais, permitindo a liberação de vírions, pela ação do produto de uma recomposição dos transcritos das ORFs E1/E4, conhecido como proteína E4 (DOORBAR et al., 1991). Ao fim de uma infecção produtiva ocorre a montagem do capsídeo viral com inserção do genoma de HPV replicado resultante da expressão tardia de L1 e L2, respectivamente (BUCK et al., 2008).

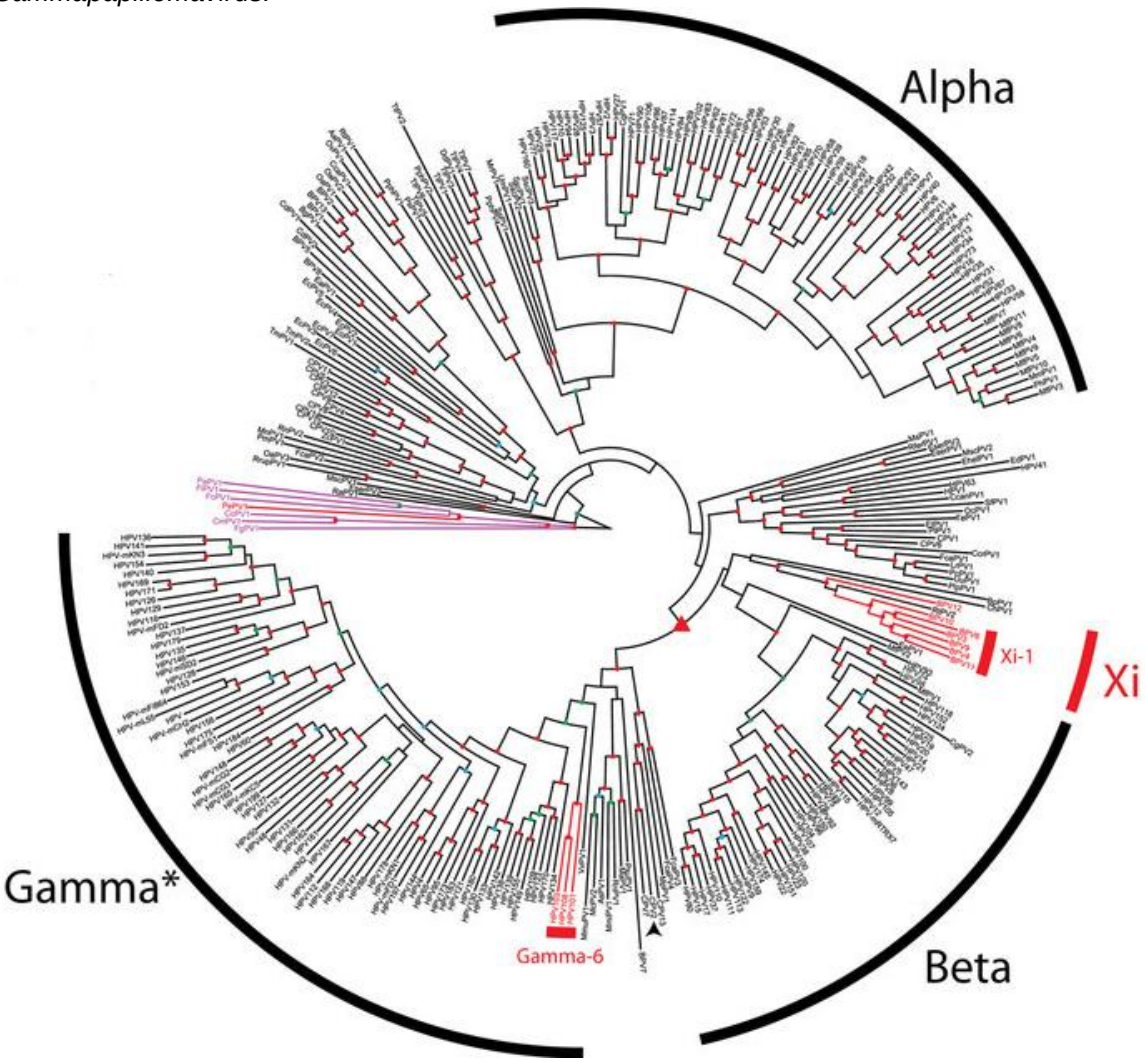
2.3 Papilomavirus: HPVs, genótipos e variantes

Os papilomavírus (PVs) pertencem à família Papilomaviridae e compreendem vírus espécie-específico que infectam não apenas humanos, mas também outros mamíferos, aves e répteis, sendo denominados de acordo com o organismo os quais infectam. BPV (*Bovine Papillomavirus*) infectam bovinos, CRPV (*Cottontail Rabbit Papillomavirus*) infectam coelhos, COPV (*Cannine Oral Papillomavirus*) e HPV (*Human Papillomavirus*) infectam humanos (SCHEURER et al., 2005).

Os PVs são classificados com base na sequência nucleotídica do gene L1, gene altamente conservado, de forma que se torna possível o agrupamento dos PVs em gêneros (sequências com acima de 60% de homologia na sequência), em espécies (quando apresentam homologia entre 71-89%) e cepas (quando diferem em 10% na sequência de L1) (DE VILLIERS et al., 2005; KALANTARI et al., 2010). Para que uma sequência possa ser agrupada em variante, esta deve diferir 1% em relação a sequência de referência e para ser considerada uma sublinhagem deve variar entre 0,5% a 1% (BURK et al., 2013).

Os PVs são classificados em 49 gêneros (Figura 3) enquanto que os HPVs são divididos em cinco gêneros: *Alphapapilomavirus* (Alfa), *Betapapilomavirus* (Beta), *Gammapapilomavirus* (Gama), *Mupapilomavirus* (Mu) e *Nupapilomavirus* (Nu) (DE VILLIERS et al., 2004; BERNARD et al., 2010). Os dois gêneros de HPV que agrupam mais tipos são classificados como Alfa e Beta, contando cerca de 90% dos tipos virais como membros de um desses dois gêneros (MUÑOZ et al., 2006). Os HPVs estão atualmente classificados em mais de 160 tipos de acordo com os seus genótipos, por meio de análise de sequência do gene L1 (DE VILLIERS et al., 2004; BRAVO et al., 2010; VAN DOORSLAER *et al.,* 2011).

Figura 3: Árvore filogenética da família *Papillomaviridae* construída com base no alinhamento das sequências dos genes E1, E2, L2 e L1. Os clados marcados em vermelho não apresentam a ORF E6 canônica, os clados em roxo indicam os vírus de aves e tartarugas que apresentam um único domínio na proteína E6. (*) Indica que alguns membros do clado ainda não foram reconhecidos como G*ammapapillomavirus.*



Fonte: Van Doorslaer, (2016).

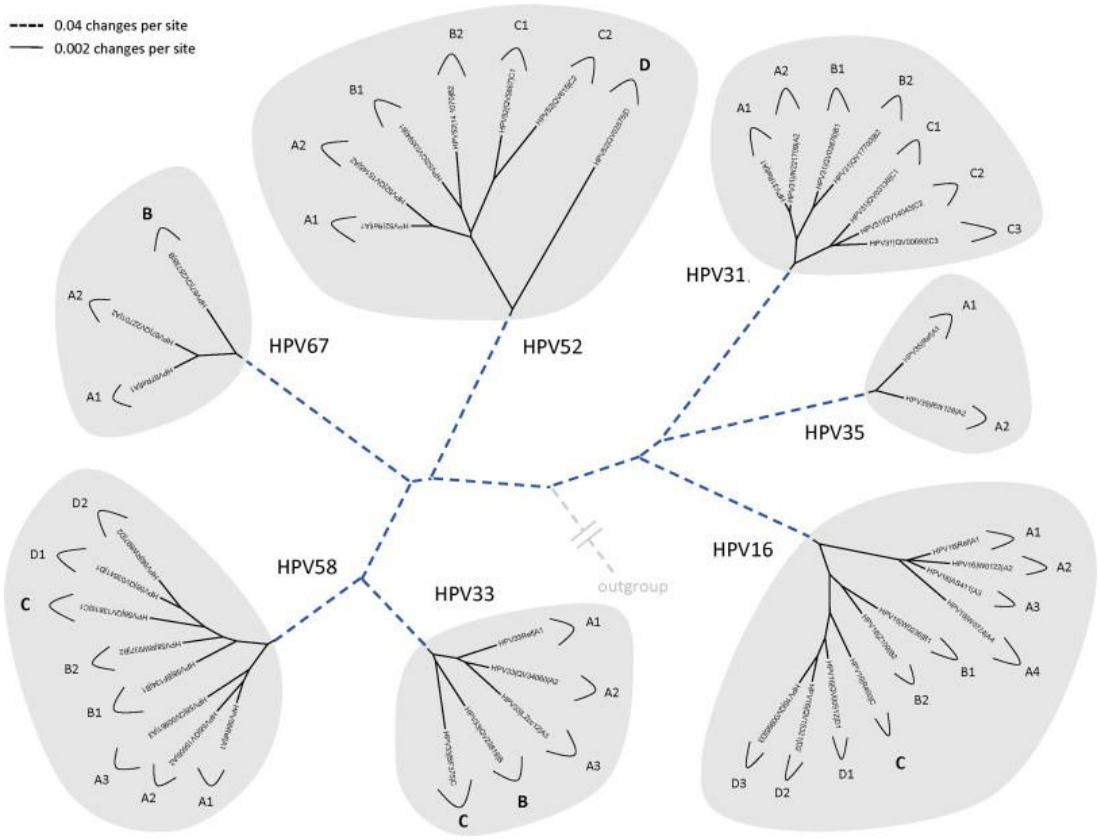
Cerca de 40 genótipos infectam o trato genital humano, sendo classificados em alto risco (HR-HPV) e baixo risco (LR-HPV), refletindo o seu potencial oncogênico (ZUR HAUSEN, 2002; DOORBAR, 2006). Os HPVs de alto risco estão associados às lesões encontradas na vagina, vulva, pênis e colo do útero e incluem os tipos 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82 (BADARACCO et al., 2000; STEVENS, 2002; GUPTA et al., 2003; GURGEL et al., 2013). Os tipos 16 e 18 são considerados os mais prevalentes, por aparecerem em 70% de todos os casos de câncer cervical (BERNARD et al.,

2010). Os HPVs de baixo risco são tipicamente implicados no surgimento de verrugas genitais e incluem os tipos 6, 11, 42, 44, 51, 53, 83 (ZUR HAUSEN, 2002; LI et al., 2012; GURGEL et al., 2013).

Com base no potencial para desenvolvimento do câncer na região genital os Alpha-Papilomavírus são divididos em altamente carcinogênico HPV-16, 18, 31, 33, 45, 51 e 52; provavelmente carcinogênico HPV-68; possivelmente carcinogênico HPVs 26, 53, 64, 65, 66, 67, 69, 70, 73, 82. Além disso, outros membros estão frequentemente associados à lesões benignas na região genital e não genital, tais como o HPV-6, 11, 40, 42, 43, 44, 54, 61, 72, 81 e 89 (SCHIFFMAN et al., 2009; CUBIE, 2013). Recentemente o HPV-16 foi reagrupado em quatro variantes (A, B, C e D) e nove sublinhagens e o HPV-31 em três variantes (A, B e C) e sete sublinhagens (Figura 4) (BURK et al., 2013).

Os HPVs do gênero Beta são mais frequentemente relacionados a infecções cutâneas, comumente assintomáticas. Entretanto, podem estarem associados ao desenvolvimento do câncer de pele em casos de imunocomprometimento e em pacientes acometidos com epidermodisplasia verruciforme (EV) hereditária (HARWOOD e PROBY, 2002; PFISTER, 2003). Os demais tipos de HPV estão distribuídos entre os gêneros, Gama, Mu e Nu, e estão correlacionados a papilomas cutâneos e verrugas sem progressão maligna (DE VILLIERS et al., 2004).

Figura 4: Árvore filogenética mostrando os tipos representativos com linhagens variantes e sublinhagens no grupo alpha-9.



Fonte: Burk et al., (2013).

A distribuição dos genótipos dos HPVs varia de acordo com as áreas geográficas estudadas (BOSCH et al., 1995; WALBOOMERS et al., 1999; CLIFFORD et al., 2005). A distribuição mundial dos tipos de HPV é alvo de vários estudos nos quais a prevalência do HPV-16 sobre todos os demais tipos parece ser uniforme. Comumente o segundo tipo mais prevalente é o HPV-18 ou 31, dependendo da região da Europa, América Central e América do Sul dependendo do país estudado. Notavelmente, a Ásia registra como os mais frequentes os HPVs 33, 52, 56, 58 e 53 (BOSCH et al., 1995; CLIFFORD et al., 2003; CLIFFORD et al., 2005; SANJOSÉ et al., 2007; CAI et al., 2009). No Brasil, o HPV-16 figura como o tipo predominante, conforme estudos de populações locais das regiões Norte, Nordeste, Sudeste e Sul do país, embora sua frequência flutue bastante em determinadas regiões quando comparados a outros tipos (CRUZ et al., 2004;BALDEZ da SILVA et al., 2009; CASTRO et al., 2011; FERNANDES et al., 2011; CHAGAS et al., 2013). Na capital do Estado de Pernambuco, Recife, o

HPV-16 acompanha o padrão de frequência mundial como o tipo mais detectado em amostras cervicais, seguido pelos tipos HPV-31, HPV-33, HPV-58, e em menor frequência o HPV-18 (LORENZATO et al., 2000; BALDEZ da SILVA et al., 2009). Em outras regiões do Brasil, como por exemplo, em São Paulo, ainda que o HPV-16 permaneça como o tipo de maior frequência, a distribuição dos demais tipos diverge consideravelmente, tendo como segundo tipo mais prevalente o HPV-56, seguido pelos HPV-52 e HPV-31 (MARTINS et al., 20016). Sugere-se que diferenças na distribuição dos HPVs podem ocorrer devido ao processo de migração de pessoas, o que contribui para uma maior diversidade populacional e comportamentos sexuais (BOSCH et al., 1995; BARZON et al., 2008).

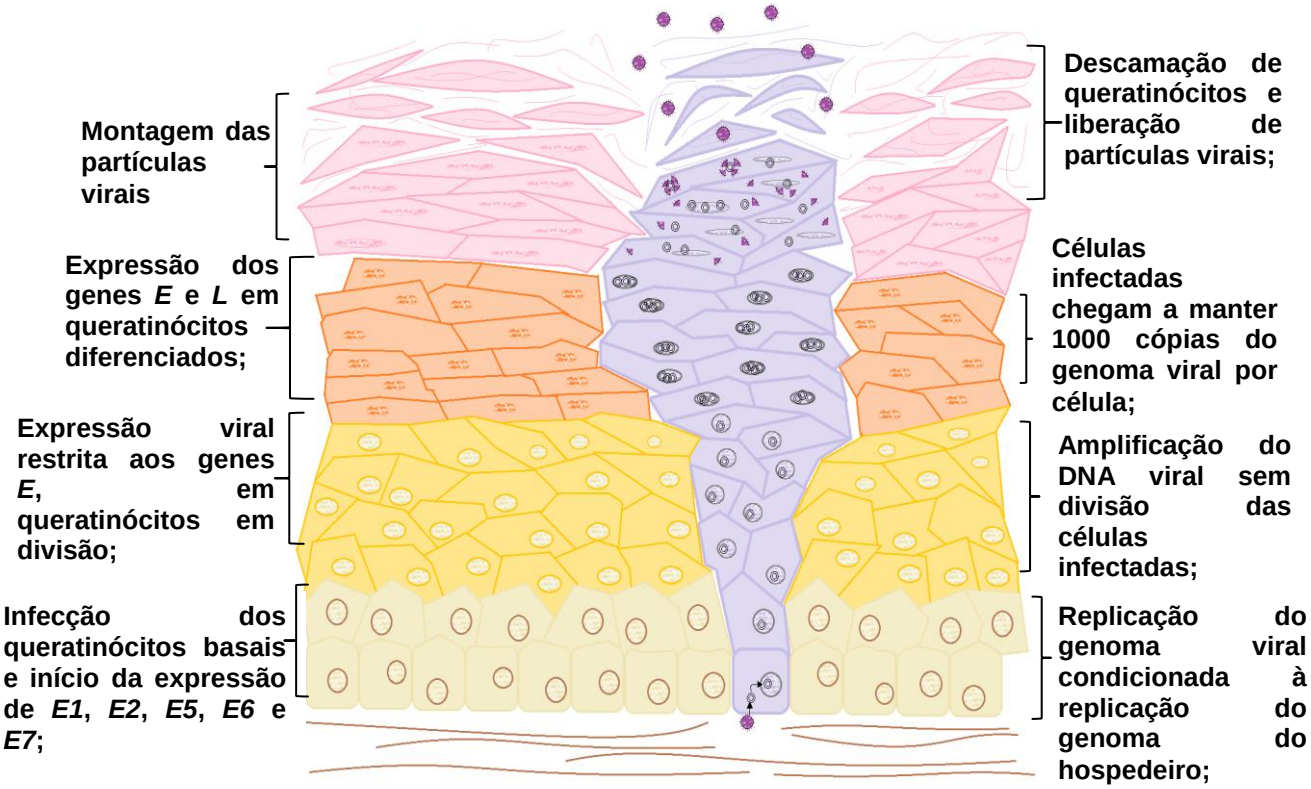
2.4 HPV: ciclo infeccioso e carcinogênese cervical

O ciclo viral está intimamente ligado com a diferenciação dos queratinócitos que são as células hospedeiras do HPV (STUBENRAUCH e LAIMINS, 1999; ZUR HAUSEN, 2002). Para o HPV ser capaz de infectar sua célula alvo é necessário o contato com a membrana basal, exposta em micro lesões ou abrasões no epitélio cervical (DOORBAR, 2005). Acredita-se que proteoglicanos de heparan sulfato (HSPGs) e outros componentes da membrana basal da mucosa anogenital, a exemplo da laminina, atuem como receptores primários para ancoragem do vírus através da interação com a proteína L1 do HPV (COMBITA et al., 2001; GIROGLOU et al., 2001; JOHNSON et al., 2009; DOORBAR et al., 2012). Estas interações resultarão em alterações conformacionais que irão expor a região aminoterminal da proteína L2, permitindo a ação da furina convertase que cliva uma região de L2 expondo o sítio de ligação para um outro co-receptor. Alguns trabalhos apontam que a alfa integrina 6 seria este co-receptor. Também é possível encontrar a atuação de receptores para fatores de crescimento que contribuam para o processo de internalização do vírus, e auxiliem no processo de transferência do genoma para o núcleo celular (EVANDER et al., 1997; LICITRA et al., 2006; SCHEURER et al., 2007; KINES et al., 2009; SURVILADZE et al., 2013), conforme revisado por Doorbar et al., (2012) .

Após a entrada do vírus na célula hospedeira, ocorre a acidificação das vesículas de endocitose, levando à desencapsidação do genoma viral e migração para o núcleo da célula. Este será estabilizado na forma de episssomas. Assim,

inicia-se o ciclo de replicação viral, com a expressão dos genes E1, E2, E4, E5, E6 e E7 (COMBITA et al., 2001; GIROGLOU et al., 2001; WOODMAN et al., 2007; JOHNSON et al., 2009; SAPP e DAY, 2009; DOORBAR et al., 2012;) (Figura 5).

Figura 5: Esquema do ciclo infeccioso ao longo de 6 a 12 semanas a partir da infecção pelo HPV.



Fonte: Cordeiro, 2015.

Após a entrada do HPV nos queratinócitos primários, as proteínas E1 e E2 irão mediar a replicação do genoma viral em baixos níveis. À medida que as células da camada basal infectadas pelo HPV entram em divisão celular, algumas células filha movem-se para o estrato superior do epitélio, iniciando o processo de diferenciação celular (FEHRMANN e LAIMINS, 2003; MCCANCE, 2005; FREITAS et al., 2012). Neste estágio, o genoma viral inicia a transcrição dos genes da fase tardia do ciclo de infecção (L1 e L2 que formarão os capsídeos dos novos vírus) os quais estão aptos a iniciar uma nova infecção. A proteína E2 do HPV desempenha um importante papel nesse processo, pois regula dois promotores no genoma viral, o promotor dependente de diferenciação p670 (localizado no gene E7) o p97 (localizado na região 3´ da LCR), responsável pela expressão

dos genes da fase inicial. Este também permite a expressão dos genes da fase tardia do vírus incluindo o E4 (facilitando o rompimento do citoesqueleto citoplasmático para a liberação dos vírions) (DOORBAR et al., 1991) e dos genes L1 e L2 (codificam os genes para o capsídeo viral). Por sua vez o sinal de poliadenilação precose (pAE) interrompe a expressão dos genes da fase tardia durante o início do processo de infecção, inibindo a expressão prematura de moléculas imunogênicas e assegurando a persistência da infecção viral.

As novas partículas virais serão liberadas com os queratinócitos diferenciados através da descamação do epitélio superior (Figura 5). Queratinócitos primários da camada basal irão servir como um reservatório do DNA viral para propagação do genoma infeccioso no epitélio (FEHRMANN e LAIMINS 2003; MCCANCE 2005; WOODMAN et al., 2007).

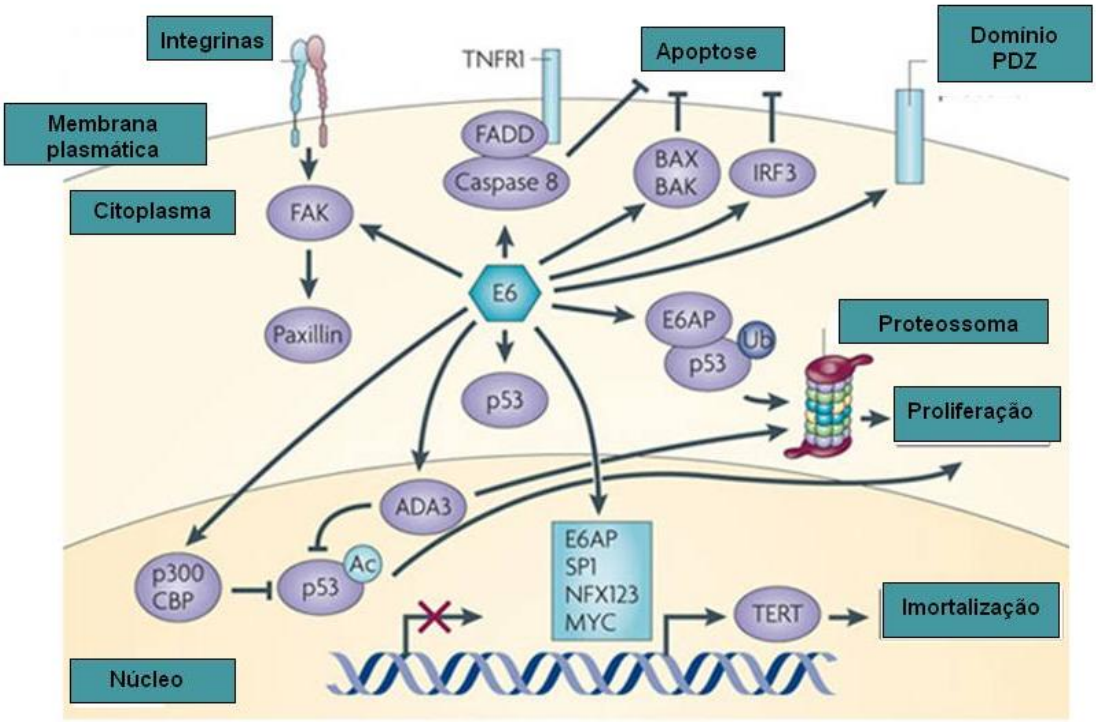
Enquanto o ciclo infeccioso leva a produção de novas partículas virais, a ocorrência de integração do genoma viral ao hospedeiro iniciando a um dos principais eventos da carcinogênese mediada pelo HPV. Para que ocorra o processo de integração do DNA viral é necessária a quebra na região de E1/E2, resultando na perda da expressão desses dois genes (MOTOYAMA et al., 2004). O produto do gene E2 atua como repressor transcricional dos genes E6 e E7 de modo que a perda de E2 resulta na superexpressão de E6 e E7 críticos no processo de imortalização celular (BOSCH et al., 1992; JEON et al., 1995).

A degradação de p53 e pRb mediada pelos produtos dos oncogenes E6 e E7 leva à instabilidade gênomica e repressão da apoptose (ZUR HAUSEN, 2002; PINTO et al., 2011). A oncoproteína E6 desempenha um papel muito importante na transformação dos queratinócitos através de sua interação com diversos alvos celulares (Figura 6), tais como a proteína p53, proteínas da família PDZ (envolvida na manutenção da polaridade celular, sinalização celular e supressão tumoral), a proteína Bak (relacionado com a liberação de fatores proapoptóticos e ativação das vias das caspases), TNF (relacionada com a via da apoptose) e a telomerase (relacionada com o escape da senescência e consequentemente morte celular (GLAUNSINGER et al., 2000; FILIPPOVA et al., 2002; FEHRMANN e LAIMINS, 2003; VOGT et al., 2006; SIMMONDS e STOREY, 2008; THOMAS e BANKS, 2008). A oncoproteína E6 se liga a uma proteína ubiquitina ligase denominada E6AP codificada pelo gene *UB3A*, formando um heterodímero E6-

E6AP, perimitindo a degradação de p53 pela via ubiquitina-proteossomo (SCHEFFNER et al., 1993).

A proteína p53 ocupa uma posição crítica na cadeia de interações proteicas para a resposta celular a diferentes tipos de estresses aos quais a célula está sujeita como, por exemplo, danos no DNA em decorrência da exposição à radiação ultravioleta (UV). Esta proteína ativa genes do mecanismo de reparo do DNA ou pode ainda sinalizar para a apoptose. A inatividade da proteína p53 resulta em perda da capacidade celular de perceber e reparar danos ao DNA, seguindo-se um aumento na frequência de mutações, aneuploidias e rearranjos cromossômicos. O acúmulo desses eventos é um dos requisitos para o desenvolvimento neoplásico e progressão para o câncer (VOUSDEN, 1993).

Figura 6: Proteínas e vias de sinalizações celulares afetadas pela oncoproteína E6.

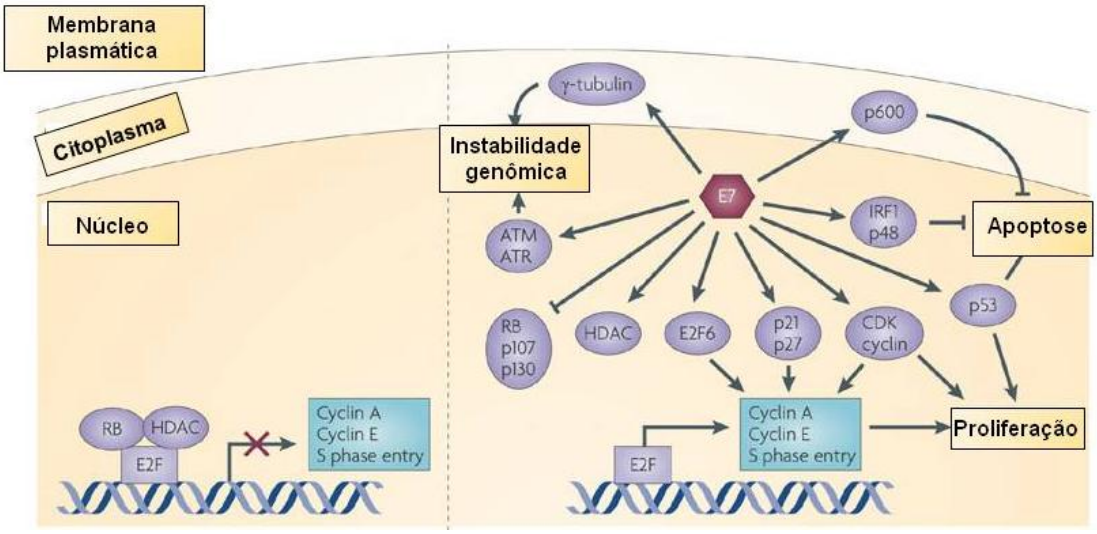


Fonte: Adaptado de Moody e Laimins (2010).

