UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUACÃO EM GENÉTICA

IMUNIZAÇÃO GENÉTICA CONTRA O CÂNCER CERVICAL BASEADO NO ONCOGENE E5 DO PAPILOMAVÍRUS HUMANO TIPO 16

MARCELO NAZÁRIO CORDEIRO

Recife - PE
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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: Prof. Antonio Carlos de Freitas

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Aprovado em 15/03/2015

Banca Examinadora:

_____

Dr. Antonio Carlos de Freitas

Universidade Federal de Pernambuco - UFPE

_____

Dr. Luis Carlos de Souza Ferreira

Universidade de São Paulo - USP

_____

Dr. Moacyr Jesus Barreto de Melo Rêgo

Universidade Federal de Pernambuco - UFPE

_____

Dra. Laura Helena Vega Gonzalez Gil

Instituto Aggeu Magalhães - IAM/FIOCRUZ

_____

Dr. Antonio Roberto Lucena Araújo

Universidade Federal de Pernambuco - UFPE

Recife - PE

2015

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"Retém a instrução e não a largues: guarda-a, porque ela é a tua vida."

Provérbios 4:13

RESUMO

Imunização genética contra o câncer cervical baseado no oncogene E5 do papilomavírus humano tipo 16

A infecção persistente pelo HPV16 está associada com cânceres cervicais anogenitais e um subconjunto de cânceres de cabeça e pescoço. E5, E6 e E7 são oncoproteínas virais que contribuem com a transformação de queratinócitos humanos. Além de potencializar a habilidade das proteínas E6 e E7 na imortalização/invasão de queratinócitos primários, as proteínas E5 de HPV de alto risco têm participação relevante nas fases precoces de transformação, como na alteração da via dependente do receptor do fator de crescimento epidermal. Entretanto, o antígeno E5 como candidato vacinal ainda não foi devidamente explorado. Ainda, as linhagens celulares mais utilizadas como modelos de desafio tumoral podem não simular os padrões de expressão dos oncogenes de HPV, já que, sua expressão é dirigida por promotores diferentes dos de HPV. Este trabalho utilizou um novo modelo de desafio para validar vacinas anti-HPV baseado em células C3, as quais apresentam a expressão do HPV sob controle de seu próprio promotor. Duas versões do oncogene E5 de HPV16 foram geradas como vacinas de DNA; uma mantendo a sequência íntegra do gene E5 e outra codificando apenas seus dois prováveis epítopos imunogênicos em duplicata. As candidatas vacinais foram submetidas a experimentos *in vivo* para demonstração de efeitos anti-tumorais contra células C3. Sob regime preventivo ou terapêutico, tumores em camundongos vacinados sofreram efeitos de imunidade celular, conforme indicado pelo acompanhamento do desenvolvimento tumoral e ensaios de ELISPOT. Os efeitos anti-tumorais elicitados pela imunização genética baseada em E5 foram equiparáveis àqueles obtidos por abordagem similar adotada por imunização genética baseada em E6 e E7. O aprimoramento técnico sobre esta abordagem deve, no futuro, resultar em perspectivas de estudos clínicos baseados na imunização genética com E5 contra o HPV e seus tumores associados.

Palavras-chave: papilomavírus humano; oncoproteína E5, imunização genética

Abstract

Genetic immunization against cervical cancer based on human papillomavirus 16 E5 oncogene

HPV16 persistent infection is associated with cervical and anogenital cancers and a subset of head and neck cancers. E5, E6 and E7 are oncoproteins that contribute to human keratinocytes viral transformation. Further enhancing E6/E7-mediated immortalization/invasion of primary keratinocytes, high risk HPV E5 proteins have significant participation in early stages of transformation, for example, by triggering epidermal growth factor receptor (EGFR)-dependent cell growth. However, E5 antigen as a vaccine candidate has not been well explored yet. In addition, the most commonly cell lines used as tumor challenge models may not properly simulate the HPV oncogene expression patterns, since its expression is directed by non-HPV promoters. This study has adopted a new challenge model based on C3 cell line to evaluate anti-HPV vaccines, which present HPV expression driven by its own promoter. Two E5-based versions were generated as DNA vaccines; an HPV16 E5 whole gene sequence and another gene, that encodes only two likely immunogenic epitopes in duplicate. Vaccine candidates were subjected to *in vivo* experiments in order to demonstrate anti-tumor effects against HPV16-expressing C3 cells-bearing mice. Under preventive or therapeutic procedure, tumors in vaccinated mice suffered cellular immunity effects, as indicated by monitoring tumor growth and ELISPOT assays. The anti-tumor effects elicited by genetic immunization based on E5 were comparable to those obtained by similar approach taken by genetic immunization based on E6 and E7. The technical improvement on this approach should, in future, results in prospects for clinical studies based on E5 genetic immunization against HPV and its associated tumors.

Key words: human papillomavirus; E5 oncoprotein; genetic immunization.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

Item	Definição
HPV	*Human Papillomavirus*
CRPV	*Cottontail Rabbit Papillomavirus*
BPV	*Bovine Papillomavirus*
DNA	*Desoxiribonucleic acid*
RNA	*Ribonucleic acid*
ORF	*Open Reading Frame*
E	*Early*
L	*Late*
LCR	*Long Control Region*
INCA	Instituto Nacional do Câncer
Kb	Kilobase
EGF	*Epidermal Growth Factor*
EGFR	*Epidermal Growth Factor Receptor*
DST	Doença Sexualmente Transmissível
HSPG	*Heparan sulfate Proteoglican*
AIDS	*Acquired Immunodeficiency Syndrome*
UBR4	*Ubiquitin Protein Ligase E3 Component n-Recognin 4*
E2F	*E2F transcription factor*
pRB	proteína do Retinoblastoma
G1	*Gap* 1
S	*Synthesis*
G2	*Gap* 2
kDa	KiloDaltons
ATP	*Adenosin tri-fosfate*
MHC-I	*Major histocompatibility complex – I*
MHC-II	*Major histocompatibility complex – II*
CD	*cluster of differentiation*
NK	*Natural Killers*
VLP	*Virus-like Particles*
IM	Injeção intra-muscular
APC	*Antigen presentation cell*
CpG	*cytosine triphosphate deoxynucleotide/ phosphodiester/guanine triphosphate deoxynucleotide*
HLA	*human leukocyte antigen*
PCR	*Polymerase Chain Reaction*
RT-PCR	*Reverse Transcriptase - Polymerase Chain Reaction*
HR-HPV	*High risk human papillomavirus*
ELISPOT	*Enzyme-linked Immuno-Spot*
ELISA	*Enzyme-linked Immune-sorbent assay*
WB	*Western blot*
IFN	*Interferon*
MPBS	*Milk in Phosphate Buffered Salin Solution*
PMA	*Phorbolmyristate acetate*
CP	*Coat protein*
IPTG	*Isopropyl β-D-1-thiogalactopyranoside*
His	Histidina
SAP	Saporina

Ni-NTA	*Niquel nitrilotriacetic acid*
Pc	*Post challenge*
CTL	*Citolitic T lymphocyte*
AdV	*Adenovirus*
ASCUS	*Atypical squamous cells of undeterminated significance*
HSIL	*High grade squamous intra-epithelial lesion*
LSIL	*Low grade squamous intra-epithelial lesion*
WHO	*World Health Organization*
SUS	Sistema Único de Saúde
AOX1	*Alcohol oxigenase 1 gene*
SDS-PAGE	*Sodium dodecil-sulfate - polyacrylamide gel electrophoresis*
PV	*Papillomavirus*

SUMÁRIO

1. INTRODUÇÃO

A família *Papillomaviridae* agrega pequenos vírus de DNA, não envelopados, responsáveis por lesões usualmente benignas em diversos grupos animais, dentre os quais, aqueles que infectam humanos e bovinos são os mais bem estudados. O papilomavírus humano (HPV) é um patógeno largamente disseminado entre a população sexualmente ativa, responsável por quadros clínicos que variam desde tumores benignos até cânceres anogenitais e cervicais. A mais recente associação do HPV a um tipo de câncer é aquela que o implica como fator predisponente ao câncer de cabeça e pescoço, o que reforça a urgência no desenvolvimento de estratégias de diagnóstico e tratamento cada vez mais eficazes. O HPV interfere no ciclo celular de queratinócitos primários, principalmente por meio de suas principais oncoproteínas, E6 e E7, compreensivelmente, as mais estudadas como alvos terapêuticos. Entretanto, evidências mais recentes ressaltam a importância de uma terceira oncoproteína, designada E5, cujas atividades reforçam o potencial transformante das demais oncoproteínas, além de modular negativamente a resposta imune contra a célula infectada.

Os estudos aqui realizados visam uma melhor compreensão acerca de estratégias de prevenção e terapia contra os papilomavírus, por meio do desenvolvimento de vacinas inovadoras. Testando o potencial imunogênico ainda pouco explorado da proteína E5, nossos resultados sugerem efeitos anti-tumorais *in vivo* de imunização genética baseada no gene E5 do HPV16. Ainda, nosso trabalho apresenta um novo modelo de desafio tumoral baseado em células C3 contendo o genoma íntegro de HPV16, o que pode fornecer dados mais precisos quanto a eficiência de uma vacina em elicitar resposta imune contra células infectadas.

2. JUSTIFICATIVA

O câncer do colo uterino é um importante problema de saúde pública no mundo, representando 9% dos casos de câncer feminino e sendo considerado como a terceira maior causa de morte em mulheres no mundo, com mais de 529.800 novos casos e 275.100 mortes por ano (Jemal *et al*., 2011; Freitas *et al*., 2012). Quando comparada aos países mais desenvolvidos, sua incidência é cerca de duas vezes maior em países menos desenvolvidos (Ministério da Saúde, 2011). Em conformidade com a portaria N° 874, de 16 de Maio de 2013, do Ministério da Saúde do Brasil, 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 inclui o câncer cervical como um alvo de combate e controle prioritário. 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. A pesquisa aqui proposta contribui com a busca pelo aumento da qualidade e expectativa de vida da população, por meio de desenvolvimento de competência técnico-científica voltada para o controle de uma doença altamente disseminada. Embora seja um grave problema de saúde pública, o câncer cervical carece de opções terapêuticas menos invasivas e debilitantes e sua prevenção depende majoritariamente de uma detecção precoce das lesões pré-malignas.

As atividades de três proteínas virais produzidas por certos tipos de papilomavírus são apontadas como as principais mediadoras da carcinogênese associada ao HPV. Pelo fato das oncoproteínas E5, E6 e E7 atuarem nas fases iniciais e/ou avançadas da transformação de queratinócitos e no desenvolvimento de lesões cervicais malignas, acreditamos que uma resposta imune elicitada

contra os epitopos imunogênicos dessas proteínas deve erradicar as células tumorais eficientemente. Diversos trabalhos de imunização genética são baseados nesses antígenos, embora ainda pouca evidência tenha sido demonstrada em relação àqueles codificados pelo oncogene E5. Outro ponto importante acerca da experimentação de vacinas de DNA contra células infectadas pelo HPV é a escolha do modelo de desafio. A maior parte das linhagens tumorais adotadas apresentam padrões de expressão dos genes de HPV que podem não simular apropriadamente uma infecção natural, uma vez que a expressão dos genes virais nessas linhagens é dirigida por promotores diferentes daqueles utilizados pelo vírus.

3. OBJETIVOS

3.1 Objetivo Geral

Desenvolver estratégia vacinal contra a infecção por papilomavírus humano baseada em imunização genética com o gene E5 de HPV16.

3.2 Objetivos Específicos

- Construir plasmídeos de expressão para células de mamíferos como candidatos a vacinas de DNA contra o antígeno E5 de HPV16;
- Avaliar a resposta imune gerada para vacinas de DNA baseadas no gene E5 de HPV16 em camundongos, frente a vacinas já avaliadas baseadas nos genes E6 e E7 de HPV16;
- Testar a resposta imune gerada por vacina de DNA contendo sequências duplicadas codificantes apenas para dois epítopos imunodominantes do gene E5 de HPV16;
- Aplicar vacinas de DNA baseadas no gene E5 de HPV16 em novo modelo de desafio tumoral baseado em células C3 contendo o genoma de HPV16;

4. REVISÃO DA LITERATURA

4.1. Papilomavírus. Estrutura e organização do genoma.

A família *Papillomaviridae* é constituída por 29 gêneros de vírus que compartilham diversas características, tais como morfologia, estrutura e tamanho de genoma e sua transcrição unidirecional (BERNARD et al., 2010). Em comum, todos os membros infectam os epitélios e/ou mucosas de alguma espécie de vertebrado (ver Tabela 1). Dentre os tipos de papilomavírus mais bem estudados, destacam-se aqueles que infectam coelhos (CRPV - *Cottontail Rabbit Papillomavirus*), bovinos (BPV – *Bovine Papillomavirus*) e humanos (HPV – *Human Papillomavirus*). Os papilomavírus são considerados estritamente espécie-específicos e, mesmo em condições experimentais, não infectam outro hospedeiro que não o seu natural (DOORBAR, 2005). Entretanto, casos de infecção cruzada foram relatados unicamente para os BPVs tipos 1 e 2, implicados no desenvolvimento de sarcóide equino em cavalos e outros equídeos (WHITE, 2014) (NASIR; CAMPO, 2008). Na notável diversidade de espécies animais nas quais algum tipo de papilomavírus já foi identificado, além dos mamíferos, incluem-se répteis (HERBST et al., 2009) e aves (TACHEZY et al., 2002). Em relação às consequências para o hospedeiro, de modo geral, os vírus podem permanecer assintomáticos ou iniciar lesões hiperproliferativas no epitélio. Por sua capacidade neoplásica, os papilomavírus são considerados potencialmente oncogênicos.

Quadro 1. Relação de 29 gêneros de papilomavírus e suas espécies hospedeiras

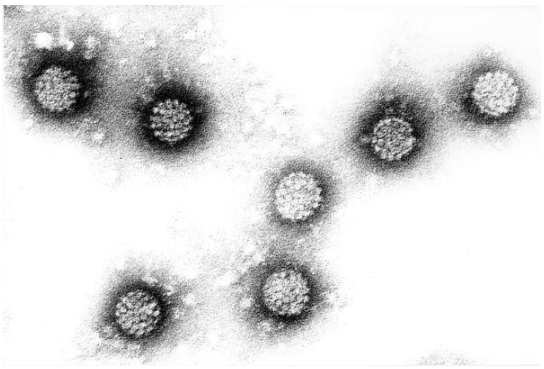
Gênero	**Hospedeiro**
Alphapapillomavirus	*Homo sapiens* *Macaca mulata*
Betapapillomavirus	*Homo sapiens* *Macaca fascicularis*
Gammapapillomavirus	*Homo sapiens*
Deltapapillomavirus	*Alces alces* *Odocoileus virginianus* *Ovis aries* *Bos taurus* *Capreolus capreolus*
Epsilonpapillomavirus	*Bos Taurus*
Etapapillomavirus	*Fringilla coelebs*
Iotapapillomavirus	*Mastomys natalensis*
Kappapapillomavirus	*Sylvilagus floridanus* *Oryctolagus cuniculus*
Lambdapapillomavirus	*Felis domesticus* *Canis familiaris*

Mupapillomavirus	*Homo sapiens*
Nupapillomavirus	*Homo sapiens*
Pipapapillomavirus	*Mesocricetus auratus*
Thetapapillomavirus	*Psittacus erithacus*
Xipapillomavirus	*Bos taurus*
Zetapapillomavirus	*Equus caballus*
Rhopapillomavirus	*Trichechus manatus latirostris*
Sigmapapillomavirus	*Erethizon dorsatum*
Taupapillomavirus	*Canis familiaris*
Upsilonpapillomavirus	*Tursiops truncatus*
Phipapillomavirus	*Capra hircus*
Chipapillomavirus	*Canis familiaris*
Psipapillomavirus	*Rousettus aegyptiacus*
Omegapapillomavirus	*Ursus maritimus*
Dyodeltapapillomavirus	*Sus scrofa*
Dyoepsilonpapillomavirus	*Francolinus leucoscepus*
Dyozetapapillomavirus	*Caretta caretta*
Dyoetapapillomavirus	*Erinaceus europaeus*
Dyothetapapillomavirus	*Felis domesticus*
Dyoiotapapillomavirus	*Equus caballus*

Fonte: Adaptado de Bernard *et al.,* (2010)

Os papilomavírus apresentam um capsídeo icosaédrico não envelopado e seu genoma está compreendido em 6,8 a 8,0Kb de uma dupla-fita de ácido desoxirribonucleico (DNA) circular (ZHENG; BAKER, 2006). O capsídeo proteico dos papilomavírus, medindo cerca de 52 a 55nm, encerra 72 capsômeros, cada qual composto pelo pentâmero da principal proteína estrutural do vírus, designada L1. Ainda como constituinte do capsídeo, 12 cópias de uma segunda proteína estrutural, denominada L2, atravessam a estrutura da partícula viral e se associam ao DNA viral, sob a forma de um nucleossomo (MODIS; TRUS; HARRISON, 2002). O genoma dos papilomavírus encontra-se associado a histonas celulares, compondo um cromossomo rudimentar (TAN et al., 1998). Esse genoma pode conter entre 8 a 10 *open reading frames* (ORFs), cujas sequências são expressas de acordo com um padrão precoce e tardio conforme a diferenciação da célula hospedeira ao longo da infecção (GRAHAM, 2010).

Figura 1. Eletromicrografia de papilomavírus.



Fonte: Hanswalter Zentgraf, German Cancer Research Center (2000)

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. Além das ORFs, o DNA viral contém uma região não codificante, denominada *Long Control Region* (LCR), a qual encerra sítios de interação com fatores transcricionais do vírus e da célula hospedeira, e a origem de replicação viral (DOLLARD; BROKER; CHOW, 1993) (BERG; STENLUND, 1997).

4.2 Papilomavírus humano e o câncer.

O câncer cervical é descrito como uma progressão maligna que tem seu início a partir de lesões na cérvice, região compreendida desde a vagina até a estreita abertura do útero. A porção onde ocorre o encontro entre a ectocervice (parte vaginal, células pavimentosas escamosas) e a endocervice (parte uterina, células colunares), caracterizada pela confluência de dois tipos epiteliais distintos, é chamada de zona de transformação. A zona de transformação ou “zona T” é o local de maior probabilidade de desenvolvimento de células anormais ou pré-cancerosas (WAGGONER, 2003). Cerca de 80% dos cânceres cervicais são derivados de células escamosas transformadas, enquanto os demais, conhecidos como adenocarcinomas, desenvolvem-se a partir de glândulas produtoras de muco do endocérvice. A tumorigênese de ambos os tipos de carcinoma já foi relacionada a infecções pelo HPV (LAX, 2011).

O câncer cervical é um tipo de câncer de desenvolvimento lento, sem manifestar sintomas precoces, apesar de ter na identificação por papiloscopia (coleta de células a partir de esfregaços da cérvice e análise microscópica) sua ferramenta de detecção precoce mais utilizada (SCHIFFMAN et al., 2000). Um estudo de acompanhamento de grupos tratados e não-tratados ao longo de 30 anos sugere que pelo menos um terço das lesões precursoras irão evoluir para o câncer invasivo (MCCREDIE et al., 2008). Pesquisas recentes estão focadas em melhorar o diagnóstico precoce de lesões cervicais precursoras, como identificação de biomarcadores teciduais e moleculares (GADDUCCI et al., 2010) (PEIRSON et al., 2013).

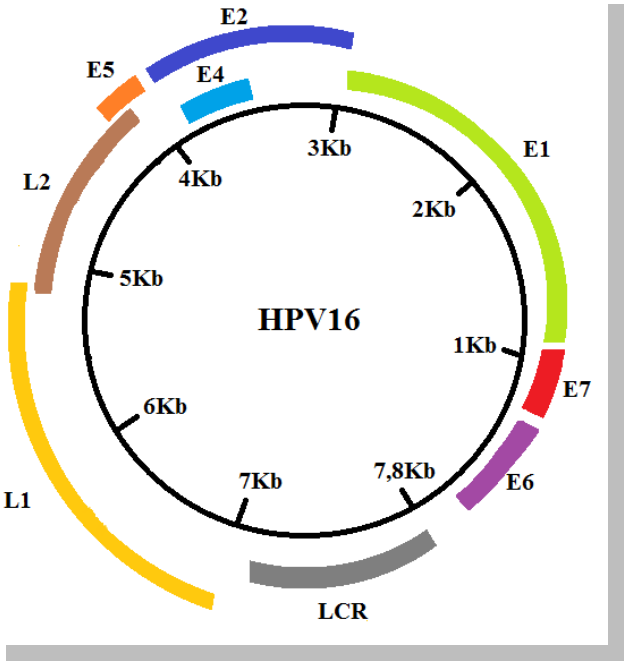
O câncer cervical é atualmente reconhecido como a terceira classe de doença maligna mais diagnosticada em mulheres no mundo (JEMAL et al., 2011). No Brasil, esta neoplasia situa-se como a terceira mais comum entre a população feminina, apenas suplantada pelo câncer de pele não melanoma e o câncer de mama, sendo a quarta causa de morte entre mulheres por câncer. Aproximadamente, 85% dos casos e 88% das mortes ocorrem em mulheres que vivem em países em desenvolvimento (FERLAY et al., 2010).

Em 1976, zur Hausen e Gissmann detectaram o DNA de papilomavírus humano em diversas amostras de verrugas genitais e cânceres cervicais (ZUR HAUSEN, 1976). Em seus trabalhos subsequentes o conjunto de dados gerados constituiu uma das associações mais bem estabelecidas entre uma infecção viral e a carcinogênese. Nas décadas seguintes, estudos combinando a detecção do DNA de HPV com a detecção de anticorpos contra suas proteínas virais implicariam mais de 98% dos casos de câncer cervical com a infecção pelo HPV (ZEHBE; WILANDER, 1997) (WALBOOMERS et al., 1999) (SCHIFFMAN et al., 2000).

A partícula viral segue o padrão morfológico dos demais papilomavírus, embora a organização do genoma pode variar em relação à quantidade de ORFs, além da função de algumas de suas proteínas. O capsídeo tipicamente icosaédrico do HPV é desprovido de envelope e compreende seu material genético em uma dupla fita de DNA circular, com cerca de 8Kb de tamanho associada a histonas celulares. Seu genoma é dividido em três regiões, LCR,

região das proteínas precoces (E1-E7) e das proteínas tardias (L1 e L2) (SCHWARZ et al., 1985). Suas ORFs codificam produtos envolvidos na regulação da replicação e transcrição viral em queratinócitos (ORFs E1 e E2) (CHIANG et al., 1992), desestabilização da matriz de queratina e liberação de vírions (recomposição dos transcritos das ORFs E1/E4) (DOORBAR et al., 1991), alteração do ciclo celular e oncogênese de queratinócitos primários (ORFs E5, E6 e E7) (DIMAIO; PETTI, 2013) (VANDE POL; KLINGELHUTZ, 2013) (ROMAN; MUNGER, 2013) e composição do capsídeo (ORFs L1 e L2) (BUCK et al., 2008) (Figura 2.).

Figura 2. Esquema referente à organização estrutural genoma do HPV16.



Fonte: Cordeiro MN (2013)

Quadro 2. Relação sumarizada das proteínas de HPV e suas principais funções.

Proteínas	Funções resumidas
E6	Bloqueio da atividade da proteína supressora tumoral p53, por degradação
E7	Inativação da proteína supressora tumoral pRb por degradação
E5	Ativação prolongada da via dependente do Fator de Crescimento Epidermal (EGF) por maior sobrevida de seu receptor;
E1	Replicação do DNA viral
E2	Regulador da transcrição viral (repressor de E6/E7);
E4	Montagem e liberação da partícula viral
L1	Principal proteína do capsídeo
L2	Proteína “menor” do capsídeo

Fonte: Stanley M (2001)

Os diversos tipos de HPV foram classificados conforme o risco de câncer cervical assumido durante a infecção. Enquanto os HPVs de baixo risco restringem seus principais sinais clínicos a verrugas genitais, os HPVs de alto risco já foram descritos como agentes etiológicos de lesões de alto grau (ZUR HAUSEN, 2002) e carcinomas de células escamosas, não apenas cervicais, como também de cabeça e pescoço (GILLISON et al., 2000) (HERRERO et al., 2003). Dentre os mais de 180 genótipos já identificados em lesões cervicais, os principais representantes daqueles considerados de alto risco, conhecidos como oncogênicos, são os tipos 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 e 82 (MUÑOZ et al., 2003) (GUAN et al., 2012). Dentre esses, o HPV 16 destaca-se como o mais prevalente no mundo, identificado em mulheres com infecção subclínica ou diagnosticadas com câncer cervical (BERNARD et al., 2010), além de ser descrito como um dos mais virulentos, devido à presença de seu genoma em cerca de 70% das neoplasias intra-epiteliais de alto grau (CLIFFORD et al., 2003).

4.3 Transmissão e Ciclo Viral.

Embora, de modo não exclusivo, o ato sexual, compreendido pelo intercurso vaginal, oral e/ou anal, é uma atividade permissiva à infecção pelo HPV. De fato, dentre alguns aspectos do comportamento sexual, o número de parceiros é destacado como um fator de risco para a prevalência de doença ano-genital e oral associada ao HPV (TROTTIER; FRANCO, 2006) (D'SOUZA et al., 2009). Estudos epidemiológicos destacam ainda que a infecção por algum tipo de HPV ocorre cedo, logo após as primeiras atividades sexuais (COLLINS et al., 2009) (WINER et al., 2003). Dentre os mais frequentes fatores de risco correlacionados com a infecção e persistência do HPV na população, destacam-se o contato sexual precoce e o alto número de parceiros sexuais (VELDHUIJZEN et al., 2010). Há evidências de que a infecção por HPV tem progressivamente aumentado nas últimas décadas, o que, para alguns, classifica a papilomatose humana como uma das doenças sexualmente transmissíveis (DST) mais prevalentes no mundo (SCHEURER; TORTOLERO-LUNA; ADLER-STORTHZ, 2005). A infecção é principalmente contraída pelo contato sexual, através do qual microtraumas na mucosa genital permitem o acesso de partículas virais ao

epitélio basal (BURCHELL et al., 2006). Nessa região, o HPV infecta os queratinócitos primários cujo programa de diferenciação celular relaciona-se a um ciclo infeccioso especialmente adaptado ao ciclo celular.

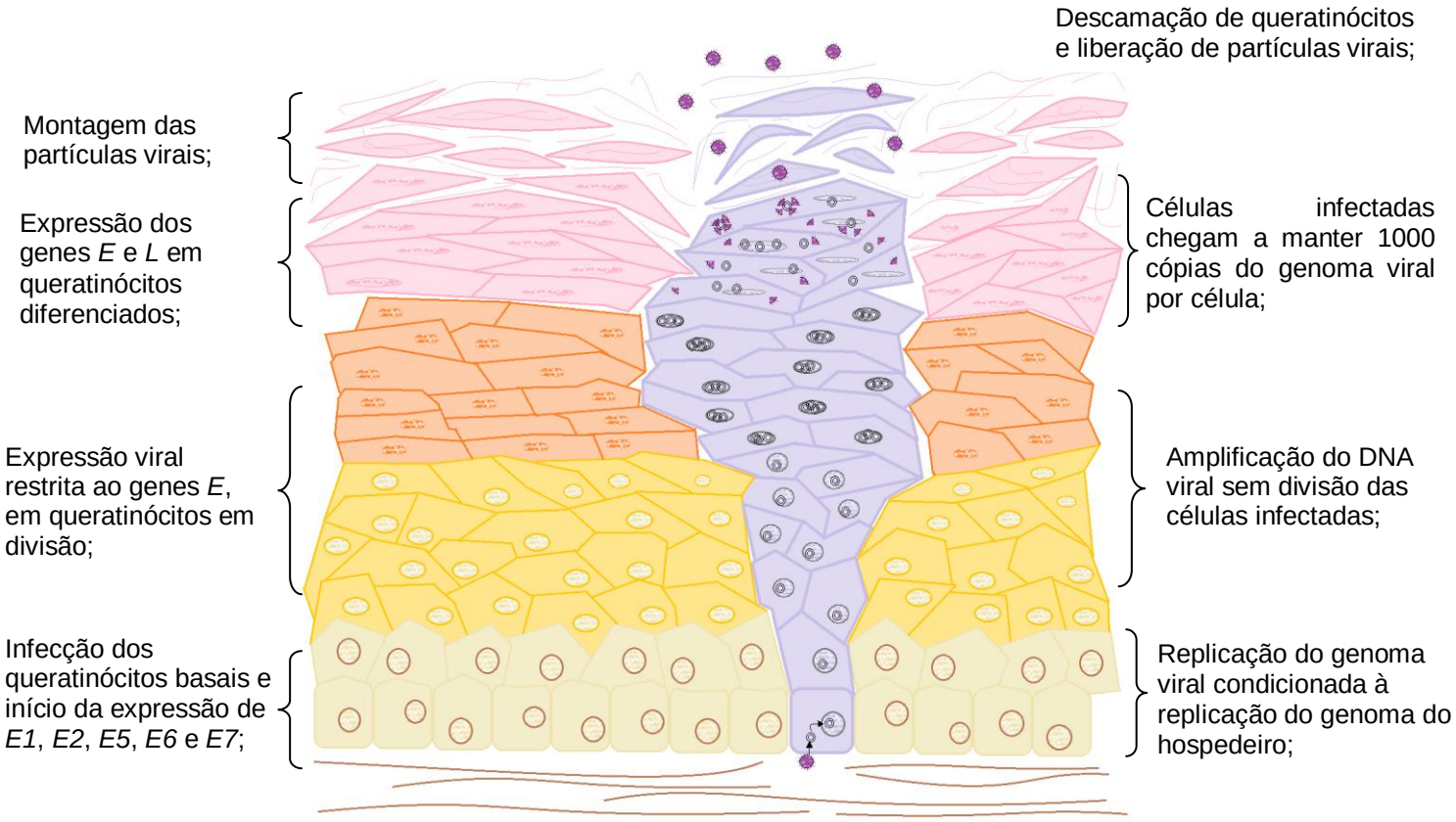
Ao ter acesso à membrana basal do epitélio, a partícula viral interage com um restrito grupo de receptores. Para a ancoragem primária da maioria dos papilomavírus, parecem ser necessários receptores proteoglicanos de heparan sulfato (HSPG) (GIROGLOU et al., 2001) (JOYCE et al., 1999), não apenas os presentes na superfície celular de queratinócitos, como também os secretados presentes na membrana basal (ROBERTS et al., 2007). Interações da proteína L1 do capsídeo com a superfície celular e mudanças conformacionais que expõem sítios de clivagem para furina na proteína L2 permitem a entrada da partícula viral na célula-alvo. Especificamente, a exposição da cadeia amino terminal da proteína L2 após clivagem na superfície celular parece ser determinante para a infectividade da partícula viral (BIENKOWSKA-HABA; PATEL; SAPP, 2009).