De modo semelhante à oncoproteína E6, a oncoproteína E7 atua na transformação de queratinócitos, por meio da interação com proteínas da família pRb (retinoblastoma), ocasionando sua degradação precoce, o que compromete sua ação inibitória sobre o fator E2F, resultando na progressão contínua para a

fase S do ciclo celular, tornando-se um estímulo para a proliferação das células infectadas (SANDAL, 2002). Além da pRb, a E7 interage com dois outros membros da família pRb, p107 e p130, as quais também regulam negativamente o fator de transcrição E2F (Figura 7) (BOULET et al., 2007). Assim, a proteína pRb exerce uma regulação negativa do ciclo celular através de sua fosforilação específica ciclo-dependente, onde a pRb desfosforilada é capaz de inibir a progressão do ciclo celular por ligação específica a E2F. Uma vez fosforilada, por quinases dependentes de ciclinas (CDK), a ligação entre pRb e E2F não ocorre, e isto resulta diretamente no estímulo da transcrição dos genes que são responsáveis pela replicação do DNA durante a fase S do ciclo celular. As proteínas E7 dos HPV de alto risco são reconhecidamente eficientes ao interagir com ciclinas A e E, interagem com complexos ciclina-quinase e anulam atividades de inibidores de quinases dependente de ciclina (CKIs), tais como p21 e p27, as quais são importantes reguladoras durante a diferenciação do epitélio (SHIN et al., 2009).

Figura 7: Proteínas e vias de sinalizações celulares afetadas pela oncoproteína E7 em células não infectadas pelo HPV à esquerda e infectadas à direita.



Fonte: Adaptado de Moody e Laimins (2010).

Apesar das oncoproteínas E6 e E7 serem as mais estudas no processo de formação nas neoplasias cervicais, E5 vem se mostrando importante neste processo. Um dos seus principais efeitos está relacionado com o aumento da meia vida do receptor de fator de crescimento epidermal, importante para a

sinalização mitogênica e proliferação celular (PEDROZA-SAAVEDRA et al., 2010).

Estudos acerca de polimorfismos nos oncogenes E6 e E7 demonstraram associações que podem ser importantes no desenvolvimento de diagnósticos, construção de vacinas e fundamentação de outros métodos terapêuticos de controle das infecções persistentes. Devido a suas atuações críticas na manutenção da infecção e progressão de lesões relacionadas ao HPV, variações de sequência das proteínas E6 e E7 podem ser relevantes uma vez que podem alterar a ligação com seus diversos parceiros de interação, incluindo efeitos sobre a degradação e fosforilação da p53 e pRB, respectivamente (VILLA et al., 2000; CHAGAS et al., 2011; CHAGAS et al., 2013).

Tratando-se particularmente das funções da proteína E6 do HPV-16, polimorfismos foram indicados como prováveis causas da capacidade alterada de diferenciação regulada pela via dependente de serina/cálcio, induzindo uma degradação mais eficiente da proteína p53. Ainda, o polimorfismo T350G da proteína E6 do HPV-16 alterou quantitativamente a ativação da via MAPK (CHAKRABARTI et al., 2004), com aumento da degradação da proteína Bax e maior interação com a E6AP, principal mediador dos efeitos celulares da proteína E6, quando comparado ao seu protótipo (LICHTIG et al., 2006). Por fim, também foi possível associar esse polimorfismo do gene E6 de HPV-16 com a persistência da infecção e progressão da lesão cervical (KAMMER et al., 2002; ZEHBE et al., 1998; ZEHBE et al., 2001; GRODZKI et al., 2006).

No que diz respeito à proteína E7 do HPV16 foi encontrada uma alteração nucleotídica que resulta em Ser31Arg e que altera a fosforilação do E7 pela Casein Kinase II (CKII) (WISE-DRAPER e WELLS, 2008). Alteração na posição 647 N29S é mais frequente em pacientes com câncer cervical (SONG et al., 1997).

2.5 Relação entre a via NF-kB e o câncer cervical

O NF-κB (fator nuclear-κB) corresponde a uma família de fatores transcricionais que se liga a sequências κB responsivas encontradas no DNA. Estes fatores são responsáveis pela expressão de genes relacionados com a resposta inflamatória, proliferação, diferenciação, adesão e apoptose. (KARIN et

al., 2002; GERONDAKIS et al., 2006; VALLABHAPURAPU e KARIN, 2009; HAYDEN e GHOSH, 2012). Estruturalmente, são heterodímeros ou homodímeros constituídos por proteínas da família Rel, incluindo cRel, RelA (p65), RelB, p50 (NF-κB1) e p52 (NF-κB2).

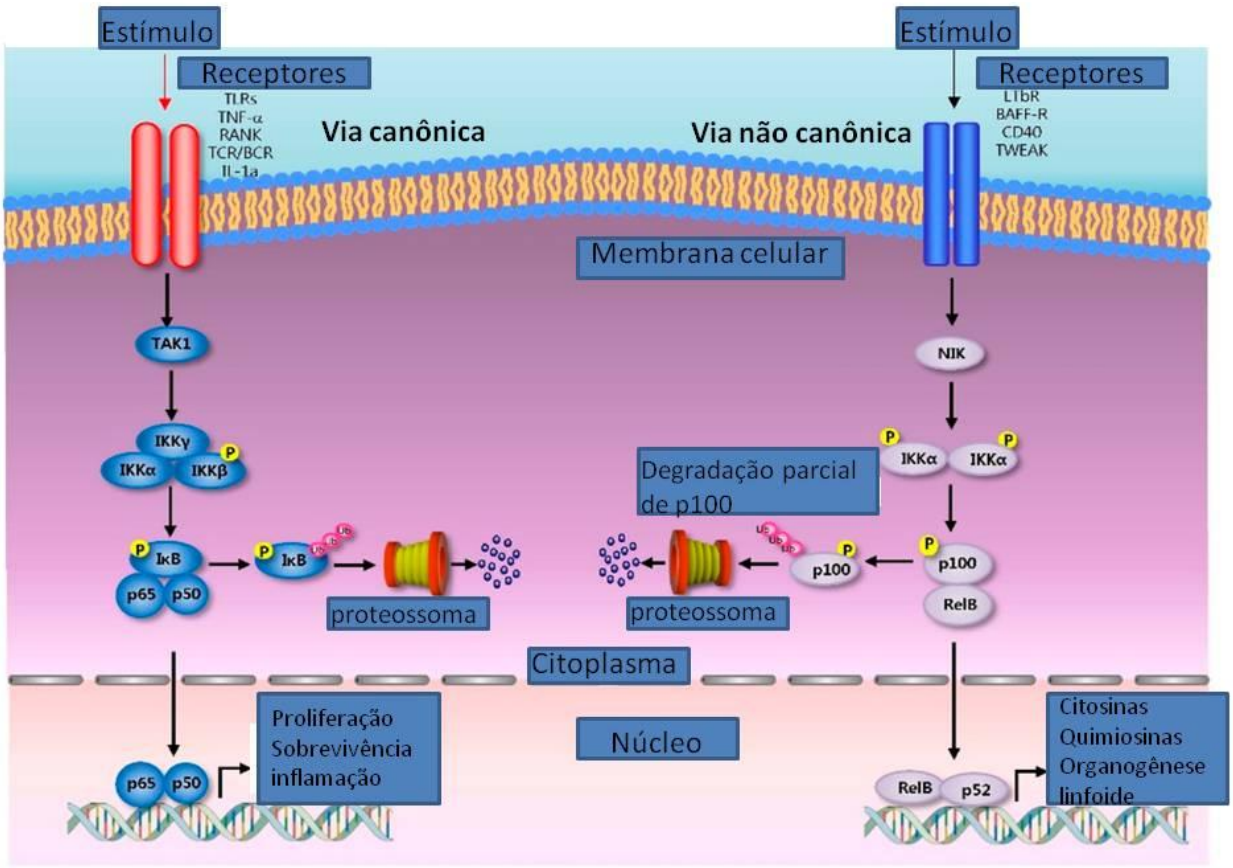
NF-κβ é essencial para as funções imunes adequadas, porém sua ativação excessiva ou constitutiva é uma das causas frequentes de quadros de inflamação crônica (LI et al., 2002). NF-κβ promove a transcrição de genes de citocinas pró-inflamatórias como interleucinas (IL-1b, IL-2, IL-6, IL-8, IL-10, IL-12) e TNF-α, além de moléculas de adesão celular como E-selectina, ICAM-1 e VCAM-1.

Existem duas vias de sinalização distintas, uma canônica e outra não-canônica, para a ativação de NF- κB (Figura 8). A via canônica é principalmente responsável pela regulação da inflamação, bem como proliferação e apoptose de células linfoides durante a resposta imune. A família de quinases IκB (IKK) consiste em três subunidades: dois componentes catalíticos, IKKα e IKKβ, e uma subunidade não-catalítica regulatória, NEMO/IKKγ. Em células não estimuladas, os dímeros de NF-κB são mantidos inativos no citoplasma, devido a interação com inibidores de NF-κB (IκB) (HAYDEN e GHOSH, 2008). O estímulo dessa via, que pode ocorrer através de receptores Toll-like, receptores de antígenos, citocinas pró-inflamatórias como o TNF- α e fatores de crescimento, estresse oxidativo ou genotóxico (CHRISTIAN et al., 2016), inicia a fosforilação do complexo IKK (MAY et al., 2000). Como resultado, o IκB fosforilado é degradado, permitindo a migração do complexo NF-κB (dímero p50/p65) para o núcleo, onde atua como fator transcricional de genes κB responsivos (KARIN e DELHASE, 2000; KARIN e LIN, 2002; GODWIN et al., 2013).

Por sua vez a via não-canônica depende da formação do complexo RelB/p52 (SOLAN et al., 2004). A via não canônica está implicada em vários processos biológicos como a organogênese linfoide, maturação e sobrevivência de células B, desta forma assegurando a montagem de uma resposta imune eficaz. Esta via é ativada por sinais de desenvolvimento que se ligam aos receptores TNF e ativam o complexo NIK/IKK1, o qual, uma vez ativado, fosforila a p100. A fosforilação resulta no processamento de p100 para p52 que se torna ativo para se ligar a RelB criando um dímero que migrará para o núcleo, ativando a expressão de genes relacionados com a resposta imune (BASAK et al., 2008).

Além de controlar a resposta imune inata e adquirida, a sinalização NF-κB tem papel crucial nos eventos celulares como apoptose, adesão, proliferação, diferenciação e resposta celular ao estresse (CHEN e GREENE, 2004; PERKINS, 2007).

Figura 8: Esquema apresentando as vias de sinalização canônica e não canônica para a ativação de NF-κB.



Fonte: Adaptado de Park e Hong (2016).

Os efeitos biológicos da ativação de NF-κB são únicos para cada tipo de célula e tecido. Na pele, NF-κB apresenta dupla função, de modo que um balanço de resposta NF-κB deve ser alcançado para a manutenção da integridade tecidual e homeostase, bem como para a habilidade do sistema imune inato responder rapidamente a potenciais ameaças (PASPARAKIS, 2009).

A ativação constitutiva da via NF-κB tem sido associada como mau prognóstico para vários tipos de cânceres entre eles linfoma (ZOU et al., 2007), leucemia (VILIMAS et al., 2007), pulmão (TEW et al., 2008), mama (CHUA et al., 2007) e ovário (ANNUNZIATA et al., 2010). A ativação desta via está relacionada a expressão de genes que promovem a proliferação celular tais como ciclinas D e

E, possibilitando a transição da fase G1 para S do ciclo celular e regula de forma negativa a expressão da proteína GADD45 relacionada ao controle do ciclo celular no *check point* G2/M (CHEN et al., 2001). Além da proliferação celular, ocorre o bloqueio da apoptose, aumento na angiogênese tumoral e potencial metastático (GUPTA et al., 2010; PRASAD et al., 2010).

Vários estudos sugerem que as oncoproteínas dos HPVs de alto risco modulam a expressão de genes responsivos a NFκB (NEES et al., 2001; HAVARD et al., 2005). A proteína E7 do HPV-16 interfere com a sinalização NF-κB, interagindo diretamente com as subunidades IKKα e IKKβ, causando a redução da atividade relacionada à sinalização NF-κB em células U2OS (SPITKOVSKY et al., 2002). Mesmo sob estímulo da citocina TNF-α, células U2OS transfectadas com o gene E7 de HPV16 foram menos responsivas, exibindo uma sinalização NF-κB atenuada (BYG et al., 2012). Por fim, o mecanismo através do qual a função da proteína E7 de HPV de alto risco reduz uma das principais vias de sinalização pró-inflamatória ainda exige mais estudos.

Certa controvérsia emerge de estudos recentes que demonstram um efeito contrário ao esperado decorrente das funções das oncoproteínas E6 e E7 sobre a via NF-κB, a depender do tipo de célula experimentado. A ativação da via NF-κB é um achado frequente em células escamosas de carcinoma, sendo uma importante evidência de transformação de células epiteliais (HUBER et al., 2004). As oncoproteínas E6 de HPVs de alto risco são capazes de ativar a via NF-κB (NEES et al., 2001; HAVARD et al., 2005; YUAN et al., 2005; JAMES et al., 2006; D'COSTA et al., 2012). O exato mecanismo ainda não está claro, embora existam indícios de que E6 possa interagir com motivos de ligação PDZ presentes em componentes da via de sinalização (JAMES et al., 2006).

Apontando em outra direção, estudos indicam que E6 de HPVs de alto risco inativam uma de-ubiquitinase, denominada CYLD, a qual causa ativação do NF-κB em condições de hipóxia (AN et al., 2008), além de interagir com NFX-91, o que também resulta em ativação de NF-κB (XU et al., 2010). Também há evidências de que ambas as proteínas E6 e E7 de HPV-38, considerado cutâneo, possam ativar NF-κB (HUSSAIN et al., 2011). A ativação de NF-κB leva a uma regulação positiva de cIAP2, um inibidor de apoptose, o que poderia se esperar conferir alguma resistência a agentes de danos ao DNA (JAMES et al., 2006; WU et al., 2010). As consequências da ativação de NF-κB por E6 são complexas e

podem depender do tipo celular em questão. É possível que a ativação desta via por E6 em células da ectocérvice aumente a proliferação, enquanto a ativação da via seja inibitória ao crescimento de células derivadas da zona de transformação, onde a maioria dos cânceres cervicais se desenvolve (VANDERMARK et al., 2012).

3. Capítulo I

Título**: Identification and characterization of human papillomavirus E7 variants in clinical samples from Northeastern Brazil**

Autores: R.C.P. Lima[1], M.N. Cordeiro[1], R.C.O. Silva[1], A.P.F. Campos[1], M.V.A. Batista[2], A.P.A.D. Gurgel[3], A. Venuti[4], B.S. Chagas[1], A.C. Freitas[1]

[1]Federal University of Pernambuco; Department of Genetics; Laboratory of Molecular Studies and Experimental Therapy (LEMTE); Pernambuco, Brazil.
[2]Department of Biology, Federal University of Sergipe, Sergipe, Brazil.
[3]Department of Engineering and Environment, Federal University of Paraiba, Paraiba – Brazil
[4]HPV-UNIT, UOSD Tumor Immunology and Immunotherapy, Regina Elena National Cancer Institute Rome, Italy.

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Identification and characterization of Human papillomavirus E7 variants in clinical samples from Northeastern Brazil

R.C.P. Lima[1], M.N. Cordeiro[1], R.C.O. Silva[1], A.P.F. Campos[1], M.V.A. Batista[2], A.P.A.D. Gurgel[3], A. Venuti[4], B.S. Chagas[1], A.C. Freitas[1*]

[1]Federal University of Pernambuco; Department of Genetics; Laboratory of Molecular Studies and Experimental Therapy (LEMTE); Pernambuco, Brazil.

[2]Department of Biology, Federal University of Sergipe, Sergipe, Brazil.

[3]Department of Engineering and Environment, Federal University of Paraiba, Paraiba – Brazil

[4]HPV-UNIT, UOSD Tumor Immunology and Immunotherapy, Regina Elena National Cancer Institute Rome, Italy

Running Title: Identification and characterization of HPV E7 variants

Corresponding author: Dr. Antonio Carlos de Freitas, Av. Prof. Moraes Rego, 1235, Cidade Universitária, 50670-901, Recife-PE, Brazil. Fax: +55 81 21268512. E-mail: acf_ufpe@yahoo.com.br.

Abstract

Cervical and a subset of head and neck cancers are caused by high-risk human papillomavirus (HPV) persistent infection. HPV oncogenicity is strongly associated with environmental factors, viral types, and their variants, but, even polymorphisms in viral oncogenes may be involved in malignant progression risk. The knowledge

about how genetic variance is distributed among populations and its relationship with high-grade lesions may be crucial to diagnosis and therapeutic strategies against HPV-related diseases. In Brazilian Northeastern patients, our study described the presence of HPV types and variants from cervical cells by PCR technique and sequencing. HPV-16 was found as the most prevalent type, followed by HPV-31 and HPV-58. Among HPV-16 variants, C and D lineages were detected mostly from high-grade cervical lesions. Furthermore, we have identified four HPV-16 E7 gene polymorphisms. A non-synonymous polymorphism was located into predicted T and B-cell epitopes, which are under positive selection. This polymorphism is also located in a loop region next to LXCXE helix domain, which is responsible for the interaction of E7 protein and pRb, crucial step in cancer development. Novel adaptive mutations could lead the virus to evade host immunological system and increase pathogenicity. These results add important data on E7 variability, which could contribute to a better understanding of HPV diversity and infection.

Keywords: HPV; Cervical cancer; E7 polymorphisms; NF-kβ

Introduction

High-risk HPV persistent infection is the major predisposing factor to high-grade cervical neoplasia formation (1). HPV types are distributed among five genera: *Alphapapillomavirus*, *Betapapillomavirus*, *Gammapapillomavirus*, *Mupapillomavirus*, and *Nupapillomavirus* (2,3).

At moment, only *Alphapapillomavirus* genus contains high-risk HPV types, including 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 (4,5). HPV

variants are classified into lineages and sub- lineages based on whole genome sequence analysis (6,7,8), which requires phylogenetic topology support and nucleotide sequence differences from 1% to 10%, and from 0.5% to 1% ranges, respectively (6). HPV-16 phylogenetic analysis has clustered together five intratypic variant groups: European (E), Asian–American (AA), African-1 (Af-1) and African-2 (Af-2), and Asiatic (As) (9,10). Recently, HPV-16 variants have been re-classified into four lineages (A, B, C and D) and nine sublineages (6).

Several studies have correlated HPV-16 variants C and D with persistent viral infection, which may be followed by malignant lesions outcomes (6). Besides functional variants with differential oncogenicity and immunogenicity, single nucleotide polymorphisms (SNPs) may be involved in variant-specific integration potential (11).

Therefore, the present study was performed to detect major viral types distribution in HPV-positive Brazilian Northeastern patients, showing the prevalence of HPV variants in this population and its phylogenetic relationships. We have also identified polymorphisms in HPV-16 E7 oncogene, which is located in predicted T and B-cell epitopes sequences. These findings support the hypothesis that polymorphic HPV genomes are under diversifying evolutionary pressure leading to variable oncogenicity and immunogenicity in the infected host. Our results add significant data to research efforts to a better understanding of HPV types and variants profile in the Brazilian population.

Materials and methods

Study Population and Ethics Statement

Samples were collected from a cohort of 318 women randomly selected during their medical appointment at the Gynecology Unit of Clinical Hospital (n=81) and Oswaldo Cruz University Hospital (n=156) at Recife, Pernambuco State, and at the Center for Integral Attention to Women's Health (n=81) at Aracaju, Sergipe State, both regional hospitals of Northeastern Brazil. This study included women with abnormal cervical cytology: 74 women with low-grade squamous intraepithelial lesions (LSIL), 86 women with high-grade squamous intraepithelial lesions (HSIL), and two women with cervical cancer. This study has been approved by the Ethics Committee and Research from Federal University of Pernambuco (CEP/CCS/UFPE N° 491/11), and the Hospital Complex Oswaldo Cruz (HUOC/PEOCAPE 64/2010) and the Center for Integral Attention to Women's Health (CEP/CCS/UFPE N° 491/11). All women signed the informed consent. Human immunodeficiency virus positive (HIV-positive) patients, pregnant, or hysterectomized patients not were included in this study.

Total Nucleic Acid Isolation and HPV16 Detection

Cervical cells have been collected by cytobrush technique. Samples were kept in PBS solution (pH 7.4) during delivery to Laboratory of Molecular Studies and Experimental Therapy (LEMTE); they were stored at −80^{o}C. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen), following manufacturer's instructions.

DNA was amplified by polymerase chain reaction (PCR) by using MDM2 primers to avoid false negative results and to ascertain the quality of DNA samples (12). HPV screening was carried out by PCR using MY09/MY11 degenerate

primers, which anneal in L1 gene region (13). HPV typing was performed by PCR through specifics primers for HPVs 16, 31 and 58.

HPV-16 E7 Gene Analysis by PCR and Sequencing

Sixty-six HPV-16 positive cervical samples were further analyzed by E7 sequence amplification by PCR with the following primers: 5'-CTCGAGATGCATGGAGATACACC-3' and 5'-GCGGCCGCATTTCTAGATTATGGTTTCTGAG -3'.

Reactions were performed to 25μL final volume. PCR reaction was composed by 50ng of DNA template, 20pmol of each primer, and 1X PCR Master Mix (Promega). PCR cycling conditions were initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C for 10 seconds, annealing at 61°C for 20 seconds, elongation at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. PCR products were analyzed by electrophoresis on agarose gel (1.5%). Amplicons were purified by Invisorb Fragment Cleanup Kit (Invitek), and nucleotide sequences were determined by fluorescent BigDye Terminator Cycle Sequencing v 3.1 3500 Genetic Analyzer (Applied Biosystems) to obtain both the forward and reverse sequences. PCR and sequencing were performed in duplicate.

Sequence Analysis

Nucleotide sequences were assembled using Pregap4 and Gap4 programs in Staden package (14). A Phred value above 30 was used as the cutoff for quality analysis. Subsequently, sequences were aligned with HPV-16 E7 reference sequence (GenBank Accession Number: K02718) using Basic Local Alignment Search Tool (BLAST) (15). Multiple sequence alignment was performed by using

ClustalW algorithm, incorporated in Mega 5.2 software (16). Phylogenetic trees were constructed using Maximum Likelihood method with Kimura 2-Parameter evolutionary model and SPR heuristic method using MEGA software version 5.2 (16). Branch support was assessed with 10,000 bootstrap replicates. Four clusters were identified as lineages A, B, C, and D. Table 1 lists the reference sequences used in this study.

T and B Cell Epitope Prediction

ProPred-1 (17) and ProPred (18) servers were used to perform T-cell epitope screening to predict MHC I and II binding regions, respectively. Bcepred server (19) was used to predict B-cell epitopes based on amino acid physicochemical properties (hydrophilicity, flexibility, polarity and exposed surface). All default parameters were utilized in this analysis.

Diversifying Selection Analysis

Maximum likelihood estimates were used to assess which type of selective pressure is associated with the polymorphisms found in E7 gene. These estimates were calculated by using several codon substitution models with CODEML program, incorporated in Phylogenetic Analysis by Maximum Likelihood package (PAML) version 4.9c (20).

Initially, the detection of positive (diversifying) selection was carried out by calculating the parameters of six different models (M0, M1, M2, M3, M7, and M8). Next, the Likelihood Ratio Test (LRT) was used to verify which model better fits the data, calculating the difference of likelihood values between nested models. This likelihood difference follows a chi-square distribution, allowing us to determine

the statistical significance between the models, which assumes that the degrees of freedom are given by the difference between the parameters of each model.

Molecular Modeling of HPV-16 E7 Protein

A amino acid reference sequence of HPV-16 E7 protein (GenBank accession number: AAA46940) was used for the determination of its 3D structure. A blast search was carried out in Protein Data Bank (PDB) using blastp algorithm for template selection. However, only small fragments of HPV-16 E7 protein was obtained.

In this context, an *ab initio* approach was established to determine the 3D structure of HPV-16 E7 protein using the Rosetta de novo protocol (21,22). Molecular modeling was carried out using Robetta server (23). The obtained models were refined by energy minimization by using ModRefiner (24). The predicted models were evaluated by PROCHECK (25) for the Ramachandran plot quality evaluation, Anolea (26), Gromos (27), QMEAN6 (28), DFire (29), DSSP (30), and Promotif (31). According to these parameters, the best model was chosen in order to assess the structural variability.

In order to identify the possible effect of a mutation in the protein tertiary structure, we used the Protein Variability Server (PVS) (32). This server evaluates the variability of a set of aligned protein sequences and plots it onto a given 3D-structure (32).

Results

HPV detection and typing.

Cervical samples were screened with L1 consensus primers. 225 (70.8%) and of these samples tested positive for HPV DNA. Further genotyping with type-

specific primers has revealed that 56 (24.9%) samples were positive for HPV-16, 37 (16.4%) positive for HPV-31, and 12 (5.3%) positive for HPV-58. From HPV-positive samples, 59 have presented more than one HPV type. The results indicated that HPV-16 and HPV-31 simultaneous presence was the most frequent (n=48/21.3%), followed by HPV-16 and HPV-58 (n=6/2.7%), and HPV-16, 31 and 58 (n=5/2.2%). HPV type could not be determined in 61 samples.

All 56 HPV16 positive samples were subjected to amplification and sequencing of the E 7 gene. Of these, 32 samples were excluded because either they did not produce an amplicon or did not have a reliable sequencing. Thus, 24 samples were analyzed for presence of polymorphisms in the HPV16 E7 gene. Among these, it was observed that 18 patients (75%) have presented HSIL, four (16.7%) have shown low-grade squamous intraepithelial lesion (LSIL), and two (8.3%) have presented cervical cancer

Phylogenetic Analysis

The evolutionary relationships among E7 sequences of HPV-16 were assessed. The phylogenetic tree has shown a lineage- based clustering of the isolates, which apparently groups HPV-16 E7 variants into four different lineages (Figure 1). The phylogenetic tree presents well-supported clusters, making the HPV-16 variants genotyping possible.

Fifteen isolates could be classified as lineage A, three as lineage B, three as lineage C, and three as lineage D. This result highlights the diversity of HPV-16 E7 protein in Northeast region of Brazil.

Genetic Variability of HPV-16 E7 Gene

Comparative analysis of E7 gene with the HPV-16 reference sequence (K02718) has revealed that eight isolates (33.3%) were HPV-16 E7 variants. Four different mutations were found in these isolates (Table 2). One non-synonymous mutation (A647G) has been described as amino acid change N29S. The other three alterations were synonymous mutations.

For the A647G (N29S) mutation, a T and B-cell epitope prediction analysis was performed to evaluate its impact on HPV-16 E7 protein immune features. It was observed that the amino acid change was located in overlapped epitopes from MHC-I binding site (24-32 CYEQLSDSS – allele HLA-A24; 21-29 DLYCYEQLS – allele HLA-A2.1; 25-33 YEQLSDSSE – alleles HLA-B*51 and HLA-B*5401) and MHC-II binding site (23-31 YCYEQLSDS – alleles DRB1_0426 and DRB1_0401; 22-30 LYCYEQLSD – alleles DRB1_0817, DRB1_0801 and DRB1_0806) peptides. The N29S polymorphism is also located inside a B-cell epitope.

Selective Pressure Analysis

The E7 protein of HPV-16 presented values of ω between 0 and 1, which means that this gene is under selection. The model that best fits the data is the one that shows higher values of log-likelihood (lnL). For E7, two models are equally good, M2 and M3, with ω = 0.1285 (Table 3).

Although HPV-16 E7 protein presented negative or purifying selection, we could assess whether specific sites of this protein are under positive or diversifying selection. LRT test indicated the presence of one positively selected site (N29S), representing a mutation with a high probability of being fixed in the viral population (Table 4).

Structural Analysis of HPV-16 E7 Mutations

According to all parameters, the best HPV-16 E7 protein model presented 95.3% of residues in most favored regions in the Ramachandran plot, which shows that we could get a good model (Figure 2). Expected values for stereochemical parameters in well-resolved structures have >90% of phi and psi angles in the core region of the plot. All other quality parameters confirm the reliability of the model (data not shown).

HPV-16 E7 structure has 98 amino acid residues, composed of one zinc-binding domain, which consists of two CXXC domains separated by 29 amino acid residues. A motif LXCXE consists of an alpha helix, which is important to interact with cellular proteins, such as pRb.

The N29S polymorphism is located in a loop region, right after a small alpha-helix domain, which contains the LXCXE motif in the N-terminal region of E7 protein (Figure 3). This loop region represents a predicted immunogenic epitope, showing that the polymorphism might have an important role in the adaptation of the virus.

Discussion

The present study has been performed in Brazilian Northeastern patients, whose cervical samples were positive for HPV in 70.8% of cases. HPV-16 was the most prevalent type, followed by HPV-31 and HPV-58. It was also possible to detect co-infection with different HPV types in the same sample. The most frequent HPV type combinations were HPV-16/HPV-31 and HPV-16/HPV-58. Four HPV-16 E7 polymorphisms have been identified at positions 647 (N29S), 732

(F57F), 789 (I76I), and 795 (T78T). N29S is a non-synonymous mutation at codon 29 that leads to an amino acid replacement in predicted epitopes of T and B-cell recognition regions. The other three mutations were synonymous. The phylogenetic tree has clustered the HPV-16 isolates into four different lineages, which demonstrates the great genetic diversity of E7 gene in the region.

Although the *Alphapapillomavirus* genus is composed by several high-risk HPV genotypes, HPV-16 is remarkable as the most common alpha-PV found in Brazil (33, 34, 35, 36, 37, 38), as well as in the world (39, 40, 41). This fact is in agreement with previous studies in Northeastern Brazil population (5, 42, 43). Besides, HPV-31 was pointed as the second most prevalent type found in Northeast region of Brazil (42, 43,44,45,46,47), and in Southern Europe population (48).

Multiple infection events are commonly reported (49), and different combinations of HPV types may be found depending on the studied population. It is hypothesized that different HPV types may synergistically act in high-grade lesion progression (50,51,52). It has already been reported that HPV16/HPV18 combination is more related to cervical lesions development risk (46). The high prevalence of HPV-16/HPV-31 combination detected in our study may suggest another oncogenic risk situation.

Different lineages could present different pathogenic outcomes. Studies indicated an increasing risk of 2 to 4-fold for CIN3 and cervical cancer development in non-European as compared to European lineages (53,54,55,56,57,58,59). Several studies have shown the association between HPV-16 lineage D infection and cervical lesion, and cervical cancer development risk (60,59, 61). Here, we observed all four different lineages (A, B, C, and D) for

E7 gene in cervical lesions. The most frequent HPV-16 E7 variant was from lineage A. This result highlights the great genetic diversity of this gene in Northeastern Brazil. E7 is an essential viral oncoprotein related to cancer development, and this genetic variability evidences its capacity to adapt and evolve with different pathogenic outcomes in the host. Although we have found all these E7 genetic variants, a previous study suggests that HPV-16 E7 gene seems to be more conserved than E6 gene (62).

Despite previous studies that have detected different HPV-16 lineages in Northeast region (47), Midwest region (36), and in Southeast region of Brazil (63), this is the first time that HPV-16 E7 variants was assessed in this country. Information about E7 gene variability of HPV-16 is still very scarce.

Variations in the genetic sequence of HPV-16 E7 protein may be significant once they may cause changes in viral protein interaction with host cellular proteins as well as interfere with pRb degradation pathway (56,44,45). Although HPV-16 E7 is considered a highly-conserved alpha-PV gene (64,65, 66,67,68), our study has identified four single nucleotide polymorphisms (SNPs) in E7 open reading frame, known as N29S, F57F, I76I, and T78T; all of them have already been described (69,70,64,71*)*. One mutation is non-synonymous (N29S - serine replaced by asparagine), and it was found in two clinical samples (8.3%). The other three mutations are synonymous, and they were found in the clinical samples three (12.5%), eight (33.33%), and seven (29.16%) samples respectively. SNP frequency results were different from those reported by Song *et al*. (1997), with HPV-16 positive cervical cancerous and noncancerous lesions obtained from Korea, in which N29S was found in 60% and the other variants were found in only 16.7% of cases.

The N29S is a common polymorphism in Asian population (70, 72,71*,73*). This E7 polymorphism frequency, however, may be quite different in other regions of the world, even within vast countries. For example, in China, its frequency may fluctuate from 14.29% (Southwest China) to 70.21% (Central and Southern China) (73). The N29S frequency may also be similar between some countries, as it has been reported in Japan (60%) (70), and Korea (60%) (71). Therefore, while N29S may achieve high frequency in Asiatic populations, its frequency in Northeastern Brazil seems to agree with those found in non-Asiatic countries, such as Congo (23%) (62), and Morocco (29%) (74).

Previous studies have reported oncogenic potential associated to N29S polymorphism (71). Two of our N29S-positive samples were included into C lineage (previously referred to as African-2 lineage), while three F57F-positive samples were classified as D lineage (previously referred as Asiatic-American lineage). Studies indicated a higher pathogenic potential of non-European lineages, nowadays called lineages B, C and D, when compared with European lineage, called lineage A (6). However, as indicated by E7/activated Ras transforming activity study in cell culture, there is no increase in the HPV-16 E7 transforming potential has been associated with N29S presence (70).

The mutation A647G (N29S) was located in T and B-cell epitopes. Following Bontkes *et al*. (75) and Zehbe *et al*. (66), the presentation of viral peptides to T-cells may be influenced by HPV polymorphisms and Human Leucocyte Antigen (HLA) polymorphisms. In HPV-16 E6 oncogene, Ellis *et al*. (76) suggested that the R10G variant might alter a B*07 binding epitope that would interfere with immune recognition by cytotoxic T lymphocytes. The L83V variant association with HLA-I (B*44, B*51 and B*57) has also been suggested to

increase from four to five-fold the risk of cancer (77). Therefore, it is possible that N29S SNP influences the immune recognition of HPV infected cells as well as evasion from the natural immune response.

In this study, it was observed that N29S SNP was under diversifying selection, which means that it has a high possibility to be fixed in this viral population. Adaptive mutations are necessary for viral evolution and adaptation to the host, showing that this SNP might be relevant for the HPV infection cycle and, consequently, pathogenicity. Structural analysis revealed that N29S polymorphism is located in a loop region, right after the LXCXE helix domain. This domain is responsible for the interaction of E7 protein and pRb, which is a crucial step in cancer development. HPV-16 E7 variants that present structural alterations in this region could alter its pathogenicity. Although N29S polymorphism is not located in the LXCXE motif, its proximity might affect the interaction between E7 and cellular proteins. However, more studies are needed to confirm this hypothesis.

It is known that novel adaptive mutations could lead the virus to adapt and evade host immunological system, increasing pathogenicity. Here the polymorphisms in HPV16-E7 gene were evaluated. These results add important data on E7 variability, which could contribute to a better understanding of HPV diversity and infection cycle, relevant to develop more efficient diagnostic and treatment methods. To the best of our knowledge, this is the first study that describes the genetic variability of HPV-16 E7 gene in the Brazilian population.

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Conflicts of interest

The authors declare have no conflict of interest.

References

1- Rositch AF, Burke AE, Viscidi RP, Silver ML, Chang K, Gravitt P. 2012. Contributions of recent and past sexual partnerships on incident human papillomavirus detection: acquisition and reactivation in older women. Cancer Res 72: 6183–6190.

2- Bernard HU, Burk RD, Chen Z, Doorslaer KV, Hausen HZ, Villiers EM. 2010. Classification of Papillomaviruses (PVs) Based on 189 PV Types and Proposal of Taxonomic Amendments. Virology 401: 70–79.

3- Villiers E De, Fauquet C, Broker TR, Bernard H. 2004. Classification of papillomaviruses. Virology 324 17– 27

4- Badaracco G, Venuti A, Morello R, Muller A, Marcante ML. 2000. Human papillomavirus in head and neck carcinomas: prevalence, physical status and relationship with clinical/pathological parameters. Anticancer Res 20:1301-5.

5- Pavla A, Diniz A, Chagas BS, Amaral CMM, Albuquerque EMB, Serra IGSS, Neto JCS, Muniz MTC, Freitas AC. 2013. Prevalence and Genetic Variability in Capsid L1 Gene of Rare Human Papillomaviruses (HPV) Found in Cervical Lesions of Women from North-East Brazil. BioMed Research International, Article ID 546354, 7 pages.

6- Burk RD, Harari A, Chen Z. 2013. Human papillomavirus genome variants. Virology 445: 232–243.

7- Chen Z, Schiffman M, Herrero R, Desalle R, Anastos K, Segondy M, Sahasrabuddhe VV, Gravitts PE, Hsing AW, Burk RD. 2011. Evolution and Taxonomic Classification of Human Papillomavirus 16 (HPV16) -Related Variant Genomes: HPV31, HPV33, HPV35, HPV52, HPV58 and HPV67. PLoS ONE 6: e20183.

8- Smith B, Chen Z, Reimers L, Doorslaer K Van, Schiffman M, Desalle R, Herreno R, Yu Kai, Wacholder S, Wang T, Burk RD. 2011. Sequence Imputation of HPV16 Genomes for Genetic Association Studies. PLoS ONE 6: e21375.

9- Ho L, Chan S, Burk RD, Das BC, Fujinaga KEI, Icenogle JP, Kahn T, Kiviat N, Lancaster W, Nazos PM, Labropoulou V, Rosenbaum SM, Norrild B, Pillai MR, Stoerker J, Syrjaenen K, Syrjaenen S, Tay SK, Villa LL, Wheeler CM, Williamson

AL, Bernard HU. 1993. The Genetic Drift of Human Papillomavirus Type 16 Is a Means of Reconstructing Prehistoric Viral Spread and the Movement of Ancient Human Populations. Journal of Virology 67:6413-6423.

10- Yamada T, Manos MM, Peto J, Greer CE, Bosch FX, Wheeler CM. 1997. Human Papillomavirus Type 16 Sequence Variation in Cervical Cancers : a Worldwide Perspective. Journal of Virology 71:2463–2472.

11- Jackson R, Rosa BA, Lameiras S, Cuninghame S, Bernard J, Floriano WB, Lamberts PF, Nicolas A, Zehbe I. 2016. Functional variants of human papillomavirus type 16 demonstrate host genome integration and transcriptional alterations corresponding to their unique cancer epidemiology. BMC Genomics 17:851.

12- Ma H, Hu Z, Zhai X, Wang S, Wang X, Qin J, Jin G, Liu J, Wang X, Wei Q, Shen H. 2006.Polymorphism in the MDM2 promoter and risk of breast cancer: a case-control analysis in a Chinese population. Cancer Lett 240:261

13- Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinsky SM.1989. Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. Cancer Cells 209–213.

14- Staden R. 1996. The Staden sequence analysis package. Mol Biotechnol. 5:233-241.

15- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol 215:403-410.

16- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5 : Molecular Evolutionary Genetics Analysis Using Maximum Likelihood ,

Evolutionary Distance , and Maximum Parsimony Methods. Mol Bio Evol 28:2731–2739.

17- Singh H, Raghava GPS. 2003. ProPred1 : prediction of promiscuous MHC Class-I binding sites. Bioinformatics 19:1009–1014.

18- Singh H, Raghava GPS. 2001. ProPred: prediction of HLA-DR binding sites. Bioinformatics 17:1236-1237.

19- Saha S, Raghava GPS. 2004. BcePred:Prediction of Continuous B-Cell Epitopes in Antigenic Sequences Using Physico-chemical Properties. In G.Nicosia, V.Cutello, P.J. Bentley and J.Timis (Eds.) ICARIS, LNCS 3239:197-204.

20- Yang Z. 2007. PAML 4: phylogenetic analysis by maximum likelihood. Mol Biol Evol 24:1586–1591.

21- Simons KT, Kooperberg C, Huang E, Baker D. 1997. Assembly of Protein Tertiary Structures from Fragments with Similar Local Sequences using Simulated Annealing and Bayesian Scoring Functions. J Mol Biol 268:209-225.

22- Bradley P, Misura KM, Baker D. 2005. Toward high-resolution de novo structure prediction for small proteins. Science 309:1868-1871.

23- Kim DE, Chivian D, Baker D. 2004. Protein structure prediction and analysis using the Robetta server. Nucleic Acids Research Vol. 32, Web Server issue

24- Xu D and Yang Zhang. 2011 Improving the Physical Realism and Structural Accuracy of Protein Models by a Two-step Atomic-level Energy Minimization. Biophysical Journal 101:2525-2534.

25- Laskowski R A, MacArthur M W, Moss D S, Thornton J M. 1993. PROCHECK: a program to check the stereochemical quality of protein structures. J Appl Cryst 26:283-291.

26- Melo F, Feytmans E. 1998. Assessing Protein Structures with a Non-local Atomic Interaction Energy. J Mol Biol 277:1141-1152.

27- van Gunsteren WF, Billeter SR, Eising AA, Hünenberger PH, Krüger P, Mark AE, Scott WRP, Tironi IG. 1996. Biomolecular Simulation: The GROMOS96 Manual and User Guide. VdF: Hochschulverlag AG an der ETH Zürich and BIOMOS b.v.: Zürich, Groningen.

28- Benkert P, Tosatto SCE, Schomburg D. 2008. QMEAN: A comprehensive scoring function for model quality assessment. Proteins 71:261–277.

29- Zhou H, Zhou Y. 2002. Distance-scaled , finite ideal-gas reference state improves structure-derived potentials of mean force for structure selection and stability prediction. Protein Science 11:2714–2726.

30- Kabsch W, Sander C. 1983. Protein Secondary Structure: Pattern Recognition of Hydrogen-Bonded and Geometrical Features. Biopolymers 22:2577-2637.

31-Hutchinson, EG, Thornton JM. 1996. PROMOTIF--a program to identify and analyze structural motifs in proteins. Protein Sci 5:212-220.

32- Garcia-boronat M, Diez-rivero CM, Reinherz EL, Reche PA. 2008. PVS : a web server for protein sequence variability analysis tuned to facilitate conserved epitope discovery. Nucleic Acids Research Vol. 36, Web Server issue.

33-Castro MM, Farias IP, Borborema-Santos CM, Correia G, Astolfi-Filho S. 2011.

Prevalence of human papillomavirus (HPV) type 16 variants and rare HPV types in the central Amazon region. Genetics and Molecular Research 10:186–96.

34- Fernandes JV, Meissner Rde V, Carvalho MG, Fernandes TA, Azevedo PR, de Azevedo JW, de Araújo JM. 2011. Human papillomavirus prevalence in women with normal cytology and with cervical cancer in Natal, Brazil. Mol Med Rep. 4:1321–1326.

35- Alencar TR, Cerqueira DM, Cruz MR da, Wyant PS, Ramalho ED, Martins CRF. 2006. New HPV-16 European and non-European variants in Central Brazil. Virus Genes 35:1–4.

36- Cruz MR, Cerqueira DM, Cruz WB, Camara GNL, Brígido MM, Silva EO, Carvalho LGS, Martins CRF. 2004. Prevalence of Human Papillomavirus Type 16 Variants in the Federal District , Central Brazil. Mem Inst Oswaldo Cruz 99: 281-282.

37- Fernandes J V, Meissner R V, Carvalho MGF, Fernandes TAAM, Azevedo PRM, Sobrinho JS, Prados JCM, Villas L. 2010. Prevalence of human papillomavirus in archival samples obtained from patients with cervical pre-malignant and malignant lesions from Northeast Brazil. BMC Research Notes 3:96.

38- de Medeiros Fernandes TAA, de Vasconcellos Meissner R, Bezerra LF, de Azevedo PR, Veríssimo Fernandes J. 2008. Human papillomavirus infection in women attended at a cervical cancer screening service in Natal, Brazil. Braz J Microbiol 39:573-578.

39- de Sanjose S, Quint WG, Alemany L, Geraets DT, Klaustermeier JE, Lloveras B, Tous S, Felix A, Bravo LE, Shin HR, Vallejos CS, de Ruiz PA, Lima MA, Guimera N, Clavero O, Alejo M, Llombart-Bosch A, Cheng-Yang C, Tatti SA, Kasamatsu E, Iljazovic E, Odida M, Prado R, Seoud M, Grce M, Usubutun A, Jain A, Suarez GA, Lombardi LE, Banjo A, Menéndez C, Domingo EJ, Velasco J, Nessa A, Chichareon SC, Qiao YL, Lerma E, Garland SM, Sasagawa T, Ferrera A, Hammouda D, Mariani L, Pelayo A, Steiner I, Oliva E, Meijer CJ, Al-Jassar WF, Cruz E, Wright TC, Puras A, Llave CL, Tzardi M, Agorastos T, Garcia-Barriola V, Clavel C, Ordi J, Andújar M, Castellsagué X, Sánchez GI, Nowakowski AM, Bornstein J, Muñoz N, Bosch FX; Retrospective International Survey and HPV Time Trends Study Group. 2010. Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. Lancet Oncol 11:1048-1056.

40- Jemal A, Edgar P, Dorell C, Noone A, Markowitz LE, Kohler B, Eheman C, Saraiya M, Bandi P, Saslow D, Cronin KAC, Watson M, Schiffman M, Henley SJ, Schymura MJ, Anderson RN, Yankey D, Edwards BK. 2013. Annual Report to the Nation on the Status of Cancer , 1975 – 2009 , Featuring the Burden and Trends in Human Papillomavirus (HPV) – Associated Cancers and HPV Vaccination Coverage Levels. JNCI Journal of the National Cancer Institute Advance.

41- Bosch FX, Burchell AN, Schiffman M, Giuliano AR, de Sanjose S, Bruni L, Tortolero-Luna G, Kjaer SK, Muñoz N. 2008. Epidemiology and natural history of human papillomavirus infections and type-specific implications in cervical neoplasia. Vaccine 10:K1–16.

42- Baldez da Silva MF, Chagas BS, Guimarães V, Katz LM, Felix PM, Miranda

PM, Lima AA, Arraes LC, Martins DB, Lima Filho JL, Stocco RC, Crovella S, Freitas AC, Beçak W. 2009. HPV31 and HPV33 incidence in cervical samples from women in recife Braz. Genet Mol Res 8:1437–1443.

43- Baldez da Silva MF, Guimarães V, Silva MA, Medeiros do Amaral CM, Beçak W, Stocco RC, Freitas AC, Crovella S. 2012. Frequency of human papillomavirus types 16, 18, 31, and 33 and sites of cervical lesions in gynecological patients from Recife Brazil. Genet Mol Res 11:462–466.

44- Chagas BS, Batista MV, Guimarães V, Balbino VQ, Crovella S, Freitas AC. 2011. New variants of E6 and E7 oncogenes of human papillomavirus type 31 identified in Northeastern Brazil. Gynecol Oncol 123:284-288.

45- Chagas BS, Gurgel AP, da Cruz HL, Amaral CM, Cardoso MV, Silva Neto Jda C, da Silva LA, de Albuquerque EM, Muniz MT, de Freitas AC. 2013. An interleukin-10 gene polymorphism associated with the development of cervical lesions in women infected with Human Papillomavirus and using oral contraceptives. Infect Genet Evol 19:32-37.

46- Chagas BS, Comar M, Gurgel AP, Paiva S, Seraceni S, de Freitas AC, Crovella S. 2015. Association Study between Cervical Lesions and Single or Multiple Vaccine-Target and Non-Vaccine Target Human Papillomavirus (HPV) Types in Women from Northeastern Brazil. PLoS One 10:e0132570.

47- Pavla A, Diniz A, Chagas BS, Medeiros C, Nascimento KCG, Leal LRS, Neto JCS, Muniz MTC, Freitas AC. 2015. Prevalence of Human Papillomavirus Variants and Genetic Diversity in the L1 Gene and Long Control Region of HPV16, HPV31 , and HPV58 Found in North-East Brazil. BioMed Research International.

48- Cento V, Rahmatalla N, Ciccozzi M, Perno CF, Ciotti M. 2011. Intratype variations of HPV 31 and 58 in Italian women with abnormal cervical cytology. J Med Virol 83:1752-1761.

49- Chaturvedi AK, Myers L, Hammons AF, Clark RA, Dunlap K, Kissinger PJ, Hagensee ME. 2005. Prevalence and Clustering Patterns of Human Papillomavirus Genotypes in Multiple Infections. Cancer Epidemiol Biomarkers Prev 14:2439–2445.

50- Dickson EL, Vogel RI, Bliss RL, Downs LS. 2013. Multiple-type HPV infections: a cross-sectional analysis of the prevalence of specific types in 309,000 women referred for HPV testing at the time of cervical cytology. Int J Gynecol Cancer 23(7).

51- Dickson EL, Vogel RI, Geller MA, Downs L. 2014. Cervical cytology and multiple type HPV infection: A study of 8,182 women ages 31–65. Gynecol Oncol 133:405–408.

52- Chaturvedi AK, Katki HA, Hildesheim A, Rodríguez AC, Quint W, Schiffman M, Doom LJV, Porras C, Wacholder S, Gonzalez P, Sherman ME, Herrero R. 2011. Human Papillomavirus Infection with Multiple Types : Pattern of Coinfection and Risk of Cervical Disease. The Journal of Infectious Diseases 203:910–920.

53- Hildesheim A, Schiffman M, Bromley C, Herrero R, Rodriguez C, Bratti C, Sherman ME, Scarpidis U, Lin QQ, Terai M, Bromley RL, Buetow K, Apple RJ, Burk RD. 2001. Human Papillomavirus Type16 Variants and Risk of Cervical Cancer. Journal of the National Cancer Institute 93:315-318.

54- Schiffman M, Rodrigues AC, Chen Z, Wacholder S, Herrero R, Hildesheim A, Desalle R, Befano B, Yu Kai, Safaeian M, Sherman ME, Morales J, Guillen D, Alfaro M, Hutchinson M, Solomon D, Castje PE, Burk RD. 2010. A population-based prospective study of carcinogenic human papillomavirus (HPV) variant lineages, viral persistence, and cervical neoplasia. Cancer Res 70:3159–3169.

55- Sichero L, Ferreira S, Trottier H, Duarte-franco E, Ferenczy A, Franco EL, Ferenczys A, Villa LL. 2007. High grade cervical lesions are caused preferentially by non-European variants of HPVs 16 and 18. Int J Cancer 120:1763–1768.

56- Villa LL, Sichero L, Rahal P, Caballero O, Ferenczy A, Rohan T. 2000. Molecular variants of human papillomavirus types 16 and 18 preferentially associated with cervical neoplasia. Journal of General Virology, 81:2959–2968.

57- Xi LF, Koutsky LA, Hildesheim A, Galloway DA, Wheeler CM, Winer RL, Ho J, Kiviat NB. 2007. Risk for High-Grade Cervical Intraepithelial Neoplasia Associated with Variants of Human Papillomavirus Types 16 and 18. Cancer Epidemiol Biomarkers Prev 16:4-10.

58- Zuna RE, Moore WE, Shanesmith R, Dunn ST, Wang SS, Schffman M, Blakey GL, Teel T. 2009. Association of HPV16 E6 variants with diagnostic severity in cervical cytology samples of 354 women in a US population. Int J Cancer 125:2609–2613.

59- Burk RD, Terai M, Gravitt PE, Brinton LA, Kurman RJ, Barnes WA, Greenbergerg MD, Hadjimichael OC, Fu L, McGowan L, Mortel R, Schwartz PE, Hildesheim A. 2003. Distribution of Human Papillomavirus Types 16 and 18 Variants in Squamous Cell Carcinomas and Adenocarcinomas of the Cervix. Cancer Research 63:7215–7220.

60- Berumen J, Ordoñez RM, Lazcano E, Salmeron J, Galvan SC, Estrada RA, Yunes E, Carranca AG, Lira GG, Campa AM. 2001. Asian-American Variants of Human Papillomavirus 16 and Risk for Cervical Cancer: a Case–Control Study. J Natl Cancer Inst 93:1325–1330.

61- Quint KD, de Koning MN, van Doorn LJ, Quint WG, Pirog EC. 2010. HPV genotyping and HPV16 variant analysis in glandular and squamous neoplastic lesions of the uterine cervix. Gynecol Oncol 117:297–301.

62- Boumba LMA , Assoumou SZ, Hilali L, Mambou JV, Moukassa D, Ennaji MM. 2014. Genetic variability in E6 and E7 oncogenes of human papillomavirus Type 16 from Congolese cervical cancer isolates. PLoS One 9:e100746.

63- Freitas LB, Chen Z, Muqui EF, Boldrini NA, Miranda AE, Spano LC, Burk RD. 2014. Human Papillomavirus 16 Non-European Variants Are Preferentially Associated with High-Grade Cervical Lesions. PLoS One. 9:e100746.

64- Icenogle JP, Sathya P, Miller DL, Tucker RA, Rawls WE Virology. 1991. Nucleotide and amino acid sequence variation in the L1 and E7 open reading frames of human papillomavirus type 6 and type 16. Virology 184:101-107.

65- Grassmann K, Rapp B, Maschek H, Petry KU. 1996. Identification of a Differentiation-Inducible Promoter in the E7 Open Reading Frame of Human Papillomavirus Type 16 (HPV-16) in Raft Cultures of a New Cell Line Containing High Copy Numbers of Episomal HPV-16 DNA. J of Virology 4:2339–2349.

66- Zehbe I, Wilander E, Delius H, Tommasino M. 1998. Human Papillomavirus 16 E6 Variants Are More Prevalent in Invasive Cervical Carcinoma than the Prototype1. Cancer Research 58:829-833.

67- Wu Y, Chen Y, Li L, Yu G, He Y. 2016. Analysis of mutations in the E6 / E7 oncogenes and L1 gene of human papillomavirus 16 cervical cancer isolates from China. Journal of General Virology 87:1181–1188.