Enquanto a proteína L1 não pode ser transportada para o núcleo e é separada do genoma viral durante o desnudamento endocítico (NELSON et al., 2000), a proteína L2 tem papel direto no escape do genoma viral do compartimento endocítico, num processo dependente de clivagem por furina (RICHARDS et al., 2006). O transporte do genoma para o núcleo também depende fortemente da ação da proteína L2, uma vez que sua interação com proteínas motoras dos microtúbulos, como a dineína, já foi demonstrada (FLORIN et al., 2006). De fato, queratinócitos primários em atividade proliferativa, numa tentativa de regenerar as fissuras da epiderme, podem representar uma condição fortuita ao transporte do genoma viral acoplado à proteína L2. Nessa condição, a intensa atividade de microtúbulos e microfilamentos pode favorecer uma translocação não apenas dependente de dineína, como também de *β*-actina (YANG; WILDEMAN; SHAROM, 2003) do complexo L2-genoma viral para o núcleo da célula. Até mesmo o fato de o envelope nuclear ser transientemente desintegrado, durante a divisão celular, pode facilitar o aporte do genoma viral no núcleo do queratinócito (PYEON et al., 2009).

Uma vez estabelecido no núcleo, o genoma viral inicia seu programa de expressão e replicação completamente dependente da maquinaria de replicação celular. De fato, nenhuma das proteínas virais apresenta atividade de polimerase, o que torna a replicação viral condicionada ao período de síntese do DNA de sua célula hospedeira durante o ciclo celular (CHOW; BROKER; STEINBERG, 2010). Uma vez que o queratinócito em seu estado terminal de diferenciação não sintetiza novas moléculas de DNA, as atividades das proteínas virais E5, E6 e E7 são especificamente adaptadas para atrasar ou prejudicar o programa de diferenciação desta célula e iniciar novos ciclos celulares sucessivamente. Quando as células basais infectadas se dividem as células-filhas podem se mover lateralmente sobre a membrana basal ou mais adiante na camada celular espinosa. Nessa camada, um subconjunto de queratinócitos aberrantemente reinicia o ciclo celular e amplifica o genoma viral de um baixo para um alto número de cópias (CHOW; BROKER; STEINBERG, 2010).

Os últimos genes virais a serem expressos são os que codificam as proteínas do capisídeo do HPV, L1 e L2. Durante a infecção produtiva, novas partículas virais são montadas e liberadas espontaneamente na camada mais apical do epitélio, momento em que o queratinócito atinge seu estado de diferenciação terminal, morre e é desintegrado do epitélio, num processo fisiológico de descamação do tecido (*anoikis*). Notavelmente, um ciclo infeccioso no qual a liberação de vírions ocorre sem lise celular patológica e sem a liberação de fatores pró-inflamatórios constitui um dos principais mecanismos pelos quais o HPV evita a detecção pelo sistema imune (KANODIA; FAHEY; KAST, 2007).

Figura 3. Esquema ilustrativo do ciclo infeccioso ao longo de 6 a 12 semanas a partir da infecção pelo HPV.



Fonte: Cordeiro MN (2013)

A regressão tumoral, que caracteriza uma lesão benigna, é resultado de uma resposta imune celular competente. A imunidade efetiva consiste de uma resposta celular contra proteínas precocemente produzidas durante a infecção viral, principalmente E2 e E6 (WOO et al., 2010), necessárias para a regressão da lesão concomitante a soro-conversão e produção de anticorpos contra a principal proteína do capsídeo, L1. De maneira geral, o próprio ciclo infeccioso do vírus é um mecanismo que responde pela ineficiente resposta imune do hospedeiro ao vírus, pois ele não gera viremia. Além do baixo nível de expressão de todos os genes virais, seu mecanismo de produção de novas partículas virais é essencialmente não citolítico (PETT et al., 2006).

4.4. Carcinogênese.

Certos fatores ambientais ou intrínsecos ao hospedeiro podem influenciar diretamente a carcinogênese associada ao HPV. Deficiências do sistema imune,

por exemplo, ocasionadas pela Síndrome da Imunodeficiência Adquirida (*Acquired Immunodeficiency Syndrome* – AIDS), polimorfismo da proteína p53, tabagismo e contraceptivos orais são descritos como alguns dos aspectos que propiciam o desenvolvimento da neoplasia intra-epitelial escamosa (PINTO; TULIO; CRUZ, 2002) (WANG et al., 2009).

Entretanto, é a situação de infecção persistente por um HPV de alto risco que resulta em uma exposição prolongada dos queratinócitos infectados às formas mais virulentas das oncoproteínas E5, E6 e E7 do HPV. Em suma, essas oncoproteínas virais estimulam a proliferação celular, sobrevivência celular e modulam a diferenciação dos queratinócitos. Um grande marco no processo oncogênico mediado pelo HPV é a integração do genoma viral ao genoma do hospedeiro. A partir deste evento, a infecção deixa de ser produtiva, pois, frequentemente, a ORF E2, cujo produto é o principal repressor transcricional dos oncogenes E6 e E7, é comprometida (COLLINS et al., 2009) (XUE et al., 2012). Como resultado, uma maior expressão de E6 e E7 potencializa a atividade de suas oncoproteínas. A expressão contínua de E6 e E7 sustenta o fenótipo de câncer, enquanto a expressão de E5 parece ser relevante apenas na fase pré-maligna, ou seja, criar as condições necessárias para iniciar a transformação celular, mas não para mantê-la. Esse fato é apoiado por evidências que sugerem uma ausência ou, pelo menos drástica redução nos níveis de expressão do gene E5 em células pós-integração do HPV (ZIEGERT et al., 2003).

As proteínas de HPV de alto risco E6 e E7 estão submentidas ao mesmo promotor, o que justifica padrões semelhantes de expressão. As proteínas E7 de HPV de alto risco interagem com membros da família de ligases de ubiquitina, como a *ubiquitin protein ligase E3 component n-recognin 4* (UBR4), e contêm um motivo de ligação (LXCXE) para membros da família de proteínas do retinoblastoma, que regulam os fatores transcricionais da família E2F. Sua atividade mais bem conhecida é o direcionamento do supressor tumoral Proteína do Retinoblastoma (pRB) à degradação via ubiquitina, o que promove expressão aumentada de todos os genes subordinados ao fator transcricional E2F, envolvido no avanço do ciclo celular de G1 para S (DYSON et al., 1989) (ZHANG; CHEN; ROMAN, 2006). O produto do gene E6 de HPV de alto risco, que pode ter sua

origem numa recomposição do gene E7 (SHAH; DOORBAR; GOLDSTEIN, 2010), sustenta o avanço do ciclo celular provocado por pela proteína E7 por bloquear a atividade de outro supressor tumoral, a p53 (WERNESS; LEVINE; HOWLEY, 1990). A proteína E6 do HPV16, após se estabilizar pela ligação com uma proteína celular designada E6AP, assume uma conformação ideal para interação com p53 (ANSARI; BRIMER; VANDE POL, 2012), sobre a qual adiciona sinais de ubiquitinação, que conduzem o complexo à degradação proteossomal (SCHEFFNER et al., 1993).

Nos últimos anos, outra oncoproteína associada a lesões causadas por infecção pelo papilomavírus tem se destacado por seu papel no processo carcinogênico associado ao HPV. A proteína E5 é uma pequena proteína (entre 9 e 7Kda) hidrofóbica com aproximadamente 84 aminoácidos, que pode ser localizada na membrana do citoplasma, do retículo endoplasmático e do aparato de Golgi (OETKE et al., 2000).

Essa proteina tem um papel importante na formação da neoplasia celular, por estar implicada na ativação constitutiva do EGFR (Receptor de Fator de Crescimento Epidermal), que por sua vez, ativa vias de transdução de sinal envolvidas na sinalização mitogênica e proliferação celular (PEDROZA-SAAVEDRA et al., 2010), impedindo o trafego de EGFR, inclusive, de modo independente de acidificação de endossomos (SUPRYNOWICZ et al., 2010).

Já foi proposto que a ligação de E5 de HPV16 a uma subunidade de uma ATPase de prótons celular (FEHRMANN; LAIMINS, 2003) é responsável pela perda de capacidade de acidificação do aparato de Golgi e endolisossomos e, consequente, perda de função dessas organelas, como observado durante a infecção pelo HPV16 (SCHAPIRO et al., 2000).

As atividades biológicas de E5 de HPV16 que podem facilitar a carcinogênese incluem a evasão do sistema de imunodetecção do hospedeiro por interferir no transporte de moléculas apresentadoras de antígenos via Complexo Principal de Histocompatibilidade classe I (MHC-I) (ASHRAFI et al., 2006) na membrana celular, promoção de crescimento celular independente de ancoragem (STRAIGHT et al., 1993) e comprometimento das junções *gap* responsáveis pela comunicação célula-célula (TOMAKIDI et al., 2000). Moléculas *cluster of differentiation* classe 1 (CD1d) interrelacionam as respostas immune inata e

adaptativa através de células *Natural Killers* (NK) contra micróbios. A regulação negativa ou *downregulation* de CD1d é utilizada por uma série de microrganismos para evadir a imunodetecção. O estudo de Miura e colaboradores, em 2010, demonstrou que CD1d é menos expressa a nível pós-traducional em células humanas transfectadas com o gene E5 de HPV16 (MIURA et al., 2010).

A participação de oncoproteína E5 de HPV16 na transformação celular ocorre nos estágios iniciais da doença (DIMAIO; MATTOON, 2001), verificando-se que, logo após a infecção, a produção da proteína E5 atinge níveis detectáveis, nas lesões intraepiteliais escamosas de baixo grau. Portanto, E5 está presente nos eventos primários da carcinogênese, onde existem poucas células tumorais e, portanto, torna-se um alvo bastante atrativo e promissor para a erradicação da progressão neoplásica (KIM; YANG, 2006) (VENUTI et al., 2011) (GANGULY, 2012).

4.5. Prevenção e terapia

A vacinação é uma abordagem de destaque como medida de combate ao câncer cervical, visando a proteção principalmente, contra os tipos de papilomavírus conceituados como de alto risco. Devido às dificuldades técnicas da propagação viral *in vitro*, a vacinação baseada em proteínas virais produzidas em organismos recombinantes, como partículas semelhantes ao vírus (VLPs), tem sido a alternativa mais utilizada na pesquisa (HARRO et al., 2001).

Duas vacinas profiláticas baseadas em VLP estão comercialmente disponíveis. A Merck & Co. é responsável pela Gardasil®, uma vacina quadrivalente, eficiente na proteção contra os tipos 6, 11 (baixo risco), 16 e 18 (alto risco) (VILLA et al., 2006), produzida em sistema de expressão heterólogo de levedura. A GlaxoSmithKline responde pela Cervarix®, uma vacina bivalente contra os HPVs tipos 16 e 18 (GARLAND et al., 2007), produzida por meio de sistema de expressão heterólogo em células de inseto. Testes das vacinas profiláticas em diversos grupos humanos demonstraram sua excelente eficácia para a proteção contra a infecção por HPV em mulheres com idade entre 16-26 anos, com eficácia clínica maior nos indivíduos que nunca tinham sido expostos aos tipos virais contemplados pelas vacinas (CUMMINGS et al., 2012).

A eficácia da vacinação é, entretanto muito menor em mulheres com infecção prevalente por HPV (PAAVONEN et al., 2009). Em um estudo de grupos

com intenção de tratamento, incluindo aqueles com infecção prevalente por HPV, a taxa de redução de lesões perianais vaginais ou vulvares causadas por HPV foi de 34%, enquanto para lesões cervicais foi de apenas 20% (GARLAND et al., 2007).

O alto custo das vacinas dificulta sua distribuição pela rede pública de saúde; sua abrangência a poucos tipos de papilomavírus, assim como sua baixa eficiência contra os casos onde já existe a infecção apontam para um baixo impacto no controle do câncer cervical por meio dessas vacinas. A vacinação profilática contra o papilomavírus humano pode ainda não oferecer uma interferência notável, a curto e médio prazo, na alta incidência de câncer cervical.

Diante deste quadro, uma estratégia terapêutica no combate à infecção pelos papilomavírus humanos e, por consequência, contra o câncer cervical constitui uma segunda frente de pesquisa. No momento, o foco do combate ao câncer cervical em mulheres já diagnosticadas com lesões consiste em aumentar as taxas de sobrevivência, principalmente por meio de terapias direcionadas em combinação com a quimio-radiação padrão(DEL CAMPO et al., 2008). A histerectomia radical com linfadenectomia pélvica e radioterapia apresentam taxas de sobrevida similares em casos de diagnóstico precoce do câncer cervical (LANDONI et al., 1997), enquanto a quimio-radioterapia é o tratamento padrão para doença local avançada (CHEMORADIOTHERAPY FOR CERVICAL CANCER META-ANALYSIS COLLABORATION (CCCMAC), 2010). Quimioterapia neoadjuvante seguida de histerectomia radical é uma recente opção terapêutica para os estágios mais avançados de câncer cervical de células escamosas (BENEDETTI-PANICI et al., 2002) (GADDUCCI et al., 2010).

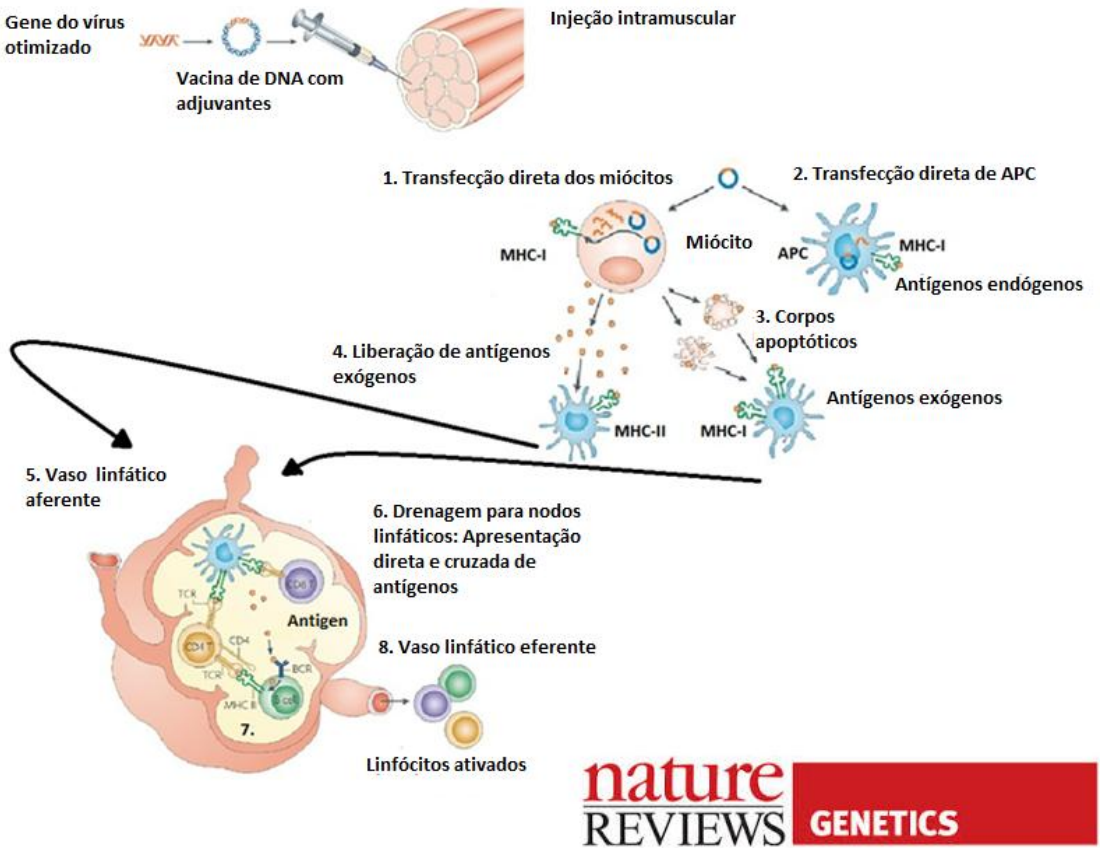
4.6. Vacinas de DNA Anti-HPV

Nas últimas décadas, uma nova abordagem terapêutica se destaca por conduzir uma opção de indução do sistema imune. Apesar de evidências em meados dos anos 80, foi em 1990 que Wolff e colaboradores publicaram resultados que indicaram a habilidade de simples plasmídeos de DNA de entrarem em células de mamíferos, quando injetados *in vivo*, resultando na síntese da proteína codificada (WOLFF et al., 1990). O plasmídeo em si não necessitou de outra alteração que não a substituição do promotor bacteriano por um promotor ativo em mamíferos. Esses primeiros resultados iniciaram uma série

de pesquisas que fomentaram o que hoje é conhecido por imunização genética, uma das estratégias mais recentes para o tratamento de doenças.

O DNA codificando um antígeno vacinal pode ser liberado no organismo por uma variedade de rotas, incluindo injeção intramuscular (IM). O antígeno é produzido dentro das células do organismo vacinado e portanto, usa a maquinaria traducional do hospedeiro. A proteína vacinal (contendo os epitopos íntegros) é processada pela via de reconhecimento antigênico do MHC-I e apresentada para reconhecimento pelo sistema imunológico, promovendo uma resposta imune celular. Desta forma, as vacinas de DNA atuam de forma semelhante às vacinas virais atenuadas, sendo capazes de efetivamente induzirem resposta humoral, a depender da co-expressão de imunomoduladores, e, principalmente, uma resposta celular, tão necessária para o sucesso de uma terapia (DUNHAM, 2002).

Figura 4. Esquema ilustrativo sobre o princípio de ação de uma vacina de DNA.



Fonte: Michele A. Kutzler & David B. Weiner (2008).

A plataforma vacinal de DNA plasmidial pode representar uma intervenção terapêutica para estágios precoces do câncer cervical ou doença associada ao HPV, uma vez que é capaz de gerar resposta imune celular para prevenir a progressão maligna (KIM et al., 2008). De fato, diversos estudos recentes sugerem antígenos virais baseados em E6 e E7, as principais oncoproteínas de HPV, administrados através de imunização genética como terapia anticânceres induzidos por HPV (CHENG et al., 2001) (PENG et al., 2004) (LESCAILLE et al., 2013) (LEE; DANISHMALIK; SIN, 2015).

Uma vacina baseada em um plasmídeo apresenta ainda uma característica interessante do ponto de vista imunológio; por conter motivos de deoxiribonucleotídeos trifosfato de citosina seguido de trifosfato de guanina (CpG) não metilados, o DNA inoculado pode ser reconhecido por receptores *Toll-like* 9, atuando assim como um adjuvante cujo processo inflamatório local atrai células do sistema imune para o local (HEMMI et al., 2000) (SHIROTA; KLINMAN, 2014). Entretanto, a maioria dos estudos reconhece a necessidade de incrementar a resposta imune elicitada contra qualquer antígeno viral, codificado na vacina, através de associação a algum imunomodulador, adjuvante molecular ou terapia auxiliar. Estudos preliminares sugerem uma melhor eficiência de vacinas terapêuticas baseadas em E7 por meio de combinação à terapias anti-câncer indicadas na clínica, como, por exemplo, o uso de agentes anti-angiogênicos (PENG et al., 2011).

Estratégias para elicitar uma resposta imune celular mais eficiente incluem, por exemplo, induzir a ativação simultânea de células TCD8+ e TCD4+ auxiliares. A possibilidade explorar ambas as vias de processamento do antígeno, através dos mecanismos de apresentação de MHC-I e MHC-II, já foi demonstrado ao se dirigir o antígeno E7 para compartimentos lisossomais, ao longo de seu processamento (JI et al., 1999). Ainda, assumindo que as células dendríticas tenham um papel determinante na eficiência da montagem de uma resposta imune contra um antígeno de HPV, durante a imunização genética, uma proposta interessante é aumentar o tempo de sobrevida de células dendríticas que tenham sido transduzidas pela vacina de DNA. Co-administração de DNA codificante para o antígeno de E7 com DNA codificante para inibidores da apoptose de células

dendríticas foi empregado com relativo sucesso em elicitar uma maior resposta celular em camundongos (KIM et al., 2003).

Ajuvantes são compostos que aumentam a duração, qualidade e magnitude da resposta imune específica a antígenos. A adição de adjuvantes a vacinas pode reduzir a quantidade de antígeno e o número de imunizações necessárias para se atingir a resposta imune desejada (DUBENSKY; REED, 2010). Nesse sentido, para se induzir uma robusta resposta imune citotóxica, um adjuvante deve provocar a imunidade inata, levando ao recrutamento, ativação e maturação de células apresentadoras de antígenos, como as células dendríticas, ou mesmo otimizar o processamento do antígeno via MHC-I ou MHC-II. Por exemplo, em ensaio clínico, uma vacina de DNA direcionada contra a oncoproteína E7 de HPV16 foi geneticamente fusionada a uma chaperona de *Mycobacterium tuberculosis*, Hsp70, para potencializar o processamento e apresentação pelo MHC I de células apresentadoras de antígenos. Detecção de resposta T específica e regressão tumoral em 3 de 9 pacientes tratados forneceram indícios dos efeitos dessa abordagem (TRIMBLE et al., 2009).

Diversas abordagens também têm o objetivo de incrementar a “entrega” da vacina de DNA, para permitir o maior acesso possível à células do sistema imune de interesse. Essas abordagens em ensaios pré-clínicos e clínicos variam desde protocolos de eletroporação *in vivo*, revestimento de partículas de ouro até conjugação a polímeros. O sistema de imunização genética via biobalística ou *gene gun*, na qual nanopartículas de ouro são revestidas da vacina de DNA e bombardeiam sob alta pressão o tecido, resultou em um dos sistemas mais eficientes de imunização genética anti-HPV. Com grande vantagem sobre a injeção intramuscular, vacinas de DNA baseadas no gene E7 de HPV16 aplicadas sob regime de biobalística, a uma dose menor e com menos repetições, gerou magnitude de resposta TCD8+ superior (TRIMBLE et al., 2003). A eletroporação *in vivo* é considerada um dos grandes avanços tecnológicos recentes por aumentar a assimilação de plasmídeos e gerar infiltração de células inflamatórias locais. A aplicação de curtos pulsos elétricos no local da injeção intramuscular já teve sua eficácia testada em estudo clínico utilizando vacinas de DNA baseadas nos genes E6 e E7 dos HPV16 e 18 (BAGARAZZI et al., 2012).

De fato, alguns testes clínicos atuais começam a demonstrar os potenciais benefícios de vacinas de DNA contra infecções e lesões relacionadas ao HPV. Um plasmídeo codificando o epítopo compreendido entre os aminoácidos 83 a 95 da proteína E7 de HPV16 antígeno leucocitário humano (HLA-A2) restrito, formulado com micropartículas de polímero biodegradável, foi desenvolvido para tratamento. A resposta imunológica e sua segurança foram avaliadas na fase I de testes clinicos sobre 12 pacientes portadores de neoplasia intra-epitelial anal, dos quais 10 exibiram resposta imune específica (KLENCKE et al., 2002). Em outro teste, essa mesma vacina induziu resposta T específica em 11 de 15 pacientes portadores de neoplasia intra-epitelial cervical 2 e 3, dos quais cinco apresentaram regressão completa da lesão (SHEETS et al., 2003).

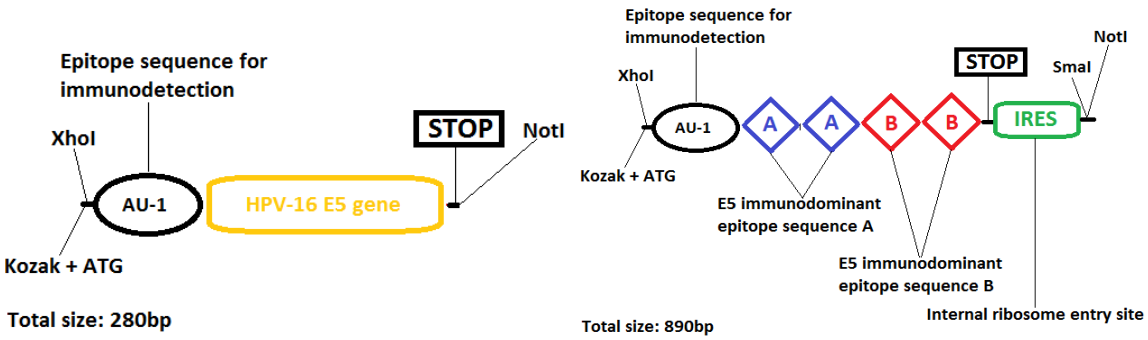
Ainda que sejam promissores, nenhuma vacina de DNA terapêutica contra o HPV se encontra licensiada, o que reforça a necessidade de estudos pré-clínicos que potencializem a eficiência em elicitar uma resposta imune adequada. Explorar novos alvos terapêuticos como a proteína E5 pode fornecer indícios para estratégias inovadoras no combate e controle da infecção pelo HPV.

5. METODOLOGIA

5.1 Descrição das vacinas de DNA

O gene sintético E5 de HPV-16 (237pb) foi construído com base na referência NP_041330.1, e seus códons foram otimizados de acordo com aqueles de uso mais frequente para mamíferos (*codon usage*) (*Epoch Biolabs*), além da inserção de sequências para epitopo AU-1, Kozak e sítios de restrição, totalizando o gene E5H16 em 280pb. Uma segunda sequência, denominada multiepitopos de E5 (224pb) também foi sintetizada, contendo apenas os dois epítopos imunodominantes, em duplicata, citados na literatura (LIU et al., 2007). A sequência multiepitopos de E5 possui ainda, em sua porção 3', um sítio interno de entrada ribossomal (*internal entry ribosome site* ou IRES) (620pb). Semelhantemente ao gene E5H16 citado, foi adicionado a sequência para o epitopo AU-1, a 5', Kozak e sítios de restrição, totalizando 890pb para o gene E5Multi. Para uma melhor compreensão sobre os genes construídos, com descrição detalhada de seus sítios de restrição, códon de início e parada, inserção do IRES e demais sequências, vide **Apêndice I**. A ilustração detalhada de cada gene, com seus respectivos sítios de restrição e sequências para detecção pode ser vista na Figura 5. Os genes E5 e E5Multi foram devidamente clonados no vetor de expressão pCI-neo (Promega™).

Figura 5. Representação ilustrativa dos genes E5H16 e E5Multi.



O gene mutante derivado do gene E6 de HPV16, denominado E6F47R (gentilmente cedido por G Travé; Strasburg, França) é deficiente para poliubiquitinação, tornando a proteína E6 mutante incapaz de degradar a p53

(RISTRIANI et al., 2009). O gene E6F47R foi fusionado ao gene da proteina do capsídeo do Potato Virus X, para a confecção de uma vacina de DNA (De Giuli Morghen, comunicação pessoal), e clonado no vetor de expressão pCDNA3.1.