68- Pande S, Jain N, Prusty BK, Bhambhani S, Gupta S, Sharma R, Batra S, Bhudev C. 2008. Human Papillomavirus Type 16 Variant Analysis of E6 , E7 , and L1 Genes and Long Control Region in Biopsy Samples from Cervical Cancer Patients in North India . Journal of Clinical Microbiology 46:1060–1066.

69- Eschle D, Diirst M, Luande J, Eberhardt C, I MP, Gissmann L. 1992. Geographical dependence of sequence variation in the E7 gene of human papillomavirus type 16. Journal of General Virology 73:1829-1832.

70- Fujinaga Y, Okazawa K, Nishikawa A, Yamakawa Y, Fukushima M, Kato I, Fujinaga K. 1994. Sequence variation of human papillomavirus type 16 E7 in preinvasive and invasive cervical neoplasias. Virus Genes 9:85-92.

71- Song YS, Kee SH, Kim JW, Park NH, Kang SB, Chang WH, Lee HP. 1997. Major sequence variants in E7 gene of human papillomavirus type 16 from cervical cancerous and noncancerous lesions of Korean women. Gynecol Oncol 66:275-281.

72- Chan PKS, Lam CW, Cheung TH, Li WWH, Lo KWK, Chan MYM, Cheung JLK, Xu LY, Cheng A. 2002. Human Papillomavirus Type 16 Intratypic Variant Infection and Risk for Cervical Neoplasia in Southern China. The Journal of Infectious Diseases 186:696–700.

73- Shang Q, Wang Y, Fang Y, Wei L, Chen S, Sun Y, Li B, Zhang F, Gui H. 2011. Human Papillomavirus Type 16 Variant Analysis of E6 , E7 , and L1 Genes and

Long Control Region in Identification of Cervical Carcinomas in Patients in Northeast China . Journal of Clinical Microbiology 49:2656–2663.

74- Qmichou Z, Ennaji MM, Amrani M, Mostafa E, Fahime E, Melloul M, Khair MM, Mzibri ME, Khyatti M, Atealeb M. 2013. Molecular Characterization of HPV16 E6 and E7 Variants among Women with Cervical Cancer in Morocco. British Microbiology Research Journal 3:692-705.

75- Bontkes HJ, Walboomers JM, Meijer CJ, Helmerhorst TJ, Stern PL. 1998. Specific HLA class I down-regulation is an early event in cervical dysplasia associated with clinical progression. Lancet 351:187-188.

76- Ellis JRM, Keating PJ, Baird J, Hounsell EF, Renouf DV, Rowe M, Hopkins D, Keen MFD, Bartholomew JS, Young LS, Stern PL. 1995. The association of an HPV16 oncogene variant with HLA-B7 has implications for vaccine design in cervical cancer Nature Medicine 1:464-470.

77- Zehbe I, Mytilineos J, Wikström I, Henriksen R, Edler L, Tommasino M. 2003. Association between human papillomavirus 16 E6 variants and human leukocyte antigen class I polymorphism in cervical cancer of Swedish women. Hum Immunol 64:538-542.

Figures:

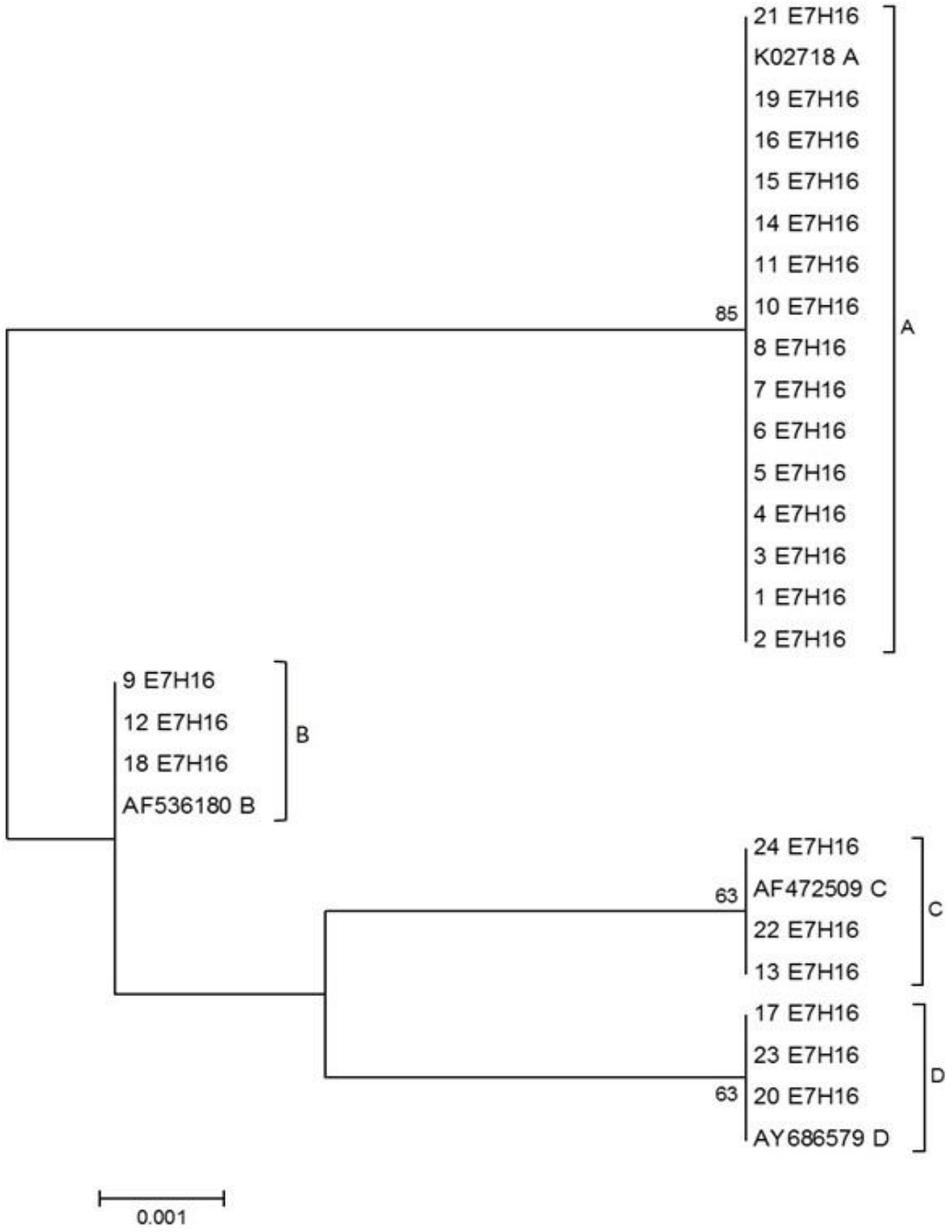

Figure 1: Unrooted Maximum Likelihood phylogenetic tree of HPV-16 variants based on E7 gene. Four clusters were identified as lineages A, B, C, and D. Reference sequences are listed in Table 1. Only bootstrap values above 50% are represented.

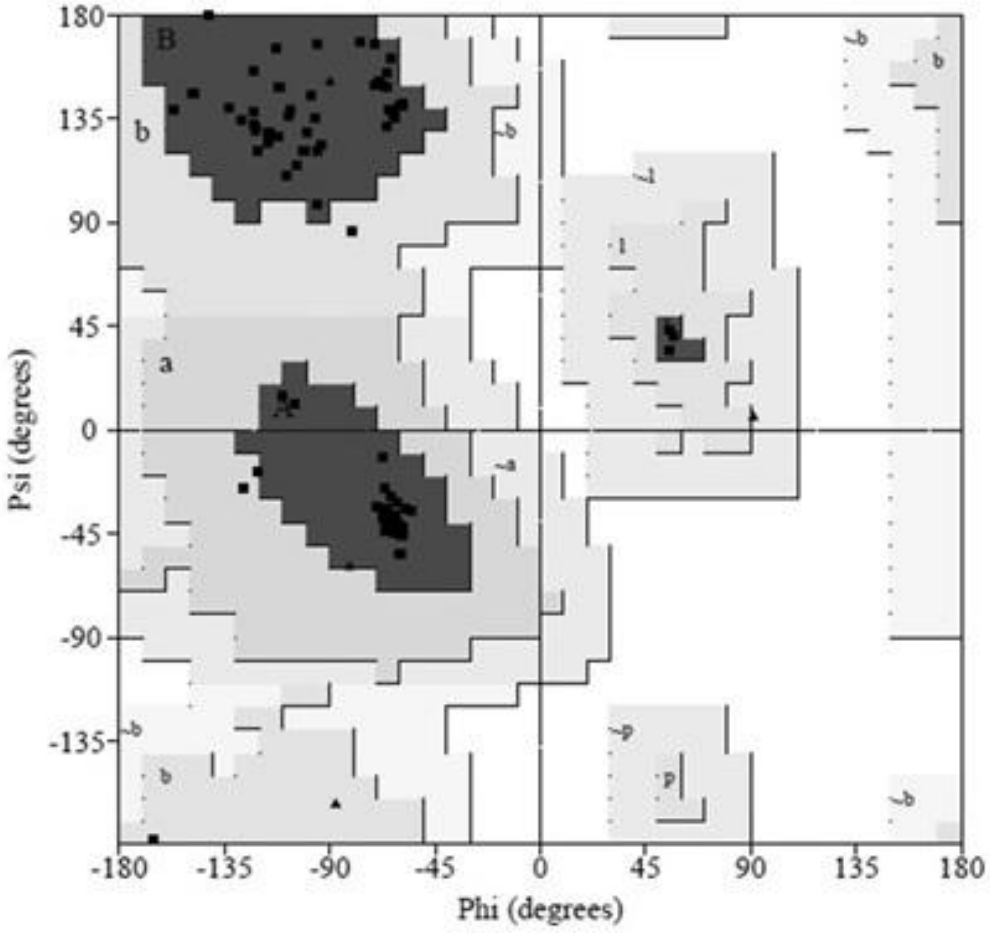

Figure 2: Ramachandran plot of E7 from HPV-16 displaying 95.3% of residues in most favored regions.

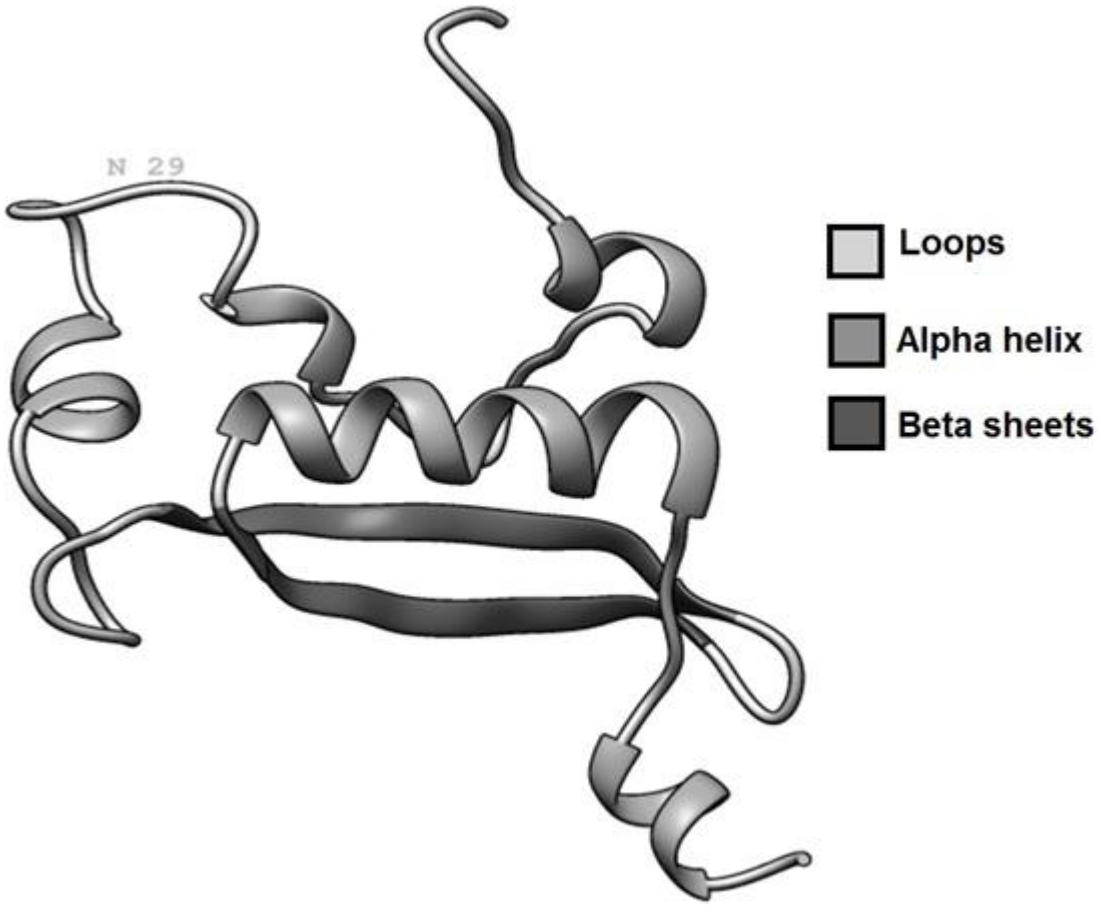

Figure 3: Representation of HPV-16 E7 protein. The N29S polymorphism is represented in the structure (N 29) in a loop region, right after a small alpha-helix domain (LXCXE) in the N-terminal region of the protein. The alpha helix is represented in gray, beta sheets in dark gray, and loops in light gray.

Tables:

Table 1. Reference genomes used in lineage classification of HPV-16 variants.

Variant Genome ID	Accession number	HPV type	Lineages
Ref	K02718	HPV-16	A
W0236	AF536180	HPV-16	B
R460	AF472509	HPV-16	C
QV15321	AY686579	HPV-16	D

Table 2. Nucleotide and amino acid changes in E7 gene of HPV-16 isolates identified in this study based on reference sequence (K02718).

Isolates	Nucleotide position in E7 gene				Cervical lesion
	647	732	789	795	
	A	T	T	T	
1	.	.	.	.	HSIL
2	.	.	.	.	HSIL
3	.	.	.	.	HSIL
4	.	.	.	.	LSIL
5	.	.	.	.	HSIL
6	.	.	.	.	LSIL
7	.	.	.	.	HSIL
8	.	.	.	.	HSIL
9	.	.	C	G	HSIL
10	.	.	.	.	HSIL
11	.	.	.	.	HSIL
12	.	.	.	.	LSIL
13	G	.	C	G	HSIL
14	.	.	.	.	HSIL
15	.	.	.	.	HSIL
16	.	.	.	.	HSIL
17	.	C	C	G	HSIL
18	.	.	C	G	Cancer
19	.	.	.	.	Cancer
20	.	C	C	G	HSIL
21	.	.	.	.	HSIL
22	.	.	C	.	HSIL
23	.	C	C	G	HSIL
24	G	.	C	G	LSIL
Reference amino acids	N	F	I	T	
Codon position	29	57	76	78	
Amino acids in E7 variants	S	F	I	T	

N – Asparagine; S – Serine; F – Phenylalanine; I – Isoleucine; T - Threonine.

Table 3: Selective pressure estimates in E7 gene of HPV-16 based on likelihood, calculated by PAML program. The model that best fits the data are in bold.

ORF	Model	lnL	ω = dN/dS*	Parameters
E7	M0	-429.243357	0.1285	ω = 0.1285
	M1	-429.242862	0.1284	p_0 = 0.99999 (p_1 = 0.00001) ω_0 = 0.12844; ω_1 = 1.00000
	M2	**-429.242860**	**0.1285**	**p_0 = 1.00000; p_1 = 0.00000 (p_2 = 0.00000) ω_0 = 0.12845; ω_1 = 1.00000; ω_2 = 1.00000**
	M3	**-429.242860**	**0.1285**	**p_0 = 0.34372; p_1 = 0.40336 (p_2 = 0.25292) ω_0 = 0.12845; ω_1 = 0.12845; ω_2 = 0.12846**
	M7	-429.243348	0.1267	p = 14.39552; q = 99.00000
	M8	-429.243172	0.1285	p_0 = 0.99999 (p_1 = 0.00001) p = 14.63292; q = 99.00000; ω_s = 1.00000

* Means that the values were determined from the arithmetic mean.

Table 4: LRT test results for the detection of sites under positive (diversifying) selection. Values in between parenthesis indicate the posterior probability of the site to be positively selected.

ORF	Model 1	Model 2	LRT	Positively selected sites
E7	M0	M3	345.913886	-
	M1	M2	0.225326	None
	M1	M3	160.488580	None
	M7	M8	0.000416	29 N (0.866)

4. Capítulo II

Artigo em fase de elaboração:

Functional evaluation of Human papillomavirus 16 E7 variants

R.C.P. Lima[1], M.N. Cordeiro[1], Dhalia R[2], R.C.O. Silva[1], T.H.A. Oliveira[1], A.P.F. Campos[1], M.V.A. Batista[2], A. Venuti[4], B.S. Chagas[1], A.C. Freitas[1*]

[1]Federal University of Pernambuco; Department of Genetics; Laboratory of Molecular Studies and Experimental Therapy (LEMTE); Pernambuco, Brazil.

[2]Virology and Experimental Therapy Laboratory, Aggeu Magalhães Research Center-CPqAM/FIOCRUZ, Recife, Brazil.

[3]Department of Biology, Federal University of Sergipe, Sergipe, Brazil.

[4]HPV-UNIT, UOSD Tumor Immunology and Immunotherapy, Regina Elena National Cancer Institute Rome, Italy

Running Title: Functional evaluation of Human papillomavirus 16 E7 variants

Corresponding author: Dr. Antonio Carlos de Freitas, Av. Prof. Moraes Rego, 1235, Cidade Universitária, 50670-901, Recife-PE, Brazil. Fax: +55 81 21268512. E-mail: acf_ufpe@yahoo.com.br

Abstract

BACKGROUND:

Cervical cancer is the third most common neoplasia in Brazilian women. Epidemiologic studies indicate that high-risk human papillomavirus (HPV) persistent infection is the major cause of cervical neoplasia. High-risk HPV E7 protein alters various aspects of the human cell cycle. Polymorphisms may lead to altered biological function with clinical consequences. Besides impairing pRB-mediated cell cycle control, E7 activities include NF-κB disturbing, which affects an anti-viral cellular critical pathway. In this study, we have performed one of the first NF-κB activity-based functional analysis of HPV16 E7 polymorphisms.

METHODS:

E7 synthetic genes have been designed with three HPV-16 E7 variants (N29S, I76I and T78T) simultaneously, with only one of each, or without any of them. These E7 genes have been cloned into pcDNA3.1 vector, and plasmids have been called all variants, N29S, I76I, T78T and 16 E7 Reference. HEK- 293 cell groups were co-transfected with an NF-κB-dependent firefly luciferase reporter, known as (κB)3-Luc plasmid, and a *Renilla* luciferase-expressing plasmid, as luminescence normalizer. NF-κB activity was measured by GloMax® 96 Microplate Luminometer. Statistical analysis were performed using ANOVA; $p < 0.05$ was considered significant. In addition, in order to access E7 mRNA expression levels from cell cultures, 24 hours post-transfection, total RNA were obtained and cDNA has been synthetized. Gene expression was normalized with GAPDH and ACT as the reference genes and the gene expression was calculated by $2^{-\Delta\Delta Cq}$.

RESULTS:

Our results have shown that HPV-16 E7-expressing cells, with all variants, have dramatically reduced NF-kB pathway activity, in comparison to 16 E7 Reference-expressing cells (NF-kB activity control group). However, none of the E7 synthetic genes with only one variant have shown significant NF-kB pathway disturbance. N29S containing E7 mRNA expression was 9-fold higher than 16 E7 ref.-expressing cells, while none of the others E7 synthetic genes with only one variant have an E7 mRNA expression 2-fold higher than 16 E7 ref.-expressing cells. Despite the most potent effect on NF-kB pathway, all variants-containing E7 mRNA expression were only 2.2-fold higher than 16 E7 ref.-expressing cells.

CONCLUSION: HPV-16 E7 gene variants in which the three polymorphisms are present may perform a strong suppressive effect on the NF-kB pathway in HPV-infected patients with implications for infection persistence and it's progression. However, the changes resulting from the polymorphisms may not necessarily be related to the differential expression levels of the variants.

1. Introduction

Human Papillomaviruses (HPVs) are one of the most studied viral causes to human cancer. Oncogenic HPV infection cycle are able to establish persistent infection, which is directly associated with high-grade cervical neoplasia formation (Rositch *et al*., 2013). High risk HPV-related carcinogenesis is promoted by E6 and E7 viral oncoproteins, whose mainly activities impairs two main tumor suppressor gene products function, *TP53* and *Rb*, respectively (Leemans, Braakhuis and Brakenhoff, 2011). However, there is a set of E6 and E7 roles that affect many others cell regulation key-proteins. For example, high-risk HPV E7 proteins can bind to cyclin/cdk kinases p21CIP1 and p27KIP1; both are two well known activators of Rb with critical roles in cell cycle progression, differentiation and cell death (Zerfass-Thome *et al*., 1996; Funk et al. 1997). On the other hand, high-risk HPV E6 proteins bind to a large variety of PDZ cellular proteins, which include proteins of cell signaling, cell adhesion, tight-junction integrity, molecular scaffolding for protein complex assembly, and possibly tumor suppressor activity (Glaunsinger *et al*., 2000).

Many viruses, including HPV, have acquired mechanisms to interfere in innate host-cell defense pathways, as showed by Thompson *et al*. (2001). TNF-α-dependent activation of pro-caspase 8 in HPV16 E7-expressing fibroblasts was severely decreased, which indicates a possible mechanism of infected cells that reach apoptosis resistance. However, it is also feasible that less studied functions of E7 oncoprotein may impair more signaling pathways, which are important in host cell anti-viral state development.

NF-κB is an essential pathway to appropriate immune functions (Hayden and Ghosh, 2012). NF-κB promotes pro-inflammatory cytokines gene transcription, such as interleukins (IL-1, IL-6, and IL-8) and TNF-α, cell adhesion molecules like E-selectin, ICAM-1 and VCAM-1 (Hoesel and Schmid, 2013).

There are two distinct signaling pathways to NF-κB activation, a canonical and a non-canonical (Gilmore, 2006). The canonical pathway is largely responsible for regulation of inflammation as well as proliferation and apoptosis of lymphoid cells during an immune response (Hoesel and Schmid, 2013;Perkins, 1997). Stimulation of this pathway by pro-inflammatory cytokines, such as TNF-α, activates phosphorylation of an IKK complex, consisting of IKKα, IKKβ and two NEMO subunits (Sun, 2011). Inhibitor IKKβ phosphorylation leads to its

degradation, which releases NF-κB that enters the nucleus and exerts its function as a transcription factor. The outcome of this chain activation is the expression of inflammatory genes (Hayden and Ghosh, 2008).

Besides controlling the innate and acquired immune response, NF-κB signaling has a crucial role in cell behavior such as apoptosis, cell adhesion, proliferation, development, cell differentiation, and the cellular stress response (Chen and Greene, 2004; Perkins, 2007).

Oncogenic HPV E7 oncoproteins impairs Rb protein family function, leading to continuous DNA replication and cell immortalization. In addition, previous studies showed that HPV16 E7 protein interfere in NF-κB signaling by direct interaction with IKKα and IKKβ subunits, which causes NF-κB signaling-related activities decreasing in U2OS cells (Spitkovsky *et al*., 2002). Even under TNF-α stimuli, HPV16 E7-expressing U2OS cells were less responsive, exhibiting attenuated NF-κB signaling (Byg *et al*., 2012).

Our unpublished data has detected polymorphisms into HPV-16 E7 oncogene from high-risk variants in Brazilian Northeastern patients. Although several reports indicate association between some HPV variants and poor prognosis of cervical cancer (Freitas *et al*., 2014; Schiffman *et al*., 2010; Villa *et al*., 2000; Zehbe *et al*., 2009) , only a handful of functional studies has tested polymorphic HPV genes, such as high-risk HPV E7. Therefore, the present study assessed polymorphism-related functional effects on the relationship between NF-κB signaling pathway and the high-risk HPV E7 expression in cell culture.

2. Materials and methods

2.1. Analysis of E7 HPV16 by PCR and Sequencing

HPV16 E7 positive cervical samples were analyzed by E7 gene sequence amplification by PCR using the following primers: 5'-CTCGAGATGCATGGAGATACACC-3' and 5'-GCGGCCGCATTTCTAGATTATGGTTTCTGAG -3'. PCR conditions were carried out as described in Lima *et al*. (2017) (submited).

PCR products were analyzed by electrophoresis in agarose gel (1.5%). Afterwards, amplicons were purified by Invisorb Fragment Cleanup Kit (Invitek) and nucleotide sequences were accessed by fluorescent BigDye™ Terminator Cycle Sequencing using v 3.1 Ready Reaction ABI PRISM (Applied Biosystems)

to obtain both forward and reverse sequences. PCR and sequencing were performed in duplicate.

2.2. Plasmid constructs and cloning

Polymorphic E7 gene, which contains single nucleotide polymorphisms (SNP) in positions 647, 789 and 795 (Figure 1), called respectively N29S, I76I and T78T (Eschle *et al.,* 1992; Fujinaga *et al.,* 1994; Icenogle *et al.,* 1991; Song *et al.,* 1997) has been amplified from HPV16-positive clinical samples and cloned into pGEM-T TA-cloning vector (Promega®). BamHI, HindIII and EcoRI restriction sites have been inserted at polymorphic E7 gene flanks, which allows cloning into pcDNA3.1 mammal expression, called “All variants” plasmid. Similarly, three versions of polymorphic E7 gene were design, each one containing only one variant, and they were cloned into pcDNA3.1 vector, which has generated the constructs N29S, I76I and T78T. The empty plasmid, pcDNA3.1, and an HPV-16 E7 reference E7-containing plasmid (16 E7 Ref.), which does not have N29S, I76I and T78T SNPs, have been used as control.

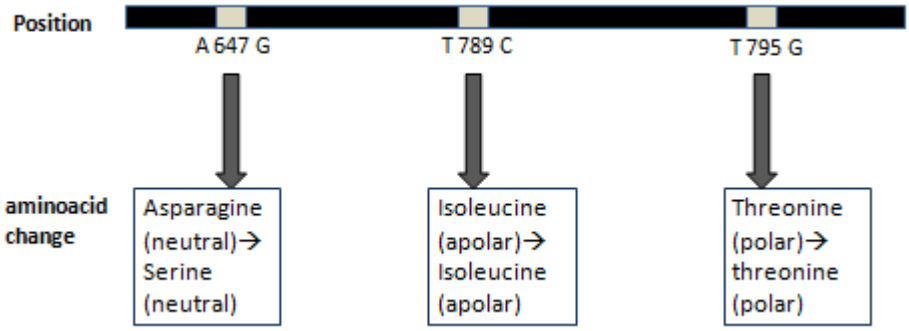

Figure 1: Nucleotide position and amino acid change description of E7HPV16 based on the reference sequence (NC_001526).

2.3. Cell culture, transfection, and TNFα stimulation

Human embryonic kidney cells (HEK-293) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) 1885 supplemented with 1% penicillin/streptomycin (5,000 U/ml), 2 mM l-glutamine, 10% FBS and 10% tetracycline approved FCS in 37ºC in humidified air atmosphere with 5% CO_2.