O gene $E7_{GGG}$, descrito por Massa et al., 2008 (MASSA et al., 2008), derivado do gene E7 de HPV16, contém três mutações pontuais em sítios de ligação à proteína pRb, o que torna seu produto incapaz de interagir com a pRb. O gene $E7_{GGG}$ foi fusionado à sequência correspondente à proteína do capsídeo do Potato Virus X, para a composição de um candidato à vacina de DNA, e clonado no vetor de expressão pCDNA3.1. Cada preparação plasmidial foi devidamente purificada de cultura bacteriana por gradiente de CsCl para obter formulações livres de endotoxinas.

5.2 Descrição do vetor de expressão bacteriana

O gene E5 foi inserido no vetor não comercial pAE (RAMOS et al., 2004), conforme os sítios de restrição adicionados por ocasião de sua síntese. O vetor pAE fusiona uma cauda de histidina (6XHIS), como segunda opção de imunodetecção, além do epitopo AU1, previamente citado. O plasmídeo resultante pAE-E5H16 foi usado como agente transformante para células quimicamente tratadas *Escherichia coli* (DH5α), conforme metodologia de clonagem molecular descrita por Sambrooks e Russel, 2001 (SAMBROOKS; RUSSEL, 2001). A composição plasmidial desejada foi verificada através de análises de restrição, *Polymerase Chain Reaction* (PCR) e sequenciamento.

5.3 Eletroporação para transformação bacteriana

As células bacterianas (DH5α) contendo os vetores pAEE5H16 ou pAEE5multi, tiveram seus plasmídeos extraídos por kit de extração (Plasmid Maxi Kit - Qiagen™). Por meio de eletroporação (1800V/5ms), em equipamento BTX-Harvard Apparatus ECM 830 Square Wave Electroporator células de *E.coli* da linhagem BL21 previamente tratadas, receberam os plasmídeos, gerando clones contendo uma ou outra construção.

5.4 Indução

As células de *E. coli* (BL21) contendo o plasmídeo de interesse foram selecionadas quanto a sua resistência à ampicilina e repicadas, obtendo-se culturas frescas para início dos ensaios de indução da expressão. Foi realizada cultura em 500mL de meio Luria-Bertani (LB), com indução por 1mM de IPTG a

23°C sob 200rpm de agitação durante 4 horas. A proteína E5 foi purificada por resina de Ni-NTA (Qiagen), de acordo com instruções do fabricante, e analizada em SDS-PAGE 23%. O immunoblotting foi realizado com anticorpo primário anti-HIS de camundongo e anticorpo secundário anti-camundongo conjugado à peroxidase (Invitrogen). A presença de bandas reativas foi revelada por quimioluminescência (ECL-kit,Amersham).

5.5 Linhagens celulares e RT-PCR

As células tumorais C3 (uma doação de CJM Melief) são células embrionárias de camundongo transformadas com o genoma completo de HPV16 e portadoras de mutação no gene *ras* (FELTKAMP et al., 1993). As células C3 são singênicas ao camundongo C57BL/6 e foram cultivadas em meio RPMI-1640 com 10% de soro fetal bovino (Life Technology).

Antes do inóculo, como desafio tumoral, as células C3 foram submetidas à tripsinização, lavadas duas vezes em tampão PBS, em ressuspendidas em solução salina para uma concentração de 5 x 10^5 células/mL. O RNA total foi extraído de células C3 em cultura pelo RNeasy Plus Mini kit (Qiagen), de acordo com instruções do fabricante. O RNA total foi pré-tratado com desoxirribonuclease I (DNase I, Amplification grade, Invitrogen), submetido à retrotranscrição, para produção de cDNA por 1 hora a 42°C, usando *primers* hexaméricos randômicos, como descrito pelo fabricante (GeneAmp RNA PCR kit Applied Biosystem). O ensaio foi realizado com e sem a transcriptase reversa, para excluir falsos positivos. O cDNA obtido foi submetido a PCR com polimerase Platinum TaqDNA (Invitrogen) por meio de *primers* específicos para o gene E5, seguindo o protocolo: 95 °C por 3 minutos, 35 ciclos de denaturação a 95°C por 50 segundos, anelamento a 55°C por 50 segundos e elongação a 72°C por 60 segundos. Os amplicons foram visualizados em gel de agarose corado com brometo de etídio. Os primers para o gene E5 foram desenhados pelo software OligoAnalyzer Tool (Integrated DNA Technologies) e sintetizados de acordo com as sequências: Forward: 5′–ATCTCGAGGC CACCATGGGA TACTGCATTC ACAATATAAC–3′ Reverse:5′- TAGCGGCCGC GAATTCTTAT CATGTGATCA GGAATCTTG – 3′.

5.6 Enzyme-linked immune-sorbent assay (ELISA)

Os anticorpos anti-E5 de HPV16 no soro foram determinados por meio de ELISA direto. Uma placa de 96 poços foi revestida a 4°C durante 16 horas com

200ng de E5 de HPV16 obtido de *E. coli* recombinante e depois bloqueada com 150ul de leite desnatado a 5% (w/v) em PBS (MPBS). Os soros foram recolhidos no dia 14 após o *boost*, diluídos em 2% de MPBS (1:100 e 1:50), e incubados durante 2 horas a 37°C. IgG total foi detectada com anticorpo caprino anti-camundongo conjugado à peroxidase de *horseradish* (HRP) (MP Biomedicals Life Sciences). A atividade enzimática foi medida através da adição do substrato 2,2-azino-bis (3-etilbenztiazolino sulfonato) e a absorvância foi lida a 405nm em um leitor de placas de microtítulo de ELISA.

5.7 IFN-gamma enzyme-linked immuno-spot assay (ELISPOT)

Precursores de células T específicos contra E5 de HPV16 foram detectados por ELISPOT para células secretoras de IFN-gama (BDTMELISPOT BD Biosciences Pharmingen) de acordo com protocolos relatados anteriormente (MIYAHIRA et al., 1995). Resumidamente, uma suspensão de esplenócitos colhidos de cada grupo de camundongos vacinados foi adicionado aos micropoços de uma placa de imunoensaio de 96 poços que foram pré-revestidos por 16 horas a 4°C com anticorpo anti-IFNgama de camundongo (BD Biosciences Pharmingen). A estimulação dos linfócitos foi realizada em triplicado a 37°C durante 72 horas, com a proteína E5. Uma mistura de acetato de phorbolmyristate (PMA) foi utilizado para detectar a capacidade de resposta celular. As placas foram incubadas com um anticorpo anti-IFN-gama de camundongo biotinilado (BD Biosciences Pharmingen) durante 4 horas à temperatura ambiente. Estreptavidina conjugada a HRP foi em seguida adicionada durante 1 hora a temperatura ambiente, e os *spots* de células marcadas foram filtrados com substrato 3-amino-9-etilcarbazole, durante 1-5 minutos. Os *spots* foram contados sob um microscópio de dissecação.

5.8 Animais e esquema de vacinação

Laboratórios Charles River forneceram camundongos C57BL/6 de 6-8 semanas do sexo feminino. Todos os procedimentos que envolvem o manuseio e sacrifício de animais foram realizados sob condições específicas isentos de agentes patogênicos no Biotério do Instituto Nacional do Câncer Regina Elena, Roma-Itália. A Comissão de Ética aprovou os protocolos desenvolvidos em conformidade com as orientações européias números 86/609/CEE e 116/92 para a proteção dos animais experimentais de laboratório e cuidados com animais de

laboratório (Ministério da Saúde, Departamento de Saúde Pública Veterinária, Nutrição e Segurança Alimentar, Protocolo 17/2006).

Dois protocolos de imunização foram adotados: o protocolo de resposta imune, em que os camundongos receberam vacinações múltiplas antes do desafio tumoral, e o protocolo de imunização terapêutica, no qual os camundongos foram desafiados com células tumorais antes da administração das vacinas. No protocolo de imunização, os animais foram vacinados com DNA plasmidial pCI-E5H16 (50µg/camundongo, IM) ou com DNA plasmidial pCI-E5Multi (50µg/camundongo IM), e receberam reforço 3 vezes a intervalos de uma semana entre as doses. Após quatro semanas os camundongos foram desafiados no flanco posterior com injeção sub-cutânea (SC) de 200µl de solução salina contendo 5 x 10^5 células tumorais C3. O crescimento do tumor foi monitorizado por inspeção visual e palpação três vezes por semana. Os animais foram pontuados como portador de tumor, quando os tumores atingiram um tamanho de aproximadamente 1 a 2mm de diâmetro.

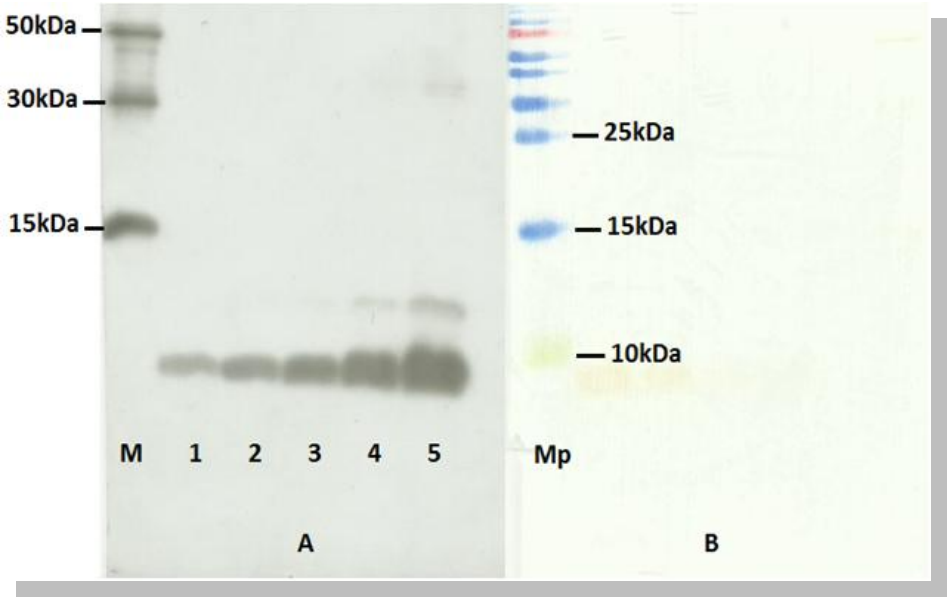
Na imunização terapêutica, 5 grupos de 5 camundongos foram desafiados por células tumorais C3 como descrito anteriormente. Três dias após o inóculo do tumor, os camundongos foram vacinados (*prime*) com preparações de DNA plasmidial por injeção intramuscular seguida de eletroporação *in vivo*. O reforço (*boost*) foi realizado uma semana após a primeira dose usando as mesmas preparações plasmídiais. O tamanho do tumor foi medido com compasso de calibre, e o volume estimado pela fórmula (comprimento x largura × 0,52). A vacinação com DNA plasmidial mediada por eletroporação foi realizada de acordo com o método descrito por Seo et al. 2009 (SEO et al., 2009). Resumidamente, os camundongos foram injetados com 100µL de solução salina contendo 50µg de cada vacina de DNA no músculo tibial da perna esquerda raspada. A injeção de DNA foi seguida imediatamente por eletroporação de onda quadrada no local usando um aparelho BTX830 (BTX Harvard Apparatus). Um eletrodo de pinças (Aparelho Harvard BTX) foi utilizado para descarregar seis pulsos de 100V/cm durante 20ms.

6. RESULTADOS

6.1 Produção de proteína E5 em bactérias

Para verificar efeitos de uma vacina baseada em E5 foi necessário obter proteína purificada para testes de rastreamento de anticorpos ou células T reativas à proteína E5. E5 é uma proteína hidrofóbica e muito pequena, representando certa dificuldade em se produzi-la em sistema de *E. coli*. No entanto, conseguimos produzir E5 de HPV16 fusionada à cauda de histidina em linhagem de *E. coli* cepa BL21. Após a indução e a lise das células por sonicação, E5 foi purificado por resina de Ni-NTA. Análise de Western Blot (WB) acusou a presença de uma proteína com o tamanho esperado, indicando a expressão correta da proteína E5 por este sistema (Figura 6). O rendimento de proteína E5 purificada, quantificado de acordo com o método de Bradford, foi de 1,68µg/mL a partir de uma cultura bacteriana de 500mL.

Figura 6. Produção da proteína E5 de HPV16; **Colunas de 1 a 5:** Diluições seriadas de proteína purificada por resina Ni-NTA a partir de extratos de bactéria recombinante. **Coluna M:** Marcador molecular 6XHIS Protein Ladder™ (Qiagen).

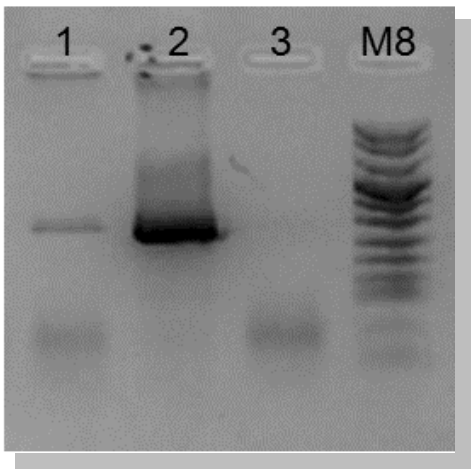


6.2 Análise da expressão de E5 de HPV16 em células tumorais C3 por RT-PCR

A linhagem C3 já foi utilizado como modelo de desafio para vacinas terapêuticas baseadas em E6/E7, por expressar esses genes, mas para ser adequada para teste de vacina terapêutica baseada em E5 não havia informações

disponíveis sobre sua expressão nesta linhagem. RT-PCR foi realizada para detectar a presença de transcritos do gene E5 de HPV16. RNAm de E5 específicos foram detectados, como mostrado na Figura 7, indicando a adequação da linhagem celular C3 como modelo tumoral pré-clínico de teste para vacinas baseadas em E5.

Figura 7. Detecção do RNAm do gene E5 de HPV16 em linhagem celular C3; **Coluna 1:** Amplicon do gene E5 obtido de cDNA produzido a partir de RNA total de cultura celular C3. **Coluna 2:** Amplicon do gene E5 obtido de DNA plasmidial pCI-E5H16, como controle positivo da reação. **Coluna 3:** RNA total de cultura celular C3 não tratado com transcriptase reversa e submetido à PCR, como controle negativo.

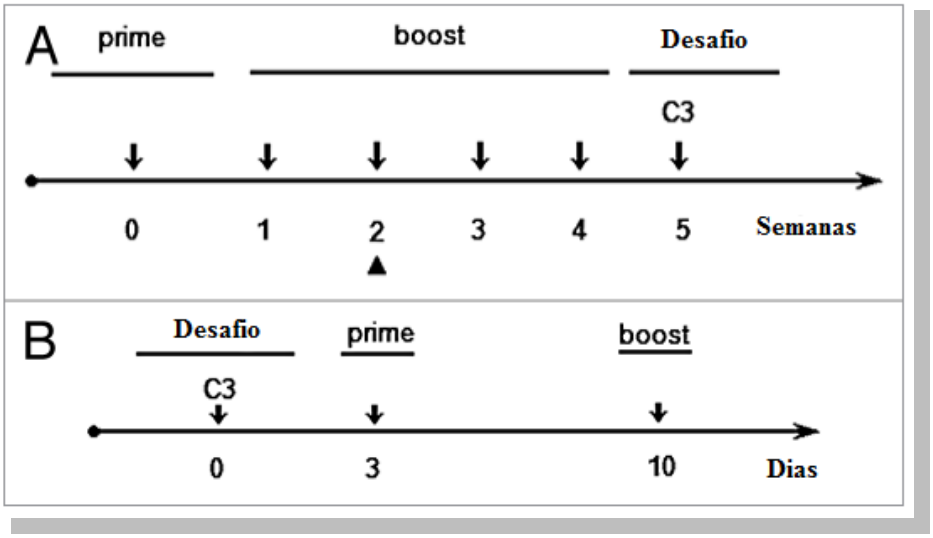


6.3 Resposta imune em camundongos imunizados com os genes E5 e E5multi

Em lesões cervicais pré-malignas, quando E5 ainda é expressa, uma vacina direcionada para células que expressam o oncogene E5 pode ser uma boa estratégia para evitar lesões pré-malignas de progredir para cânceres cervicais invasivos. Nosso trabalho gerou duas construções diferentes como candidatos a vacina genética: o gene E5 completo e uma versão modificada sem atividade oncogênica. De fato, a proteína E5 nativa pode ser prejudicial a seres humanos devido a sua atividade oncogênica, e, portanto, foi desenhado um gene baseado no oncogene E5, o E5Multi, que contém apenas duas sequências previamente descritas (LIU et al., 2007) (CHEN et al., 2004), que codificam epítopos imunogênicos, em duplicata. Desta forma, o gene E5Multi codifica um antígeno que deve apresentar imunogenicidade aumentada e, entretanto, se qualquer atividade oncogênica.

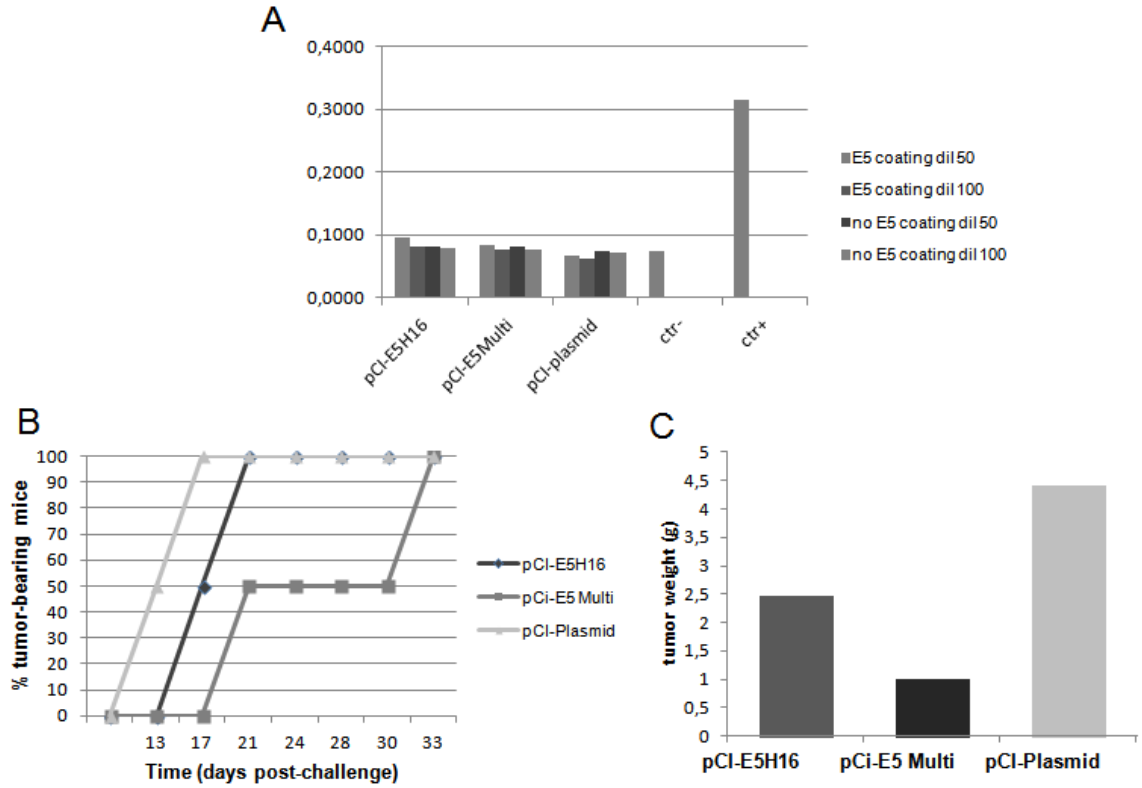
Os efeitos imunológicos das vacinas baseadas em E5 foram verificados em camundongos C57BL/6 com o esquema de vacinação *prime/boost* descrito na Figura 8A. Após 14 dias, o soro dos animais vacinados foi recolhido e testado para a presença de anticorpos específicos contra E5. Nenhum anticorpo circulante foi detectado no ELISA com proteínas E5 obtidas de *E. coli* (Figura 9A).

Figura 8. Esquema de imunização preventiva e terapêutica *prime/boost*; **A:** Esquema de imunização preventiva, onde a flecha escura representa o ponto de coleta de sangue para ELISA. **B:** Esquema de imunização terapêutica.



No entanto, uma semana após o último reforço, os camundongos foram desafiados com 5 x 10^5 células tumorais C3 e o volume do tumor foi medida três vezes por semana com um compasso. Como mostrado na Figura 9B, a vacinação com base no gene E5 e, em particular, a vacinação com o gene E5Multi, retardou significativamente o desenvolvimento do tumor induzido por célula C3, enquanto o inóculo com plasmídeo vazio não teve nenhum efeito anti-tumoral. Os tumores foram colhidos cinco semanas após o desafio e os seus pesos foram medidos. Os tumores de animais vacinados com os plasmídeos contendo os genes E5 e E5Multi foram quase 2 ou 4 vezes menores do que os tumores colhidos do grupo-controle, respectivamente (Figura 9C).

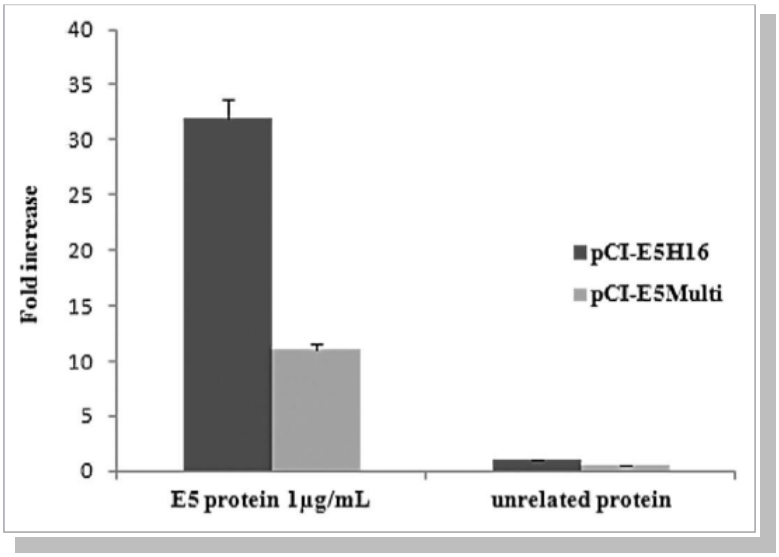
Figura 9. Imunização preventiva baseada no gene E5 de HPV16; **A:** ELISA, com dados expressos em média para cada grupo de animais sob desvio padrão para mais ou para menos. **B:** Crescimento tumoral. **C:** Carga tumoral; Cinco semanas após o desafio, os animais foram sacrificados, seus tumores foram excisados e mensurados em balança analítica. Dados estão representados em média para cada grupo de animais sob desvio padrão para mais ou para menos.



6.4 Imunização baseada no gene E5 e E5Multi induz resposta de células T em camundongos vacinados

Uma vez que as células T citotóxicas CD8+ têm um papel reconhecido como efetores em respostas anti-tumorais, nós sugerimos que a resposta anti-tumoral nos animais sem anticorpos específicos foi induzida por células T produtoras de IFN-gama especificamente ativadas por reconhecimento do antígeno E5 via imunização genética. Cinco semanas após o desafio com células C3, os baços foram colhidos a partir de camundongos imunizados no esquema de *prime/boost* descrito na Figura 8A. Esplenócitos obtidos foram testados em ensaio ELISPOT para produção de INF-gama. A proteína E5 produzida em *E. coli* foi utilizada para ativar os linfócitos anti-E5. Células secretoras de IFN-gama foram visualizadas e contadas como *spots* sob microscópio de dissecação.

Figura 10. Resposta imune mediada por células em animais vacinados; A produção de IFN-gama foi mensurada em ELISPOT após estímulo antigênico específico com proteína E5. Dados estão representados como aumento de vezes de resposta contra a proteína E5, comparado com o grupo controle, e correspondem às médias de todos os grupos animais com desvio padrão para mais ou menos.



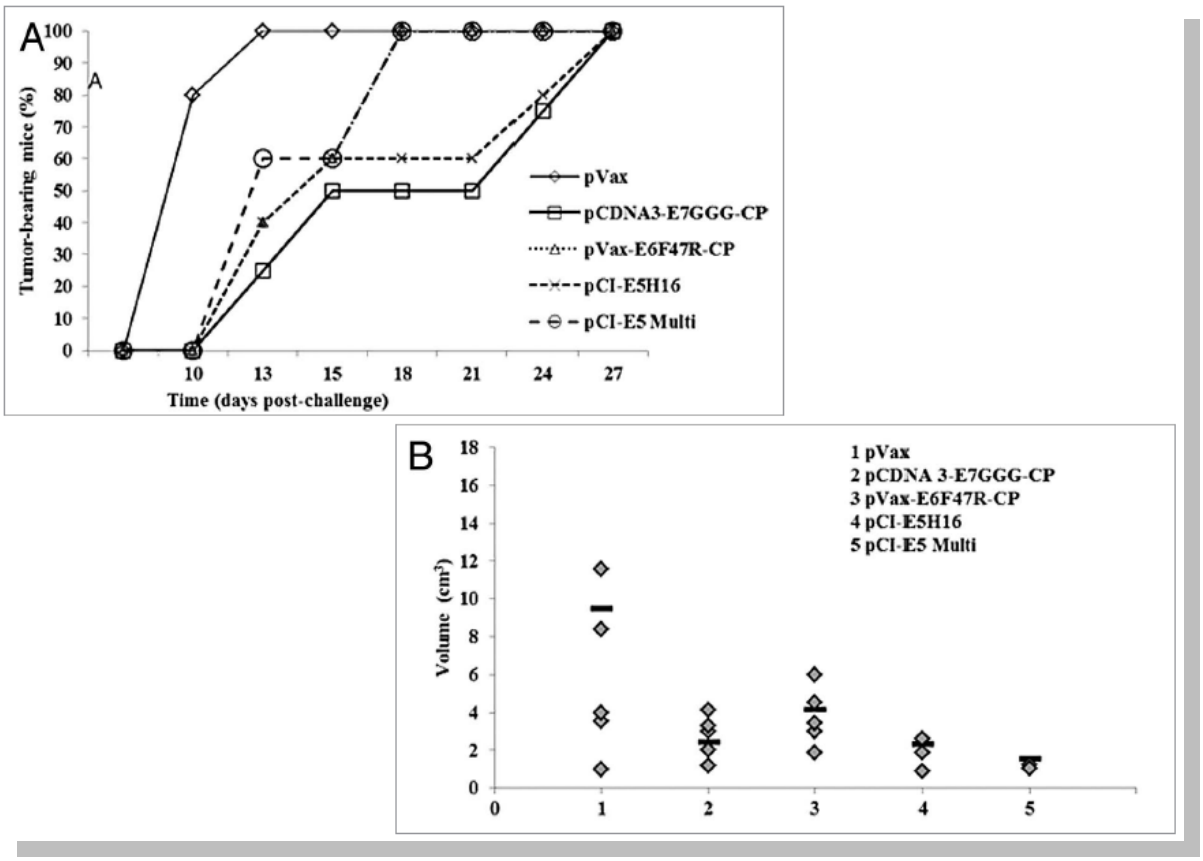
A pontuação mais alta foi obtida em camundongos vacinados com o DNA plasmidial pCI-E5H16, apresentando resposta de células T específica contra E5 cerca de 30 vezes mais elevada do que a observada em camundongos-controle, como mostrado na Figura 10. Além disso, a vacina E5Multi apresentou resposta de células T 10 vezes maior que o controle. Estes resultados indicam que a resposta específica mediada por células T contra a proteína E5 pode ser gerada em camundongos vacinados com os genes E5 e E5Multi.

6.5 Imunização terapêutica

A atividade imunogênica verificada destas novas vacinas anti-E5 levou-nos a examinar seu potencial terapêutico no modelo pré-clínico de desafio com células C3. Além disso, duas vacinas já produzidas contra as proteínas E6 e E7 de HPV16, pCDNA3-E7$_{GGG}$-CP e pCDNA3-E6F47RCP, respectivamente, foram utilizadas para comparação. Depois de injetar as células C3 em camundogos C57BL/6, vacinação (*prime*) e reforço (*boost*) foram realizados nos dias 3 e 10, respectivamente (Figura 8B). Como mostrado na Figura 11A, o desenvolvimento do tumor foi atrasado até ao dia 13 pós-desafio em 75% dos animais tratados com pCDNA3-E7$_{GGG}$CP, em 60% dos camundongos vacinados com pCDNA3-E6F47R-CP e pCI-E5H16, e em 40% dos camundongos vacinados com pCI-

E5Multi. No mesmo dia, 100% dos camundongos imunizados com o vetor vazio desenvolveram tumores. Ao dia 21, o percentual de camundongos livres de tumor era de 50% no grupo vacinado com pCDNA3-$E7_{GGG}$-CP e 40% no grupo vacinado com pCI-E5H16 (Figura 11A). Após 4 semanas, todos os animais foram afetados por tumores, mas as diferenças na carga tumoral foram detectadas por medição do volume do tumor. Os camundongos vacinados com o gene E7 demonstraram a presença de tumores pequenos, confirmando dados relatados em um estudo anterior (MASSA et al., 2008) e, interessantemente, os camundongos vacinados com o gene E5 mostraram tumores ainda menores (Figura 11B).

Figura 11. Crescimento tumoral após imunização terapêutica baseada em E5, E6 e E7; A: Crescimento tumoral. B: Carga tumoral. (━) representa o valor médio para cada grupo de animais.



7. DISCUSSÃO

Vacinas baseadas em DNA foram investigadas como promissoras abordagens terapêuticas contra células infectadas com HPV, com potencial progressão maligna, devido a sua segurança, facilidade de produção com alto grau de pureza e capacidade de fornecer expressão estável do antígeno codificado. A imunização genética por meio de plasmídeos está fundamentada na produção de antígenos virais de forma não replicativa, sem poder infeccioso e não oncogênica, capaz de provocar, através de células apresentadoras de antígenos, resposta imune citotóxica e/ou humoral específica. Nesse contexto, apresentamos um estudo pré-clínico focado na imunização genética baseada num alvo terapêutico pouco explorado, o gene *E5* de HPV16.

O primeiro ponto a se discutir é a importância de *E5* como alvo terapêutico. Enquanto a expressão de *E6* e *E7* de HPV de alto risco é mantida ao longo da infecção e mesmo nos estágios mais avançados de malignização celular, o que os qualifica como alvos terapêuticos, a expressão de *E5* é frequentemente perdida ou dificilmente detectada após o evento de integração (CHANG et al., 2001). Esse fato sugere que a imunidade, ainda que eficientemente induzida pela proteína viral E5, não seria capaz de combater células cancerosas. Entretanto, a relevância cada vez maior das atividades de E5 em criar condições celulares para a transformação de queratinócitos infectados, qualifica a escolha deste antígeno como uma estratégia promissora para a terapia contra a infecção pelo HPV e prevenção da progressão de lesões pré-cancerosas para câncer invasivo.

Como parte da estratégia de construção dos plasmídeos candidatos vacinais, optamos neste trabalho por otimizar os códons da sequência do antígeno E5 de HPV16, recompondo o gene conforme os códons preferencialmente utilizados por células de mamíferos, além de inserir sequência consenso Kozak. De fato, muitos estudos acerca das atividades biológicas de E5 de HPV foram dificultados pelos baixos níveis de expressão em linhagens celulares estáveis. Foi relatado que 33 dos 83 aminoácidos codificados no gene E5 de HPV16 dependiam de códons raramente utilizados por genes de mamíferos genes (DISBROW et al., 2003). O mesmo trabalho obteve um aumento da produção proteica entre 6 a 9 vezes, em células derivadas de fibroblastos de

macacos (COS), após o gene *E5* de HPV16 ser submetido à otimização de códons, o que suporta sua otimização neste trabalho. A otimização de códons e a adição de sequência Kozak são adaptações comumente empregadas no desenho de vacinas de DNA, como incremento de produção do antígeno e aumento de sua imunogenicidade, como já observado na avaliação de imunização genética baseada em E6 e E7 em ensaios pré-clínicos e clínicos (BAGARAZZI et al., 2012).

Outra modificação importante na sequência de antígenos de HPV para a construção de vacinas de DNA é a quebra de atividade oncogênica exigida para a segurança da vacina. Trabalhos prévios relatam mutações inseridas no gene E7 de HPV16, para perda de sua capacidade de interação com membros da família pRB (TRIMBLE et al., 2009) (SMAHEL et al., 2001). Nosso trabalho construiu um gene correspondente a potenciais epitopos imunodominantes da proteína E5 em um dos candidatos a imunização genética, designado E5Multi, comprometendo a atividade oncogênica de E5, mas mantendo sua imunogenicidade. O antígeno E5Multi foi elaborado com base nas sequências correspondentes aos epitopos VCLLIRPLL e YIIFVYIPL, o primeiro relatado em estudo pré-clínico utilizando vacina de peptídeo em camundongos C57BL (CHEN et al., 2004) e o segundo obtido de sequência de aminoácidos preditas *in silico* e testadas quanto a indução de resposta T citotóxica de camundongos transgênicos (LIU et al., 2007).

Em paralelo, nosso trabalho também produziu a proteína E5 de HPV 16 em sistema de expressão bacteriano, a qual foi purificada através de resina Ni-NTA e utilizada como estímulo específico em ensaios *Enzyme-Linked ImmunoSpot* (ELISPOT) posteriores. Curiosamente, a substituição de códons por aqueles preferencialmente utilizados por células de mamíferos pode não ter sido prejudicial para a expressão em *Escherichia coli*. Enquanto trabalhos anteriores, utilizando o gene E5 de HPV16 não-otimizado, recorreram a técnicas de purificação por cromatografia de alta afinidade (YANG; WILDEMAN; SHAROM, 2003) ou purificação por adsorção à *beads* de glutationa-agarose (KELL et al., 1994), com rendimentos variáveis e apropriados a seus objetivos, obtivemos cerca de 1g de proteína E5, partindo de uma cultura de 500mL.

A maior parte dos estudos pré-clínicos acerca de vacinas terapêuticas contra a infecção por HPV ou contra neoplasias associadas ao HPV adota um dos modelos mais bem descritos de desafio tumoral, designado TC-1. Resumidamente, trata-se de uma linhagem de células primárias de pulmão de camundongos C57BL/6, imortalizada pela transdução por vetor retroviral contendo os genes E6 e E7 de HPV16. Além disso, as células imortalizadas foram transformadas com o gene ativo *c*-Ha-*ras* (LIN et al., 1996). Desde então, essa linhagem celular tem sido largamente empregada como desafio no teste de efeitos anti-tumorais de candidatos vacinais baseados nos antígenos E6 e E7 de HPV16.

Entretanto, movidos pela necessidade de criar modelos factíveis para se avaliar efeitos anti-tumorais de candidatos vacinais baseados em outros alvos terapêuticos de HPV, alguns grupos optaram por adaptar o modelo abordado na linhagem TC-1. Vacinas anti-tumorais específicas contra o antígeno E5 de HPV16 demandam a capacidade de elicitar resposta imune contra células que expressem o gene *E5*. Uma abordagem já empregada para se testar a eficácia da vacinação com adenovírus expressando E5 de HPV16 foi a transfecção da linhagem TC-1 por um plasmídeo contendo o gene *E5* de HPV16 subordinado ao promotor do citomegalovírus (CMV) (LIU et al., 2000). No entanto, essa adaptação leva a uma condição de super expressão de *E5* que não condiz com padrões de expressão observados durante o ciclo infeccioso do HPV em sua célula hospedeira natural. Na tentativa de avaliar um modelo mais preciso como desafio tumoral para nossas candidatas vacinais, adotamos a linhagem celular C3, desenvolvida a partir de células embrionárias de camundongos transfectadas com o genoma íntegro de HPV16 e portadora do oncogene *ras* ativado (KANODIA; DA SILVA; KAST, 2008). Nosso ensaio de RT-PCR indicou algum nível de expressão do gene *E5* de HPV16, dirigida pelo promotor próprio de HPV, nessa linhagem celular, o que a torna adequada como desafio tumoral para o desenvolvimento de vacinas terapêuticas anti-HPV baseadas em E5.

Nosso primeiro ensaio pré-clínico obedeceu um esquema preventivo de imunização, a partir do qual não foi possível observar níveis detectáveis de anticorpos anti-E5 de HPV16 por meio de *Enzyme Linked Immuno Sorbent Assay*

(ELISA). Contudo, observou-se que o desenvolvimento tumoral foi menos robusto nos animais vacinados com as candidatas codificando *E5* e *E5Multi*, nos quais alguns tumores tiveram surgimento retardado e apresentaram menor massa em relação aos animais vacinados com plasmídeo vazio. Esse fato pode indicar um efeito anti-tumoral fundamentado em resposta imune citotóxica contra células expressando E5 de HPV16. Os escassos trabalhos de imunização genética com *E5* ou baseada em peptídeos de E5, de fato, apontam para uma forte dependência de linfócitos $TCD8^{+}IFN\text{-}\gamma^{+}$ para prevenção e regressão de tumores que expressam E5 de HPV16 (LIU et al., 2000), (CHEN et al., 2004) (LIAO et al., 2013).

Curiosamente, uma das primeiras tentativas de se obter resposta imune fundamentada na ativação de linfócitos T por uma vacina de DNA baseada em E5 de HPV16, sob uma mesma abordagem (vetor vaccinia) válida para vacinas baseadas em E6 e E7, resultou em falha (MENEGUZZI et al., 1991). Esta falha pode ser explicada pela expressão não detectada de E5 nas células tumorais adotadas como modelo de desafio. Na etapa seguinte de nosso estudo, empregamos um esquema terapêutico de imunização, no qual os efeitos anti-tumorais das candidatas vacinais E5 e E5Multi foram comparados àqueles promovidos por vacinas de DNA baseadas nos genes E6 e E7 sob um mesmo modelo de desafio (C3). O procedimento de imunização foi realizado por meio de injeção intramuscular seguida de eletroporação *in vivo*, cuja padronização técnica está demonstrada no Apêndice II. A eletroporação consiste em uma tentativa de se obter uma indução mais robusta de resposta citotóxica, por aumentar o número de células receptoras do plasmídeo, além de ocasionar um processo inflamatório local. Entretanto, conforme já observado em estudos anteriores com vacinas baseadas em genes de HPV (PENG et al., 2011), ajustes de pulsos e voltagem aplicada, além da quantidade de DNA inoculado podem ser críticos para a indução da resposta imune. ELISPOT conduzido a partir de esplenócitos recuperados dos animais vacinados demonstrou ativação específica de linfócitos $TCD8^{+}$ produtores de IFN-γ ao estímulo proporcionado pela proteína E5 de HPV16, reforçando indícios de imunidade celular ativa contra os tumores. Novamente, os tumores nos animais imunizados geneticamente com E5 e E5Multi exibiram desenvolvimento inferior quando comparados aos animais controle.

O emprego de adjuvantes, frequentemente presente em estudos de imunização genética, permite potencializar os possíveis efeitos anti-tumorais de vacinas anti-HPV, por exemplo, orquestrando um processo inflamatório local que permita um maior influxo de células do sistema imune (KHALLOUF; GRABOWSKA; RIEMER, 2014). Os efeitos anti-tumorais apresentados pelos animais dos grupos E5 e E5Multi foram semelhantes àqueles verificados para vacinas E6 e E7, as quais contêm o adjuvante *potato virus X coat protein* (CP) geneticamente fusionado, cujo efeito potencializador sobre a imunogenicidade já foi demonstrado (MASSA et al., 2008). Ainda que as vacinas baseadas em E5 fossem desprovidas deste adjuvante, o volume médio dos tumores nos animais com elas vacinados manteve-se levemente abaixo do volume médio dos tumores observados nos animais dos grupos E6 e E7.

Os dados acima apresentados agregam evidências que fundamentam E5 como um alvo terapêutico promissor, em cuja abordagem pode permitir o desenvolvimento de vacinas que previnam a malignização associada ao HPV. Novos experimentos sobre os efeitos induzidos pela imunização genética baseada em E5, sob esquema preventivo e terapêutico, podem, por exemplo, melhor caracterizar a resposta imune através de citometria de fluxo, conforme diversos trabalhos já demonstraram (DINIZ et al., 2010) (MUSIL et al., 2014). Logicamente, novas técnicas de incremento da resposta imune elicitada por E5 devem incluir, por exemplo, escolha de adjuvantes adequados, otimização de protocolos de vacinação, como eletroporação *in vivo*, e desenho de vacinas capazes de participar da terapia contra os demais tipos de HPV, aproveitando a similaridade de epitopos compartilhada pelas proteínas E5 de HPV16 e HPV31 (DIMAIO; PETTI, 2013). Outra abordagem ainda a ser explorada seria os efeitos combinados da imunização genética contemplando o antígeno E5 associado aos antígenos E6 e E7.

8. CONCLUSÕES

O estudo pré-clinico que começa a ser desenvolvido com esse trabalho fornece indícios sobre aplicabilidade de uma estratégia vacinal baseada no gene E5 de HPV16. Utilizando o recurso da imunização genética, foi possível explorar parte do potencial imunológico exibido pelo antígeno E5, ainda passível de grande aperfeiçoamento. O modelo de desafio tumoral aqui utilizado mostrou-se adequado no sentido de responder à vacinação numa condição similar aos padrões de expressão esperados para os genes de HPV. O aprimoramento técnico sobre esta abordagem deve, no futuro, resultar em perspectivas de estudos clínicos baseados na imunização genética com E5 contra o HPV e seus tumores associados.

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10. ANEXO I

Artigo de Tese

Título: Anti-tumor effects of genetic vaccines against HPV major oncogenes

Autores: Marcelo Nazário Cordeiro[1], Francesca Paolini[2], Silvia Massa[3], Gianfranca Curzio[2], Elena Illiano[4], Anna Jéssica Duarte Silva[1], Rosella Franconi[3], Massimiliano Bissa[4], Carlo De Giuli Morghen[4], Antonio Carlos de Freitas[1] e Aldo Venuti[2]

Filiação:

1 Universidade Federal de Pernambuco; Departamento de Genética; Laboratório de Estudos Moleculares e Terapia Experimental (LEMTE); Pernambuco, Brasil

2 Regina Elena National Cancer Institute; Laboratory of Virology – HPV-UNIT; Rome, Italy

3 ENEA; Italian National Agency for New Technologies, Energy and Sustainable Economic Development; C.R. Casaccia, UT BIORAD-FARM; Rome, Italy

4 Department of Medical Biotechnologies and Translational Medicine; Università di Milano; Milan, Italy

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RESEARCH PAPER

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Anti-tumor effects of genetic vaccines against HPV major oncogenes

Marcelo Nazário Cordeiro[1,†], Francesca Paolini[2,†], Silvia Massa[3], Gianfranca Curzio[2], Elena Illiano[4], Anna Jéssica Duarte Silva[1], Rosella Franconi[3], Massimiliano Bissa[4], Carlo De Giuli Morghen[4], Antonio Carlos de Freitas[1], and Aldo Venuti[2,*]

[1]Federal University of Pernambuco; Department of Genetics; Laboratory of Molecular Studies and Experimental Therapy (LEMTE); Pernambuco, Brazil; [2]Regina Elena National Cancer Institute; Laboratory of Virology – HPV-UNIT; Rome, Italy; [3]ENEA; Italian National Agency for New Technologies, Energy and Sustainable Economic Development; C.R. Casaccia, UT BIORAD-FARM; Rome, Italy; [4]Department of Medical Biotechnologies and Translational Medicine; Università di Milano; Milan, Italy

[†]These authors contributed equally to this work.

Keywords: Human Papillomavirus, DNA vaccines, HPV16 E5, C3 cell line, immunologic response

Abbreviations: ELISPOT, Enzyme-linked Immuno-spot; ELISA, Enzyme-linked immune-sorbent assay; ORF, open reading frame; LCR, long control region; pRb, protein of retinoblastoma; i.m., intramuscularly; p.c., post challenge; HPV, Human papillomavirus; HR, high-risk; EGFR, epidermal growth factor receptor; WB, western blot; RT-PCR, reverse transcription polymerase chain reaction; INF, Interferon; SAP, saporin; CP, coat protein; s.c., sub-cutaneous; His, histidine; Ni-NTA, nickel-nitrilotriacetic acid; IPTG, isopropyl-beta-D-1-thiogalactopyranoside; MPBS, milk in Phosphate Buffered Saline; PMA, phorbolmyristate acetate; E5H16, HPV16 E5 gene

Expression of HPV E5, E6 and E7 oncogenes are likely to overcome the regulation of cell proliferation and to escape immunological control, allowing uncontrolled growth and providing the potential for malignant transformation. Thus, their three oncogenic products may represent ideal target antigens for immunotherapeutic strategies. In previous attempts, we demonstrated that genetic vaccines against recombinant HPV16 E7 antigen were able to affect the tumor growth in a pre-clinical mouse model. To improve this anti-HPV strategy we developed a novel approach in which we explored the effects of E5-based genetic immunization. We designed novel HPV16 E5 genetic vaccines based on two different gene versions: whole E5 gene and E5Multi. The last one is a long multi epitope gene designed as a harmless E5 version. Both E5 genes were codon optimized for mammalian expression. In addition, we demonstrated that HPV 16 E5 oncogene is expressed in C3 mouse cell line making it an elective model for the study of E5 based vaccine. In this mouse model the immunological and biological activity of the E5 vaccines were assessed in parallel with the activity of anti-E7 and anti-E6 vaccines already reported to be effective in an immunotherapeutic setting. These E7 and E6 vaccines were made with mutated oncogenes, the E7GGG mutant that does not bind pRb and the E6F47R mutant that is less effective in inhibiting p53, respectively. Results confirmed the immunological activity of genetic formulations based on attenuated HPV16 oncogenes and showed that E5-based genetic immunization provided notable anti-tumor effects.

Introduction

Human papillomavirus (HPV) is a well-recognized, world-wide-spread, sexually transmitted virus, counting for a severe burden for human health.[1-3] In particular, HPV types classified as "high-risk" (HR) play a significant role in human carcinogenesis.[4,5] In fact, persistent infection by HR-HPVs is one of the cervical cancer-associated factors best described,[6] and in addition, HR-HPVs, in particular HPV16, are associated with a subset of head and neck cancers.[7,8] HPVs comprise a large family of double-stranded DNA viruses, whose genome is organized into three regions: early genes (E1 to E7), late genes (L1 and L2) and long control region (LCR) containing a variety of cis-elements that regulate viral replication and gene expression.[9] E5, E6 and E7 are viral oncogenes and their expression induces immortalization and transformation. In particular, the HR-E5 is able to enhance the ability of HR-E6 and HR-E7 to immortalize primary human keratinocytes and to increase the motility and invasiveness of human keratinocyte cell lines.[10]

Although the precise mechanism remains unclear and has been relatively less defined than that of E6 and E7, E5 may play a role in keratinocyte carcinogenesis. Indeed, while several HR-E6 and HR-E7 activities have consequences on host cell life cycle (i.e., E6 and E7 inactivate p53 and pRb, respectively)[11,12] only in recent few years HR-E5 has been recognized as a possible relevant oncoprotein and as an interesting therapeutic target.[13] Many, but not all papillomaviruses encode E5 proteins and the E5 ORF is often deleted in cervical carcinoma cells indicating that it may not

*Correspondence to: Aldo Venuti; Email: venuti@ifo.it
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be essential in maintaining the transformed status.[14] Nevertheless HR-E5 can be involved in early phases of transformation by altering growth factor pathway, and by increasing the levels of epidermal growth factor receptor (EGFR) on the cell surface.[15-17]

In addition, E5 actively induces immunological escaping, favoring the persistence of infection.[18,19] According to the relevance of their activities, HR-E6, HR-E7, and HR-E5 could be suitable therapeutic targets for immunotherapy against HPV-associated tumors. Although several approaches have demonstrated the potential of E6 and E7-based genetic immunization,[20-22] few data are available with respect to E5.[23] Hence, in premalignant lesions, where E5 is still expressed, an E5 vaccine may be a good strategy to cure and to prevent premalignant lesions from progressing into invasive cervical cancers. However, the potential of E5 protein as a tumor vaccine candidate has not been identified yet.[24] In addition, the utilized challenge models are mostly based on cell lines that may not precisely simulate HPV oncogene expression patterns during natural infection, because viral gene expression is driven by non-HPV promoters.[25] A more accurate model of HPV associated cancer could be the C3 cell line in which the expression of all viral genes is under control of HPV promoter.[26]

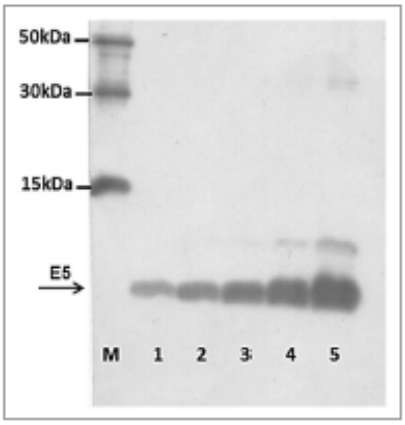

Figure 1. HPV 16 E5 peptide production. Immunoblotting of purified bacterial extracts was performed as in Material and Methods. Lanes 5 to 1–0.5 dilution of purified bacterial extracts; Lane M–6xHIS Protein Ladder (Qiagen).

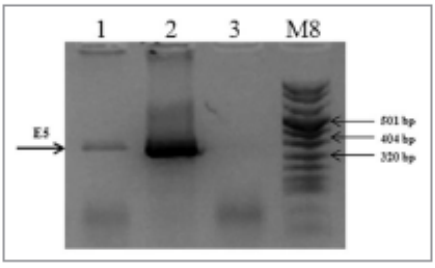

Figure 2. mRNA expression of the E5 gene in C3 cell line. Total cellular RNA was extracted from C3 cell line. cDNAs were synthesized by RT-PCR and specific amplified products were obtained with specific primers as described in "Materials and Methods". Lane 1, E5 gene amplified from C3 RNA; Lane 2, E5 gene amplified from pCIE5 plasmid, as positive control; Lane 3, total C3 RNA without reverse transcriptase, as negative control; M, molecular weight marker VIII (Roche).

Our previous data have demonstrated the induction of antitumor activity in a safe setting by vaccines based on two mutated, non-transforming forms of HPV16 E7 (E7GGG)[27,28] and E6 (E6F47R) (De Giuli Morghen, Personal Communication). Hereby, we present the effects of novel HPV16 E5 genetic vaccines based on two different gene versions: whole E5 gene and E5Multi. The last one is a long multi epitope gene designed as a harmless E5 version.

Results

E5 protein production in bacteria

To check the activity of an E5 vaccine it is important to have at least a purified protein for ELISA tests. E5 is a very hydrophobic and small protein that it is difficult to produce in *E.coli* system. Nevertheless we succeeded in its production by transfection of *E. coli* BL21 strain with His-tag E5-recombinant pAE-plasmid. After induction and cell lysis by sonication, His-tag E5 fusion protein was purified by Ni-NTA resin. Western Blot (WB) analysis showed the presence of a protein of the expected size, indicating the correct expression of E5 by this system (**Fig. 1**).The yield of purified E5 protein, quantified according to the Bradford method, was 1.68 µg/µL (500 µL total) from a 500 mL bacterial culture.

Analysis of E5 gene expression in C3 tumor cells by RT-PCR

C3 cell line was already utilized as challenge model for E6/E7 therapeutic vaccines but for E5 therapeutic vaccine no information was available regarding the E5 expression. RT-PCR was performed to detect the presence of viral transcripts encoding the E5 protein. E5-specific transcripts were revealed, as shown in **Figure 2**, indicating the possibility to utilize C3 cell line as preclinical model for E5-based vaccines.

New recombinant genetic vaccines

In premalignant lesions, when E5 is still expressed, a vaccine targeted to E5-expressing cells may be a good strategy to prevent premalignant lesions from progressing to invasive cervical cancers. We designed two different constructs to be utilized as genetic vaccine: the whole E5 gene and a synthetic harmless version. Indeed, the E5 protein can be harmful in humans due its oncogenic activity, and therefore we designed an E5-based gene, the E5Multi, which contains two previous described coding sequences for immune epitopes, in duplicate.[29,30] By this way, this E5Multi gene should increase the immunogenicity of the antigen and in the meantime could eliminate any possible oncogenic activity. An illustrated scheme of E5 and E5Multi genes is described in **Figure 3**.

Immune response in mice immunized with E5 and E5-Multi

The HPV16 E5 gene (E5H16) and the E5Multi sequence were cloned into the pCI vector, as described in Material and Methods. The immunological effects of the E5-based vaccines were ascertained in C57BL/6 mice with the prime/boost schedule described in **Figure 4A**. After 14 days, serum was collected and tested for the presence of specific antibodies against E5. No

circulating antibodies were detected in our ELISA assay with *E.coli*-produced E5 protein (**Fig. 5A**). Nevertheless, one week after the last boost the mice were challenged with $5x10^5$ C3 tumor cells and the tumor volume was measured three times a week by caliper. As shown in **Figure 5B**, E5 gene-based vaccination, and in particular the vaccination with pCI-E5Multi plasmid, delayed significantly C3 cell-induced tumor development, while inoculation of empty pCI plasmid had no effect. Tumors were collected five weeks after challenge and their weights were measured. Tumors of E5 and E5Multi vaccinated mice were almost 2 or 4 times smaller than tumors collected from control group, respectively (**Fig. 5C**).

E5 and E5Multi gene-based immunization elicits T cell response in vaccinated mice

Since CD8+ cytotoxic T cells have a recognized role as effectors in anti-cancer responses we hypothesized that the anti-tumor response in the animal with no specific antibodies was induced by E5 specific INF-gamma producing T cells. Five weeks after C3 challenge, spleens were collected from mice immunized with the prime/boost schedule described in **Figure 4A**. Collected splenocytes were tested in INF-gamma ELISPOT assay. The *E.coli*-produced E5 protein was used to activate anti-E5 lymphocytes. INF-gamma secreting cells were visualized and counted as spots under dissecting microscope.

The highest score was obtained in pCI-E5H16 vaccinated mice, exhibiting E5-specific T cell response approximately 30-fold higher than that recorded in control mice vaccinated with the empty vector as shown in **Figure 6**. Moreover, pCI-E5Multi vaccine showed 10-fold increase of E5-specific T cell response respect to the control. These results indicate that specific T cell-mediated response against the E5 protein can be generated in E5 and E5Multi-vaccinated mice.

Therapeutic immunization

The ascertained immunological activity of these new anti-E5 vaccines prompted us to determine the therapeutic potential of these DNA vaccines in the C3 pre-clinical model. In addition, two vaccines already produced against the E7 and E6 proteins, the pcDNA3-E7GGG-CP and the pcDNA3-E6F47R-CP, respectively, were utilized for comparison. After injecting the C3 cells into naïve C57Bl6 mice, prime and boost immunizations were performed on days 3 and 10, respectively (**Fig. 4B**). As shown in **Figure 7A**, tumor appearance was delayed to day 13 post challenge (p.c.) in 75% of the pcDNA3-E7GGG-CP treated animals, in 60% of mice vaccinated with pcDNA3-E6F47R-CP and pCI-E5H16, and in 40% of the pCI-E5Multi vaccinated mice. By the same day, 100% of the mice immunized with the empty pVAX vector developed tumors. In addition, pcDNA and pCI plasmids, used as controls, produced same results with 100% of animals bearing tumors on the same day p.c. (data not shown). By day 21, tumor free mice were 50% in pcDNA3-E7GGG-CP vaccinated group and 40% in pCI-E5H16 vaccinated group (**Fig. 7A**). After 4 weeks all the animals were tumor affected but differences in tumor burden were detected by measuring tumor volume. The E7 vaccinated mice showed the presence of only small-volume tumors confirming data reported in a previous study[27] and, interestingly, E5-vaccinated mice showed even smaller tumors (**Fig. 7B**).

Discussion

In previous reports we showed that immunization against the E7 protein of HPV16 trigger a complete immune response, which inhibited E7-expressing tumor growth in mouse models.[27,31-34] Genetic vaccine with E7 recombinant DNA vaccine[27,31,32] as well as plant-produced E7 recombinant protein.[33,34] demonstrated the same antitumor activity. In the present study, we explored the possibility to utilize the E5 protein of HPV16 as new therapeutic target of immunization, by demonstrating that E5-based genetic immunization could induce specific CD8+ T-cell responses.