Cell transfection was performed using Polyfect reagent (Qiagen), according to manufacturer's protocol. Cells were incubated with TNFα (10 ng/ml) for 6 hours. Stimulation was broken by replacing the medium with cold PBS, followed by cell lysis using Passive Lysis Buffer (Promega, Madison, WI, USA), and stored at -80^{o}C.

2.4. Luciferase assays

HEK-293 cells were seeded in 6-well plates at 5×10^{5} cells per well. All cell groups were co-transfected with an NF-κB-dependent firefly luciferase reporter construct with three NF-κB binding sites (κB) upstream of the luciferase gene, (κB)3-Luc (1µg) (BCCMTM/LMBP, Gent, Belgium) and a renilla luciferase-expressing plasmid (1 ng), as luminescence normalizer. Twenty four hours after the transfection, cells were incubated with TNFα (10 ng/ml) during 6 hours. Groups of HEK-293 cells were transfected with 0.4 pmol of each polymorphic E7 contruct (All variants, N29S, I76I and T78T), HPV-16 E7 reference (16 E7 Ref.) or pcDNA3.1 empty vector. NF-κB activity was measured by GloMax® 96 Microplate Luminometer (Promega®) using dual luciferase reporter assay system (Promega). In order to ensure comparable transfection efficiencies, results were normalized by renilla firefly luminecense. The readings for firefly and renilla were normalized to protein levels detected in the sample by Bradford protein assay, and the fold activation was determined by assigning 1 to the empty vector.

2.5. Real Time qPCR

Total RNA was obtained by RNeasy mini kit (Qiagen) and cDNA has been synthesized by Improm® Reverse Transcription kit (Promega), according to manufacturer's instructions. qPCR reactions were performed by QuantiTect SYBR Green® PCR kit (Qiagen) and primers and cDNA concentrations were the same as the ones described by Leitão et al., (2014). The cycle protocol was 95°C for 15min, 40 denaturation cycles at 95°C for 10s and reference genes annealing at 55°C for 30s, while E7 gene annealing at 60°C. Melting curve protocol has gradually varied from 65 to 95°C.

Gene expression normalization has adopted GAPDH and ACT reference genes. Each reaction was performed in three biological replicates including a

negative control for each gene. Gene expression was calculated according to Livak and Schmittgen, (2001).

2.6. Statistical analysis

Statistical analysis was used the software GraphPad Prism (version 6.0). Normality test D'Agostino-Pearson was used to check the distribution of the data for the luminescence data. Statistical analysis was performed using the one-way analysis of variance (ANOVA) followed by Bonferroni correction post-test. $p < 0.05$ was considered significant.

3. Results

3.1. HPV16 E7 containing all variants (N29S, I76I and T78T) attenuates NF-κB activity

In order to investigate the effect of polymorphic E7 and each one of its variants on NF-κB pathway, HEK-293 cells were transfected with four versions of variants E7-coding plasmid (All variants, N29S, I76I and T78T). Their effects on NF-κB pathway were compared with those found in HPV-16 E7- Reference expressing cells (16 E7 Ref.), which were transfected with empty plasmid-transfected cells.

In this study, we evaluated the influence of HPV16 E7 variants on HEK-293 NF-kB pathway. Expression of HPV16 E7, that presents simultaneously the three variants, N29S, I76I, T78T, has dramatically reduced NF-kB pathway activity to 50-fold below NF-kB pathway activity of the cell control (Figure 2), and 25-fold below the 16 E7 Ref.-expressing cells NF-kB pathway activity. None of the E7 versions with only one variant (N29S, I76I or T78T) have shown downregulation of NF-kB pathway activity, which were not significantly different from the control cells. However, each E7 gene with a single variant has no statistically significant depression on NF-kB pathway in relation to 16 E7 Ref.-expressing cells NF-kB pathway activity (Table 1).

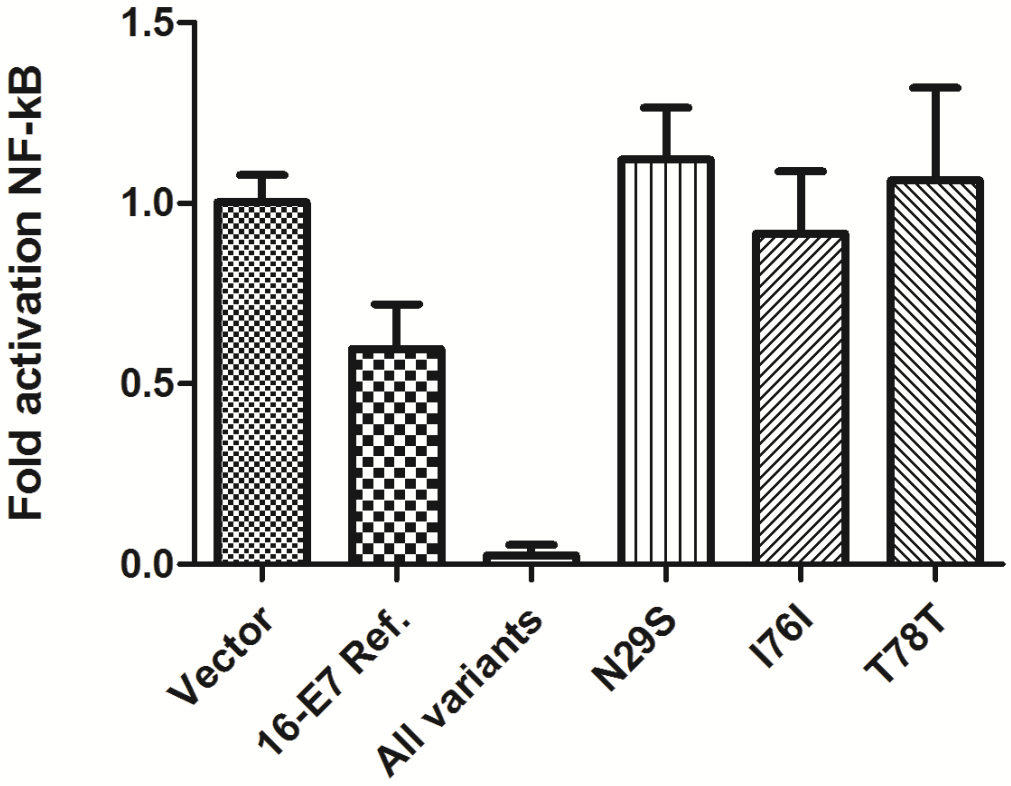

Figure 2: HPV16 E7 effect on NF-κB activity. All groups of HEK-293 cells were transfected with (κB)3-Luc luminescence reporter plasmid and pRenilla luminescence normalizer plasmid. Each group was transfected with one of the variants E7 constructs (All variants, N29S, I76I and T78T) or with HPV-16 E7 Reference construct (16-E7 Ref.). Cells were harvested after 24 hours post-transfection; whole cell extracts were prepared and the firefly luciferase and renilla luciferase activities were measured. HEK-293 cells transfected with an empty vector were used as a control. The NF-κB fold induction was determined. Bars represent the mean of each condition performed in duplicate. Error bars indicate a mean standard error.

3.2. HPV-16 E7 containing N29S variant presented the highest E7 mRNA expression

HEK-293 cells were transfected with four E7 synthetic genes (All variants, N29S, I76I and T78T). Their expressions were compared with those found in HPV-16 E7 reference-expressing cells, which were transfected with pcDNA-w/oE7variant, and those found in empty plasmid-transfected cells.

In E7 mRNA expression evaluation, we found that N29S-containing gene was 9-fold higher than HPV-16 E7 reference expression (Figure 3). The other E7 synthetic genes All variants, I76I, and T78T, have presented 2.2-fold, 1.9-fold and 1.4-fold higher expression values, respectively, than the HPV-16 E7 reference.

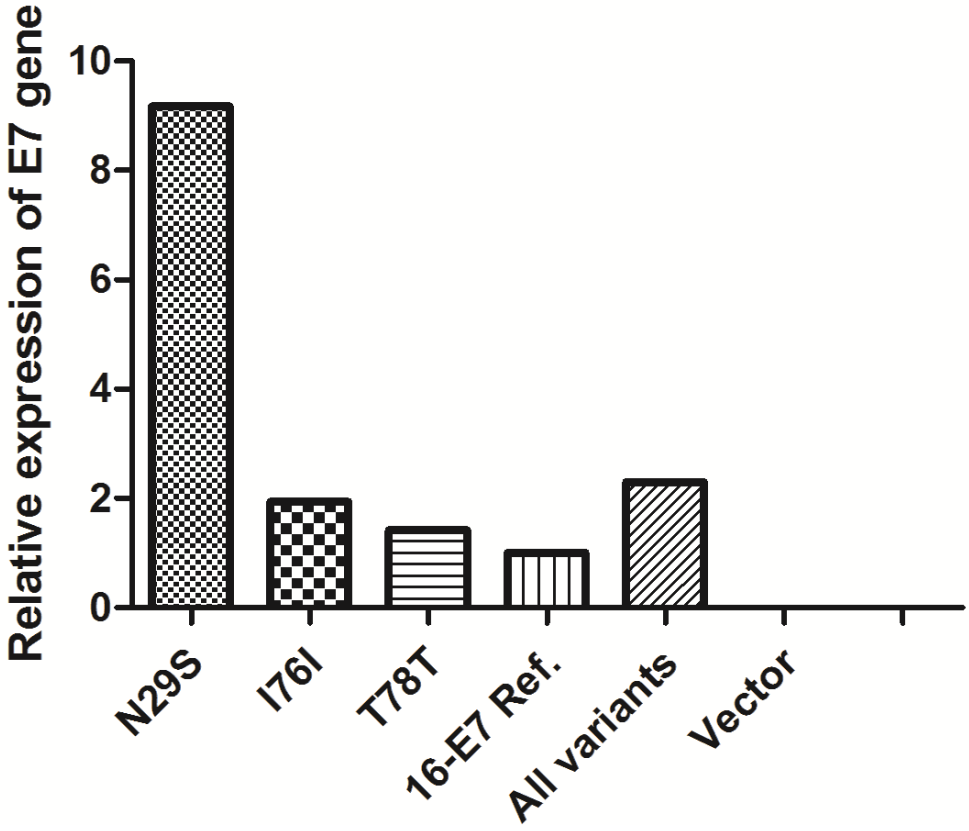

Figure 3: HPV-16 E7 mRNA expression. All groups of HEK-293 cells were transfected with one of the polymorphic E7 constructs (All variants, N29S, I76I and T78T) or with HPV-16 E7 Reference construct (16-E7 Ref.). Cells were harvested after 24 hours post-transfection total RNA were obtained and cDNA was synthetized. Gene expression normalization used GAPDH and ACT reference genes. Each reaction was performed in three biological replicates including a negative control for each gene. Gene expression was calculated by $2^{-\Delta\Delta Cq}$.

4. Discussion

In this study, we evaluated the influence of HPV-16 E7 variants expression in HEK-293 cell culture NF-kB pathway. Three HPV-16 E7 polymorphisms (N29S, I76I and T78T) found in circulating HPV variants from northeastern brazilian population had been chosen, although all of them have already been described in previous works (Eschle *et al*., 1992; Fujinaga *et al*., 1994; Icenogle *et al*., 1991; Song *et al*., 1997).

Several studies show association between non-European variants of HPV-16 and -18 and increased risk of cervical intraepithelial lesion (Aho *et al*., 2004, Gagnon *et al*., 2004, Sichero *et al*., 2007, Xi *et al*., 2007). Despite oncogenic potential being a possible consequence of intratypic variability (Freitas *et al*., 2014; Schiffman *et al*., 2010), rare studies have tried HPV polymorphisms from a

functional perspective. Our work provides one of the first evidences that HPV-16 E7 polymorphisms may have significant effects on NF-kB pathway, which is a critical component of antiviral cellular response and inflammation. While cell culture transfected with HPV-16 E7 gene without polymorphisms have presented a competent NF-kB activation, under stimulation, the simultaneous expression of three variants (N29S, I76I and T78T) within an HPV-16 E7 synthetic gene have dramatically reduced NF-kB pathway activity. Curiously, none of the HPV-16 E7 synthetic gene harboring only one variant (N29S, I76I or T78T) have showed NF-kB pathway downregulation.

It is possible that a single polymorphism can affect the biological function of a whole protein. A well characterized polymorphism is found in European variants harboring HPV-16 E6 gene, where a substitution 350T to G leads to a non-synonymous mutation L83V (Bernard *et al*., 2006). While several studies suggest an association between L83V variant and increased risk of persistent infection and cytological progression to cervical intraepithelial neoplasia (CIN) grade 2/3 and squamous cell carcinoma (Grodziki *et al*., 2006; Lee *et al*., 2008, Sathish *et al*., 2005), others have not found the same association (del Refugio González-Losa *et al*., 2004; Lizano *et al*., 2006). However, a link between L83V and risk of malignant disease has been provided by some functional studies. HPV-16 E6 harbouring L83V activates MAPK signaling through Rap1, while E6 protein without this polymorphism activates MAPK signaling through Ras (Chakrabarti *et al*., 2004). L83V variant is more efficient eliciting Bax degradation (Asadurian *et al*., 2007) and resistance to serum and calcium-triggered differentiation of primary human keratinocytes (Lichtig *et al*., 2006).

Although more than 20 single nucleotide polymorphisms have been described to HPV-16 E7 variants (Boumba *et al*., 2015; Fujinaga *et al*.,1994, Vaeteewoottacharn *et al*., 2003), and some of them have been associated to high grade CIN (Song *et al*., 1997; Wu *et al*., 2006), little may be understood about its functional outcome. HPV-31 E7 variants showed no significant difference when compared to the prototype in the levels of degradation of pRb (Ferenczi *et al.*, 2015). HPV-18 E7 mutagenesis has been performed and only negatively charged serine residues mutation within pRb binding regions and casein kinase II (CKII) phosphorylation sites were able to change the cell proliferation in differentiated keratinocytes organotypic cultures (Chien *et al*., 2000). Nevertheless, N29S

variant is commonly found in European, African or Asian lineages and its role in cancer development remains unclear. A recent study suggests an increased transforming activity associated to variant HPV-16 E7 harboring N29S polymorphism. Zine El Abidine et al. (2017) have demonstrated that N29S mutation creates a new phospho-acceptor site, increasing E7 interaction with some cellular targets, like TATA Box Binding Protein (TBP) and pRb family.

Studies have shown that HPV16 E7 protein interferes with NF-kB signaling by interacting directly with IKKα and IKKβ subunits, causing reduction in NF-kB signaling-related activity in U2OS cells (Spitkovsky *et al*., 2002). Our data corroborates with Byg *et al*. (2012), which observed that even under a TNF-α stimulation, HPV E7-expressing cells were less responsive, exhibiting an attenuated NF-kB signaling. As a novel altered function resulting from N29S, the strong suppressor effect on NF-kB in our work has been observed only under simultaneous expression of three E7 variants (N29S, I76I and T78T), what may suggest a strictly synergic activity between at least two or three of them. It is also possible that N29S transcripts confer some advantage to E7 mRNA, once we found that N29S was 9-fold higher than HPV-16 E7 reference expression. Finally, the mechanism by which the E7 gene polymorphisms reduce a major pro-inflammatory signaling pathway still requires further studies.

This study provides one of the first evidence that these polymorphisms may be relevant to NF-κB signaling pathway. HPV-16 E7 gene variants in which the three polymorphisms are present may perform a strong suppressive effect on the NF-kB pathway in HPV-infected patients with implications to infection persistence and progression. However, our mRNA evaluation indicates that changes resulting from the polymorphisms may not necessarily be related to the differential expression levels of the variants. Our work collaborates with a research effort on HPV infection profiles in the population and provides important data for a better understanding of how variants can be distinguished under its clinical consequences.

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Conflicts of interest

The authors declare that they have no conflict of interest.

5- References

Aho J, Hankins C, Tremblay C, Forest P, Pourreaux K, Rouah F, Coutlé F. (2004) Genomic Polymorphism of Human Papillomavirus Type 52 Predisposes toward Persistent Infection in Sexually Active Women. J Infect Dis 190 (1): 46-52.

Asadurian Y, Kurilin H, Lichtig H, Jackman A, Gonen P, Tommasino M, Zehbe I, Sherman L (2007) Activities of human papillomavirus 16 E6 natural variants in human keratinocytes.J Med Virol. 79(11):1751-60.

Bernard HU, Calleja-Macias IE, Dunn ST (2006) Genome variation of human papillomavirus types: Phylogenetic and medical implications. Int. J. Cancer 118(5) 1071–1076

Boumba LM, Assoumou SZ, Hilali L, Mambou JV, Moukassa D, Ennaji MM (2015) Genetic variability in E6 and E7 oncogenes of human papillomavirus Type 16 from Congolese cervical cancer isolates. Infect Agent Cancer.10:15.

Byg LM, Vidlund J, Vasiljevic N, Clausen D, Forslund O, Norrild B (2012) NF-κB signalling is attenuated by the E7 protein from cutaneous human papillomaviruses.Virus Res. 169(1):48-53.

Chakrabarti O, Veeraraghavalu K, Tergaonkar V, Liu Y, Androphy EJ, Stanley MA, Krishna S (2004) Human papillomavirus type 16 E6 amino acid 83 variants enhance E6-mediated MAPK signaling and differentially regulate tumorigenesis by notch signaling and oncogenic Ras.J Virol. 78(11):5934-45.

Chen LF, Greene WC (2004) Shaping the nuclear action of NF-kappaB. Nat Rev Mol Cell Biol. 5(5):392-401.

Chien WM, Parker JN, Schmidt-Grimminger DC, Broker TR, Chow LT (2000) Casein kinase II phosphorylation of the human papillomavirus-18 E7 protein is critical for promoting S phase entry. Cell Growth Diff 11: 425–435.

del Refugio González-Losa M, Laviada Mier y Teran MA, Puerto-Solís M, García-Carrancá A (2004) Molecular variants of HPV type 16 E6 among Mexican women with LSIL and invasive cancer.J Clin Virol. 29(2):95-8.

Eschle D, Diirst M, Luande J, Eberhardt C, I MP, Gissmann L (1992) Geographical dependence of sequence variation in the E7 gene of human papillomavirus type 16. Journal of General Virology 73:1829-1832.

Ferenczi A, Gyöngyösi E, Szalmás A, László B, Kónya J, Veress G. (2016) Phylogenetic and functional analysis of sequence variation of human papillomavirus type 31 E6 and E7 oncoproteins. Infect Genet Evol. 43:94-100.

Freitas LB, Chen Z, Muqui EF, Boldrini NAT, Miranda AE, Spano LC, et al. (2014) Human Papillomavirus 16 Non-European Variants Are Preferentially Associated with High-Grade Cervical Lesions. PLoS One.

Fujinaga Y, Okazawa K, Nishikawa A, Yamakawa Y, Fukushima M, Kato I, Fujinaga K (1994) Sequence variation of human papillomavirus type 16 E7 in preinvasive and invasive cervical neoplasias. Virus Genes 9:85-92.

Gagnon S, Hankins C, Tremblay C, Forest P, Pourreaux K, Coutlé F (2004) Viral polymorphism in human papillomavirus types 33 and 35 and persistent and transient infection in the genital tract of women. J. Infect. Dis. 190: 1575-1587.

Gilmore TD (2006) Introduction to NF-kappaB: players, pathways, perspectives. Oncogene. 25(51):6680-4.

Grodzki M, Besson G, Clavel C, Arslan A, Franceschi S, Birembaut P, Tommasino M, Zehbe I (2006) Increased risk for cervical disease progression of French women infected with the human papillomavirus type 16 E6-350G variant.Cancer Epidemiol Biomarkers Prev. 15(4):820-2.

Hayden MS, Ghosh S (2012) NF-κB, the first quarter-century: remarkable progress and outstanding questions. Genes Dev. 26(3):203-34.

Hoesel B, Schmid JA (2013) The complexity of NF-κB signaling in inflammation and cancer. Mol Cancer. 2;12:86.

Icenogle JP, Sathya P, Miller DL, Tucker RA, Rawls WE (1991) Nucleotide and amino acid sequence variation in the L1 and E7 open reading frames of human papillomavirus type 6 and type. Virology 184(1):101-7.

Lee K, Magalhaes I, Clavel C, Briolat J, Birembaut P, Tommasino M, Zehbe I. (2008) Human papillomavirus 16 E6, L1, L2 and E2 gene variants in cervical lesion progression.Virus Res. 131(1):106-10.

Leemans CR, Boudewijn JM, Braakhuis & Ruud H, Brakenhoff (2011) The molecular biology of head and neck cancer. Nature Reviews Cancer 11, 9-22.

Leitão Mda C, Coimbra EC, de Lima Rde C, Guimarães Mde L, Heráclio Sde A, Silva Neto Jda C, de Freitas AC (2014) Quantifying mRNA and MicroRNA with qPCR in Cervical Carcinogenesis: A Validation of Reference Genes to Ensure Accurate Data PLoS One. 3,9(11).

Lichtig H, Algrisi M, Botzer LE, Abadi T, Verbitzky Y, Jackman A, Tommasino M, Zehbe I, Sherman L (2006) HPV16 E6 natural variants exhibit different activities in functional assays relevant to the carcinogenic potential of E6.Virology. 350(1):216-27.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25(4):402-8.

Lizano M, De la Cruz-Hernández E, Carrillo-García A, García-Carrancá A, Ponce de Leon-Rosales S, Dueñas-González A, Hernández-Hernández DM, Mohar A (2006) Distribution of HPV16 and 18 intratypic variants in normal cytology, intraepithelial lesions, and cervical cancer in a Mexican population.Gynecol Oncol. 102(2):230-5.

Perkins ND (2007) Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat Rev Mol Cell Biol. 8: 49-62. 10.1038/nrm2083.

Rositch AF, Koshiol J, Hudgens MG, Razzaghi H, Backes DM, Pimenta JM, Franco EL, Poole C, Smith JS (2013) Patterns of persistent genital human papillomavirus infection among women worldwide: A literature review and meta-analysis. Int J Cancer 133:1271–1285.

Sathish N, Abraham P, Peedicayil A, Sridharan G, Chandy G (2005) HPV 16 E6 sequence variations in Indian patients with cervical neoplasia. Cancer Lett. 8;229(1):93-9

Schiffman M, Rodriguez AC, Chen Z, Wacholder S, Herrero R, Hildesheim A, et al. (2010) A population-based prospective study of carcinogenic human papillomavirus variant lineages, viral persistence, and cervical neoplasia. Cancer Res. 70:3159–3169.

Sichero L, Ferreira S, Trottier H, Duarte-Franco E, Ferenczy A, Franco EL, Villa LL (2007) High grade cervical lesions are caused preferentially by non-European variants of HPVs 16 and 18. Int. J. Cancer 120 (8): 1763–1768.

Song YS, Kee SH, Kim JW, Park NH, Kang SB, Chang WH, Lee HP (1997) Major sequence variants in E7 gene of human papillomavirus type 16 from cervical cancerous and noncancerous lesions of Korean women. Gynecol Oncol 66:275-281.

Spitkovsky D, Hehner SP, Hofmann TG, Moller A, Schmitz ML (2002) The human papillomavirus oncoprotein E7 attenuates NF-kappa B activation by targeting the Ikappa B kinase complex. Biol Chem 277: 25576–25582.

Sun SC (2011) Non-canonical NF-κB signaling pathway. Cell Res. 2011 Jan;21(1):71-85.

Thompson DA, Zacny V, Belinsky GS, Classon M, Jones DL, Schlegel R, Münger K (2001) The HPV E7 oncoprotein inhibits tumor necrosis factor alpha-mediated apoptosis in normal human fibroblasts. Oncogene 20, 3629 ± 3640.

Vaeteewoottacharn K, Jearanaikoon P, Ponglikitmongkol M (2003) Co-mutation of HPV16 E6 and E7 genes in Thai squamous cervical carcinomas. Anticancer Res. 23(2C):1927-31.

Villa LL, Sichero L, Rahal P, Caballero O, Ferenczy A, Rohan T, et al. (2000) Molecular variants of human papillomavirus types 16 and 18 preferentially associated with cervical neoplasia. J Gen Virol. 81:2959–2968.

Xi LF, Koutsky LA, Hildesheim A, Galloway DA, Wheeler CM, Winer RL, Ho J and Kiviat NB (2007) Risk for high-grade cervical intraepithelial neoplasia associated with variants of human papillomavirus types 16 and 18. Cancer Epidemiol. Biomarkers Prev. 16, 4-10

Zehbe I, Richard C, DeCarlo CA, Shai A, Lambert PF, Lichtig H, Tommasino M, Sherman L (2009) Human papillomavirus 16 E6 variants differ in their

dysregulation of human keratinocyte differentiation and apoptosis. Virology. 2009 383(1):69-77.

Zerfass-Thome K, Zwerschke W, Mannhardt B, Tindle R, Botz JW, Jansen-Durr P (1996) Inactivation of the cdk inhibitor p27KIP1 by the human papillomavirus type 16 E7 oncoprotein. Oncogene. 13:2323–2330.

Zine El Abidine A, Tomaic V, Bel Haj Rhouma R, Massimi P, Guizani I, Boubaker S, Ennaifer E, Banks L (2017) A naturally occurring variant of HPV-16 E7 exerts increased transforming activity through acquisition of an additional phospho-acceptor site. Virology 500:218-225.

5. DISCUSSÃO GERAL

O estudo aqui apresentado detectou o genoma do HPV em 70.8% dos escovados cervicais. Dentre os tipos identificados, o HPV-16 figurou como o mais frequente em nossa população. A maior prevalência do HPV-16 evidente em nosso estudo corrobora com os resultados obtidos por outros estudos que perfilam a disseminação do HPV na população brasileira (Alencar *et al*., 2006; Castro *et al*., 2011; Cruz *et al*., 2004; Fernandes *et al*., 2010;Fernandes *et al*., 2011; de M Fernandes *et al*., 2008), concordando ainda com aqueles que já observaram, a nível global, a prevalência do HPV-16 (Bosch *et al.,* 2008; De Sanjose *et al.,* 2010; Jemal *et al.,* 2013). Essa prevalência na população também prevalece no Nordeste (Baldez *et al.,* 2009; Baldez *et al.,* 2012; Gurgel *et al.,* 2013).

Em se tratando do segundo tipo mais prevalente em escala global, existe uma variação de acordo com a região ou grupo populacional estudado entre os HPVs tipo 18, 31 ou 56. O segundo tipo de HPV mais prevalente apontado em nosso estudo foi o HPV-31, conforme observado em outros estudos no Nordeste do Brasil (Baldez da Silva *et al.,* 2009, 2012; Chagas *et al.,* 2011, 2013, 2015; Gurgel *et al.,* 2015) e, semelhante ao perfil observado no sul da Europa (Cento *et al.,* 2011). Em outras regiões do Brasil, como por exemplo, em São Paulo, ainda que o HPV-16 permaneça como o mais prevalente, a distribuição dos demais tipos diverge consideravelmente, tendo como segundo tipo mais prevalente o HPV-56, seguido pelos HPV-52 e HPV-31 (Martins *et al*., 2016). Essas diferenças na prevalência dos tipos de HPV podem ocorrer devido ao processos diferenciais de migração de pessoas, que interferiram na composição da diversidade populacional em cada região (Bosch *et al.,* 1995; Barzon *et al.,* 2008).