In any vaccine preparation and in particular in genetic vaccine one of the key issue is the production of possible high level of antigen in the host. To achieve a high expression of antigen after genetic vaccination, HPV 16 E5 gene was codon-optimized for the expression in mammalian cell in accordance to previous studies, showing 6 to 9-fold enhanced expression by codon optimization.[35]

In order to evaluate our E5-based vaccines, we needed to produce the E5 protein for the immunological testing and to identify a suitable pre-clinical mouse model. The first point was achieved by succeeding in producing the E5 protein in a bacterial system. Even without a proper codon adaptation for *E. coli* expression, our bacterial system was able to provide a suitable amount of E5 protein. Besides the well-known HPV E5-associated hydrophobicity,[36] this protein has an intrinsic trend to aggregate as

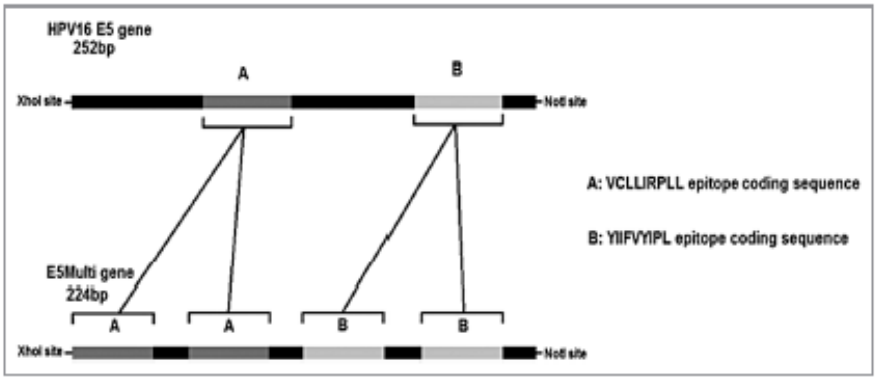

Figure 3. HPV16 E5 and E5Multi genes. The schematic representations of the E5 HPV16 gene (252 bp) with the position of CTL epitope sequences (cassettes A and B) and of the synthetic E5Multi gene (224 bp) with the position of the duplicated CTL epitope sequences (cassettes A and B) are shown together with the restriction sites for directional cloning. The aminoacid sequences of both epitopes are also indicated.

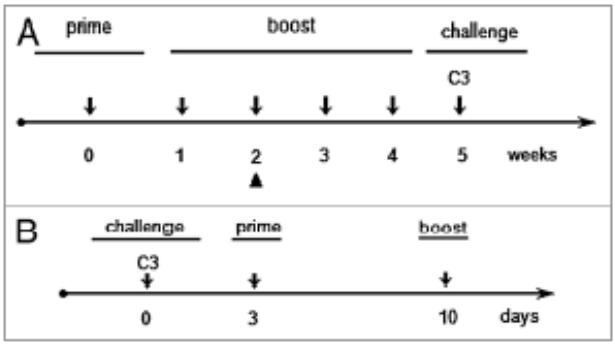

Figure 4. Prime/boost schedule for preventive or therapeutic immunizations. Five mice per group were used in both immunization schedules. (**A**) Preventive immunization schedule; the arrowhead (▲) indicates the day of blood sampling for ELISA test. (**B**) Therapeutic immunization schedule.

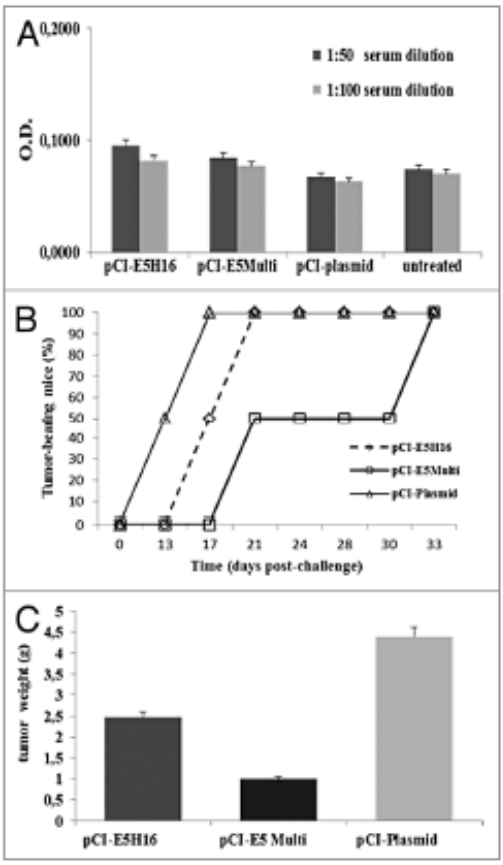

Figure 5. Preventive immunization with E5-based vaccines. (**A**) ELISA assay. Sera were collected from mice on day 14 after last boost and analyzed by E5 specific ELISA as described in "Materials and Methods". Data are expressed as means for each group of mice ± SD (**B**) Cancer growth. Tumor development was assessed by palpation three times per week after C3 cell challenge. (**C**) Tumor burden. Five weeks after challenge mice were sacrificed and excised tumors weighted. Data are represented as mean of all of the mice in the groups ± SD.

an oligo-hexamer when produced by heterologous expression systems that may represent a challenge for downstream purification procedure.[37] Indeed, two chromatography steps were necessary to perform structural analysis of the HPV-16 E5 protein.[38] For our goal an highly purified protein was not necessary and a single purification under denaturing conditions provided enough E5 protein for the preparation of the ELISA tests.

The second key point was achieved by identifying a suitable pre-clinical mouse model. The TC-1 cell line widely used as pre-clinical model for therapeutic vaccines[25,34] is not a valid option to investigate an E5-based genetic immunization because this model does not express HPV16 E5 gene.[22] Other alternative models were created by inducing an overexpression of E5 in tumor cells, which is not mimicking the low E5 expression in natural host cells.[24] Thus, our analysis of C3 cell showing an expression of E5 mRNA under the control of the viral promoter indicated that C3 cell line was a suitable pre-clinical model for the study of E5 vaccines.

No anti-E5 antibody were detected in the sera of vaccinated mice, but tumor weight was smaller in E5H16 or E5Multi vaccinated mice than in controls, suggesting that cellular immune response is involved in controlling tumor growth. Moreover, tumor appearance was delayed in a considerable percentage of mice vaccinated with pCI-E5H16 and pCI-E5Multi. Furthermore in the therapeutic setting, the number of mice that remained tumor-free after three weeks was still 40% in the pCI-E5H16 group, whereas pcDNA3-E7GGG-CP vaccination group had 50% mice that were tumor-affected by the same date. Interestingly, the E5-vaccinated mice, in particular using E5Multi preparations, had even smaller-volume tumors than pcDNA3-E7GGG-CP vaccinated animals. In addition, our ELISPOT assay indicated that this anti-tumor effect is mediated by anti-E5 CD8+ T cells, which were induced by our E5-based genetic immunization. Taken together our data suggest that our E5 vaccines can produce an immunological response that in turn may affect tumor growth. However the vaccines against the other E6 and E7 oncogenes seem to be more effective in inducing an antitumor effect. These differences may account for the presence of CP sequences in the recombinant E7 and E6 vaccines. We already developed a strategy to enhance the potency of HPV DNA vaccines by fusing the attenuated HPV16 E7GGG gene to the Potato Virus X coat protein gene (pcDNA3/E7GGG-CP).[27] The same HPV16 E7GGG without the CP sequence was totally ineffective against the E7-expressing tumor model, indicating that genetic vaccines require adjuvant sequences.[27] The same HPV16 E7GGG gene was also fused to a DNA sequence encoding the inactive mutant of saporin protein (SAP-KQ) from *Saponaria officinalis*,[28] increasing the $CD4^+/CD8^+$ T cell immune response against E7. Moreover, many other studies employed immune enhancing and modulating molecules, such as calreticulin,[20] herpes glycoprotein,[22] histone deacetylase inhibitor,[39] and other molecules (for a review see ref. 40) in order to elicit a stronger immune response against HPV antigens by DNA vaccines. Thus, many different strategies can be utilized to improve the genetic vaccine activity. In this work, our E5-based genetic vaccines were produced without any fusion to adjuvant sequences, but the preparations

were still able to induce CD 8+ T- mediated immune response, suggesting that these E5 antigens are particularly immunogenic. In alternative the electroporation delivering might be particularly efficacious in increasing the activity of genetic vaccines even without adjuvant molecules. Indeed, the reported data on HPV16 E7GGG were collected in mice vaccinated i.m.[27] Nevertheless it is likely that a strong antitumor activity could be induced by co-expression of adjuvant molecules such as CP or saporin.[27,28] The demonstrated activity of our genetic vaccines against the three HPV 16 oncogenes opens new therapeutic perspective of combinatorial vaccination such as prime-boost regimens against different HPV oncogenes. This new strategy could act in a synergistic way with other immunomodulators and could be utilized in the near future to cure patients with HPV infected cells.[41] Continuing progress in these efforts may allow the cure of HPV-associated lesions.

Materials and Methods

Plasmids

The E5 HPV16 gene (252 bp) (NCBI Reference Sequence: NC_001526.2) was codon optimized for mammal expression using IEBD Analysis Resources. The Db-restricted CTL epitope sequence (25–33aa:VCLLIRPLL) and HLA-A 0201-restricted CTL epitope sequence (63–71aa:YIIFVYIPL) from HPV16 E5 gene were duplicated to produce an immunogenic harmless version of the E5 gene, theE5Multi (224 bp). Each sequence was inserted into the pCI-neo expression vector (Promega Corporation) between XhoI and NotI restriction sites. pCI-neo empty vector was used as negative control. Plasmid integrity was verified by DNA sequencing. The E6F47R mutant (kindly provided by G Travé; Strasburg, France) that is defective for polyubiquitination and subsequent degradation of p53[42] was fused to Potato Virus X coat protein gene (CP), in order to obtain a novel recombinant genetic vaccine (De Giuli Morghen, Personal Communication). The pcDNA3-E7GGG-CP was constructed by fusing the HPV16 E7GGG sequence, mutated in the binding site for pRb,[31] to the 3′ end of the CP gene as already described by Massa et al.[27] In each experiment all plasmid preparations were purified by CsCl gradient to obtain endotoxin-free products.

Cell lines

The C3 tumor cells (a gift of C.J.M. Melief) are B6 embryonic mouse cells transformed with the whole HPV 16 genome and EJ ras.[26] The C3 cells are syngeneic to C57BL/6 mice and were cultured in RPMI-1640 with 10% fetal bovine serum (Life Technology). Before inoculation, C3 cells were harvested by trypsinization, washed twice, and resuspended in saline solution at 5×10^5 cells/ml.

RT-PCR

RNA was extracted from C3 cells by the RNeasy Plus Mini kit (Qiagen), according to the manufacturer's instructions. Total RNA pre-treated with deoxyribonuclease I (DNase I, Amplification grade, Invitrogen) was retro-transcribed into cDNA for 1 h at 42 °C using a random hexamer primer kit as described by the manufacturer (GeneAmp RNA PCR kit Applied Biosystem). The assay was performed both with and without reverse transcriptase to exclude the presence of contaminating DNA. The synthesized cDNA underwent PCR with Platinum TaqDNA polymerase (Invitrogen) and specific primer for the E5 gene using the following protocol: 95 °C for 3 min, then 35 cycles of denaturation at 95 °C for 50 s, annealing at 55 °C for 50 s and elongation at 72 °C for 60 s. The amplified products were resolved in ethidium bromide stained agarose gels. The E5 primers utilized were designed by the OligoAnalyzer Tool (Integrated DNA Technologies) software and synthesized according the following sequences:

Forward:5′–ATCTCGAGGC CACCATGGGA TACTGCATTC ACAATATAAC–3′ Reverse:5′-TAGCGGCCGC GAATTCTTAT CATGTGATCA GGAATCTTG – 3′.

E5 protein

The HPV16 E5 gene was inserted into pAE bacterial expression vector[43] and produced by *Escherichia coli* (BL21 strain) expression system in 500 mL LB medium. Induction was performed with 1mM IPTG at 23 °C and shaking at 200 rpm during 4 h. E5 protein was purified by Ni-NTA resin (Qiagen), according to the manufacturer's instructions and analyzed by 23% SDS-PAGE. Immunoblotting was performed with primary anti-His antibody (Invitrogen) and secondary peroxidase-conjugated anti-mouse antibody (Invitrogen). The presence of reacting bands were revealed by chemoluminescence (ECL-kit,Amersham).

Enzyme-linked immune-sorbent assay (ELISA)

The anti-HPV16 E5 serum antibodies were determined by a direct ELISA assay. A 96-well plate was coated at 4 °C overnight with200 ng of *E.coli*-derived HPV16 His-E5 and then blocked with 150 µl of 5% (w/v) no-fat dry milk in PBS (MPBS). Sera were collected on day 14 after boost, diluted in 2% MPBS (1:100 and

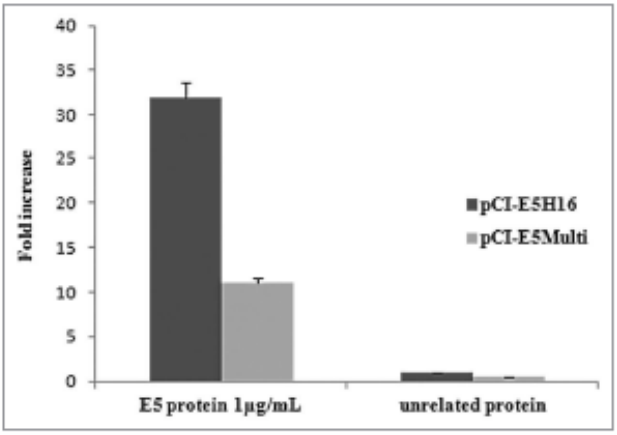

Figure 6. Cell-mediated immune responses in vaccinated mice. C57BL/6 mice were immunized by intramuscular administration of the various genetic vaccines as described in "Materials and Methods". IFN-gamma production was measured in an ELISPOT assay after specific antigenic stimulation with E5 protein. Data are presented as fold-increase responses to the E5 protein, compared with mice vaccinated with empty vector, and they represent the means of all of the mice in the groups ± SD.

1:50), and incubated for 2 h at 37 °C. Total IgGs were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG(H-L) (MP Biomedicals Life Sciences). Enzymatic activity was measured by adding 2,2-azino-bis(3-ethylbenzthiazoline sulfonate) substrate and absorbance was read at 405 nm on an ELISA microtiter plate reader.

IFN-gamma enzyme-linked immuno-spot assay (ELISPOT)

HPV16 E5-specific T-cell precursors were detected by ELISPOT for IFN-gamma-secreting cells (BDTMELISPOT BD Biosciences PharMingen) according to previous reported protocols.[44] Briefly, a single-cell suspension of splenocytes harvested from each group of vaccinated mice was added to microtiter wells that were pre-coated overnight at 4 °C with anti-mouse-IFN-gamma antibody (BD Biosciences PharMingen).Lymphocyte stimulation was performed in triplicates at 37 °C for 72 h, with the E5 protein. A mixture of phorbolmyristate acetate (PMA) was used to detect cell responsiveness. The plates were incubated with a biotinylated anti-mouse IFN-gamma-antibody (BD Biosciences PharMingen) for 4 hours at room temperature. Streptavidin–HRP was then added for 1 hour at room temperature, and the cell spots stained with filtered 3-amino-9-ethylcarbazole substrate, for 1–5 min. The spots were counted under a dissecting microscope.

Animals and vaccination schedule

Charles River Laboratories supplied 6–8 weeks female C57BL/6 mice. All procedures involving handling and sacrifice of animals were performed under specific pathogen-free conditions at the Animal House of the Regina Elena National Cancer Institute. The Ethical Committee approved the protocols developed in accordance with the European guidelines no. 86/609/CEE and 116/92 for the protection of laboratory experimental animals and laboratory animal care (Ministry of Health, Department for Veterinary Public Health, Nutrition and Food Security, Protocol17/2006). Two immunization protocols were followed: the immune response protocol, in which the mice received multiple vaccinations prior tumor challenge, and the therapeutic immunization protocol, in which the mice were challenged with C3 tumor cells before administration of the vaccines. In the immunization protocol, animals were primed with the recombinant pCI-E5H16 (50 µg/mouse, i.m.) or with the recombinant pCI-E5Multi (50 µg/mouse i.m), and boosted 3 times at 1 week intervals with the same preparations. After four weeks mice were challenged in the flank by sub-cutaneous (s.c.) injection of 200 µl of saline solution containing 5×10^5 C3 tumor cells. Tumor growth was monitored by visual inspection and palpation three times a week. Animals were scored as tumor bearing when tumors reached a size of approximately 1 to 2 mm in diameter.

In the therapeutic immunization, 5 groups of 5 mice were challenged by C3 tumor cells as above described. Three days after the tumor challenge, the mice were primed with electroporation-mediated DNA vaccines. Boosting was performed one week after the priming using same plasmid preparations. Tumor size was measured with caliper, and the volume estimated by the formula (width^2 × length × 0.52). Electroporation-mediated DNA vaccination was performed according to the method described by Seo et al.[45] Briefly, mice were injected with 100 µL of saline solution containing 50 µg of each DNA vaccine into the *tibialis* muscle of the shaved left leg. DNA injection was followed immediately by square wave electroporation at the injection site using a BTX830 apparatus (BTX Harvard Apparatus). A tweezers electrode (BTX Harvard Apparatus) was used to deliver six pulses at 100 V/cm for 20 ms.

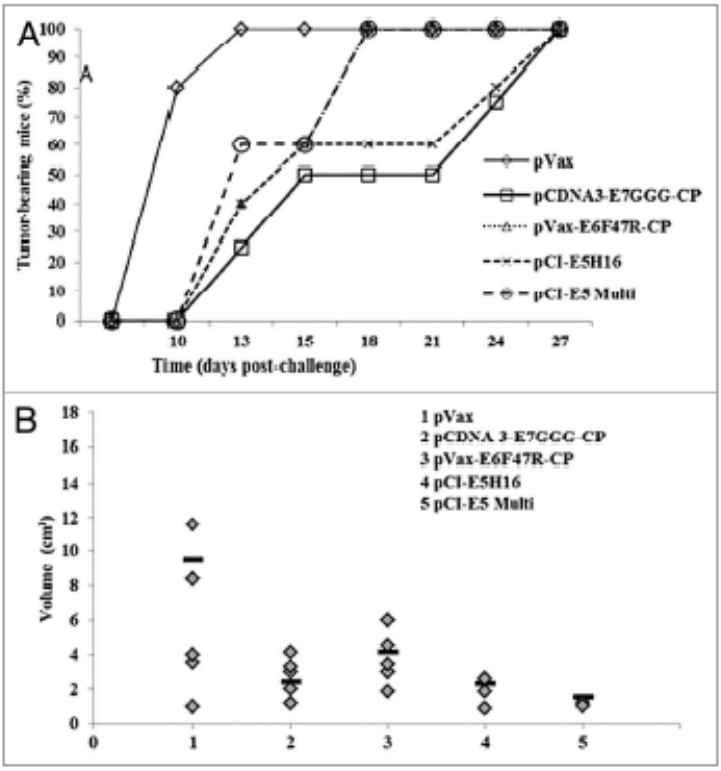

Figure 7. Tumor growth after therapeutic E5, E6 and E7 immunization. Fifty micrograms of plasmid DNA were administered by electroporation on days 3 and 10 with either the indicated vaccine preparations or saline solution as control. (**A**) Cancer growth. Tumor development was assessed by palpation three times per week. (**B**) Tumor burden. Tumor volume was measured 4 wk p.c. and calculated as in "Material and Methods". Bars (▬) represent the mean value per each mouse group.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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11. ANEXO II

Artigo de colaboração

Título: Vaccine Strategies against Human Papillomavirus: A Discussion Focused on Developing Countries

Autores: Antonio Carlos de Freitas, Filipe Colaço Mariz, Eliane Campos Coimbra, **Marcelo Nazário Cordeiro** e André Luiz Santos de Jesus

Filiação:

Universidade Federal de Pernambuco; Departamento de Genética; Laboratório de Estudos Moleculares e Terapia Experimental (LEMTE); Pernambuco, Brasil

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Review Article Open Access

Vaccine Strategies against Human Papillomavirus: A Discussion Focused on Developing Countries

Antonio Carlos de Freitas*, Filipe Colaço Mariz, Eliane Campos Coimbra, Marcelo Nazário Cordeiro and André Luiz Santos de Jesus

Laboratory of Molecular Studies and Experimental Therapy, Department of Genetics, Center of Biological Science, Universidade Federal de Pernambuco, Rua Prof. Nelson Chaves s/n, Cidade Universitária 50732-970, Recife – PE, Brazil

Abstract

Cervical cancer is the second most common form of cancer among women, and responsible for 274,000 deaths each year, most of which occur in developing countries. Persistent infection with high-risk human papillomavirus (HR-HPV) is an essential factor in the development of cervical cancer and also a contributory factor in other types of cancer. The current prophylactic HPV vaccines provide protection against the -16 and -18 genotypes which are most commonly associated with cervical cancer worldwide. However, the increased costs of these vaccines inhibit their implementation in developing countries, affecting their viability. Moreover, a therapeutic vaccine is needed for women who are already infected by HPV and/or affected by HPV-related cancer. A number of innovative approaches to combat and treat HPV infection are currently being studied and some of these will be consider in this work, together with the development of new vaccines, especially in seriously affected areas located in developing countries. At the same time, there will be a discussion of the issues involved in carrying out effective HPV vaccination programs; these will take account of financial constraints, the lack of adequate infrastructure and the competing priorities, that are found in the surrounding social context of the developing countries.

Keywords: Human papillomavirus; Cervical cancer; Prophylactic vaccines; Therapeutic vaccines; HPV vaccination

Introduction

Every year about 500,000 women develop cervical cancer and 274,000 die from this disease throughout the world, resulting in a mortality rate of approximately 55% [1-4]. Over 80% of these deaths occur in developing countries [4,5]. Worldwide, cervical cancer affects around 1.4 million women, and the highest incidence rates are found in Africa and Latin America, while India has the largest number of cases (20%) [4]. While among developed countries the 5-years patient survival rate ranges from 51% to 66%, in developing countries, where cases only tend to the diagnosed in a relatively advanced stage, this survival rate is about 40%. The world average is estimated as being 49% [3].

According to World Health Organization (WHO), persistent infection by Human Papillomavirus (HPV) is the main risk factor for developing cervical cancer [6]. The relationship between cervical cancer and HPV infection has been established by epidemiological and functional studies [7,8], in which the virus was detected in more than 99.7% of squamous cell carcinoma [9,10] and in 94-100% of cervical adenocarcinoma and adenosquamous carcinoma [11,12]. However, it takes several years for the cervical cancer to become established. At least 15 oncogenic HPV types have been identified as high-risk (HR-HPV), among which -16 and -18 genotypes are detected in more than 70% of all cases of cervical cancer [13]. The monitoring of the uterine cervix and removal of premalignant lesions can result in significant decline in the mortality rates reported worldwide. Apart from skin cancer, cervical cancer shows the greatest scope for prevention and cure when diagnosed early [3].

It should be mentioned here that screening programs are necessary to ensure that cervical cancer is prevented to a satisfactory degree, but in developing countries these programs are performed in precarious manner. Prophylactic vaccination is a potential means of supplementing the screening programs, and helping to reduce the burden of cervical cancer by preventing HPV infection [14]. The search for preventive vaccines against HPV infection has been the object of studies for several decades [15]. Although the production of inactivated or attenuated HPV virions on a large scale is difficult to reproduce in vitro, because the productive life-cycle is dependent on epithelial differentiation [16,17], the emergence of new technologies has intensified the development of these vaccines [18]. The HPV prophylactic vaccines already licensed are based on these methodologies.

Since a large number of people are already infected with HPV, and current treatments have a low rate of effectiveness, a therapeutic approach is needed to treat patients with advanced lesions. Therapeutic HPV vaccines form a part of more recent studies, and are still undergoing preclinical testing and clinical studies [19].

The aim of this paper is to review the current status of vaccines that are being evaluated for HPV infection and hence for cervical cancer. These innovative technologies to combat infection are argued, especially given the challenges posed to its availability in developing countries. Finally, we focus on the need to discuss the importance of implementing an effective program involving currently licensed vaccines against HPV for the control of cervical cancer and other HPV-related cancers in developing countries and the poorest regions of the world.

***Corresponding author:** Antonio Carlos de Freitas, Laboratory of Molecular Studies and Experimental Therapy, Department of Genetics, Center of Biological Science, Universidade Federal de Pernambuco, Cidade Universitária 50732-970 Recife – PE, Brazil, Tel: (81) 21268520 and (81) 2126-8569; Fax: (81) 2126-8522; E-mail: acf_ufpe@yahoo.com.br; antonio.freitas@pq.cnpq.br

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The Current Situation with Regard to Prophylactic HPV Vaccines

Principles and current formulations

About 120 HPV genotypes have been identified and more than 15 have been shown to induce tumors that can progress to carcinomas [20]. In particular, 53.5%, 17.2% and 6.7% of cervical cancer cases worldwide are attributed to HPV -16, -18 and -45 respectively. The remaining 12 oncogenic genotypes (-31, -33, -52, -58, -35, -59, -56, -51, -39, -68, -73 and -82, in descending order of importance) cause 30% of all the cases of cervical cancer [18].

At present, there are two licensed preventive vaccines against HPV, both composed of macromolecular structures formed by the L1 subunits, called virus-like particles (VLPs), which are morphologically similar to the virion. These current HPV vaccines comprise Gardasil® from Merck & Co., Inc. [21], composed of four VLP types (HPV-6, -11, -16, and -18), and Cervarix® from GlaxoSmithKline, UK. [22], which is bivalent and combines two VLP types (HPV-16 and -18). The need to produce multivalent vaccines is attributed to type-specific immune response, which is due to the L1 immunodominant epitopes that are located in the hypervariable regions of the loops that compose the protein [23]. In addition, neutralizing antibodies recognize conformational (nonlinear) epitopes, which result in a greater binding specificity [24]. Nevertheless, weak cross- reactivity is observed with other HPV types, leading to certain protective effects [25,26].

According to reports from the major phase III trials, the vaccines prevent from 97 to 98% of infections caused by HPV-16 and -18 [27]. Investigators from the FUTURE (Females United to Unilaterally Reduce Ecto/Endocervical Disease) II study found a 98% protection rate after a 3-year follow-up period. Gardasil® is recommended for 11-12 year old girls (who are expected not to have been sexually exposed) [28]. Females in the age range of 9-26 years may also be vaccinated and, recently, vaccination has been shown to be efficacious in women in the age range of 24-45 years who have not already been infected with the relevant HPV types. Cervarix®, on the other hand, is licensed for use among women up to 45 years old in Australia and an immunogenicity study showed 100% seroconversion in women up to the age of 55 [28]. Despite the efficacy that has been observed, the exact duration of antibody protection is unknown, although the longest follow up study so far has shown high antibody levels up to 7.3 years after vaccination with Cervarix® [28].

Attempts to develop alternatives for prophylactic HPV vaccines

Perhaps, the most serious concern about the licensed prophylactic vaccines is their high cost (up to $120 per dose, and 3 doses are required), which makes it difficult for them to be widely available in developing countries, where the vast majority of cervical cancer cases occur [29]. In view of this, several attempts have been made to establish other biotechnological HPV VLP production platforms that can fill the gap that exists in the demand and supply [30]. These measures can reduce the price of currently licensed vaccines, and increase their global penetration [30].

The VLPs can be obtained through the production of L1 protein in heterologous expression systems using mammalian cells [31], plants [32], bacteria [33], insects [34] and yeasts [24]. Both the bivalent and quadrivalent HPV vaccines contain VLPs produced from insect cells (*Trichoplusia ni Hi5* infected with recombinant baculovirus) or *Saccharomyces cerevisiae* yeast, respectively. Since 2007, several investigators have reported the expression and purification of HPV L1 protein in *Pichia pastoris*, and characterized the system as a potential vaccine platform in view of its low cost and the high levels of recombinant protein expression reported in the last 15 years [35]. In a similar way, our research group has been working with the HPV -16, -18 and -33 L1 expression in *P. pastoris* [36,37].

All these studies in the *Pichia* system employ commercial vectors and exploit the potential of the *AOX1* inducible promoter, with a wide range of expression results. In 2005, de Almeida and colleagues [38] isolated and characterized the *P. pastoris* constitutive *PGK1* promoter, which showed higher expression levels than the *AOX1* promoter. Currently, our group has been working on the expression of HPV proteins by employing a non-commercial vector based on this constitutive promoter and the partial results are promising (unpublished data). The main distinguishing feature of this work involves the use of a non-commercial vector and an alternative expression system to those already being employed or that adopted by Merck and GlaxoSmithKline. This avoids the payment of royalties tied to the use of commercial vectors and allows the possibility of making a patent application and thus, having exclusive rights for use in the future production of HPV vaccines.

The role of universities is essential in the production of medicines and vaccines and their participation has been critical in providing the basis for the creation of HPV vaccines [39-41]. The main problem is that, in the past, every public research entity involved in the initial development of these vaccines obtained licenses for the exclusive commercial use of this technology, and this has created a major obstacle to laboratories in developing countries that seek to draw on the new technology. Additionally, all of the business partners involved were based in the developed world [42]. This meant that there was little incentive to develop vaccine for low-cost sale in developing countries.

Technology transfer mechanisms are essential to ensure low-cost access to these new vaccines in developing countries, as well as for the funding of research by foundations with adequate resources. One study of manufacturers in developing country found that there were various arrangements between multi-national corporations (MNCs) and academic research institutions [42]. In the case of Indian and Brazilian vaccine manufacturers, this reliance on technology transfer allows a greater freedom to operate and more equitable benefits. These Indian and Brazilian vaccine manufacturers found that, on average 28 percent of new antigens were being produced, either by in-house research activities or by technology transfer [42].

With regard to this, the financial support by the Bill & Melinda Gates Foundation for the development of a new generation of HPV vaccines is also very important. Public institutions are taking the lead in developing these vaccines and among the participating research institutes is the Ludwig Cancer Center in Brazil [43].

A Pan-HPV vaccine

Another drawback of the available vaccines against HPV is their failure to provide protection against other carcinogenic genotypes which account for about 30% of cervical cancer cases reported worldwide. In addition, the prevalence of HR-HPV [44] is subject to geographical variation, which is the case, for example, in Brazil. Although there are no clear statistics on the prevalence of HPV infection in the sexually-active population in various states and regions, several studies confirm the high prevalence of HPV-16 in all Brazilian regions,

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whereas in the case of the second most relevant HPV type, the degree of incidence differs in each region. For example, HPV-31 and -33 are more commonly found than -18 in the Northeast and Midwest regions, while in the North, South and Southeast regions, HPV-18 appears as the second most prevalent type [45-48]. This clearly shows that there is a need to develop a second generation of prophylactic vaccines against HPV, with a larger spectrum of action [49].

As well as the L1 protein, which is the main structural unit of the viral capsid, the PVs have another capsid protein called L2. This contains an N-terminal region that was found to be conserved among the HPV genotypes, and suggests that there is a potential peptide that can cause a cross-immune reaction. In confirmation of this, animals immunized with the HPV L2 protein produced neutralizing antibodies for a broad HPV spectrum [50,51]. This observation contrasts sharply with the type-specific protection induced by L1 and suggests the possibility of a simple pan-HPV prophylactic vaccine, based on the L2 protein [49,52,53].

However, some caveats must be noted. The neutralizing antibody titers produced by vaccination with L2 are much lower than those produced by vaccination with L1 VLPs. Since VLPs, as well as natural immunogens, have proved to be efficient carriers of peptides, DNA and small molecules to dendritic cells [52], some researchers have produced VLPs based on both L1 and L2 proteins to increase the immunogenicity of L2 and extend protection to more genotypes. Unfortunately, the immunogenicity of L2 is subdominant to L1 and, in the context of an L1/L2 VLP or even in serological studies of natural infection; the neutralizing antibodies are produced almost exclusively against the L1 protein [54].

In 2010, Jagu and colleagues [53] evaluated different vaccine formulations with L2 polypetides (different HPV fused neutralizing epitopes) in animals. In accordance with the findings of the authors, the formulations containing L2 polypeptides with L1 capsomeres were found to be the vaccine with the highest potential. This may be an inexpensive strategy, because lowered the production costs of the vaccine by employing a system based on *Escherichia coli*, to expand the immunity against HPV. However, the L2-based prophylactic vaccines have not been tested on patients and when providing protection, it was unclear how long this immunity would last [53].

Therapeutic Vaccine Against Cervical Cancer

A vaccine for the prevention of cervical cancer is very important in public health. However, many women (an estimated figure of 5 million) are already infected by HPV and several of them will develop invasive cervical cancer [54]. The two HPV vaccines currently in use cannot combat the lesion or cancer that has already established itself and, thus, it is essential to seek different strategies that address the major antigens that are active in the transformation by the virus, as well as the cellular pathways involved [55,56].

HPV oncoproteins that are early expressed in the viral cycle and responsible for malignant progression of the lesion, are the most suitable targets in developing therapeutic vaccines because they stimulate the cellular response to the transformed cells [57,58]. Several studies of therapeutic vaccines against cervical cancer are being conducted and almost all of them are using E6 and E7 proteins. Some of these are already undergoing pre-clinical and clinical trials and include the use of peptides or proteins, recombinant vaccines with live virus vectors, cell-based vaccines and DNA vaccine [59,60].

Two of the first therapeutic approaches to HPV vaccines were called TA-CIN and TA-GW and consisted of HPV-16 L2 protein fused to the E6 and E7 oncoproteins and HPV-6 L2 protein fused to E7, respectively, both produced in bacterial cells [61,62]. Although these approaches potentially have a preventive and therapeutic effect on cervical lesions and genital warts, it was only the results obtained from the TA-GW vaccine that were found to be promising due to the considerable amount of neutralizing antibodies, interferon gamma and IL5 produced, as well as the proliferation of antigen-specific T cells [63,64]. Two different therapeutic approaches examined the use of chimeric VLPs (cVLPs), which are structures formed of capsid proteins (L1 or L1/L2) fused to various viral epitopes or polypeptides [17]. Both protective and therapeutic responses were achieved through immunization with cVLP containing E7 [65] or a polypeptide composed of E1, E2 and E7 fused protein of HPV-16 [17].

Genetic immunization: promising results

Tests involving genetic immunization by introducing viral DNA into organisms have shown to be a very attractive candidate for antigen-specific immunotherapy, because it can express high levels of antigen in the cells where it was introduced [66,67]. After the vector vaccine has been injected in the body, the antigen is then produced through the transcriptional machinery of the host [68], and induce both types of immunity: humoral and cellular ($CD8^+$ T cells and $CD4^+$ T cells, respectively) [68,69].

However, some studies show low immunogenicity, which can be explained by the introduction of genetic material in non-specific cells and the difficulty found in replicating or spreading this to the neighboring cells *in vivo*. For this reason, there have been a large number of studies aimed at enhancing the DNA vaccine through the improving of the DNA delivery systems and DNA sequence (codon optimization and/or fusion with other genes for the improvement of vaccinal antigens) [70-78].

Recently, two vaccines have been tested for cervical cancer. One is the ZYC-101a vaccine (MGI Pharma, MA, USA), which encodes multiple E6 and E7 epitopes (for activation of cytotoxic T lymphocytes - CTLs) of HPV-16 and -18. This vaccine was tested through the administration of three intramuscular doses and the results showed that significantly more types of CIN-2/3 lesions were successfully treated in young women (under 25) than in those who received placebo doses [79,80]. Another example of DNA vaccine, called Sig/E7detox/HSP70, was administered in Phase I clinical trials. This vaccine encodes a signal sequence, fused to an E7 mutated form (E7detox) for loss of affinity for pRB, which in turn is fused to heat shock protein (HSP70). The results of the tests carried out with patients who had high-grade CIN-2/3 lesions showed an E7-specific immune response of cytotoxic cells ($CD8^+$ T), with no adverse effects. A lesion regression was observed in 3 of the 9 vaccinated patients [81].

The E5-based therapeutic vaccines also hold out good prospects. An adenovirus vector (AdV) carrying an E5 gene has been tested in mice with an E5-expressing tumoral cell line (TC-1/E5) and showed a reduction of the tumor [82]. Another vaccine, which comprises a potential Db-restricted CTL peptide of the HPV-16 E5 gene fused to CpG motifs (DNA sequences found in bacteria and used as vaccine adjuvant) showed better results than the previous recombinant adenovirus vaccine (rAd-E5) [83,84]. These studies show that E5 is recognized by the immune system as a tumor antigen and supports the hypothesis that this oncogene is a good candidate for the eradication

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of premalignant lesions, since this viral protein is more active in the early stages of cervical cancer [85,86]. Following this line of argument, our group has been working on a therapeutic strategy based on genetic immunization with the HPV-16 E5 gene (unpublished data).

The Impact of HPV Vaccination on Developing Countries

Over 80% of new cases of cervical cancer diagnosed each year occur in developing countries and it is estimated that this percentage will rise to 90% by 2020 [4]. In developing world, cervical cancer is the biggest single cause of years of life lost from cancer, because affects relatively young women. The 5-year survival rate of patients observed in these countries is less than 50%, while in developed countries it reaches 66% [87].

According to the American Cancer Society, the introduction of Pap smear screening in prevention programs in the United States reduced deaths from cervical cancer by almost 75% between 1955 and 1992 [88]. Similarly, the incidence of cervical cancer and mortality rates sharply declined in Europe (particularly in Nordic countries) and Canada due to the implementation of cervical cytology in health care, most notably in population-based screening programs [27]. The establishment of cervical cancer trial programs requires a large number of well-trained professionals and persistent public funding to support the requisite infrastructure [30,89]. The cytology-based cervical screening needs to be conducted regularly and systematically using an organized approach, with quality systems ensuring sufficient uptake, laboratory services and continuous improvement, in order to have substantial impact [90]. Unfortunately, the developing countries have experienced great difficulty in doing this in a satisfactory manner.

In the developing countries (and even in some developed or middle-income countries), the situation in rural areas can cause people great difficulty in gaining access to the health services. The accessibility of health services in these areas is far too low to guarantee desired impact overall [27]. At the same time, it should be noted that the conventional methods of detection, such as Pap smears and liquid based cytology, have been found to yield contradictory results. In a clinically-controlled study, Clavel and colleagues [91] conducted tests that showed sensitivity of 68% and 88%, respectively, for HSIL. In view of this, it is essential to test for DNA hybridization of HR-HPV, as a supplementary test for women over 30 years old and in cases of dubious Papanicolaous test (atypical squamous cells of undetermined significance, ASCUS) [92], although this inflate even more the cost of the procedure.

The HPV vaccines provide different means of prevention, and theoretically, they are more practical in the poorest areas, because they make it easier to control cervical cancer [90]. Since 2007, there have been two prophylactic vaccines against HPV. Both have the potential to prevent 70% of cases of cervical cancer and 90% of genital warts in the next 10-20 years [93]. In a shorter period (5-10 years), it is estimated that the implementation of an effective HR-HPV vaccination program can prevent 30% of the infections caused by these virus types, as well as 40-50% of cytological abnormalities and 50-60% of HSIL [93]. Some mathematical models suggest that it would take 40-60 years of vaccination to see significant reductions in all HPV-related diseases worldwide, including non-genital disease [94]. However, the introduction of prophylactic vaccines in mass vaccination programs by developing countries is impracticable due to their high cost. As a result, the expected impact of these vaccines on the reduction of cervical cancer will not be achieved if current economic conditions persist.

The choice of a target group: cultural, ethical and educational factors

Another key issue is the choice of a target population for vaccination. First, it should be noted that the success of vaccination (or even a screening program) partly depends on the decision made by the objects of the intervention or other parties (such as parents, other family members, leaders of communities). In particular cases of HPV, attitudes toward vaccination tend to be more positive among people who have more information about the vaccine, and the causal link between HPV and cancer. People with a low social status in developing countries, living in the rural regions, are the least well off in the case of HPV-related diseases [27]. Although some experts regard HPV vaccination as a prevention strategy in low-income areas, a campaign to heighten awareness among the people is needed by the authorities to ensure it is provides effective coverage.

With regard to the specific case of HPV vaccination, with a strait window of applicability (before sexual debut), it is advisable to decide early on if the vaccine will be offered only to females or to both sexes. Additionally, delivering HPV vaccines effectively in primary prevention programs raises various issues and challenges, ranging from financial constraints regarding access to questions of cultural or religious acceptability, unlike those faced by other routine childhood vaccination programs [27,90].

While the average age of "sexual debut" differs across populations, most countries recommend primary HPV vaccination between the ages of 10 and 14. Several studies have shown that there is an increased immune response with high antibody titers during pre-puberty, compared to post-puberty [90]. Nevertheless, the vaccination of adolescents in some regions of the world raises moral, social, religious and ethical questions, such as in India, where pre-marital sex is socially unacceptable [95]. In that country, cervical cancer cases comprise 25% of cases worldwide and the implementation of these vaccines against HPV could reduce the incidence of cervical cancer by 60-100% [92]. However, a preliminary survey carried out with the parents of 9-16 year old girls showed that most of them believe that these vaccines could encourage early sex and promiscuity, which is disapproved in India. This could cause social stigmatizing and undermine the family´s prestige [95].

Other fundamental questions about the provision of vaccines to pre-adolescent/adolescent girls entail concerns about how best to access this group. Since routine vaccination of pre-adolescents and adolescents is less common than infant vaccination in developing countries, the health care provision of this group is generally less structured. While the delivery of public health interventions (including immunization) to this age group in many developed countries occurs most effectively through school-based services, developing countries often lacks any infrastructure or experience in school-based delivery vaccine [90]. Possible strategies to reach adolescent girls in school-based approaches are contracting out non-government organizations or the inclusion of the private sector. PATH (Program for Appropriate Technology in Health) is leading HPV vaccine implementation schemes in four countries – India, Vietnam, Uganda and Peru – and shows that a high coverage has been achieved, between 80 and 95% [90,96].

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On the other hand, although boys cannot develop cervical cancer, they can be infected by HPV and suffer from other diseases related to the virus, such as anogenital and oropharyngeal cancers. Vaccinating males is currently not recommended by the WHO, but some experts believe that the impact of herd immunity on female cancers and other HPV-related cancers may need to be reconsidered. The cost benefits of vaccinating both sexes are still under investigation and there is evidence that the vaccination of males with the quadrivalent vaccine reduces the risk of HPV transmission to their partners, as well as the HPV-6/11-associated disease burden [27,97,98].

Type replacement

There has also been much discussion about the potential risk of new oncogenic genotypes emerging from the introduction of vaccination programs based on currently licensed vaccines. Previously work reported the increased risk of acquiring co-infections over time [99]. The herd immunity and resulting reduction in the prevalence of HPV-16 and -18 could create the required ecological niche for type replacement, and give to the HR-HPV non-targeted by the vaccines a competitive advantage over those two types [99]. Women who are vaccinated, in the belief that they are protected against all types of HPV, could adopt a high-risk sexual behavior and be at risk of infection by oncogenic genotypes, besides the HPV-16 and -18.

Data recently published by our group [48] suggest that, in Pernambuco (Brazil), while HPV-31 (15.49%) shows a much higher incidence than HPV-18 (2.82%), which has the same incidence as HPV-33, there was also a high percentage of concurrent infections between HPV -16/-31 (70.67%) and between HPV -16/-33 (18.66%). In addition, among women infected with HPV-31, there was a greater frequency of HSIL when compared with low-grade lesions, contrary to what was observed in women infected with HPV-16. Finally, it was observed that when the HPV-16 infection was combined with HPV-31, the lesions tended to be more severe. Thus, these results suggest that the vaccines currently available cannot meet the requirements of different populations such as those of Pernambuco (Brazil). Some studies have found that the bivalent vaccine provides little protection against HPV-31 and -33, but nothing can be inferred about its effectiveness when it is observed that these genotypes are, at least, as prevalent as those that it covers [48]. These vaccines could further allow the spread of other oncogenic genotypes, which in certain populations assume a more malignant form, as seems to be the case with HPV-31 in the population of Pernambuco (Brazil).

Some aspects of the Brazilian experience

The screening programs for the prevention of cervical cancer are widely available and the self-reported healthcare coverage by the Pap test is generally suited to the conditions in Brazil. The decline in the incidences of mortality attributable to cervical cancer in the last two decades has run parallel with an improvement in the screening practices, which were introduced in the 1980s and has been refined since 1998 [100-102]. From 2003 to 2008, the frequency rate of women between 25 and 59 years old who reported having had at least one Pap test in the previous three years, rose from 25% to 84.6% [101], despite the fact that there are wide variations in levels of income. However, in the rural areas of North and Northeast regions (areas with restricted access to screening programs), mortality rates are still rising. Overall, the incidence of cervical cancer is still very high, with rates close to those countries with the highest incidence, like Peru and some African countries [101,102].

In Brazil, the National Immunization Program has been very successful and achieved the highest immunization coverage rates in the world, without the need to resort to coercive strategies. All the vaccines are offered free of charge at the public health centers. This is largely due to the existence of centenary research centers, such as Adolfo Lutz Institute, Bio-Manguinhos and Butantan Institute, which produce most of the vaccines offered by the SUS (The Brazilian Unified Health System) [103]. The HPV vaccines, in contrast, are only available in private clinics, but recent public-private partnerships have increased the supply by offering large discounts to low-income and middle-income patients, with medical care provided by charity institutions that form part of the Holy House of Mercy Fraternity [103].

Although a constant improvement in screening programs is being made by the Brazilian authorities, in a desire to increase social equity, the number of women infected by HPV and with cervical lesions remains high [102]. Added to this is the fact that Brazil has one of the highest incidences of penile cancer in the world, especially in less developed regions of the country [104]. This situation is untenable and the Brazilian Ministry of Health has fostered the development of therapeutic strategies for HPV vaccines aimed at national needs. Apart from the fact that DNA vaccines have been showing promising results for immunotherapy, their application has an advantage, especially for developing countries, since this approach is cost-effective and simple to produce in large quantities, while at the same time, it can be distributed in poor areas of difficult access, due to the stability of DNA even in high temperatures [66,67]. Running with this, alternative prophylactic vaccines are being undertaken and take account the relevant HPV type that is prevalent in Brazil.

Conclusion and New Perspectives

The training of technical and scientific competence in the development of vaccines with the aid of advanced technologies, particularly those involving genetic engineering, is essential for the control of HPV infection and cervical cancer. As a preventive vaccine for cancer, there is community demand for the vaccine in many countries. To what extend decision-makers perceive and are able to respond to this demand will vary depending on competing priorities, available resources and how health-care decisions are made in particular countries [90]. While many governments, together with international health organizations and pharmaceutical companies, are urgently attempting to cooperate and increase the accessibility of the HPV vaccines that are currently available [92], great attempts have been made in many developing countries to establish alternative vaccine platforms, which not only increase competition and help drive down costs, but also enable them to bridge the demand and supply gap [30]. Researchers in South Africa, Brazil, China and India have experienced different expression systems with promising results for a second generation of prophylactic vaccines based on VLPs. The Brazilian Ministry of Health, which has an extensive National Immunization Program [105], estimates that U.S.$ 1.857 billion will be necessary to pay for vaccinating 11-12 year-old girls with the quadrivalent HPV vaccine, in addition to the $750 million currently being invested in the program. For countries with a gross domestic product of less than $1000 per capita, the cost of the HPV vaccine will not be cost-effective unless it is as low as $1 to $2 [106]. Each 5-year delay in establishing a global vaccination program against HPV can lead to the deaths of 1.5 to 2 million people in developing countries due to cervical cancer [107]. In view of this, the search for strategies to reduce the cost of HPV vaccination should become a priority.

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12. ANEXO III

Artigo de Colaboração

Título: Expression of the bovine papillomavirus type 1, 2 and 4 L1 genes in the yeast Pichia pastoris

Autores: ALS Jesus[1], FC Mariz[1], HM Souza[1], MN Cordeiro[1], EC Coimbra[1], MCG Leitão[1], LM Nascimento[2], RC Stocco[3], W Beçak[3,4] e AC Freitas[1]

Filiação:

[1] Universidade Federal de Pernambuco; Departamento de Genética; Laboratório de Estudos Moleculares e Terapia Experimental (LEMTE); Pernambuco, Brasil

[2] Departamento de Microbiologia, Centro de Pesquisa Aggeu Magalhães, Pernambuco, Brasil

[3] Laboratório de Genética, Instituto Butantan, São Paulo, Brasil

[4] Departamento de Ciências Biológicas, Universidade da Integração Latino-Americana, Paraná, Brasil

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Expression of the bovine papillomavirus type 1, 2 and 4 L1 genes in the yeast *Pichia pastoris*

A.L.S. Jesus[1], F.C. Mariz[1], H.M. Souza[1], M.N. Cordeiro[1], E.C. Coimbra[1], M.C.G. Leitão[1], L.M. Nascimento[2], R.C. Stocco[3], W. Beçak[3,4] and A.C. Freitas[1]

[1]Departamento de Genética, Universidade Federal de Pernambuco, Recife, PE, Brasil
[2]Departamento de Microbiologia, Centro de Pesquisa Aggeu Magalhães, Recife, PE, Brasil
[3]Laboratório de Genética, Instituto Butantan, São Paulo, SP, Brasil
[4]Departamento de Ciências Biológicas, Universidade da Integração Latino-Americana, Foz do Iguaçu, PR, Brasil

Corresponding author: A.C. Freitas
E-mail: antonio.freitas@pq.cnpq.br

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ABSTRACT. Papillomaviruses are known to cause benign or malignant lesions in various animals. In cattle, bovine papillomavirus (BPV) is the etiologic agent of papillomatosis and neoplasia of the upper gastrointestinal tract and urinary bladder. Currently, there are no standard diagnostic tests or prophylactic vaccines. Protection against papillomavirus infection is conferred by neutralizing antibodies directed towards the major structural protein L1. These antibodies can be efficiently induced by immunization with virus-like particles that are formed spontaneously after L1 gene expression in recombinant systems. The yeast *Pichia pastoris* is known to provide an efficient system for expression of proteins due to reduced cost and high levels of protein production. We evaluated *P. pastoris* for expression of the L1 gene from BPV1, BPV2 and BPV4. After methanol induction, the recombinants were able to produce L1 proteins of the three different BPV types. To increase heterologous L1 protein levels, a codon optimization strategy was used for production under bioreactor conditions. The BPV1 L1

protein was identified by monoclonal antibody anti-6xHis. This is the first report of BPV L1 expression in yeast.

Key words: Bovine papillomavirus; *Pichia pastoris*; L1 gene

INTRODUCTION

Papillomaviruses are a heterogeneous group of viruses associated with specific epithelial lesions. They are small, double-stranded DNA viruses related to a wide variety of proliferative lesions of epithelial origin that are associated with different oncogenic processes in humans and other vertebrates (zur Hausen, 2002). Papillomavirus infection begins in basal cells that are accessed through microabrasions resulting from various forms of physical trauma (Campo, 2002).

In cattle, bovine papillomavirus (BPV) is the etiologic agent of cutaneous papillomatosis and neoplasia of the upper gastrointestinal tract and urinary bladder. BPV has been studied as an infectious agent, as an animal model to investigate the relationship between papillomavirus and its natural hosts, and as a model for human papillomavirus vaccination studies (Campo, 2006). Eleven BPV types have been characterized associated with specific lesions with various histopathological characteristics. The bovine papillomatosis is an important disease leading to economic depreciation of animals and the deterioration of the appearance of the animal and animal leather. The lesions may progress to cancer due to the synergistic action of genetic or environmental co-factors (Leal et al., 2003; Borzacchiello and Roperto, 2008). Although papillomaviruses are considered strictly species specific, BPV1 and, less frequently, BPV2 are recognized as the most important etiological agents in the development of fibroblastic skin tumors, or equine sarcoids, which affect horses, donkeys, and mules (Chambers et al., 2003; Bogaert et al., 2008; Nasir and Campo, 2008).

The main strategies to develop vaccines directed to papillomaviruses are related to the virus capsid proteins L1 and L2. The L1 protein comprises 90% of the capsid structure and is organized in pentamers associated with L2 protein (Campo, 2006). When expressed in eukaryotic and some prokaryotic systems, L1 can self-assemble into a structure similar to that of the viral capsid. These virus-like particles (VLPs) (Kirnbauer et al., 1992; Campo, 2006) are morphologically indistinguishable from native virions and preserve the necessary conformational epitopes to induce high titers of neutralizing antibodies (Dupuy et al., 1999; Palker et al., 2001). Because the VLPs do not contain the viral genome, they are not infectious or oncogenic and thus represent an excellent target for the development of prophylactic vaccines against papillomavirus. The expression of papillomavirus capsid protein has been reported in several heterologous expression systems such as bacteria, yeast, baculovirus-infected insect cells, transgenic plants, and mammalian cells (Liu et al., 2005; Aires et al., 2006; Park et al., 2008). BPV VLPs produced in insect cells have been described as providing potent prophylactic vaccines against BPV4 infection (Kirnbauer et al., 1996).

During the last several decades, a *Pichia pastoris* expression system has been used successfully to produce various recombinant proteins. This system offers many advantages, such as tight regulation of the promoter of the alcohol oxidase 1 gene (*AOX1*) by methanol, easy growth at high cell densities, high levels of protein production at the intra- or extracellular level, and the opportunity to perform posttranslational modifications typically associated

with higher eukaryotes (Macauley-Patrick et al., 2005). The aim of this work was to evaluate a *P. pastoris* expression system for BPV1, BPV2, and BPV4 L1 gene expression and consequent protein production.

MATERIAL AND METHODS

Construction of expression vectors

Escherichia coli TOP10 (Invitrogen, São Paulo, Brazil) was used as a host strain for plasmid cloning experiments. For expression in *P. pastoris*, the wild-type X-33 strain (Mut$^+$, His$^+$) and pPICZA expression vector were purchased from Invitrogen as part of an EasySelect® *Pichia* Expression kit. DNA manipulations were performed according to standard protocols recommended by Sambrook et al. (1989).

The L1 genes were amplified with polymerase chain reaction (PCR) from the complete viral genomes of BPVs 1, 2, and 4 with Platinum *Taq* DNA polymerase High Fidelity (Invitrogen). Specific primers were designed based on complete BPV sequences deposited in GenBank. The forward primers contained the yeast Kozak consensus sequence and a restriction site for *Eco*RI in the 5' end. The reverse primers had a restriction site for *Xho*I in the 5' end. The amplified L1 fragments were initially cloned into a pGEM-T Easy vector (Promega, Wisconsin, USA). After digestion with *Eco*RI and *Xho*I enzymes (New England Biolabs, Massachusetts, USA), the inserts were cloned in frame into the pPICZA expression vector. Then, *E. coli* TOP10 were transformed and plated onto low-salt Luria-Bertani medium (0.5% yeast extract, 0.5% NaCl, 1% tryptone, pH 7.5) supplemented with 25 µg/mL Zeocin® (Invitrogen). The presence and orientation of the insert were analyzed with PCR, enzyme digestion, and sequencing (ABI3100 sequencer, Applied Biosystems). The L1 proteins were expressed fused to a 6xHis tag at the C-terminus in the pPICZA vector.

In addition, the BPV1 L1 gene was synthesized with codons optimized for expression in *P. pastoris* yeast. Codon usage analyses of the heterologous gene and the host organism were performed using the online program Graphical Codon Usage Analyzer (http://gcua.schoedl.de). We also analyzed the GC content of the heterologous gene using Generunner for Windows version 3.05 (Hasting Software). The codon-optimized L1 gene was cloned into the pPICZA expression vector, and the presence of the insert was confirmed with PCR, enzyme digestion, and sequencing.

Transformation of *P. pastoris* and selection of recombinants

P. pastoris X-33 was transformed through electroporation with the expression plasmids previously linearized with the *Sac*I enzyme (New England Biolabs) using a Multiporator system (Eppendorf, Hamburg, Germany). The X-33 strain transformed with the empty pPICZA plasmid was used as a negative control. Immediately after the pulse, cold 1 M sorbitol was added and the cells were incubated for 2 h at 30°C. The transformants were selected on plates with YPDS medium (1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol) containing 100 µg/mL Zeocin® and incubated for 2-3 days at 30°C. The presence of the integrated expression plasmids in the transformants was confirmed using colony PCR according to manufacturer recommendations with primers complementary to the 5' and 3' region of *AOX1*.

Expression of L1 genes in *P. pastoris* in shake flasks

The positive recombinants for colony PCR were grown in minimal glycerol medium (MGY; 1% glycerol, 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 0.002% biotin) in baffled flasks at 28°C for 24 h under agitation. For induction of L1 gene expression, the cells were centrifuged, transferred to minimal methanol medium (the same components as those of MGY with glycerol replaced by methanol 0.5%), and the culture was kept at 28°C under vigorous agitation for 96 h. To maintain the induction of recombinant protein, methanol was added to the culture every 24 h to a final concentration of 2%.

Expression of L1 genes in *P. pastoris* in a bioreactor

Cultivations were performed in a 1.3-L bioreactor (BioFlo 110, New Brunswick Scientific, USA) at 28°C and an agitation of 350 rpm. Pre-cultures were inoculated into in 50 mL MGY medium in shake flasks. The cells were grown to an optical density at 600 nm of 3 before adding the inoculum of 50 mL to the bioreactor to reach a working volume of 500 mL MGY medium. After 24 h of biomass generation and total consumption of glycerol in the medium, methanol was added every 24 h to a final concentration of 1% (v/v) to induce protein expression for 48 h. The glycerol and methanol feed batches during bioreactor cultivation were carried out according to the *Pichia* fermentation protocol (Invitrogen). Aeration was kept constant at 1.0 vvm, and pure oxygen was supplied as needed. The medium pH was adjusted and controlled at 6.0 with the addition of 28% (v/v) ammonium hydroxide. Foam formation was suppressed by the addition of antifoam reagent (Antifoam C; Sigma-Aldrich, São Paulo, Brazil).

P. pastoris cell lysis

Yeast cells were harvested by centrifugation, washed, and resuspended in breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM PMSF, 1 mM EDTA, 5% glycerol). An equal volume of 0.5-mm acid-washed glass beads (Sigma-Aldrich) was added to the tubes, and a total of 10 cycles of vortexing and incubation on ice for 30 s each were performed. The samples were centrifuged, and the supernatant was transferred to a new tube.