Eventos de coinfecção por mais de um tipo HPV de alto risco têm sido reportados na literatura (Chaturvedi *et al.,* 2005), não diferindo dos nossos resultados de detecção conjunta dos HPV-16/HPV-18 e HPV-16/HPV-31. Alguns estudos procuram verificar se esses HPVs de alto risco poderiam estar atuando sinergicamente no desenvolvimento do câncer cervical (Chaturved *et al.,* 2011Dickson *et al.,* 2013), embora essa questão ainda permaneça controversa.

Estudos focados em caracterização de variantes vêm demonstrando diferenças nos seus potenciais oncogênicos (Burk *et al*., 2013; Villa *et al*., 2000).

A compreensão dos efeitos destas variantes poderia contribuir para o esclarecimento de fatores genéticos relacionados ao desenvolvimento de lesões de alto grau e câncer em uma determinada população (Freitas *et al*., 2014).

Vários estudos apontam para um aumento no risco de desenvolvimento de NIC3 e câncer cervical de duas a quatro vezes maior em variantes não européias quando comparada as linhagens européias (Burk *et al*, 2013; Hildesheim *et al.,* 2001; Schiffman *et al*, 2010; Sichero *et al*, 2007; Villa *et al*. 2000; Zuna *et al*, 2009). Em nosso trabalho, encontramos exemplares com cada uma das variantes atualmente descritas na literatura (A, B, C e D), sendo a variante européia (A) a mais encontrada em nossa população, à semelhança dos achados de Cruz e colaboradores (2004) e Rabelo Santos e colaboradores (2006). Outros trabalhos compõem o esforço de se estabelecer um perfil de distribuição do HPV na população brasileira, indicando inclusive as diferentes variantes disseminadas ao longo das regiões do Brasil: Nordeste (Gurgel *et al.,* 2015), Centro Oeste (Alencar *et al*., 2007; Cruz *et al.,* 2004) e Sudeste (Freitas *et al.,* 2014; Villa *et al*., 2000). Adicionalmente aos demais estudos realizados no Brasil, nosso estudo é o primeiro a classificar as variantes descritas com base em análise do oncogene E7.

A oncoproteína E7 é essencial para o desenvolvimento do câncer cervical, atuando na transformação de queratinócitos, por meio da interação com proteínas da família pRb (retinoblastoma) comprometendo sua ação inibitória sobre o fator E2F, resultando na progressão contínua para a fase *S* do ciclo celular. Em suma, a atividade oncogênica do proteína E7 em HPVs de alto risco se fundamenta na no estímulo para a proliferação celular (Sandal, 2002). Dada a importância do gene E7 para o processo de carcinogênese cervical, estudos de variabilidade genética buscam elucidar aspectos biológicos envolvidos na maior persistência da infecção por HPVs de alto risco, uma vez que estas alterações podem refletir em modificações no potencial de interação entre E7 e seus parceiros de interação, como por exemplo a pRb. Elucidar essa propriedade de proteínas E7 em variantes de HPV de maior risco ou propensão a neoplasias de alto grau também seria significativo para o desenvolvimento de métodos terapêuticos inovadores mais eficientes (Chagas *et al.,* 2011; Chagas *et al.,* 2013; Villa *et al.,* 2000).

O gene E7 é considerado altamente conservado entre os *Alpha papillomavirus* (Grassmann *et al.,* 1996; Icenogle *et al.,* 1991; Pande *et al.,* 2008;

Wu *et al.,* 2006; Zehbe *et al.,* 1998). No nosso estudo estudo, foram identificados quatro SNPs no gene E7 nas seguintes posições: 647, 732, 789 e 795, e, de acordo com o aminoácido alterado e o códon, podem ser denominados de N29S, F57F, I76I, e T78T, respectivamente. Todas estas alterações foram descritas na literatura (Eschle *et al.,* 1992; Fujinaga *et al.,* 1994; Icenogle *et al.,* 1991; Song *et al.,* 1997). Dentre as alterações encontradas, apenas uma delas foi considerada não sinônima (ocorreu a troca do aminoácido) situada no códon 29, envolvendo a troca de uma serina por uma asparigina (N29S). Esse evento de troca foi encontrado em duas amostras clínicas, correspondendo a 8.3% dentre o total de 24 amostras positivas para o gene E7 encontradas em nosso estudo. As demais alterações F57F, I76I, e T78T foram encontradas em três (12.5%), oito (33.33%) e sete (29.16%) amostras clínicas, respectivamente.

Nosso resultado difere daquele apresentado por Song e colaboradores (1997), no qual, na população da Coréia, em pacientes com e sem lesões cervicais, obteve-se uma frequência do polimorfismo N29S de 60%, enquanto que os demais SNPs ultrapassaram uma frequência de 20%. De fato, vários estudos apontam para uma alta frequência do N29S em populações asiáticas, conforme registrado por diversos estudos, como por exemplo, no Japão com 60% (Fujinaga *et al.,* 1994), na região central e sudeste da China com 70.21% (Chan *et al.,* 2002; Shang *et al.,* 2011). Contrastando com essas frequências, mas de modo mais semelhante aos nossos resultados, trabalhos em populações não asiáticas, como no Congo (Boumba *et al.,* 2015) e em Marrocos (Qmichou *et al.,* 2013), aponta-se frequências de N29S de 23% e 29%, respectivamente.

As amostras positivas para o SNP N29S identificadas em nosso estudo foram classificadas como pertencentes a linhagem C (linhagem Africana-2). Curiosamente, a linhagem C, como parte daquelas descritas como não européias (B, C e D), é considerada como de maior potencial oncogênico quando comparada a linhagem A (Burk *et al.,* 2013).

Nosso trabalho localizou a mutação presente na posição 647 (N29S) em regiões de epítopos de células T e B. Eventos similares foram descritos por Bontkes e colaboradores (1998) e Zehbe e colaboradores (1998), onde é sugerido que a apresentação de peptídeos virais por células T pode ser influenciada por polimorfismos em genes do HPV e polimorfismos em antígenos leucocitários humanos (HLA). No oncogene E6 do HPV-16 foi sugerido que o

polimorfismo em R10G pode alterar um epítopo de ligação B*07 que poderia interferir com o reconhecimento de linfócitos T citotóxicos (Ellis *et al*. 1995). Ainda que não tenha sido objetivo do presente trabalho, é possível que N29S possa influenciar o reconhecimento imunológico de células infectadas pelo HPV, bem como a evasão da resposta imune natural.

Neste estudo, observamos que o N29S apresenta pressão seletiva diversificada, o que poderia significar uma alta probabilidade de fixação deste polimorfismo na população viral. Mutações adaptativas são necessárias para a evolução viral e adptação ao hospedeiro, mostrando que este SNP poderia ser relevante para o ciclo de infecção e patogênese viral. A análise estrutural revelou que o polimorfismo está situado dentro de uma região de *loop*, próximo ao domínio da hélice LXCXE. Este domínio é responsável pela interação entre E7 e pRb. Proteínas com alterações estruturais nesta região podem apresentar patogenicidade alterada, hipótese para a qual são necessários novos estudos.

Estudos funcionais com variantes de E7 são raros. Embora pelo menos seis variantes do E7 de HPV-16 tenham sido identificadas até agora, seus polimorfismos podem não gerar produtos funcionalmente diferentes. Verificou-se que, pelo menos para o seu principal parceiro de interação, as variantes E7 de HPV-31 não apresentaram diferenças significativas em comparação com seu protótipo para degradar os níveis de pRb (Ferenczi *et al*., 2015).

A mutagênese do gene E7 de HPV-18 realizada pelo trabalho de Chien e colaboradores (2000) mostrou que apenas a mutação do resíduo de serina por resíduos carregados negativamente nas regiões de ligação de pRb e fosforilação de caseína quinase II (CKII) foram capazes de alterar a proliferação celular em culturas organotípicas de queratinócitos diferenciados. Em nosso estudo, a avaliação funcional do HPV-16 E7 para induzir a desregulação da via NF-kB mostrou inibição significativa apenas quando três polimorfismos estão presentes no gene E7.

A ativação da via NF-kB é um achado frequente no carcinoma de células escamosas e é uma evidência importante da transformação de células epiteliais (Huber *et al*., 2004). Nossos resultados apresentam uma diminuição significativa na via de sinalização de NF-kB em cultura celular, onde o gene E7 polimórfico, que contém polimorfismos nas posições 647, 789 e 795 é expresso quando comparado ao controle. Estudos mostraram que a proteína E7 de HPV-16

interfere na sinalização de NF-kB, interagindo diretamente com as subunidades IKKa e IKKβ, causando uma redução na atividade relacionada com a sinalização NF-kB em células U2OS (Spitkovsky *et al*., 2002). Nossos dados corroboram com Byg *et al*. (2012), onde mesmo com a estimulação com TNF-α, as células expressando E7 do HPV-16 eram menos responsivas, exibindo uma sinalização NF-kB atenuada. Finalmente, o mecanismo pelo qual a função do polimorfismo do gene E7 reduz uma das principais vias de sinalização pró-inflamatórias ainda requer mais estudos.

6. Conclusões

A alta incidência de pacientes positivas para o HPV foi reafirmada com detecção em cerca de 70.8% dos escovados cervicais coletados;

Os HPVs de alto risco mais incidentes foram os tipos 16, 31 e 58;

A detecção simultânea de mais de um tipo de HPV na mesma amostra também é um evento recorrente, dentre os quais, as combinações mais frequentes foram 16 e 31, 16 e 58, e 16, 31 e 58;

As análises filogenéticas realizadas revelaram que todas as linhagens do HPV-16 (A, B, C e D) compões o perfil de infecção na população estudada;

Variantes do oncogene E7 do HPV-16 (N29S, F57F, I76I e T78T) já descritas na literatura foram identificadas, mas apenas a variante com alteração no codon 29 (N29S) resultou na substituição do aminoácido asparigina por serina;

N29S está localizado em sítios de epítopos de reconhecimento para células T e/ou B, além de estar próximo ao domínio de interação à pRB, LXCXE;

Em nossa análise funcional, células HEK-293 transfectadas com o gene E7 de HPV-16 apresentando as três variantes (N29S, I76I e T78T) sofreram um forte efeito repressor da via NF-kB quando comparadas àquelas tranfectadas com o gene E7 sem estas variantes;

O mesmo efeito não foi observado em células HEK-293 transfectadas com o gene E7 de HPV-16 apresentando apenas uma da variantes;

O RNAm de E7 em células transfectadas apenas com a variante N29S foi mais expresso que o gene E7 sem variantes;

Nenhuma das variantes gerou um códon raro para humanos;

Apenas a variante I76I gerou um códon mais eficiente para humanos;

Apenas o E7 all variants apresentou menos estruturas secundárias, o que poderia refletir em um RNA lido mais eficientemente.

REFERÊNCIAS BIBLIOGRÁFICAS

AMARAL, CM; CETKOVSKÁ, K; GURGEL, AP; CARDOSO, MV; CHAGAS, BS; PAIVA JÚNIOR, SS; DE LIMA, RDE C; SILVA-NETO, JC; SILVA, LA; MUNIZ, MT; BALBINO, VQ; FREITAS, AC. MDM2 polymorphism associated with the development of cervical lesions in women infected with Human papillomavirus and using of oral contraceptives. **Infect Agent Cancer**. 9:24. 2014.

AN, J; MO, D; LIU, H; VEENA, MS; SRIVATSAN, ES; MASSOUMI, R; RETTIG, MB. Inactivation of the CYLD deubiquitinase by HPV E6 mediates hypoxia-induced NF-kappaB activation. **Cancer Cell**. 14(5):394-407. 2008.

ANNUNZIATA, CM; STAVNES, HT; KLEINBERG, L; BERNER, A; HERNANDEZ, LF; BIRRER M, J; et al. Nuclear factor kappaB transcription factors are coexpressed and convey a poor outcome in ovarian cancer. **Cancer** 116, 3276–328410.1002/cncr.25190. 2010.

APPLE, RJ; ERLICH, HA; KLITZ, W; MANOS, MM; BECKER, TM; WHEELER, CM. HLA DR-DQ associations with cervical carcinoma show papillomavirus-type specificity. **Nat Genet.** 6(2):157-62.1994.

BADARACCO G, VENUTI A, MORELLO R, MULLER A, MARCANTE ML (2000) Human papillomavirus in head and neck carcinomas: prevalence, physical status and relationship with clinical/pathological parameters. Anticancer Res. 20(2B):1301-5.

BAKER, TS; NEWCOMB, WW; OLSON, NH; COWSERT, LM; OLSON, C; BROWN, JC. Structures of bovine and human papillomaviruses. Analysis by cryoelectron microscopy and three-dimensional image reconstruction. **Biophys J** 60: 1445-1456. 1991.

BALDEZ DA SILVA, MF; CHAGAS, BS; GUIMARÃES, V; KATZ, LM; FELIX, PM; MIRANDA, PM; LIMA, AA; ARRAES, LC; MARTINS, DB; LIMA, FILHO JL; STOCCO, RC; CROVELLA, S; FREITAS, AC; BEÇAK, W. HPV31 and HPV33 incidence in cervical samples from women in Recife, Brazil.**Genet Mol Res**. 8(4):1437-43. 2009.

BALDEZ DA SILVA, MF; GUIMARÃES, V; SILVA, MA; MEDEIROS DO AMARAL, CM; BEÇAK, W; STOCCO, RC; FREITAS, AC; CROVELLA, S. Frequency of human papillomavirus types 16, 18, 31, and 33 and sites of cervical lesions in gynecological patients from Recife Brazil. **Genet. Mol**. **Res**., 11 , pp. 462–466. 2012.

BARZON, L; GIORGI, C; BUONAGURO, FM; PALÙ, G. Guidelines of the Italian Society for Virology on HPV testing and vaccination for cervical cancer prevention. **Infect Agent Cancer**. 3:14. 2008

BASAK, S; SHIH, VF; HOFFMANN, A. Generation and activation of multiple dimeric transcription factors within the NF-κB signaling system. **Mol Cell Biol.** 28:3139–3150. 2008

BASEMAN, JG; KOUTSKY, LA; The epidemiology of human papillomavirus infections. **J Clin Virol**. 32(1):16-24. 2005.

BERG, M; STENLUND, A. Functional interactions between papillomavirus E1 and E2 proteins. **J Virol**. 71: 3853–3863. 1997.

BERNARD, HU; BURK, RD; CHEN, Z; VAN DOORSLAER, K; ZUR HAUSEN, H; DE VILLIERS, EM. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. **Virology**. 401(1):70-9. 2010.

BONTKES, HJ; WALBOOMERS, JM; MEIJER, CJ; HELMERHORST TJ; STERN, PL. Specific HLA class I down-regulation is an early event in cervical dysplasia associated with clinical progression. **Lancet.** 351(9097):187-8.1998.

BOSCH FX, MUÑOZ N. The viral etiology of cervical cancer. **Virus Res**.89(2):183-90. 2002.

BOSCH FX, MANOS MM, MUÑOZ N, SHERMAN M, JANSEN AM, PETO J, SCHIFFMAN MH, MORENO V, KURMAN R, SHAH KV. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. **J Natl Cancer Inst**. 87(11):796-802. 1995.

BOULET, G; HORVATH, C; VANDEN BROECK, D; SAHEBALI S; BOGERS, J. Human papillomavirus: E6 and E7 oncogenes. **Int J Biochem Cell Biol**. 39(11):2006-11. 2007.

BOUMBA, LMA; ASSOUMOU, SZ; HILALI, L; MAMBOU, JV; MOUKASSA, D; ENNAJI ,MM. Genetic variability in E6 and E7 oncogenes of human papillomavirus Type 16 from Congolese cervical cancer isolates. **PLoS One.** 9(7): e100746. 2014.

BRAVO, IG; DE SANJOSÉ, S; GOTTSCHLING, M. The clinical importance of understanding the evolution of papillomaviruses. **Trends Microbiol**. 18(10):432-8. 2010.

BRAVO, IG; FÉLEZ-SÁNCHES, M. Papillomaviruses Viral evolution, cancer and evolutionary medicine.**Evol Med Public Health**.(1): 32–51. 2015.

BUCK, CB; CHENG, N; THOMPSON, CD; LOWY, DR; STEVEN, AC; SCHILLER, JT; TRUS, BL. Arrangement of L2 within the Papillomavirus Capsid. **J Virol.** 82: 5190–5197. 2008.

BURK, RD; HARARI, A; CHEN, Z. Human papillomavirus genome variants. **Virology**. 445(1-2):232-43. 2013.

BYG, LM; VIDLUND, J; VASILJEVIC, N; CLAUSEN, D; FORSLUND, O; NORRILD B. NF-κB signalling is attenuated by the E7 protein from cutaneous human papillomaviruses.**Virus Res**. 169(1):48-53. 2012.

CASTRO, MM; FARIAS, IP; BORBOREMA-SANTOS, CM; CORREIA, G; ASTOLFI-FILHO, S. Prevalence of human papillomavirus (HPV) type 16 variants and rare HPV types in the central Amazon region. **Genetics and molecular research: GMR**.10 (1):186–96. 2011.

CENTO, V; RAHMATALLA, N; CICCOZZI, M; LO PRESTI, A; PERNO, CF; CIOTTI, M. Human papillomaviruses 53 and 66: clinical aspects and genetic analysis.**Virus Res.** 163(1):212-22. 2011.

CHAGAS, BS; BATISTA, MV; GUIMARÃES, V; BALBINO, VQ; CROVELLA, S; FREITAS, AC. New variants of E6 and E7 oncogenes of human papillomavirus type 31 identified in Northeastern Brazil. **Gynecol Oncol.** 123(2):284-8. 2011.

CHAGAS, BS; GURGEL, AP; DA CRUZ, HL; AMARAL, CM; CARDOSO, MV; SILVA NETO, JDA C; DA SILVA, LA; DE ALBUQUERQUE, EM; MUNIZ, MT; DE FREITAS, AC. An interleukin-10 gene polymorphism associated with the development of cervical lesions in women infected with Human Papillomavirus and using oral contraceptives. **Infect Genet Evol.** 19:32-7. 2013.

CHAKRABARTI, O; VEERARAGHAVALU, K; TERGAONKAR, V; LIU, Y; ANDROPHY, EJ; STANLEY, MA; KRISHNA, S. Human Papillomavirus Type 16 E6 Amino Acid 83 Variants Enhance E6-Mediated MAPK Signaling and Differentially Regulate Tumorigenesis by Notch Signaling and Oncogenic Ras. **J Virol**, 78 (11): 5934–5945. 2004.

CHAN, PKS; LAM, CW; CHEUNG, TH; LI, WWH; LO, KWK; CHAN, MYM; CHEUNG, JLK; XU, LY; CHENG, A. Human Papillomavirus Type 16 Intratypic Variant Infection and Risk for Cervical Neoplasia in Southern China. **The Journal of Infectious Diseases**. 186:696–700. 2002.

CHATURVEDI, AK; ENGELS, EA; PFEIFFER, RM; HERNANDEZ, BY; XIAO, W; KIM, E; JIANG, B; GOODMAN, MT; SIBUG-SABER, M; COZEN, W; LIU, L; LYNCH, CF; WENTZENSEN, N; JORDAN, RC; ALTEKRUSE, S; ANDERSON, WF; ROSENBERG, PS; GILLISON, ML. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. **J Clin Oncol**. 29: 4294–4230. 2011.

CHATURVEDI, AK; KATKI, HA; HILDESHEIM, A; RODRÍGUEZ, AC; QUINT, W; SCHIFFMAN, M; DOOM, LJV; PORRAS, C; WACHOLDER, S; GONZALEZ, P; SHERMAN, ME; HERRERO, R. Human Papillomavirus Infection with Multiple Types : Pattern of Coinfection and Risk of Cervical Disease. **The Journal of Infectious Diseases**. 203:910–20.2011.

CHEN, LF; FISCHLE, W; VERDIN, E; GREENE, WC. Duration of nuclear NF-kappaB action regulated by reversible acetylation. **Science.** 31;293(5535):1653-7. 2001.

CHEN, LF; GREENE, WC. Shaping the nuclear action of NF-kappaB. **Nat. Rev. Mol. Cell. Biol.** 5, 392–401. 2004.

CHIANG, CM; USTAV, M; STENLUND, A; HO, TF; BROKER, TR; CHOW, LT. Viral E1 and E2 proteins support replication of homologous and heterologous papillomaviral origins. **Proc. Natl. Acad.Sci. U.S.A.** 89:5799 –5803. 1992.

CHIEN, WM; PARKER, JN; SCHMIDT-GRIMMINGER, DC; BROKER, TR; CHOW, LT. Casein kinase II phosphorylation of the human papillomavirus-18 E7 protein is critical for promoting S phase entry. **Cell Growth Diff.** 11: 425–435. 2000.

CHRISTIAN, F; SMITH, EL; CARMODY, RJ. The Regulation of NF-κB Subunits by **Phosphorylation Cells.**18;5(1). 2016.

CHUA, HL; BHAT-NAKSHATRI, P; CLARE, SE; MORIMIYA, A; BADVE, S; NAKSHATRI, H. NF-kappaB represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. **Oncogene** 26, 711–72410.1038. 2007.

CLEVELAND, JL; JUNGER, ML; SARAIYA, M; MARKOWITZ, LE; DUNNE, EF; EPSTEIN, JB. The connection between human papillomavirus and oropharyngeal squamous cell carcinomas in the United States: implications for dentistry. **J Am Dent Assoc.**142(8):915-24. Review. Erratum in: **J. Am. Dent .Assoc.** 142(9):1005-6. 2011.

CLIFFORD, GM; GALLUS, S; HERRERO, R; MUÑOZ, N; SNIJDERS, PJ; VACCARELLA, S; ANH, PT; FERRECCIO, C; ET AL., IARC. HPV Prevalence Surveys Study Group. . Worldwide distribution of human papillomavirus types in cytologically normal women in the International Agency for Research on Cancer HPV prevalence surveys: a pooled analysis. **Lancet.** 366(9490):991-82005.

CLIFFORD, GM; SMITH, JS; AGUADO, T; FRANCESCHI, S. Comparison of HPV type distribution in high-grade cervical lesions and cervical cancer: a meta-analysis. **Br. J. Cancer.** 89(1):101-5. 2003.

COMBITA, AL; TOUZÉ, A; BOUSARGHIN, L; SIZARET, PY; MUÑOZ, N; COURSAGET, P. Gene transfer using human papillomavirus pseudovirions varies according to virus genotype and requires cell surface heparan sulfate. **FEMS Microbiol Lett.** 204(1):183-8. 2001.

CORDEIRO, MN. Imunização genética contra o câncer cervical baseado no oncogene E5 do papilomavírus humano tipo 16. Tese (doutorado) – **Universidade Federal de Pernambuco**. Centro de Ciências Biológicas. Genética. 2015.

CRUZ, MR; CERQUEIRA, DM; CRUZ, WB; CAMARA, GNL; BRÍGIDO, MM; SILVA, EO; CARVALHO, LGS; MARTINS, CRF. Prevalence of Human Papillomavirus Type 16 Variants in the Federal District , Central Brazil. **Mem Inst Oswaldo Cruz**. Rio de Janeiro, Vol. 99(3): 281-282. 2004.

CUBIE, HA. Diseases associated with human papillomavirus infection. **Virology.** 445(1-2):21-34. 2013.

D'COSTA, ZJ; JOLLY, C; ANDROPHY, EJ; MERCER, A; MATTHEWS, CM; HIBMA, MH. Transcriptional repression of E-cadherin by human papillomavirus type 16 E6. **PLoS One.** 7(11):e48954. 2012.

DE SANJOSE, S; ALMIRALL, R; LLOVERAS, B; FONT, R; DIAZ, M; MUÑOZ, N; CATALÀ, I; MEIJER, CJ; SNIJDERS, PJ; HERRERO, R; BOSCH, FX. Cervical human papillomavirus infection in the female population in Barcelona, Spain. Sex **Transm Dis.** 30(10):788-93. 2003.

DE SANJOSÉ, S; DIAZ, M; CASTELLSAGUÉ, X, CLIFFORD, G; BRUNI, L; MUÑOZ, N; BOSCH, FX. Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis. **Lancet. Infect. Dis.** 7(7):453-9. Review. 2007.

DE VILLIERS, EM; FAUQUET, C; BROKER, TR; BERNARD, HU; ZUR HAUSEN, H. Classification of papillomaviruses. **Virology.** 324(1):17-27. Review. 2004.

DE VILLIERS, EM; SANDSTROM, RE; ZUR HAUSEN, H; BUCK, CE. Presence of papillomavirus sequences in condylomatous lesions of the mamillae and in invasive carcinoma of the breast. **Breast Cancer Res.** 7(1):1-11. 2005.

DICKSON, EL; VOGEL, RI; BLISS, RL; DOWNS, LS. Multiple-type HPV infections: a cross-sectional analysis of the prevalence of specific types in 309,000 women referred for HPV testing at the time of cervical cytology. **Int. J. Gynecol. Cancer**. 23(7). 2013.

DICKSON, EL; VOGEL, RI; GELLER, MA; DOWNS, L. Cervical cytology and multiple type HPV infection: A study of 8,182 women ages 31–65. **Gynecol. Oncol.** 133(3): 405–408. 2014.

DIMAIO, D; PETTI, LM. The E5 proteins. Virology 445:99–114. 2013.

DOLLARD, SC; BROKER, TR; CHOW, LT. Regulation of the human papillomavirus type 11 E6 promoter by viral and host transcription factors in primary human keratinocytes. **J. Virol.** 67(3):1721-6. 1993.

DOORBAR, J; ELY, S; STERLING, J; MCLEAN, C; CRAWFORD, L. Specific interaction between HPV 16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. **Nature.** 352, pp. 824–827.1991.

DOORBAR, J. The papillomavirus life cycle. **J. Clin. Virol.**.32(1):S7-15. Review. 2005.

DOORBAR, J. Molecular biology of human papillomavirus infection and cervical cancer. **Clin. Sci.** (Lond). 110(5):525-41. Review. 2006.

DOORBAR, J; QUINT, W; BANKS, L; BRAVO, IG; STOLER, M; BROKER, TR; STANLEY, MA. The biology and life-cycle of human papillomaviruses. **Vaccine.** 30(5):55-70. 2012.

EFIRD, JT; TOLAND, AE; LEA, CS; PHILLIPS, CJ. The combined influence of oral contraceptives and human papillomavirus virus on cutaneous squamous cell carcinoma. **Clin. Med. Insights. Oncol.** 5:55-75. 2011.

ELLIS JRM, KEATING PJ, BAIRD J, HOUNSELL EF, RENOUF DV, ROWE M, HOPKINS D, KEEN MFD, BARTHOLOMEW JS, YOUNG LS, STERN PL 1995. The association of an HPV16 oncogene variant with HLA-B7 has implications for vaccine design in cervical cancer. **Nature Medicine** 1, 464 - 470

ESCHLE D, DIIRST M, LUANDE J, EBERHARDT C, I MP, GISSMANN L (1992) Geographical dependence of sequence variation in the E7 gene of human papillomavirus type 16. **Journal of General Virology**. 73, 1829-1832

EVANDER M, FRAZER IH, PAYNE E, QI YM, HENGST K, MCMILLAN NA.(1997) Identification of the alpha6 integrin as a candidate receptor for papillomaviruses. **J. Virol.** 71(3):2449-56.

FEHRMANN F, LAIMINS LA (2003) Human papillomaviruses: targeting differentiating epithelial cells for malignant transformation. **Oncogene.** 22(33):5201-7. Review

FERENCZI, A; GYÖNGYÖSI, E; SZALMÁS, A; LÁSZLÓ, B; KÓNYA, J; VERESS, G. Phylogenetic and functional analysis of sequence variation of human papillomavirus type 31 E6 and E7 oncoproteins.**Infect. Genet. Evol.** 43:94-100. 2016.

FERNANDES, JV; MEISSNER, RV; CARVALHO, MGF; FERNANDES, TAAM; AZEVEDO, PRM; SOBRINHO, JS; PRADOS, JCM; VILLAS, L. Prevalence of human papillomavirus in archival samples obtained from patients with cervical pre-malignant and malignant lesions from Northeast Brazil. **BMC Research Notes** 3:96. 2010.

FERNANDES, JV; MEISSNER, RDE V; CARVALHO, MG; FERNANDES, TA; AZEVEDO, PR; DE AZEVEDO, JW; DE ARAÚJO, JM. Human papillomavirus prevalence in women with normal cytology and with cervical cancer in Natal, Brazil. **Mol. Med. Rep.** 4: 1321–1326. 2011.

FILIPPOVA, M; SONG, H; CONNOLLY, JL; DERMODY, TS; DUERKSEN-HUGHES, PJ. The human papillomavirus 16 E6 protein binds to tumor necrosis factor (TNF) R1 and protects cells from TNF-induced apoptosis.**J. Biol. Chem.** 277(24):21730-9. 2002.

FREITAS, AC; GURGEL, AP; CHAGAS, BS; COIMBRA, EC; DO AMARAL, CM. Susceptibility to cervical cancer: an overview. **Gynecol. Oncol.** 126(2):304-11. 2012.

FUJINAGA, Y; OKAZAWA, K; NISHIKAWA, A; YAMAKAWA, Y; FUKUSHIMA, M; KATO, I; FUJINAGA, K. Sequence variation of human papillomavirus type 16 E7 in preinvasive and invasive cervical neoplasias. **Virus Genes.** 9(1):85-92. 1994.

GHANEM, KG; DATTA, SD; UNGER, ER; HAGENSEE, M; SHLAY, JC; KERNDT, P; HSU, K; KOUTSKY, LA. The association of current hormonal contraceptive use with type-specific HPV detection. **Sex Transm Infect.** 87(5):385-8. 2011

GERONDAKIS, S; GRUMONT, R; GUGASYAN, R; WONG, L; ISOMURA, I; HO W; BANERJEE, A. Unravelling the complexities of the NF-κB signalling pathway using mouse knockout and transgenic models. **Front. Oncol.** 3: 120. 2013.

GIROGLOU, T; FLORIN, L; SCHÄFER, F; STREECK, RE; SAPP, M. Human papillomavirus infection requires cell surface heparan sulfate. **J. Virol.** 75(3):1565-70. 2001.

GLAUNSINGER, BA; LEE, SS; THOMAS, M; BANKS, L; JAVIER, R. Interactions of the PDZ-protein MAGI-1 with adenovirus E4-ORF1 and high-risk papillomavirus E6 oncoproteins. **Oncogene.** 19(46):5270-80. 2000.

GODWIN, P; BAIRD, AM; HEAVEY, S; BARR, MP; O'BYRNE, KJ; GATELY, K. Targeting Nuclear Factor-Kappa B to Overcome Resistance to Chemotherapy **Front. Oncol.** 3:120. 2013.

GRAHAN, SV. Human papillomavirus: gene expression, regulation and prospects for novel diagnostic methods and antiviral therapies. **Future Microbiol.** 5(10): 1493–1506. 2010

GRASSMANN, K; RAPP, B; MASCHEK, H; PETRY, KU. Identification of a Differentiation-Inducible Promoter in the E7 Open Reading Frame of Human Papillomavirus Type 16 (HPV-16) in Raft Cultures of a New Cell Line Containing High Copy Numbers of Episomal HPV-16 DNA. **J. of Virology**, p. 2339–2349. 1996.

GRODZKI, M; BESSON, G; CLAVEL, C; ARSLAN, A; FRANCESCHI, S; BIREMBAUT, P; TOMMASINO, M; ZEHBE, I. Increased risk for cervical disease progression of French women infected with the human papillomavirus type 16 E6-350G variant. **Cancer Epidemiol Biomarkers Prev.** 15(4):820-2. 2006.

GUPTA, S; TAKHAR, PP; DEGENKOLBE, R; KOH, CH; ZIMMERMANN, H; YANG, CM; GUAN SIM, K; HSU, SI; BERNARD, HU. The human papillomavirus type 11 and 16 E6 proteins modulate the cell-cycle regulator and transcription cofactor TRIP-Br1. **Virology.** 317(1):155-64. 2003

GUPTA, SC; SUNDARAM, C; REUTER, S; AGGARWAL, BB. Inhibiting NF-κB activation by small molecules as a therapeutic strategy. **Biochim Biophys Acta.** 1799(10-12):775-87. 2010.

GURGEL, AP; CHAGAS, BS; DO AMARAL, CM; ALBUQUERQUE, EM; SERRA, IG; SILVA NETO, JDA C; MUNIZ, MT; DE FREITAS AC. Prevalence and genetic variability in capsid L1 gene of rare human papillomaviruses (HPV) found in cervical lesions of women from North-East Brazil. **Biomed. Res. Int.** 2013:546354. 2013

GURGEL, AP; CHAGAS, BS; DO AMARAL, CM; NASCIMENTO, KC; LEAL, LR; SILVA NETO, J DA C; CARTAXO MUNIZ, MT; DE FREITAS, AC. Prevalence of human papillomavirus variants and genetic diversity in the L1 gene and long control region of HPV16, HPV31, and HPV58 found in North-East Brazil. **Biomed. Res. Int**. 130828. 2015.

HARMSEL, B; SMEDTS, F; KUIJPERS, J; VAN MUYDEN, R; OOSTERHUIS, W; QUINT, W. Relationship between human papillomavirus type 16 in the cervix and intraepithelial neoplasia. **Obstet Gynecol.** 93(1):46-50.1999.

HARWOOD, CA; PROBY, CM. Human papillomaviruses and non-melanoma skin cancer. **Curr. Opin. Infect.** Dis.15(2):101-14. Review. 2002

HAVARD, L; RAHMOUNI, S; BONIVER, J; DELVENNE, P. High levels of p105 (NFKB1) and p100 (NFKB2) proteins in HPV16-transformed keratinocytes: role of E6 and E7 oncoproteins. **Virology.** 331(2):357-66. 2005

HAYDEN, MS; GHOSH, S. Shared principles in NF-kappaB signaling. **Cell**. 132, 344–362. 2008.

HAYDEN, MS; GHOSH, S. NF-κB, the first quarter-century: remarkable progress and outstanding questions.**Genes Dev.** 26(3):203-34. doi: 10.1101/gad.183434.111. 2012

HILDESHEIM, A; HERRERO, R; CASTLE, PE; WACHOLDER, S; BRATTI, MC; SHERMAN, ME; LORINCZ, AT; BURK, RD; MORALES, J; RODRIGUEZ, AC; HELGESEN, K; ALFARO, M; HUTCHINSON, M; BALMACEDA, I; GREENBERG, M; SCHIFFMAN, M. HPV co-factors related to the development of cervical cancer: results from a population-based study in Costa Rica. **Br. J. Cancer.** 84(9):1219-26. 2001.

HOORY, T; MONIE, A; GRAVITT, P; WU, TC. Molecular epidemiology of human papillomavirus. **J. Formos Med. Assoc.** 107(3):198-217. 2008.

HOPMAN, EH; ROZENDAAL, L; VOORHORST, FJ; WALBOOMERS, JM; KENEMANS, P; HELMERHORST, TJ. High risk human papillomavirus in women with normal cervical cytology prior to the development of abnormal cytology and colposcopy. **BJOG.** 107(5):600-4. 2000.

HUBER, MA; BEUG, H; WIRTH, T. Epithelial-mesenchymal transition: NF-kappaB takes center stage. **Cell Cycle.** 3(12):1477-80. 2004.

HUSSAIN, I; FATHALLAH, I; ACCARDI, R; YUE, J; SAIDJ, D; SHUKLA, R; HASAN, U; GHEIT, T; NIU, Y; TOMMASINO, M; SYLLA, BS. NF-kappaB protects

human papillomavirus type 38 E6/E7-immortalized human keratinocytes against tumor necrosis factor alpha and UV-mediated apoptosis. **J Virol.** 85(17):9013-22. (2011).

ICENOGLE JP, SATHYA P, MILLER DL, TUCKER RA, RAWLS WE Nucleotide and amino acid sequence variation in the L1 and E7 open reading frames of human papillomavirus type 6 and type. Virology 184(1):101-7. (1991)

INCA. Estimativa 2016: Incidência de Câncer no Brasil. **Ministério da Saúde, Instituto Nacional do Câncer**. Rio de Janeiro. Brasil. 2016. Disponível em http://www.inca.gov.br/estimativa/2016.

JAMES, MA; LEE, JH; KLINGELHUTZ, AJ. HPV16-E6 associated hTERT promoter acetylation is E6AP dependent, increased in later passage cells and enhanced by loss of p300. **Int. J. Cancer.** 119(8):1878-85. 2006.

JEON, S; ALLEN-HOFFMANN, BL; LAMBERT, PF. Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. **J. Virol.** 69(5):2989-97. 1995

JOHNSON, KM; KINES, RC; ROBERTS, JN; LOWY, DR; SCHILLER, JT; DAY, PM. Role of heparan sulfate in attachment to and infection of the murine female genital tract by human papillomavirus. **J. Virol.** 83(5):2067-74. 2009.

KALANTARI, M; CHASE, DM; TEWARI, KS; BERNARD, HU. Recombination of human papillomavirus-16 and host DNA in exfoliated cervical cells: a pilot study of L1 gene methylation and chromosomal integration as biomarkers of carcinogenic progression. **J. Med. Virol.** 82(2):311-20. 2010.

KÄMMER, C; TOMMASINO, M; SYRJÄNEN, S; DELIUS, H; HEBLING, U; WARTHORST, U; PFISTER, H; ZEHBE, I. Variants of the long control region and the E6 oncogene in European human papillomavirus type 16 isolates: implications for cervical disease. **Br. J. Cancer.** 86(2):269-73. 2002

KARIN, M; CAO, Y; GRETEN, FR; LI, ZW. NF-kappaB in cancer: from innocent bystander to major culprit. **Nat. Rev. Cancer.** 2002 Apr;2(4):301-10. 2002.

KARIN, M; DELHASE, M. The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. **Semin. Immunol.** 12(1):85-98. 2000.

KARIN, M; LIN, A. NF-kappaB at the crossroads of life and death.**Nat. Immunol.** 3(3):221-7. 2002.

KINES, RC; THOMPSON, CD; LOWY, DR; SCHILLER, JT; DAY, PM. The initial steps leading to papillomavirus infection occur on the basement membrane prior to cell surface binding. **Proc. Natl. Acad. Sci U S A**. 106(48):20458-63. 2009.

LICHTIG, H; ALGRISI, M; BOTZER, LE; ABADI, T; VERBITZKY, Y; JACKMAN, A; TOMMASINO, M; ZEHBE, I; SHERMAN, L. HPV16 E6 natural variants exhibit

different activities in functional assays relevant to the carcinogenic potential of E6. **Virology.** 350(1):216-27. 2006.

LICITRA, L; PERRONE, F; BOSSI, P; SUARDI, S; MARIANI, L; ARTUSI, R; OGGIONNI, M; ROSSINI, C; CANTÙ, G; SQUADRELLI ,M; QUATTRONE, P; LOCATI, LD; BERGAMINI, C; OLMI, P; PIEROTTI, MA; PILOTTI, S. High-risk human papillomavirus affects prognosis in patients with surgically treated oropharyngeal squamous cell carcinoma. **J. Clin. Oncol.** 24(36):5630-6. 2006.

LI, J; MEI, J; WANG, X; HU, L; LIN, Y; YANG, P. Human papillomavirus type-specific prevalence in women with cervical intraepithelial neoplasm in Western China. **J Clin Microbiol.** 50(3):1079-81. 2012.

LI, Q; VERMA, IM. NF-kB regulation in the immune system. **Nat Rev Immunol.** 2: 331-367. 2002.

LORENZATO, F; HO, L; TERRY, G; SINGER, A; SANTOS, LC; DE LUCENA BATISTA, R; LUBAMBO, T. The use of human papillomavirus typing in detection of cervical neoplasia in Recife (Brazil). **Int. J. Gynecol. Cancer.** 10(2):143-150. 2000.

LOWY, DR; SCHILLER, JT. Reducing HPV-associated cancer globally.**Cancer Prev Res (Phila)**. 5(1):18-23. 2012.

MACLAUGHLAN, SD; LACHANCE, JA; GJELSVIK, A. Correlation between smoking status and cervical cancer screening: a cross-sectional study.**J Low Genit Tract Dis.** 15(2):114-9. 2011.

MACIAG, PC; SCHLECHT, NF; SOUZA, PS; ROHAN, TE; FRANCO, EL; VILLA, LL. Polymorphisms of the human leukocyte antigen DRB1 and DQB1 genes and the natural history of human papillomavirus infection. **J. Infect. Dis.** 186(2):164-72. 2002.

MARTINS, TR; MENDES DE OLIVEIRA, C; ROSA, LR; DE CAMPOS CENTRONE, C; RODRIGUES, CL; VILLA, LL; LEVI, JE. HPV genotype distribution in Brazilian women with and without cervical lesions: correlation to cytological data. **Virol J.** 12;13:138. 2016

MCCANCE, DJ. Transcriptional regulation by human papillomaviruses. **Curr Opin Genet Dev.** 15(5):515-9. Review. 2005

MODIS, Y; TRUS, BL; HARRISON, SC. Atomic model of the papillomavirus capsid. **The EMBO Journal.** 21 (18): 4754-4762. 2002

MOKTAR A, SINGH R, VADHANAM MV, RAVOORI S, LILLARD JW, GAIROLA CG, GUPTA RC Cigarette smoke condensate-induced oxidative DNA damage and its removal in human cervical cancer cells. **Int J Oncol.** 39(4):941-7. 2011.

MOODY, CA; LAIMINS, LA. Human papillomavirus oncoproteins: pathways to transformation. **Nat. Rev. Cancer.** 10(8):550-60. 2010.

MOTOYAMA, S; LADINES-LLAVE, CA; LUIS VILLANUEVA, S; MARUO, T. The role of human papilloma virus in the molecular biology of cervical carcinogenesis. **Kobe J. Med. Sci**. 50(1-2):9-19. Review. 2004.

MUÑOZ, N; BOSCH, FX; DE SANJOSÉ, S; HERRERO, R; CASTELLSAGUÉ, X; SHAH, KV; SNIJDERS, PJ; MEIJER, CJ. International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. Epidemiologic classification of human papillomavirus types associated with cervical cancer. **N. Engl. J. Med.** 348(6):518-27. 2003.

MUÑOZ, N. New perspective for cervical cancer prevention based on human papillomavirus. **Biomedica.** 26(4):471-4. 2006

NEES, M; GEOGHEGAN, JM; HYMAN, T; FRANK, S; MILLER, L; WOODWORTH, CD. Papillomavirus type 16 oncogenes downregulate expression of interferon-responsive genes and upregulate proliferation-associated and NF-kappaB-responsive genes in cervical keratinocytes. **J Virol.** 75(9):4283-96. 2001.

PANDE, S; JAIN, N; PRUSTY, BK; BHAMBHANI, S; GUPTA, S; SHARMA, R; BATRA, S; DAS, BC. Human papillomavirus type 16 variant analysis of E6, E7, and L1 genes and long control region in biopsy samples from cervical cancer patients in north India. **J. Clin. Microbiol.** 46(3):1060-6. 2008.

PARK, MH; HONG, JT. Roles of NF-κB in Cancer and Inflammatory Diseases and Their Therapeutic Approaches. **Cells.** 29;5(2). 2016.

PASPARAKIS, M. Regulation of tissue homeostasis by NF-kappaB signalling: implications for inflammatory diseases. **Nat Rev Immunol.** 9: 778–788. 2009.

PEDROZA-SAAVEDRA, A; LAM, EW; ESQUIVEL-GUADARRAMA, F; GUTIERREZ-XICOTENCATL, L. The human papillomavirus type 16 E5 oncoprotein synergizes with EGF-receptor signaling to enhance cell cycle progression and the down-regulation of p27(Kip1).**Virology.** 400(1):44-52. 2010.

PEIRSON, L; FITZPATRICK-LEWIS, D; CILISKA, D; WARREN, R. Screening for cervical cancer: a systematic review and meta-analysis. **Syst. Rev.** 2:35. 2013.

PERKINS, ND. Integrating cell-signalling pathways with NF-kappaB and IKK function. **Nat Rev Mol Cell Biol**. 8: 49–62. 2007.

PFISTER, H; FUCHS, PG; MAJEWSKI, S; JABLONSKA, S; PNIEWSKA, I; MALEJCZYK, M. High prevalence of epidermodysplasia verruciformis-associated human papillomavirus DNA in actinic keratoses of the immunocompetent population. **Arch. Dermatol. Res.** 295(7):273-9. 2003.

PFISTER, H. Chapter 8: Human papillomavirus and skin cancer. **J. Natl. Cancer Inst. Monographs** (31):52-56. 2003.

PINTO, VM; GOLUB, JE; TANCREDI, MV; ALENCAR, RS; MIRANDA, AE. Cervical cytology and histopathologic abnormalities in women living with AIDS in São Paulo, Brazil. **J Acquir Immune Defic Syndr.** 57(3):212-6. 2011.

QMICHOU, Z; ENNAJI, MM; AMRANI, M; MOSTAFA, E; FAHIME, E; MELLOUL, M; KHAIR, MM; MZIBRI, ME; KHYATTI, M; ATEALEB, M. Molecular Characterization of HPV16 E6 and E7 Variants among Women with Cervical Cancer in Morocco. **British Microbiology Research Journal** .3(4): 692-705. 2013.

RABELO-SANTOS, SH; VILLA, LL; DERCHAIN, SF; FERREIRA, S; SARIAN, LO; ANGELO-ANDRADE; LA; DO AMARAL, WESTIN, MC; ZEFERINO, LC. Variants of human papillomavirus types 16 and 18: histological findings in women referred for atypical glandular cells or adenocarcinoma in situ in cervical smear. **Int J Gynecol Pathol.** 25(4):393-7.2006.

ROMANA, A; MUNGER, K. The papillomavirus E7 proteins. **Virology**. 445:138–168. 2013

SANDAL, T. Molecular aspects of the mammalian cell cycle and cancer. **Oncologist.** 7(1):73-81. 2002.

SAPP, M; DAY, PM. Structure, attachment and entry of polyoma- and papillomaviruses. **Virology.** 384(2):400-9. 2009.

SAAVEDRA, KP; BREBI, PM; ROA, JC. Epigenetic alterations in preneoplastic and neoplastic lesions of the cervix. **Clin Epigenetics.** 4(1):13. 2012.

SHANG, Q; WANG, Y; FANG, Y; WEI, L; CHEN, S; SUN, Y; LI, B; ZHANG, F; GUI H. Human Papillomavirus Type 16 Variant Analysis of E6 , E7 , and L1 Genes and Long Control Region in Identification of Cervical Carcinomas in Patients in Northeast China. **J. of Clinical. Microbiology**. p. 2656–2663. 2011.

SCHEURER, ME; GUILLAUD, M, TORTOLERO-LUNA, G; FOLLEN, M; ADLER-STORTHZ, K. Epidemiologic modeling of cervical dysplasia with molecular and cytopathological markers. **Gynecol Oncol.**

SCHEFFNER, M; HUIBREGTSE, JM; VIERSTRA, RD; HOWLEY, PM. The HPV-16 E6 and E6-AP Complex Functions as a Ubiquitin-Protein Ligase in the Ubiquitination of p53. **Cell.** 75: 495–505. 1993.

SCHEURER, ME; TORTOLERO-LUNA, G; ADLER-STORTHZ, K. Human papillomavirus infection: biology, epidemiology, and prevention. **Int J Gynecol Cancer.** 15(5):727-46. Review. 2005.

SCHIFFMAN, M; RODRIGUEZ, AC; CHEN, Z; WACHOLDER, S; HERRERO, R; HILDESHEIM, A; DESALLE, R; BEFANO, B; YU, K; SAFAEIAN, M; SHERMAN, ME; MORALES, J; GUILLEN, D; ALFARO, M; HUTCHINSON, M; SOLOMON, D; CASTLE, PE; BURK, RD. A population-based prospective study of carcinogenic

human papillomavirus variant lineages, viral persistence, and cervical neoplasia. **Cancer Res.** 70(8):3159-69. 2010.

SCHWARZ, E; FREESE, UK; GISSMANN, L; MAYER, W; ROGGENBUCK, B; STREMLAU, A; ZUR HAUSEN, H. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. **Nature**. 314:111–4.1985.

SHIN, MK; BALSITIS, S; BRAKE, T; LAMBERT, PF. Human papillomavirus E7 oncoprotein overrides the tumor suppressor activity of p21Cip1 in cervical carcinogenesis. **Cancer Res.** 69(14):5656-63. 2009

SICHERO, L; FERREIRA, S; TROTTIER, H; DUARTE-FRANCO, E; FERENCZY, A; FRANCO, EL; VILLA, LL. High grade cervical lesions are caused preferentially by non-European variants of HPVs 16 and 18.**Int J Cancer.** 120(8):1763-8. 2007.

SIMMONDS, M; STOREY, A. Identification of the regions of the HPV 5 E6 protein involved in Bak degradation and inhibition of apoptosis. **Int J Cancer.** 123(10):2260-6. 2008.

SNIJDERS, PJ; VAN DEN BRULE, AJ; JACOBS, MV; POL, RP; MEIJER, CJ. HPV DNA detection and typing in cervical scrapes. **Methods Mol Med.** 119:101-14. 2005.

SOLAN, NJ; MIYOSHI, H; CARMONA, EM; BREN, GD; PAYA, CV. RelB cellular regulation and transcriptional activity are regulated by p100. **J Biol Chem.** 277:1405–1418. 2002.

SONG, YS; KEE, SH; KIM, JW; PARK, NH; KANG, SB; CHANG, WH; LEE, HP. Major sequence variants in E7 gene of human papillomavirus type 16 from cervical cancerous and noncancerous lesions of Korean women. **Gynecol. Oncol.** 66(2):275-81.1997.

SPITKOVSKY, D; HEHNER, SP; HOFMANN, TG; MOLLER, A; SCHMITZ, ML. The human papillomavirus oncoprotein E7 attenuates NF-kappa B activation by targeting the Ikappa B kinase complex. **Biol Chem**. 277: 25576–25582. 2002.

STANLEY, MA. Human papillomavirus and cervical carcinogenesis. **Best Pract Res Clin Obstet Gynaecol.** 15(5):663-76. Review. 2001.

STEENBERGEN, RD; DE WILDE, J; WILTING, SM; BRINK, AA; SNIJDERS, PJ; MEIJER, CJ. HPV-mediated transformation of the anogenital tract. **J. Clin. Virol.** 32(1): 25-33. 2005.

STEVENS, LM. JAMA patient page. Papillomavirus. JAMA. 287(18):2452. Stubenrauch F, Laimins LA Human papillomavirus life cycle: active and latent phases. **Semin Cancer Biol**.9(6):379-86. 1999.

SURVILADZE, Z; STERK, RT; DEHARO, SA; OZBUN, MA. Cellular entry of human papillomavirus type 16 involves activation of the phosphatidylinositol 3-

kinase/Akt/mTOR pathway and inhibition of autophagy. **J Virol.** 87(5):2508-17. 2013

TAN, SH; BARTSCH, D; SCHWARZ, E; BERNARD, HU. Nuclear matrix attachment regions of human papillomavirus type 16 point toward conservation of these genomic elements in all genital papillomaviruses. **J. Virol**. 72(5):3610-22. 1998.

TEW, GW; LORIMER, EL; BERG, TJ; ZHI, H; LI, R; WILLIAMS, CL. SmgGDS regulates cell proliferation, migration, and NF-kappaB transcriptional activity in non-small cell lung carcinoma. **J. Biol. Chem.** 283, 963–97610.1074. 2008.

THOMAS, M; NARAYAN, N; PIM, D; TOMAIĆ, V; MASSIMI, P; NAGASAKA, K; KRANJEC, C; GAMMOH, N; BANKS, L. Human papillomaviruses, cervical cancer and cell polarity. **Oncogene.** 27(55):7018-30. 2008.

TRIMBLE, CL; PIANTADOSI, S; GRAVITT, P; RONNETT, B; PIZER, E; ELKO, A; WILGUS, B; YUTZY, W; DANIEL, R; SHAH, K; PENG, S; HUNG, C; RODEN, R; WU, TC; PARDOLL, D. Spontaneous regression of high-grade cervical dysplasia: effects of human papillomavirus type and HLA phenotype. **Clin. Cancer Res.** 11(13):4717-23. 2005.

VALLABHAPURAPU, S; KARIN, M. Regulation and function of NF-kappaB transcription factors in the immune system. **Annu Rev Immunol.** 27:693-733. **2009.**

VANDEPOL, SB; KLINGELHUTZ, AJ. Papillomavirus E6 oncoproteins. **Virology** 445:115–137. 2013.

VANDERMARK, ER; DELUCA, KA; GARDNER, CR; MARKER, DF; SCHREINER, CN; STRICKLAND, DA; WILTON, KM; MONDAL, S; WOODWORTH, CD. Human papillomavirus type 16 E6 and E 7 proteins alter NF-kB in cultured cervical epithelial cells and inhibition of NF-kB promotes cell growth and immortalization. **Virology.** 30;425(1):53-60. 2012.

VAN DOORSLAER, K; BERNARD, HU; CHEN, Z; DE VILLIERS, EM; ZUR HAUSEN, H; BURK, RD. Papillomaviruses: evolution, Linnaean taxonomy and current nomenclature. **Trends Microbiol.** 19(2):49-50. 2011.

VAN MUYDEN, RC; TER HARMSEL, BW; SMEDTS, FM; HERMANS, J; KUIJPERS, JC; RAIKHLIN, NT; PETROV, S; LEBEDEV, A; RAMAEKERS, FC; TRIMBOS, JB; KLETER, B; QUINT, WG. Detection and typing of human papillomavirus in cervical carcinomas in Russian women: a prognostic study. **Cancer.** 85(9):2011-6. 1999.

VILLA, LL; SICHERO, L; RAHAL, P; CABALLERO, O; FERENCZY, A; ROHAN, T; FRANCO, EL. Molecular variants of human papillomavirus types 16 and 18 preferentially associated with cervical neoplasia. **J. Gen Virol.** 81(12):2959-68. 2000.

VILIMAS, T; MASCARENHAS, J; PALOMERO, T; MANDAL, M; BUONAMICI, S; MENG, F. et al. Targeting the NF-kappaB signaling pathway in Notch1-induced T-cell leukemia. **Nat. Med.** 13, 70–7710.1038/nm1524. 2007.

VOGT, M; BUTZ, K; DYMALLA, S; SEMZOW, J; HOPPE-SEYLER, F. Inhibition of Bax activity is crucial for the antiapoptotic function of the human papillomavirus E6 oncoprotein. **Oncogene.** 25(29):4009-15. 2006.