Protein purification

After induction with methanol, the yeast cells were recovered, and the protein extract was obtained. The purification of the recombinant protein was performed on a nickel column with a ProBond® Purification System kit (Invitrogen) following manufacturer recommendations.

RT-PCR

Yeast cells were recovered, and total RNA was extracted from the samples using an SV Total RNA Isolation System kit (Promega) following manufacturer recommendations. The L1 messenger RNA (mRNA) was detected using RT-PCR with an Improm-II Reverse Transcription System (Promega). The amplification was performed with specific primers that amplify a 500-bp internal fragment from BPV1, 2, and 4 L1 genes.

Dot blot assay

For dot blot analysis, protein adsorption was carried out by spotting approximately 10 µL protein extract on a nitrocellulose membrane. The membrane was dried and then blocked with 2.5% nonfat dry milk and incubated with antibody anti-6xHis alkaline phosphatase conjugated (Sigma-Aldrich) at a dilution of 1:20,000. After 3 washes with PBS 0.05% Tween 20, the membranes were revealed with nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate (NTB/BCIP®) substrate (Sigma-Aldrich).

SDS-PAGE and Western blot assay

The proteins were boiled in SDS-loading buffer (10% 2-mercaptoethanol, 4% SDS, 0.004% bromophenol blue, 20% glycerol, 0.125 M Tris-HCl, pH 6.8) for 10 min and separated using 12.5% polyacrylamide gel electrophoresis (PAGE). The gel was stained with Coomassie brilliant blue. For the Western blot, proteins were transferred onto PVDF membranes (Millipore, São Paulo, Brazil). The membranes were blocked in 5% nonfat dry milk for 1 h and incubated overnight at 4°C with antibody anti-6xHis alkaline phosphatase conjugated at a dilution of 1:1000. The membranes were then washed 3 times with PBS 0.05% Tween 20 for 10 min. The L1 protein detection was performed using the VISIGLO® chemiluminescent substrate (Amresco, Ohio, USA).

RESULTS

Expression of BPV1, 2, and 4 L1 genes in *P. pastoris*

BPV1, 2, and 4 L1 genes were cloned into the pPICZA expression vector under the control of an inducible *AOX1* promoter. *P. pastoris* transformed with these constructs were grown in medium with methanol and analyzed for L1 gene expression and protein production. After 72 h of methanol induction, recombinant *P. pastoris* were analyzed for BPV1, 2, and 4 L1 gene expression using RT-PCR. A 500-bp fragment of the L1 gene was detected (Figure 1), indicating L1 transcription. As a genomic DNA contamination control, a reverse transcription reaction without reverse transcriptase enzyme was performed for the samples analyzed (see Figure 1).

For analysis of L1 protein production in recombinant *P. pastoris*, the protein extract was analyzed using SDS-PAGE. Although the protein profile showed no signal of degradation, the band related to the L1 protein (~55 kDa) could not be visualized (Figure 2). The production of L1 protein was confirmed by dot blot assay using anti-6xHis antibody in the protein extract of the same recombinants positive for BPV1, 2, and 4 L1 gene transcription (Figure 3). The L1 protein was detected in protein extracts of the clones in the amounts of protein tested (0.3, 0.6, and 1.2 µg). The X-33/pPICZL1B4 clone displayed a weak signal at the levels evaluated; however, a new dot blot using larger amounts of protein extract (1.7, 3.4, and 6.8 µg) demonstrated better results, confirming the production of BPV4 L1 protein in the protein extract of *P. pastoris* clones (see Figure 3). Several Western blot assays using anti-6xHis antibody were then performed, with no satisfactory results.

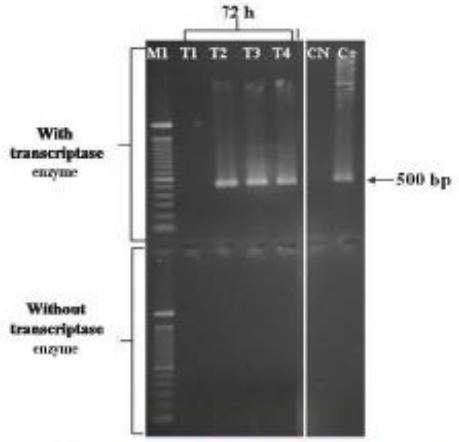

Figure 1. RT-PCR of recombinant *Pichia pastoris* after 72 h of methanol induction. The mRNAs of BPV1, 2 and 4 L1 genes were detected using specific primers that amplify a 500-bp fragment on the central portion of the nucleotide sequence, encoding L1 protein. *Lane M1* = 100-bp DNA ladder (Promega); *lane T1* = X33/pPICZA clone (empty vector, negative control of L1 expression); *lanes T2*, *T3* and *T4* = X33/pPICZAL1B1, X33/pPICZAL1B2 and X33/pPICZAL1B4 clones, respectively; *lane CN* = control reaction (without DNA); *lane C+* = plasmid used as positive control reaction. The upper side corresponds to the RT-PCR experimental test. The lower side corresponds to the control experiment of genomic DNA contamination, in which the reverse transcription reaction was performed without the reverse transcriptase enzyme.

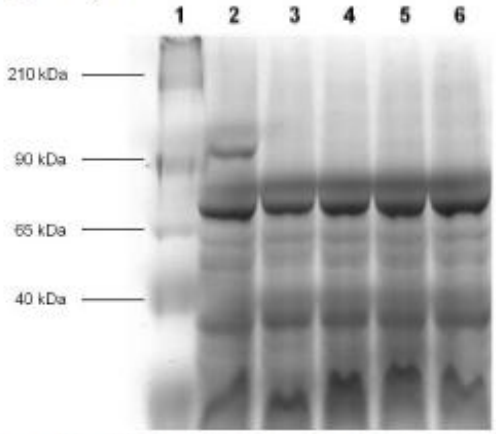

Figure 2. SDS-PAGE of recombinant *Pichia pastoris* after 72 h of methanol induction. The yeast cells were lysed after cultivation in medium with methanol and the soluble protein extract was fractionated on 12.5% SDS-PAGE (around 40 µg/lane). *Lane 1* = Color Burst Electrophoresis Marker (Sigma); *lane 2* = protein extract of recombinant *P. pastoris* GS115/pPICZ*LacZ*, positive control of intracellular expression in *P. pastoris* provided by the EasySelect® *Pichia* Expression Kit (Invitrogen); *lanes 3*, *4* and *5* = protein extract of recombinant *P. pastoris* X33/pPICZL1B1, X33/pPICZL1B2 and X33/pPICZL1B4, respectively; *lane 6* = protein extract of recombinant *P. pastoris* X33/pPICZA, empty vector, negative control of L1 expression. The figure shows that protein degradation did not occur, but the L1 protein band (~55 kDa) could not be verified. Instead, it was possible to detect the band corresponding to β-galactosidase protein (119 kDa). The gel was stained with Coomassie brilliant blue.

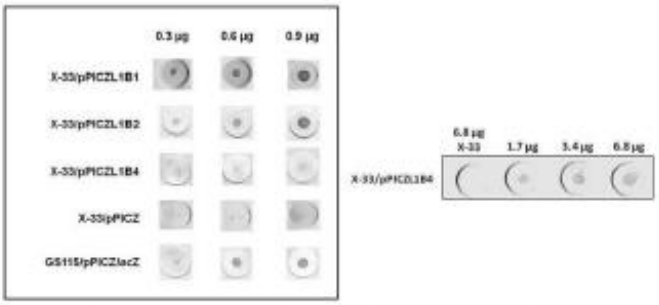

Figure 3. Detection of BPV1, 2 and 4 L1 proteins in recombinant *Pichia pastoris* after 72 h of methanol induction. Dot blots using antibody anti-6xHis alkaline phosphatase conjugated were performed to verify the L1 protein production, which has a C-terminal 6-histidine tag. The assay detected BPV1, 2 and 4 L1 proteins in recombinant *P. pastoris* in the three amounts of proteins tested (0.3, 0.6 and 1.2 µg), while in the X-33/pPICZ clone it was not detected. Although the reaction from the X-33/pPICZL1B4 clone protein extract presented low intensity, the BPV4 L1 protein was observed with greater intensity when tested with larger amounts of protein extract (1.7, 3.4 and 6.8 µg). The assay also was positive in protein extract from *P. pastoris* GS115/pPICZ*LacZ*, provided by the EasySelect® *Pichia* Expression Kit (Invitrogen), which produces β-galactosidase protein fused to 6xHis tag. The samples were diluted and were applied to nitrocellulose membrane. The dot blot was revealed with NBT/BCIP.

Expression of BPV1 L1 gene codon-optimized for *P. pastoris*

After analysis of L1 gene expression in 3 types of BPV, the BPV1 L1 gene was selected for nucleotide sequence modification based on *P. pastoris* preferred codons to increase the expression levels of L1 protein. The codon-optimized gene cloned into the pPICZA vector was successful expressed after cultivation in a bioreactor. Gene transcription was verified using RT-PCR (data not shown) and protein production by Western blot, which detected a 56-kDa band corresponding to the L1 protein (Figure 4). Higher-molecular-weight proteins were also observed. These bands may be of L1 protein dimers.

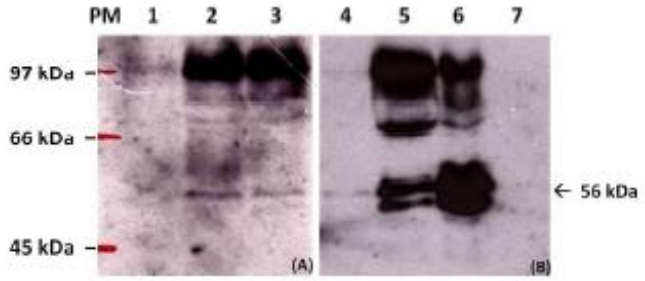

Figure 4. Detection of the recombinant BPV1 L1 protein (56 kDa) in *Pichia pastoris* by Western blot. Proteins were separated by SDS-PAGE and transferred onto PVDF membrane for Western blot assay using antibody anti-6xHis alkaline phosphatase conjugated. **A.** Protein extract was obtained at 0, 24, and 48 h after methanol induction. *Lane PM* = Protein molecular marker LMW (GE Healthcare); *lane 1* = expression of the recombinant L1 protein at 0 h; *lane 2* = expression of the recombinant L1 protein at 24 h; *lane 3* = expression of the recombinant L1 protein at 48 h. **B.** BPV1 L1 protein was purified from protein extract obtained after 48 h of methanol induction using the ProBond® Purification System. *Lane 4* = First elution of the purified recombinant L1 protein; *lane 5* = second elution; *lane 6* = third elution; *lane 7* = fourth elution.

DISCUSSION

BPV is distributed worldwide in cattle herds and is recognized as the etiologic agent associated with various benign and malignant tumors and major economic losses. Currently, no vaccine or effective treatment exists to control these infections. Traditionally, prophylactic vaccines for animal viruses are produced with live attenuated or inactivated viruses. Papillomaviruses cannot be cultured *in vitro* for the production of virions or viral proteins, which would be a source of antigens. The developing papillomavirus vaccines are related to the technology of genetic engineering. This report is the first of heterologous expression of BPV genes in yeast. In this work, BPV1, 2, and 4 L1 genes were expressed in *P. pastoris* under control of the methanol-inducible *AOX1* promoter. After optimization of the BPV1 L1 gene and cultivation of recombinant *P. pastoris* in a bioreactor, we detected the 56-kDa L1 protein with Western blot.

The methylotrophic yeast *P. pastoris* has become a highly successful system for the production of a variety of heterologous proteins (Daly and Hearn, 2005; Dummer et al., 2009; Yu et al., 2009); however, many proteins have been produced with varying degrees of success for reasons that remain unclear. Some reports have shown that AT-rich regions of heterologous genes can interrupt transcription, which considerably reduces protein production (Romanos, 1995; Boettner et al., 2007). In *Saccharomyces*, a premature termination of transcription in TA-rich sequences such as TTTTTATA, which resembles a sequence in human immunodeficiency virus 1 gp 120, has been verified to cause premature mRNA termination when expressed in *Pichia* (Scorer et al., 1993). In this study, we used RT-PCR to check the transcription of BPV1, 2 and 4 L1 genes from recombinant *P. pastoris*, confirming that the clones were positive for L1 mRNA transcription.

SDS-PAGE was carried out with various protein concentrations to assess whether degradation of protein extract occurred, and although the protein profile indicated that degradation did not occur, the band related to the L1 protein (55-58 kDa) was not visualized. To detect the presence of heterologous protein in the intracellular extract of recombinant yeasts, we initially performed a dot blot assay using an anti-His antibody to identify the 6xHis tag fused to L1 protein. Western blots were performed with positive samples for RT-PCR and dot blot using anti-His antibody, but the heterologous protein signal was not seen, which could be related to low levels of heterologous gene expression.

Enhancing expression levels in *P. pastoris* has required optimization of the heterologous gene through an increase in GC content and the replacement of rare codons with more frequent ones (Bazan et al., 2009; Kotzé et al., 2011). Eukaryotic genes are GC rich and thus more efficiently translated than are viral genes, which are generally rich in AT (Haas et al., 1996). Sinclair and Choy (2002) have shown that heterologous production can have limited success despite high levels of transcription. Thus, the authors compared a codon-optimized construct to a construct with altered GC content and, surprisingly, found that the latter provided higher translation efficiency in *P. pastoris*. Zhou et al. (1999) have observed that the BPV1 L1 gene contains rare translational codons. They therefore replaced the rare codons with codons more commonly used and compared the expression levels of a wild-type and a codon-modified BPV1 L1 gene in transient transfection experiments. Both produced high levels of L1 mRNA in the cytoplasm of cells, but only the codon-modified gene produced detectable levels of L1 protein. In the present study, data obtained from the analysis of BPV1, 2, and

4 L1 sequences revealed a difference of 10-13% between the codon usage of the L1 gene and those of the *P. pastoris* yeast. It is convenient, then, to emphasize that low levels of expression may be associated with the presence of rare codons in relation to the expression host. After codon optimization of BPV1 L1 gene, the codon usage frequency between the wild-type and the codon-optimized gene was reduced from 13 to 8.5%. This small reduction seems to be relevant to the success of heterologous expression in *P. pastoris*.

The levels of L1 protein expression detected in our results may even be related to the conditions adopted in the bioreactor for yeast cultivation or in the induction of the *AOX1* promoter. Although the expression of heterologous proteins in *P. pastoris* can be successfully performed in flasks, production levels are higher when bioreactors are used because parameters such as pH, aeration, and feeding rates of carbon sources can be controlled (Macauley-Patrick et al., 2005; Kotzé et al., 2011). In addition, the metabolism of methanol requires high levels of oxygen; thus, the expression of genes regulated by the *AOX1* promoter is negatively affected by oxygen limitation (Cereghino et al., 2002). Kotzé et al. (2011) have reported a failure to detect the HPV16 L1 gene intracellularly in *P. pastoris* using flasks. Significant levels of expression were detected only when the gene was expressed in a bioreactor. Conversely, Bazan et al. (2009) have expressed a codon-optimized HPV16 L1 gene in *P. pastoris* using flasks; however, they applied a strategy using a non-integrative plasmid expression system. Unlike integrative systems, episomal plasmids have some important disadvantages such as clonal instability, which requires continuous antibiotic selection.

In the Western blotting, we visualized some bands higher than 56 kDa, the expected molecular weight for the L1 protein. These bands probably correspond to dimers of L1 (Kirnbauer et al., 1996; Modis et al., 2002). Interestingly, we observed that after the recombinant protein was purified, we could recover a larger amount of the L1 monomer, possibly owing to the treatment with denaturing buffer from the purification kit.

The present study shows the viability of the *P. pastoris* system for the production of BPV L1 protein, a natural candidate for the development of vaccination strategies against BPV infection. In addition, the technology of BPV VLP production may be useful in the development of diagnostic strategies and vehicles with which to deliver epitopes or genes as well as for the design of interaction studies between papillomaviruses and their hosts.

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13. ANEXO IV

Artigo de Colaboração

Título: Recent insights into Bovine Papillomavirus

Autores: Freitas AC, Silva MAR, Jesus ALS, Mariz FC, **Cordeiro MN**, Albuquerque BMF e Batista MVA

Filiação:

Universidade Federal de Pernambuco; Departamento de Genética; Laboratório de Estudos Moleculares e Terapia Experimental (LEMTE); Pernambuco, Brasil

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Review

Recent insights into Bovine Papillomavirus

Freitas, A. C.*, Silva M. A. R., Jesus, A. L. S., Mariz, F. C., Cordeiro, M. N., Albuquerque, B. M. F. and Batista, M. V. A

Laboratory of Molecular Studies and Experimental Therapy, Department of Genetics, Center for Biological Sciences, Federal University of Pernambuco, Recife, PE, Brazil.

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Bovine papillomaviruses (BPV) are double-stranded DNA viruses that infect the cutaneous and mucosal epithelia inducing hyperplasic lesions in cattle. BPV is the etiologic agent of the papillomatosis and neoplasia of the upper gastrointestinal tract and urinary bladder. The benign and malignant tumors caused by BPV are emergent diseases important for beef and dairy cattle in the world. Although BPV associated tumors have veterinary and agricultural relevance, they have also been studied as a relevant model of human papillomavirus (HPV). Recent studies in BPV biology have shown a great diversity of BPV types and new putative BPV types infecting and co-infecting the herd in several parts of the world. This review will briefly summarize the genomes and structure of BPV and the bovine papillomatosis; will describe in greater detail the genotypic diversity, BPV cross-species infection, relevant aspects of BPV and co-infection and its possible routes of transmission. These new approaches about BPV may be very useful to understand the oncogenic potential of the virus, the relationship between virus and co-factors, and the development of anti-viral vaccines.

Key words: Bovine papillomavirus, co-infection, virus transmission, BPV diversity.

INTRODUCTION

Papillomaviruses (PVs) are a diverse group of small, nonenveloped, circular double-stranded DNA viruses that occur in a broad range of animal species belonging to the amniotes, including humans (Antonsson and Hansson, 2002). Bovine papillomavirus induces diseases of considerable veterinary importance in farm animals, but has also an enormous value as an in vivo model for HPV. They infect the epithelia of vertebrates, where they can cause neoplasias or persist asymptomatically. After being assorted in the old family Papovaviridae, PVs were re-designated as a distinct family, Papillomaviridae (van Regenmortel et al., 2000). BPVs are a heterogeneous group of epitheliotropic viruses that recognize bovines as its classical host. Twelve BPV types have been characterized and classified into three genera: *Deltapapillomavirus* (BPV-1 and -2), *Epsilonpapillomavirus* (BPV-5 and -8) and *Xipapillomavirus* (BPV-3, -4, -6, -9, -10, -11 and -12), and an as yet unassigned PV genus (BPV-7) (Bernard et al., 2010; Hatama et al., 2011; Zhu et al., 2011). The bovine papillomatosis is an important disease leading to economic depreciation of animals, deterioration of the appearance and of the animal leather. The lesions may progress to cancer due to the synergistic action of genetic or environmental co-factors (Borzacchiello and Roperto, 2008; Leal et al., 2003). Recent insights into BPV biology open new fields of discussion about co-infection, cross-species infection, and transmission of these viruses.

GENOMES STRUCTURE OF BOVINE PAPILLOMAVIRUS

BPV genomes comprise nearly 8 Kb, which includes a long control region (LCR), early (E) and Late (L) genes (Figure 1). The LCR (about 500-1000 nucleotides) contains transcriptional regulatory sequences and the replication origin (Munger and Howley, 2002).

There are six early genes, all of them expressed

*Corresponding author. E-mail: acf_ufpe@yahoo.com.br.

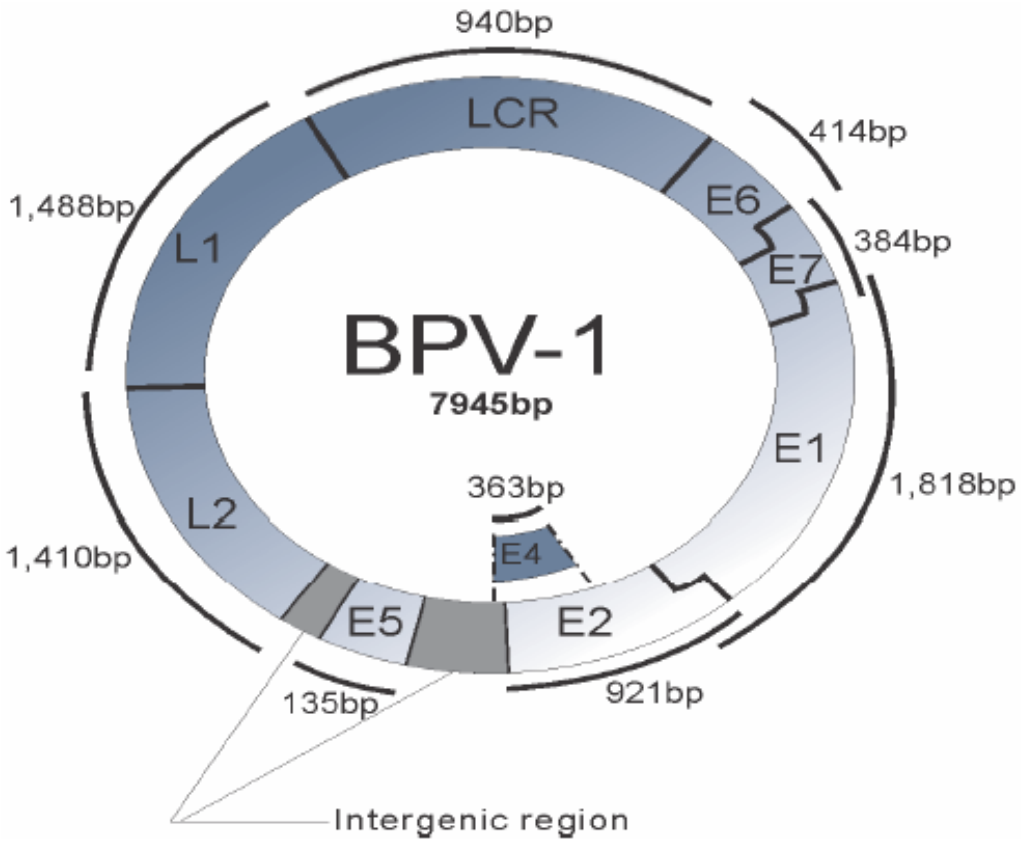

Figure 1. Genomic organization of BPV-1 showing the genomic positions of viral genes E6, E7, E1, E2, E4, E5, L2, L1, and the long control region (LCR) between L1 and E6 genes; as well as the intergenic regions between E2 and E5, and between E5 and L2.

according to the viral life cycle into the host cell. The E1 protein has helicase activity and plays its role on the viral replication (Lambert, 1991). The E2 gene product is responsible for recognition and ligation to the replication origin and, furthermore, it has mitotic chromosome binding activity in order to ensure equal distribution of viral episomes among daughter-cells (Baxter et al., 2005). The E4 gene, completely overlapping E2 gene but in a different reading frame, produces a small protein profusely found in keratinocytes cytoplasm during productive replication (Anderson et al., 1997). Three early proteins are necessary for BPV-mediated carcinogenic process, so, called oncoproteins: E5, E6 and E7 (Nasir and Campo, 2008). E5 is a membrane-associated hydrophobic protein, which plays a role on disrupting cellular growth control. BPV E6 protein is known to have a multitude of binding partners and activities on the virus life cycle. In *Xipapillomavirus*, E6 gene is replaced by an E5-like gene, which was initially defined as E8.

Nevertheless, the comprehension that most (although not all) of the functions of this protein are shared with BPV-1 E5, prompted its redefinition as E5 (Campo, 2006). The E7 protein appears to cooperate with E5 and E6 for cellular transformation, whose production with the two others oncoproteins increases the transformation efficiency (Bohl et al., 2001).

The viral capsid structure is constituted by two proteins (Modis et al., 2002; Xu et al., 2006) encoded by the late genes in viral genomes. The L1 gene is useful for classification and construction of phylogenetic trees (Bernard, 2005). The capsid is formed by 360 copies of L1 protein, organized as 72 capsomers (pentameric assembled), and 12 copies of L2 protein. Although being present in less number, L2 minor capsid protein is necessary for viral morphogenesis (Modis et al., 2002).

BPV DIVERSITY

The present PV diversity can be explained by multiple evolutionary mechanisms (Gottschling et al., 2007). Virus host-divergence is an important evolutionary force,

however this force solely cannot explain the evolution of PVs and their diversity, thus alternative mechanisms such as within-host virus duplication, recombination, viral sorting, or viral adaptation after a host switch, may therefore contribute considerably to explain the PV diversification (Shah et al., 2010; Gottschiling et al, 2011).

Although it is not very clear how these evolutionary mechanisms act on PV diversity, several robust methods have been used. Gottschling et al. (2007) used a rigorous phylogenetic approach, which took into consideration the choice of appropriate outgroups, as well as the assessment of confidence values of internal nodes. A robust study has used the method of importance sampling to Bayesian divergence time estimation, which indicates prior divergence of at least 6 PVs lineages associated with an ancestral mammalian host (Shah et al., 2010). Gottschiling et al, (2011) used different statistical approaches to assess topological and branch-length congruence, evidencing the importance of alternative mechanisms other then codivergence. Another statistical approach based on entropy was used to assess the evolution of PVs, showing that hot spots in the genome could be used as markers in order to infer PV phylogeny (Batista et al., 2011). These robust phylogenetic analyses provide the basis for contemporary classification of PVs, which is very important for any medical and veterinary researches.

The understanding of PV diversity is limited, probably underestimated. As there are more than 150 sequenced HPV genomes, less than 50 non-human papillomavirus species have been isolated and sequenced. So, more new PV types should be sequenced to increase our knowledge about PV evolution. The diversity of subtypes and variants could show a more detailed and refined scenario of PV diversification, increasing insights into the representativeness of each PV type. When it comes to BPV, 12 types are currently well described and about 14 new putative types were isolated (Antonsson and Hansson, 2002; Ogawa et al., 2004; Campo, 2006; Ogawa et al., 2007; Tomita et al., 2007; Claus et al., 2008; Hatama et al., 2008; 2011; Zhu et al., 2011).

In phylogenetic analysis, BPVs are found in at least three distantly related lineages. First, BPV-1, BPV-2, BPV-5 and BPV-8 form a paraphyletic group with OvPV-1 and OvPV-2, which infects a close related host. Other PVs that infect Artiodactyla are also close relatives of those BPVs. However, this group is clustered together with equine and canine PVs. Second, BPV-3, BPV-4, BPV-6, BPV-9, BPV-10, BPV-11 and BPV-12 are grouped together with caprine PV (ChPV-1). This group is related with a large cluster that involves human, canine and rodent PVs. BPV-7, an unclassified PV, has an uncertain phylogenetic position, which makes it difficult to infer its relatedness to other PVs.

Therefore, all this diversity found in PVs that infect one host (*Bos taurus*) is a case of evolutionary incongruence between host and PV phylogeny, indicating that co-divergence alone cannot explain the PV diversity (Gottschling et al., 2007, 2011; Shah et al., 2010).

Some conflicting phylogenetic positions of types within *Xipapillomavirus*, which includes some BPV types, have been shown when analyzing early or late genes phylogenies (García-Vallvé et al., 2005; Köhler et al., 2011). In general, the topological inconsistency between early and late genes phylogenies have been explained with ancient recombination events (Gottschling et al., 2007; Shah et al., 2010). This also could be the explanation for the contradicting positions of BPVs within *Xipapillomavirus*. For BPVs, at least three lineages seem to originate the currently known types. These lineages probably passed through a prior divergence process preceding the host divergence. This could also explain the proximity of BPVs to PVs that infect distantly related hosts. In addition, zoonotic transmission of PVs is rare event but it occurs in BPVs as they were found in zebras, horses and buffaloes (Silvestre et al., 2009; van Dyk et al., 2009; Bogaert et al., 2010a). Other evolutionary mechanisms could be associated with BPV diversification, however sampling is still a limiting factor.

BPV DETECTION AND DISTRIBUTION

BPV DNA is detected by a variety of polymerase chain reaction (PCR)-based techniques. These PCRs are based frequently on the detection of one or two BPV types using degenerated or type-specific primers. Genotyping is performed either by real-time detection (Rai et al., 2011) or by sequence analysis (Brandt et al., 2008) or restriction fragment length polymorphism (RFLP) analyses (Carr et al., 2001) of the generated PCR fragments. Consensus primers capable of identifying potentially more than two BPV types have also been described (Ogawa et al., 2004). Besides, PCR assays, designed originally for the detection of human papillomaviruses have been used to genotype different BPV types (Antonsson and Hansson, 2002; Ogawa et al., 2004). PCR assays using degenerate primers that amplify partial fragments of the L1 gene, followed by sequencing, have suggested the existence of numerous yet uncharacterized BPV types in cattle herds from diverse geographical regions. Using the primers FAP59/FAP64 and MY09/MY11, 12 putative new BPV types were detected in teat skin warts and healthy teat skin of cattle from Japan and Sweden (Antonsson and Hansson, 2002; Ogawa et al., 2004).