VOUSDEN, K. Interactions of human papillomavirus transforming proteins with the products of tumor suppressor genes. **FASEB J.** 7(10):872-9. Review. 1993.

WALBOOMERS, JM; JACOBS, MV; MANOS, MM; BOSCH, FX; KUMMER, JA; SHAH, KV; SNIJDERS, PJ; PETO, J; MEIJER, CJ; MUÑOZ, N. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide.**J Pathol.** 189(1):12-9. 1999.

WARD, KK; BERENSON, AB; BREITKOPF, CR. Passive smoke exposure and abnormal cervical cytology in a predominantly Hispanic population. **Am J Obstet Gynecol.** 204(3):213.e1-6. 2011.

WHO/ICO Information Center on HPV and Cervical Cancer (HPV Information Centre) Human papillomavirus and related cancers in Brazil. 2015.

WISE-DRAPER, TM; WELLS, SI. Papillomavirus E6 and E7 proteins and their cellular targets. **Front Biosci.** 13:1003-17. 2008.

WOODMAN, CB; COLLINS, SI; YOUNG, LS. The natural history of cervical HPV infection: unresolved issues. **Nat Rev Cancer**. 7(1):11-22. 2007.

WU, HH; WU, JY; CHENG, YW; CHEN, CY; LEE, MC; GOAN, YG; LEE, H. cIAP2 upregulated by E6 oncoprotein via epidermal growth factor receptor/phosphatidylinositol 3-kinase/AKT pathway confers resistance to cisplatin in human papillomavirus 16/18-infected lung cancer. **Clin Cancer Res.** 16(21):5200-10. 2010.

WU, Y; CHEN, Y; LI, L; YU, G; HE, Y. Analysis of mutations in the E6 / E7 oncogenes and L1 gene of human papillomavirus 16 cervical cancer isolates from China. **Journal of General Virology.**, 87, 1181–1188. 2016.

XI, LF; SCHIFFMAN, M; KOUTSKY, LA; HE, Z; WINER, RL; HULBERT, A; LEE, SK; KE, Y; KIVIAT, NB. Persistence of newly detected human papillomavirus type 31 infection, stratified by variant lineage. **Int. J. Cancer.** 132(3):549-55. 2012.

XU, M; KATZENELLENBOGEN, RA; GRANDORI, C; GALLOWAY, DA. NFX1 plays a role in human papillomavirus type 16 E6 activation of NFkappaB activity. **J Virol.** 84(21):11461-9. 2010.

YUAN, H; FU, F; ZHUO, J; WANG, W; NISHITANI, J; AN, DS; CHEN, IS; LIU, X. Human papillomavirus type 16 E6 and E7 oncoproteins upregulate c-IAP2 gene expression and confer resistance to apoptosis. **Oncogene.** 24(32):5069-78. 2005.

ZEHBE, I; TACHEZY, R; MYTILINEOS, J; VOGLINO, G; MIKYSKOVA, I; DELIUS, H; MARONGIU, A; GISSMANN, L; WILANDER, E; TOMMASINO, M. Human papillomavirus 16 E6 polymorphisms in cervical lesions from different European populations and their correlation with human leukocyte antigen class II haplotypes. **Int. J. Cancer.** 94(5):711-6. 2001.

ZEHBE, I; VOGLINO, G; DELIUS, H; WILANDER, E; TOMMASINO, M. Risk of cervical cancer and geographical variations of human papillomavirus 16 E6 polymorphisms. **Lancet.** 352(9138):1441-2. 1998.

ZEHBE, I; WILANDER, E; DELIUS, H; TOMMASINO, M. Human Papillomavirus 16 E6 Variants Are More Prevalent in Invasive Cervical Carcinoma than the Prototype1. **Cancer research**. 58. 829-833. 1998.

ZHENG, ZM; BAKER, CC. Papillomavirus Genome structure, expression, and post-transcriptional regulation. **Front Biosci.** 11: 2286–2302. 2006.

ZIELINSKI, GD; BAIS, AG; HELMERHORST, TJ; VERHEIJEN, RH; DE SCHIPPER, FA; SNIJDERS, PJ; VOORHORST, FJ; VAN KEMENADE, FJ; ROZENDAAL, L; MEIJER, CJ. HPV testing and monitoring of women after treatment of CIN 3: review of the literature and meta-analysis. **Obstet Gynecol Surv.** 59(7):543-53. 2004.

ZOU, P; KAWADA, J; PESNICAK, L; COHEN, JI. Bortezomib induces apoptosis of Epstein-Barr virus (EBV)-transformed B cells and prolongs survival of mice inoculated with EBV-transformed B cells. **J. Virol.** 81, 10029–1003610.1128/JVI.02241-06 .2007.

ZUNA, RE; MOORE, WE; SHANESMITH, R; DUNN, ST; WANG, SS; SCHFFMAN, M; BLAKEY, G; TEEL, T. Association of HPV16 E6 variants with diagnostic severity in cervical cytology samples of 354 women in a US population. **Int. J. Cancer.** 125(11): 2609–2613. 2009

ZUR HAUSEN, H. Papillomavirus infections--a major cause of human cancers. **Biochim. Biophys Acta**. 1288(2):55-78. Review. 1996.

ZUR HAUSEN, H. Papillomaviruses and cancer: from basic studies to clinical application. **Nat Rev Cancer.** 2(5):342-50. Review. 2002.

ANEXOS

ANEXO 1

Artigos publicados em colaboração

Amaral et al. Infectious Agents and Cancer 2014, 9:24
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INFECTIOUS AGENTS AND CANCER

RESEARCH ARTICLE Open Access

MDM2 polymorphism associated with the development of cervical lesions in women infected with Human papillomavirus and using of oral contraceptives

Carolina MM Amaral[1], Katerina Cetkovská[2], Ana PAD Gurgel[1], Marcus V Cardoso[3], Bárbara S Chagas[1], Sérgio SL Paiva Júnior[3], Rita de Cássia Pereira de Lima[1], Jacinto C Silva-Neto[4], Luiz AF Silva[5], Maria TC Muniz[6], Valdir Q Balbino[3] and Antonio C Freitas[1*]

Abstract

Background: The *MDM2* gene is the major negative regulator of p53, a tumor suppressor protein. Single nucleotide polymorphism in promoter region of *MDM2* gene leads to increased expression resulting in higher levels of MDM2 protein. This event increases the attenuation of the p53 pathway. Polymorphisms in this gene can interfere in the regulation of cellular proliferation. We evaluated whether *MDM2* SNP309 (rs2278744) associated or not with the use of oral contraceptive can heighten susceptibility to development of cervical lesions in women HPV infected.

Methods: *MDM2* SNP309 (rs2278744) was genotyped in a total of 287 patients using the PCR-RFLP technique. The results were analyzed by UNPHASED v.3.121 and SNPStats programs.

Results: The three groups (SIL, LSIL and HSIL) showed no significant differences in either genotype or allelic frequencies for *MDM2* polymorphisms, except when HSIL was compared with LSIL ($p = 0.037$; OR = 1.81). Furthermore, in the analysis of contraceptives, a significant association was found between the use of contraceptives and the *MDM2* variant in the development of high-grade cervical lesions for the TG genotype ($p = 0.019$; OR = 2.21) when HSIL was compared with control. When HSIL was compared with LSIL ($p = 0.006$; OR = 2.27).

Conclusion: The results of this study suggest that *MDM2* SNP309 might be a good marker for assessing the progression of LSIL to HSIL. In addition, they also show that oral contraceptives alone, did not have any effect on the progression or development of cervical lesions. However, they may act synergistically with *MDM2* SNP309 (rs2278744) and HPV infection in the development of cervical lesions.

Keywords: *MDM2*, HPV, Oral contraceptives, Cervical cancer

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PLOS ONE

Quantifying mRNA and MicroRNA with qPCR in Cervical Carcinogenesis: A Validation of Reference Genes to Ensure Accurate Data

Maria da Conceição Gomes Leitão[1], Eliane Campos Coimbra[1], Rita de Cássia Pereira de Lima[1], Mariléa de Lima Guimarães[2], Sandra de Andrade Heráclio[3], Jacinto da Costa Silva Neto[4], Antonio Carlos de Freitas[1]*

1 Laboratory of Molecular Studies and Experimental Therapy (LEMTE), Department of Genetics, Center for Biological Sciences, Federal University of Pernambuco, Pernambuco, Brazil, 2 Clinical Hospital of Federal University of Pernambuco, Pernambuco, Brazil, 3 Institute of Integral Medicine Prof. Fernando Figueira, Pernambuco, Brazil, 4 Molecular and Cytological Research Laboratory, Department of Histology, Federal University of Pernambuco, Pernambuco, Brazil

Abstract

A number of recent studies have catalogued global gene expression patterns in a panel of normal, tumoral cervical tissues so that potential biomarkers can be identified. The qPCR has been one of the most widely used technologies for detecting these potential biomarkers. However, few studies have investigated a correct strategy for the normalization of data in qPCR assays for cervical tissues. The aim of this study was to validate reference genes in cervical tissues to ensure accurate quantification of mRNA and miRNA levels in cervical carcinogenesis. For this purpose, some issues for obtaining reliable qPCR data were evaluated such as the following: geNorm analysis with a set of samples which meet all of the cervical tissue conditions (Normal + CIN1 + CIN2 + CIN3 + Cancer); the use of individual Ct values versus pooled Ct values; and the use of a single (or multiple) reference genes to quantify mRNA and miRNA expression levels. Two different data sets were put on the geNorm to assess the expression stability of the candidate reference genes: the first dataset comprised the quantities of the individual Ct values; and the second dataset comprised the quantities of the pooled Ct values. Moreover, in this study, all the candidate reference genes were analyzed as a single "normalizer". The normalization strategies were assessed by measuring $p16^{INK4a}$ and miR-203 transcripts in qPCR assays. We found that the use of pooled Ct values, can lead to a misinterpretation of the results, which suggests that the maintenance of inter-individual variability is a key factor in ensuring the reliability of the qPCR data. In addition, it should be stressed that a proper validation of the suitability of the reference genes is required for each experimental setting, since the indiscriminate use of a reference gene can also lead to discrepant results.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

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* Email: acf_ufpe@yahoo.com.br

Chapter 13
Human Papillomavirus-Related Cancers

Antonio Carlos de Freitas, Ana Pavla Almeida Diniz Gurge, Bárbara Simas Chagas, Carolina Medeiros do Amaral, Eliane Campos Coimbra, Elyda Gonçalves de Lima, Jacinto da Costa Silva Neto, Maria da Conceição Gomes Leitão, and Rita de Cássia Pereira de Lima

Abstract Cancer is a public health problem occupying the first and second place in number of deaths in developed and developing countries, respectively. Since the last century, the relationship between infection and cancer has been established in animals and more recently in several human cancers. Currently known that 15–20 % of cancers in the world of infectious origin, many of them related to viral infections. The human papillomavirus (HPV) stands out for its association with confirmed cervical cancer and the large volume of evidence that relate to the head and neck cancer. In addition, there is evidence of their relationship with breast cancers, lung and prostate. However, they are still required more detailed research that aim to clarify the possible mechanisms involved in these processes related to carcinogenic HPV.This chapter discusses the main molecular characteristics of HPV and its relationship with cancers using for this the infective models described by recent studies, the mechanisms of tumor progression, forms of diagnosis and therapy.

[AU1] ## Abbreviations

CDK	cyclin-dependent kinase
CIN	cervical intraepithelial neoplasia
COX-2	cyclooxygenase-2

[AU2] A.C. de Freitas (✉) • A.P.A.D. Gurge • B.S. Chagas • C.M. do Amaral
E.C. Coimbra • E.G. de Lima • M. da Conceição Gomes Leitão
R. de Cássia Pereira de Lima
Laboratory of Molecular Studies and Experimental Therapy, Department of Genetics,
Federal University of Pernambuco,
CidadeUniversitária, MoraesRêgo S/N, Recife, Pernambuco 50670-901, Brazil

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NCW2, a Gene Involved in the Tolerance to Polyhexamethylene Biguanide (PHMB), May Help in the Organisation of β-1,3-Glucan Structure of *Saccharomyces cerevisiae* Cell Wall

Carolina Elisabeth[1,2] · Rita de Cássia Pereira de Lima[2] · Will de Barros Pita[2,3] · Marcos Antonio de Morais Jr[2,4]

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Abstract In the present work, we provide biological evidences supporting the participation of *NCW2* gene in the mechanism responsible for cell tolerance to polyhexamethylene biguanide (PHMB), an antifungal agent. The growth rate of yeast cells exposed to this agent was significantly reduced in *dncw2* strain and the mRNA levels of *NCW2* gene in the presence of PHMB showed a 7-fold up-regulation. Moreover, lack of *NCW2* gene turns yeast cell more resistant to zymolyase treatment, indicating that alterations in the β-glucan network do occur when Ncw2p is absent. Computational analysis of the translated protein indicated neither catalytic nor transmembrane sites and reinforced the hypothesis of secretion and anchoring to cell surface. Altogether, these results indicated that *NCW2* gene codes for a protein which participates in the cell wall biogenesis in yeasts and that Ncw2p might play a role in the organisation of the β-glucan assembly.

✉ Marcos Antonio de Morais Jr
marcos.morais@ufpe.br
http://www.ufpe.br/mam

[1] Interdepartmental Research Group in Metabolic Engineering, Federal University of Pernambuco, Recife 50670-901, Brazil

[2] Department of Biochemistry, Federal University of Pernambuco, Recife 50670-901, Brazil

[3] Department of Antibiotics, Federal University of Pernambuco, Recife 50670-901, Brazil

[4] Department of Genetics, Universidade Federal de Pernambuco, Av. Moraes Rego, 1235 Cidade Universitária, Recife, PE 50670-901, Brazil

Introduction

In *Saccharomyces cerevisiae*, more than 30 genes have been reported to encode cell wall proteins (CWPs) and their coordinated expression represents a fundamental step for the assembly of a functional cell wall [1, 9, 12]. A set of compensatory alterations in gene expression and in the composition and architecture of the cell wall do occur when the cell wall integrity is compromised, and these alterations result in cells that are more resistant to further cell wall perturbations [1]. The regulation of these genes is controlled by the *PKC SLT2* cell wall integrity (CWI) mechanism, including those encoding GPI-anchored proteins Cwp1p, Sed1p, Pst1p, Crh1p and Ecm14p [1, 6, 12, 18]. The open reading frame YLR194C was also reported to encode a putative GPI-anchored protein associated to cell envelope that participates in the cell wall biogenesis [17]. It is up-regulated in response to the fungicide caspofungin [16], to high hydrostatic stress [8], to the biocide polyhexamethylene biguanide (PHMB) [6] and to medium acidification caused by sulphuric acid [4]. Moreover, its expression is increased in *gas1*Δ and *fks1*Δ mutant cells [17], which pointed to a role for YLR194C protein in a compensatory mechanism in the maintenance of the β-glucan network structure. This ORF was recently named *NCW2* gene for 'Novel Cell Wall protein 2' (F. Philippsen, personal communication to SDG database in 2013). To date, no clear phenotype has been associated to the *dncw2* strain. In the present work, we provide the first biological (genetic and physiological) evidences supporting the involvement of Ncw2p in the mechanism responsible for cell tolerance to the damaging agent PHMB. In addition, we showed that lack of Ncw2p protein turns cell more resistance to zymolyase. Altogether, our results propose a function for Ncw2p as auxiliary in the organisation of the

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Curriculum vitae (Lattes)

Rita de Cássia Pereira de Lima
Curriculum Vitae

Dados pessoais

Nome Rita de Cássia Pereira de Lima

Formação acadêmica/titulação

2013 Doutorado em Programa de Pós-Graduação em Genética.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil
Título: Identificação, Caracterização e Avaliação Funcional de Variantes do Oncogene E7 do Papilomavírus Humano 16
Orientador: Antonio Carlos de Freitas
Co-orientador: Bárbara Simas Chagas
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

2011 - 2013 Mestrado em Genética.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil
Título: Caracterização da interação de proteínas envolvidas em estresse em S. cerevisiae, Ano de obtenção: 2013
Orientador: Marcos Antonio de Morais Junior
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

2005 - 2009 Graduação em Bacharelado em Ciências Biológicas.
Universidade Federal da Paraíba, UFPB, Joao Pessoa, Brasil
Título: Aspectos ecológicos e caracterização genotípica de endossimbiontes dinoflagelados de Siderastrea stellata(Cnidaria, scleractinia) nos recifes costeiros do Cabo Branco, João Pessoa, PB
Orientador: Roberto Sassi
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

2005 - 2009 Graduação em Licenciatura em Ciências Biológicas.
Universidade Federal da Paraíba, UFPB, Joao Pessoa, Brasil

Formação complementar

2013 - 2013 Curso de curta duração em PCR em Tempo Real: Princípios Básicos e Aplicações. (Carga horária: 60h).
Universidade Federal de Pernambuco, UFPE, Recife, Brasil

Atuação profissional

1. Universidade Federal de Pernambuco - UFPE

Vínculo institucional

2013 - Atual	Vínculo: Bolsista , Enquadramento funcional: Bolsista Doutorado-CAPES , Carga horária: 40, Regime: Dedicação exclusiva
2011 - 2013	Vínculo: Bolsista , Enquadramento funcional: Bolsista de Mestrado- CNPq , Carga horária: 40, Regime: Dedicação exclusiva

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2016

Menção Honrosa no XXI Encontro de Genética do Nordeste, Sociedade Brasileira de Genética.

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Produção bibliográfica

Artigos completos publicados em periódicos

1. AMARAL, CAROLINA MM; CETKOVSKÁ, KATERINA; GURGEL, ANA PAD; CARDOSO, MARCUS V; CHAGAS, BÁRBARA S; PAIVA JÚNIOR, SÉRGIO SL; **DE LIMA, RITA**; SILVA-NETO, JACINTO C; SILVA, LUIZ AF; MUNIZ, MARIA TC; BALBINO, VALDIR Q; FREITAS, ANTONIO C
MDM2 polymorphism associated with the development of cervical lesions in women infected with Human papillomavirus and using of oral contraceptives. Infectious Agents and Cancer. , v.9, p.24 - , 2014.

2. LEITÃO, MARIA DA CONCEIÇÃO GOMES; COIMBRA, ELIANE CAMPOS; **LIMA, RITA DE CÁSSIA PEREIRA DE**; GUIMARÃES, MARILÉA DE LIMA; HERÁCLIO, SANDRA DE ANDRADE; SILVA NETO, JACINTO DA COSTA; DE FREITAS, ANTONIO CARLOS
Quantifying mRNA and MicroRNA with qPCR in Cervical Carcinogenesis: A Validation of Reference Genes to Ensure Accurate Data. Plos One. , v.9, p.e111021 - , 2014.

3. ELSZTEIN, CAROLINA; **DE LIMA, RITA DE CÁSSIA PEREIRA**; DE BARROS PITA, WILL; DE MORAIS, MARCOS ANTONIO
NCW2, a Gene Involved in the Tolerance to Polyhexamethylene Biguanide (PHMB), May Help in the Organisation of β-1,3-Glucan Structure of Saccharomyces cerevisiae Cell Wall. Current Microbiology (Print). , v.73, p.341 - 345, 2016.

Capítulos de livros publicados

1. SILVA, M. A. R.; **LIMA, R. C. P.**; CORDEIRO, M. N.; BORZACCHIELLO, G.; FREITAS, ANTONIO C
Bovine Papillomavirus In: Molecular Detection of Animal Viral Pathogens.1 ed.Boca Raton : CRC Press, 2016, p. 727-734.

2. DE FREITAS, ANTONIO CARLOS; Gurgel, Ana Pavla Almeida Diniz; Chagas, Bárbara Simas; do Amaral, Carolina Maria Medeiros; COIMBRA, ELIANE CAMPOS; de Lima, Élyda Gonçalves; da Costa Silva Neto, Jacinto; da Conceição Gomes Leitão, Maria; **de Cássia Pereira de Lima, Rita** Human Papillomavirus-Related Cancers In: Infection and Cancer: Bi-Directorial Interactions.1 ed. : Springer International Publishing, 2015, p. 243-291.

Trabalhos publicados em anais de eventos (resumo)

1. CAMPOS, A. P. F.; CORDEIRO, M. N.; SILVA, A. J. D.; **LIMA, R. C. P.**; CARDOZO, G. H. A.; MACEDO, L. S.; JESUS, A. L. S.; FREITAS, A.C. DESENVOLVIMENTO DE VACINA DE DNA BASEADA NO GENE L2 DO PAPILOMAVÍRUS BOVINO In: XXI Encontro de Genética do Nordeste, 2016, Recife.
XXI Encontro de Genética do Nordeste. , 2016.

2. **LIMA, R. C. P.**; SILVA, R. C. O.; SANTOS, D. L.; SALGADO, C.; CORDEIRO, M. N.; NASCIMENTO, K. C. G.; GURGEL, A. P. A. D.; OLIVEIRA, T. H. A.; CHAGAS, B. S.; FREITAS, A.C. FUNCTIONAL STUDY OF ONCOPROTEIN E7 VARIANTS OF HUMAN PAPILLOMAVIRUS TYPE 16 IN BRAZIL NORTHEASTERN In: VII SINATER – International Symposium on Diagnostics and Therapeutics, 2016, Recife.
VII SINATER – International Symposium on Diagnostics and Therapeutics. , 2016.

3. **LIMA, R. C. P.**; CHAGAS, B. S.; CORDEIRO, M. N.; SILVA, R. C. O.; JESUS, A. L. S.; CAMPOS, A. P. F.; SILVA, A. J. D.; FREITAS, ANTONIO C. Identificação e avaliação funcional de polimorfismos no gene E7 do HPV-16 em amostras clínicas do Nordeste do Brasil In: 52 Congresso da Sociedade Brasileira de Medicina Tropical, 2016, Macéio.
52 Congresso da Sociedade Brasileira de Medicina Tropical. , 2016.

4. **LIMA, R. C. P.**; CORDEIRO, M. N.; SILVA, R. C. O.; CAMPOS, A. P. F.; SANTOS, D. L.; GURGEL, A. P. A. D.; VENUTI, A.; CHAGAS, B. S.; FREITAS, A.C. IDENTIFICATION, CHARACTERIZATION AND FUNCTIONAL EVALUATION OF HPV16 E7 POLYMORPHISMS IN NF-kB SIGNALING PATHWAY In: XXI Encontro de Genética do Nordeste, 2016, Recife.
XXI Encontro de Genética do Nordeste. , 2016.

5. **LIMA, R. C. P.**; SANTOS, D. L.; SILVA, R. C. O.; SALGADO, C.; CORDEIRO, M. N.; CHAGAS, B. S.; FREITAS, A.C. IDENTIFICATION OF E7 POLYMORPHISMS OF HUMAN PAPILLOMAVIRUS TYPE 16 IN CERVICAL SAMPLES OF WOMEN IN NORTHEASTERN BRAZIL In: VII SINATER – International Symposium on Diagnostics and Therapeutics, 2016, Recife.
VII SINATER – International Symposium on Diagnostics and Therapeutics. , 2016.

6. NASCIMENTO, K. C. G.; GURGEL, A. P. A. D.; QUIXABEIRA, D. C. A.; SILVA, R. C. O.; **LIMA, R. C. P.**; SILVA JUNIOR, A. H. P.; CHAGAS, B. S.; LIMA, E. G.; OLIVEIRA, T. H. A.; CORDEIRO, M. N.; SILVA NETO, J.C; FREITAS, A.C. INCIDÊNCIA DO PAPILOMAVÍRUS HUMANO (HPV) EM SANGUE PERIFÉRICO DE PACIENTES SAUDÁVEIS E COM LESÕES CERVICAIS, NO ESTADO DE PERNAMBUCO, BRASIL In: XXI Encontro de Genética do Nordeste, 2016, Recife.
XXI Encontro de Genética do Nordeste. , 2016.

7. CHAGAS, B. S.; QUIXABEIRA, D. C. A.; Gurgel, Ana Pavla Almeida Diniz; NASCIMETO, K. C.; **LIMA, R. C. P.**; SILVA, R. C. O.; SILVA NETO, J.C; FREITAS, ANTONIO C. Distribuição de polimorfismo de base única (SNPs) no gene da Glutationa S-Transferase P1 (GSTP1) entre pacientes com lesão cervical e infectadas pelo Papilomavírus Humano In: 19° Congresso Brasileiro de Oncologia Clínica, 2015, Foz do Iguaçu.
Oncologia sem fronteiras. , 2015.

8. CHAGAS, B. S.; QUIXABEIRA, D. C. A.; Gurgel, Ana Pavla Almeida Diniz; NASCIMETO, K. C.; **LIMA, R. C. P.**; SILVA, R. C. O.; SILVA NETO, J.C; FREITAS, A.C. Distribuição de polimorfismo de base única (SNPs) no gene do fator de necrose tumoral (TNFA) (-238) entre pacientes com lesão cervical e infectadas pelo Papilomavírus humano In: 19° Congresso Brasileiro de Oncologia

Clínica, 2015, Foz do Iguaçu.
Oncologia sem fronteiras. , 2015.

9. CURZIO, G.; CORDEIRO, M. N.; PAOLINI, F.; MASSA, S.; ILLIANO, E.; **LIMA, R. C. P.**; FRANCONI, R.; BISSA, M.; MORGHEN, C. G.; FREITAS, A. C.; VENUTI, A. GENETIC IMMUNIZATION AGAINST HPV-16 E5 In: 30th International Papillomavirus Conference & Clinical and Public Health Workshops, 2016, Lisboa.
30th International Papillomavirus Conference & Clinical and Public Health Workshops. , 2015.

10. **LIMA, R. C. P.**; Chagas, Bárbara Simas; CORDEIRO, M. N.; SILVA, R. C. O.; FREITAS, ANTONIO C
Identificação e avaliação funcional de polimorfismos no gene E7 do HPV-16 em amostras clínicas no Nordeste do Brasil In: V Jornada de Pós-Graduação em Genética da UFPE, 2015, Recife.
V Jornada de Pós-Graduação em Genética da UFPE. , 2015.

11. **LIMA, R. C. P.**; COIMBRA, E.C; LEITÃO, M.C.G; FREITAS, A.C. Identificação de biomarcadores e alvos terapêuticos para o câncer cervical In: Terceira Jornada de Pós Graduação em Genética da UFPE, 2013, Recife.
Anais da Terceira Jornada de Pós Graduação em Genética da UFPE. Recife: UFPE, 2013.

Trabalhos publicados em anais de eventos (resumo expandido)

1. **LIMA, R. C. P.**; SILVA, R. C. O.; CORDEIRO, M. N.; SANTOS, D. L.; SALGADO, C.; NASCIMENTO, K. C. G.; GURGEL, A. P. A. D.; OLIVEIRA, T. H. A.; CHAGAS, B. S. FREITAS, AC. ANÁLISE FUNCIONAL DE POLIMORFISMOS DOS ONCOGENES E6 E E7 DOS PAPILOMAVÍRUS HUMANO TIPOS 16 E 31 EM AMOSTRAS CLÍNICAS DO NORDESTE BRASILIEIRO. In: 4 Encontro Brasileiro para Inovação Terapêutica, 2016, Recife.
4 Encontro Brasileiro para Inioação Terapêutica. 2016.