Bovine papillomavirus has been widely found in cattle worldwide. Cases have been reported in the incidence of BPV in cattle in Europe, America, Asia and Oceania. BPV-1, -6, -8 and -10 were found in bovine warts from a German cowshed (Schmitt et al., 2010). In Japan, heifers were found to have benign teat tumors causing by BPV-6 (Maeda et al., 2007). In another work, Ogawa et al. (2004)

detected BPV-1, -3, -5 and -6 in papilloma specimens. Bovine cutaneous warts were reported from India and identified as BPV types 1 and 2 (Singh et al., 2009; Pangty et al., 2010) and recently Rai et al. (2011) identified BPV-10 in teat warts from cattle at a dairy farm in India. Cattle from Brazil have also been investigated for the presence of BPV. It was identified BPV-1, -2, -6 and -8 in skin warts of cattle from southern Brazil (Claus et al., 2007; Sá e Silva et al., 2010). Results from our group, in northeastern Brazil, also revealed the presence of ten different types of BPV in the samples, with the exception of BPV-7 (Carvalho et al., in press).

As considered before, BPV is also associated with cancer in cattle. BPV-4 infection and associated tumors of upper GI tract have been found in Brazil, the Nasampolai Valley of Kenya, Western Highlands of Scotland and in southern Italy (Jarrett et al., 1978; Borzacchiello et al., 2003). Field cases of urinary bladder cancer in cattle associated with BPV-1 and -2 infections were reported in continental Europe, Azores Islands, some regions of Kenya, Brazil, New Zealand, India and China (Borzacchiello and Roperto, 2008).

A similar investigation revealed notable diversity among BPV types detected in papillomas of four cattle herds in southern Brazil. The study identified four putative new BPV types designated as BPV/BR-UEL2 to BPV/BR-UEL5 (Claus et al., 2008). Phylogenetic analysis using complete L1 ORF sequences revealed that the one of the isolates was similar with BPV-4 (78%), which suggested its classification in the genus *Xipapillomavirus* (Lunardi et al., 2010).

In a work of our group it was also detect possible new types and variants in samples from herd in northeastern Brazil, in which sequence analyses indicated the presence of two isolates (BPV/UFPE01 and BPV/UFPE02) of a putative new BPV-11 subtype (unpublished data). These two novel isolates are also closely related to BPV-4, and to the strains BPV/BR-UEL2 and BPV/BR-UEL3 described by Claus et al. (2008). Currently, the group continues the analysis of new BPV DNA sequences from cutaneous warts with very promising results for the identification of new types of BPV in Brazilian cattle.

BOVINE PAPILLOMATOSIS

Bovine papillomatosis is an infectious disease worldwide distributed among herds. The BPV is responsible for this contagious illness, whose remarkable clinical sign is the hiperproliferative lesions, known as papillomas, on cutaneous tissue and mucosa (Campo, 2006). Despite being primarily considered epitheliotropic, BPV DNA has already been isolated from peripheral blood mononuclear cells, milk, urine, seminal fluid and sperm cells of animals infected with BPV-1, BPV-2 and BPV-4 (Carvalho et al., 2003; Yaguiu et al., 2006, 2008; Roperto et al., 2008, Lindsey et al., 2009).

Similarly to others papillomatosis, it is usually observed the spontaneous regression of lesions, defined as benign proliferative neoplasm (Jelínek and Tachezy, 2005). Many times its importance is not verified by many owners and veterinarians badly clarified. Much more than an esthetic issue, bovine papillomatosis has recently grown in importance due its association with cancer and immunosuppression conditions (Campo, 2002). This infection, according to the viral type and environmental co-factors, causes distressing symptoms in cattle, as cutaneous fibropapillomas (BPV-1, and -2), cancer of the upper gastrointestinal tract (BPV-4), papillomatosis of teats and udder (BPV-1, -5, -6, -9 and 10) and penis (BPV-1) and cancer of the urinary bladder (BPV-1 and -2) and cutaneous papillomas (BPV-8) (Borzacchiello and Roperto, 2008) (Figure 2). Even the benign progression demands attention, once hyperplasic lesions may depreciate the pelt in affected animals; when located in the udder, it may lead to secondary infections and lactation problems. In fact, Campo (2006) related several economic consequences, as cows with teat papillomas cannot be milked, young calves cannot suckle, and often the peduncolated papillomas snap off, the sites become infected and mastitis may ensue with distortion of the milk canals. Animals can also develop extensive papillomas in the upper gastrointestinal tract and, consequently, present difficulty to feed and breathe, resulting in a debilitated animal that may come to death (Campo, 1997).

At the moment, there is no vaccine or effective treatment for the control of papillomatosis. There are few BPV treatments available with levels of success varying between 15-50%. However, it was evident the economic unavailability to repeat the treatment in animals that did not recover after the first therapeutic intervention (Silva et al., 2004).

BPV AND CO-INFECTION

Some reports describe the occurrence of co-infection with different types of BPV worldwide. In Japan, Ogawa et al. (2004) verified the presence of BPV in up to four BPV types and putative new BPV types in the same papilloma in the Japanese herd. In Brazil, the simultaneous presence of BPV-1 and -2 was detected in the same lesion (Yaguiu et al., 2006, 2008; Lindsey et al., 2009). Also, it was found five different combination of multiple BPV infection in cattle (Claus et al., 2009). Co-infection with BPV-1 and -2 was described in India (Leishangthem et al., 2008; Pangty et al., 2010) and co-infection with BPV-1 and -11 was assessed using a multiplex BPV genotyping assay in bovines in Germany (Schmitt et al., 2010) and in Brazil using specific BPV primers (Carvalho et al., in press).

Co-infection of FeSarPV, a new putative PV type

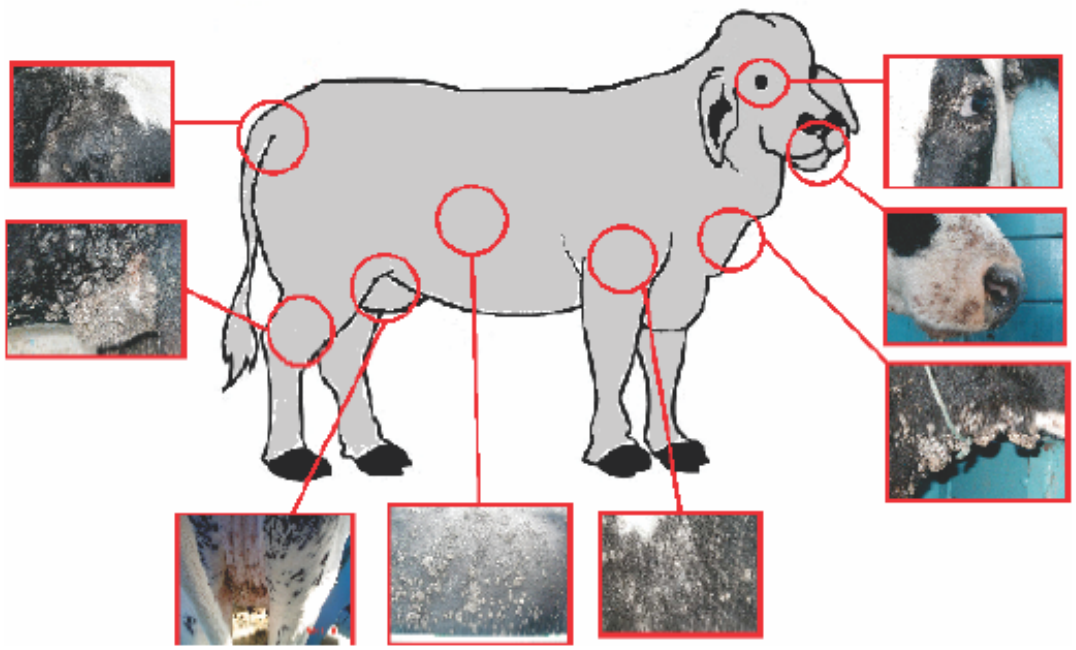

Figure 2. Schematic view of bovine papillomatosis in different sites of the cow.

related to Delta-PVs, and BPV-2 was described in New Zealand (Munday and Knight, 2010) and Brazil (Silva et al., unpublished data). In healthy cattle, the papillomas normally regress, but in cattle which have been fed on bracken fern (*Pteridium aquilinum*), there is a good correlation between persistent papillomatosis and cancer (Campo, 1997). However, the persistence of skin warts has been seen in a large number of animals (Claus et al., 2009). As several animals are constantly affected with warts in diverse body parts, it could be consequence of co-infection, which could lead to fall of immune response and prevent the regression of the lesions.

During several years, most of the first six well characterized BPVs have been described as causative agents of specific lesions in distinct body sites of bovines: BPV-1 has caused teat frond and penile fibropapillomas; BPV-2 has been described as the agent of common warts and esophageal fibropapillomas; BPV-3 and -8 in the epithelial papillomas of the skin; BPV-4 has been described as the agent of papillomas of the alimentary canal, showing specificity to the mucous epithelium; BPV-5 has caused rice grain fibropapillomas of the udder, and BPV-6 has been isolated from teat frond papillomas, BPV-9 and -10 has been associated to epithelial squamous papillomas of the udder (Campo, 1997; Borzacchiello and Roperto, 2008). However, in the late years, a diversity of multiple bovine papillomaviral infection has been described in bovine and other mammals (Ogawa et al., 2004; Bogaert et al., 2008; Claus et al., 2009; van Dyk et al., 2011) suggesting that certain viral types are not restricted as previously thought. Claus et al. (2009) observed the occurrence of several BPV types in a specific anatomical region; the detection of the same viral type in distinct body sites and determining papillomas with diverse gross aspects; and lesions with similar morphological characteristics caused by distinct papillomavirus.

Besides cutaneous warts lesions in cattle, the presence of more than one putative new BPV type was also observed in the normal skin (Ogawa et al., 2004). The simultaneous presence of BPV-1 and -2 was demonstrated in others bovine tissue such as blood and reproductive cells (Yaguiu et al., 2006, 2008; Diniz et al., 2009; Lindsey et al., 2009). BPV-1 and -2 was found co-infecting giraffe (van Dyk et al., 2011), zebra (van Dyk et al., 2009) and horse (Bogaert et al., 2008).

According to Schmitt et al. (2010), the occurrence of diverse co-infection by BPV in a single sample suggests that natural competition of different BPV types may not occur on the skin.

However, it is not clear if all BPV types founded in the lesion are transcriptionally active. Detection of viruses in apparently latency may be a result of evasion from the immune system (Schmitt et al., 2010). Nonetheless, the distribution of BPV types appeared to resemble the situation known from skin HPV types, where co-infections of more than 10 genotypes are detected frequently at very low copy numbers (Antonsson et al., 2000). In preliminary data obtained by our research group, we found very low copy number of BPV in cutaneous lesion

co-infected by several viral types (unpublished data).

TRANSMISSION OF BPV

Currently, little is known about how the disease is transmitted between animals. About this important question, it is known that confined populations are more vulnerable because virus dissemination may occur by direct (animal to animal) or indirect (contaminated objects) contact (Hama et al., 1988; Nasir and Campo, 2008). Besides the established skin–skin pathway, another via like arthropod vector and vertical trans-mission has been suggested (Freitas et al., 2003; Finlay et al., 2009). However these alternatives via of transmission might be less efficient (Bravo et al., 2010).

The increasing interest of studying BPV in the blood revealed this tissue as a source of spreading to BPV through non-epithelial tissues and fluids (Stocco dos Santos et al., 1998; Freitas et al., 2007). This hypothesis may be corroborated by the detection of BPV in different tissues and cells, including reproductive sites as oocytes, ovary, uterus, cumulus cells, and uterine lavage (Freitas et al., 2003; Yaguiu et al., 2006; Lindsey et al., 2009). The vertical transmission of BPV has been suggested (Stocco dos Santos et al., 1998; Freitas et al., 2003; Yaguiu et al., 2008). Also for humans, it has been shown that HPV- infected women can transmit the infection to the fetus by transplacental mechanisms (Rombaldi et al., 2008).

The mechanism behind the transmission of BPV to/between no specific hosts is not clear. Recent findings of BPV in epidermis and formation of L1 capsomers of equine sarcoid and active-BPV in normal skin of equine (Bogaert et al., 2008; 2010a, b; Brandt et al., 2011) could help explaining the occurrence of equine sarcoid in animals kept far away from any bovine virus source, especially when living in close contact with other affected equids (Brandt et al., 2011). It is believed that flies can be a vector for BPV and transmit the virus between bovine and horses (Nasir and Campo, 2008; Finlay et al., 2009). However, there is no further information about this virus–vector– host system. The zoonotic potential and the medical implications for the corresponding transmission route need to be explored (Bravo et al., 2010). Alternatively, BPV infection may be transmitted via stable management practices, or passed into existing wounds from contaminated pasture. Considerably more research is necessary to investigate all of these possibilities (Chambers et al., 2003). The mechanism of transmission of BPV in a cattle herd and to other mammals should be most studied since BPVs are disseminated infecting and co-infecting these animals due to its plasticity.

BPV AND CROSS-SPECIES INFECTION

Although PVs have been described as specie-specific (Campo, 2006) some PVs infect a variety of hosts. PVs appear to be widespread and have been found in a large number of vertebrate species and are assumed to have co-evolved with their hosts (Bernard, 1994; Antonsson and McMillan, 2006). Strict host specificity of PVs might act as a barrier that prevents close physical contact between different viruses, but a series of PVs infect a variety of phylogenetically distant hosts (Bravo et al., 2010). Virtually all mammalian species are hosts for one or more papillomaviruses (Sundberg et al., 2001). BPV can infect cattle but also infect close relatives of cattle such as buffalo (Silvestre et al., 2009; Pangty et al., 2010) and giraffe (van Dyk et al., 2011) causing fibropapillomas and bladder lesions (Pathania et al., 2011). Moreover, these viruses naturally infect more distantly related species, such as tapirs (Kidney and Berrocal, 2008), horses (Bogaert et al., 2008), sable antelope (van Dyk et al., 2011), and zebras living either in zoos (Löhr et al., 2005) or in the wild (van Dyk et al., 2009) causing sarcoids, and fibrosarcomas when inoculated into rodents (Robl and Oslon, 1968). Also, a variant of BPV-8 can induce papillomas in Bison (Literak et al., 2006) (Figure 3).

FeSarPV, primarily identified feline sarcoid, was verified in bovine fibropapillomas and dermatitis by Munday and Knight, (2010). It has been suggested that FeSarPV is a bovine PV causing a non-productive cross-infection in felines as well as BPV-1 and BPV-2 causes sarcoids in equids. Recently, it was found L1 capsomers in epidermis of equine with sarcoid suggesting a productive infection by BPV (Brandt et al., 2011). A newly proposed BPV type BRUEL- 4 (Claus et al., 2008) was identified in a sarcoid tumor of a horse, revealing a new viral type associated with equine sarcoid (Sá e Silva et al., 2010).

The ability of BPV-1 to infect related hosts can be a result of human domestication of cattle and horses or a phenotypic acquisition driven by vector-mediated interspecies transmission (Finlay et al., 2009; Gottschling et al., 2011). Thus, ecological changes happened concomitantly in the different hosts may have increased their susceptibility to BPV cross-infection and/or have simply increased the frequency of physical contact between them to grant BPV improved access to a potential new host (Gottschling et al., 2011).

CONCLUSION

Bovine papillomavirus is a group of viruses extensively studied in the last years. BPV has always been considered as an excellent experimental model to investigate HPV infection and carcinogenesis. It is also useful to understand the oncogenic potential of the virus, the relationship between virus and co-factors, and the development of anti-viral vaccines. In this review, we broach new insights into the mechanisms of BPV co-infection, cross-species infection and transmission.

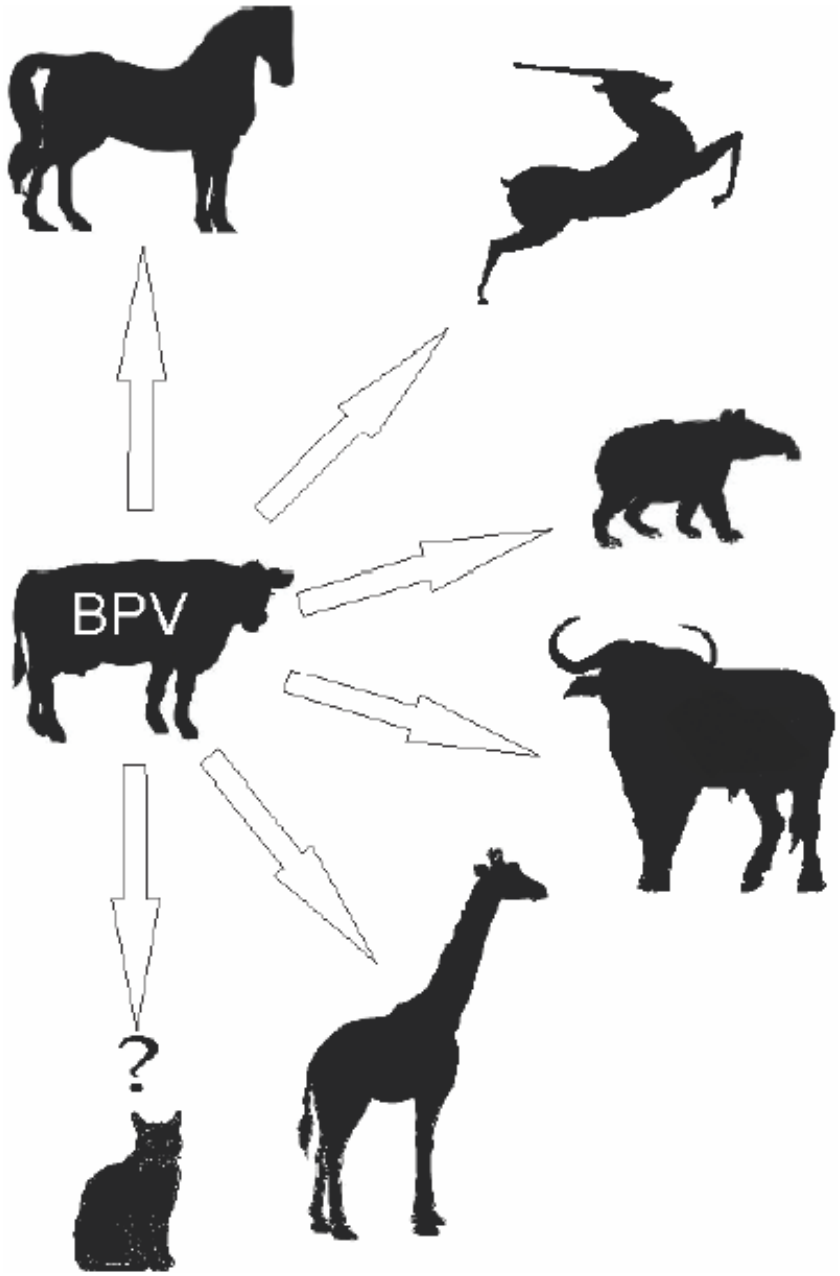

Figure 3. Schematic presentation of cross-infection caused by BPV. BPV DNA was found in close and distant related hosts: horse, sable antelope, tapir, buffalo, giraffe and possible cat.

New aspects involving the mechanisms of BPV transmission and cross-species infection have broken some paradigms about these viruses. The BPV status as an epitheliotropic and species-specific viruses can no longer be seen that way. The heterologous BPV infection has been consistently documented by several research groups worldwide, as well as the evidence of the presence of the virus in non-epithelial tissues.

The co-infection by multiple BPV has also generated interesting discussions. The occurrence of several BPV types in a specific anatomical region suggests that both multiple papillomavirus infections and high viral diversity can be frequent in cattle. The identification of multiple BPV infections may contribute to the understanding of the

epidemiological, clinical, and immunological features of cutaneous papillomatosis in cattle. Particularly for the immunological approach, this multiple infection brings important implications when is considered immunization strategies to eradicate papillomatosis, since the introduction of vaccines against a single BPV type may contribute to the spread of other genotypes able to cause skin lesions with similar morphological characteristics in cattle.

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14. APÊNDICE I

Descrição detalhada dos genes baseados em E5 de HPV16 utilizados nas contruções vacinais pCI-E5H16 e pCI-E5Multi;

Sequência da proteína E5 de HPV16:

YCIHNITGVLFALLCVLLCVCLLIRPLLLSVSTYTSLIILVLLLWITAASAFRCFIVYIIF
VYIPLFLIHTHARFLIT

CVLLCVCLLIRPLLLSVST
Epítopo A destacado em vermelho

RCFIVYIIFVYIPLFLIHT
Epítopo B destacado em verde

Sequência do gene E5 de HPV16

5'ATGTACTGCATCCACAACATTACTGGCGTGCTTTTTGCTTTGCTTTTGTGTG
CTTTTGTGTGTCTGCCTATTAATACGTCCGCTGCTTTTGTCTGTGTCTACATAC
ACATCATTAATAATATTGGTATTACTATTGTGGATAACAGCAGCCTCTGCGTTT
AGGTGTTTTATTGTATATTATATTTGTTTATATACCATTATTTTTAATACATAC
ACATGCACGCTTTTTAATTACATAA3'

Sequência do gene E5Multi

AABB

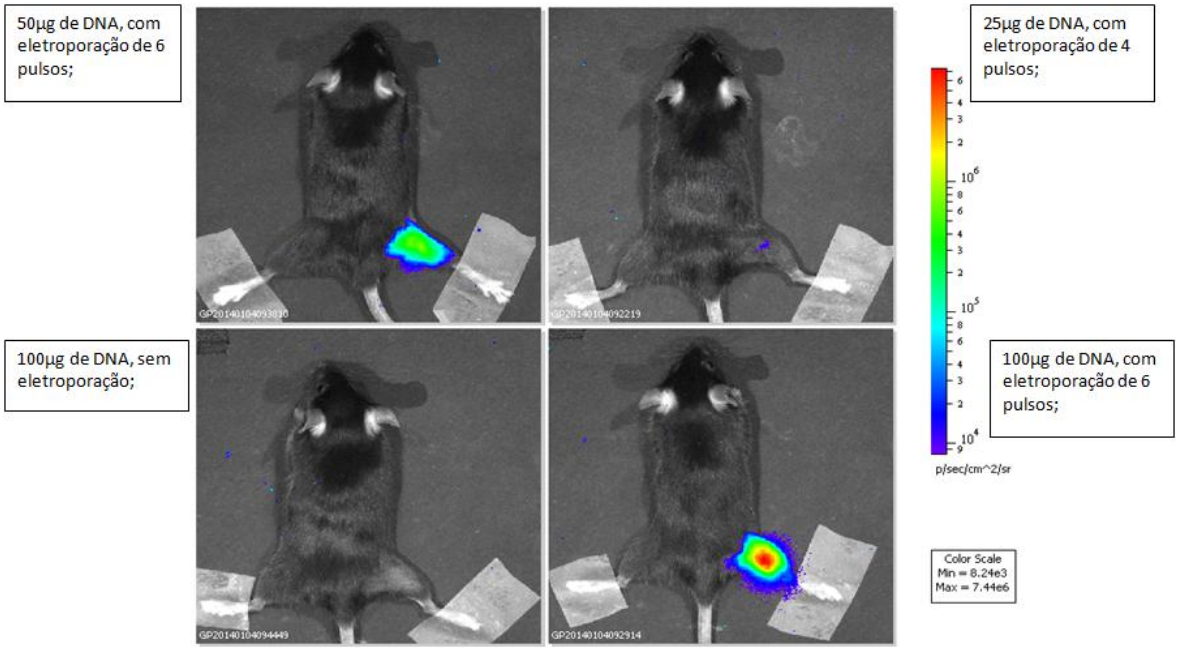
5'ATGTGTGTGCTTTTGTGTGTCTGCCTATTAATACGTCCGCTGCTTTTGTCTG
TGTCTACATGTGTGCTTTTGTGTGTCTGCCTATTAATACGTCCGCTGCTTTTGT
CTGTGTCTACAAGGTGTTTTATTGTATATTATATTTGTTTATATACCATTATT
TTTAATACATAAGGTGTTTTATTGTATATTATATTTGTTTATATACCATTATTT
TTAATACATATAA3'

15. APÊNDICE II

Padronização de técnida de eletroporação in vivo para inoculo de vacinas de DNA plasmidial em camundongos C57BL/6

Utilizando o plasmídeo pSV2-Luc, que contém o gene da luciferase de *firefly*, realizamos o seguinte ensaio para definir o protocolo mais adequado de imunização por meio de eletroporação *in vivo*. Resumidamente, um grupo de quatro camundongos foi anestesiado e a região de escolha para o procedimento foi depilada e esterilizada com etanol 70%; Cada animal foi inoculado com uma quantidade predefinida de DNA plasmidial em solução salina estéril por injeção intramuscular na *tibia femuralis*; Logo em seguida, cada camundongo foi submetido à eletroporação de 100V por 5ms, usando eletrodos *tweezers* do equipamento BTX830 (BTX Harvard Apparatus, Holliston, MA), conforme especificado na Figura 4. Após 24 horas, foi realizada injeção intraperitoneal do substrato para dosagem da luminescência;

Figura 12. Padronização do procedimento de eletroporação *in vivo*.



16. CURRICULUM VITAE (LATTES)

Dados pessoais

Nome Marcelo Nazario Cordeiro
Filiação Elias Alves Cordeiro e Débora Nazário Cordeiro
Nascimento 11/08/1983 - Recife/PE - Brasil
Carteira de Identidade 6298837 SDS - PE - 04/06/1999
CPF 059.109.184-43
Passporte sh-414701

Endereço residencial Av. Presidente Castelo Branco, 6866
Candeias - Jaboatão dos Guararapes
CEP: 54460025, PE - Brasil
Telefone: 081 96662155

Endereço eletrônico
e-mail para contato: biocellus@gmail.com
e-mail alternativo: biocellus@yahoo.com.br

Formação acadêmica/titulação

2011 Doutorado em Genética.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil
com **período sanduíche** em Istituto Nazionale Tumori Regina Elena (Orientador : Aldo Venuti)
Título: Desenvolvimento de Estratégia de Imunização Genética contra o Câncer de Colo de Útero Baseada no Gene E5 do Papilomavírus Humano Tipo 16
Orientador: Antonio Carlos de Freitas
Co-orientador: Aldo Venuti
Bolsista da: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

2008 - 2010 Mestrado em Inovação Terapêutica.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil
Título: Estudo comparativo de expressão intra- e extracelular da proteína L1 do papilomavírus bovino tipo 2 em células de *Pichia pastoris*, Ano de obtenção: 2010
Orientador: Antonio Carlos Freitas
Bolsista da: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

2004 - 2008 Graduação em Biomedicina.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil
Título: Construção do vetor de expressão pPICZAαL1B2 para produção da proteína L1 do papilomavírus bovino tipo 2
Orientador: Antonio Carlos de Freitas

Formação complementar

2011 - 2011 Curso de curta duração em Introdução à técnica de interferência por RNA.
Universidade de São Paulo, USP, São Paulo, Brasil
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

Atuação profissional

1. Universidade Federal de Pernambuco - UFPE

Vínculo institucional

2011 - Atual Vínculo: Aluno de Doutorado, Enquadramento funcional: Pesquisador, Regime: Parcial

Atividades

02/2006 - 11/2006 Estágio, Centro de Ciências Biológicas
Estágio:
Monitoria voluntária na Disciplina de Microbiologia e Imunologia

07/2004 - 08/2006 Estágio, Centro de Ciências Biológicas, Departamento de Biofísica e Radiobiologia
Estágio:
Iniciação Científica

Projetos

Projetos de pesquisa (2011 – 2015)

DESENVOLVIMENTO DE ESTRATÉGIA DE IMUNIZAÇÃO GENÉTICA CONTRA O CÂNCER DE COLO DE ÚTERO BASEADO NO GENE E5 DO PAPILOMAVÍRUS HUMANO TIPO 16
Descrição: Constitui parte de um esforço mundial de combate a uma das principais causas de mortalidade entre mulheres sexualmente ativas, o câncer de colo de útero. O projeto visa o desenvolvimento de uma das estratégias vacinais mais modernas e promissoras, uma vacina de DNA. Trata-se da elaboração de um modelo experimental de terapia gênica contra infecções pré-malignas causadas pelo HPV-16.
Situação: Em andamento Natureza: Projetos de pesquisa
Alunos envolvidos: Doutorado (3);
Integrantes: Marcelo Nazario Cordeiro; Filipe Colaço Mariz; Antonio Carlos de Freitas (Responsável);
Financiador(es): Universidade Federal de Pernambuco-UFPE

Idiomas

Inglês Compreende Bem, Fala Bem, Escreve Bem, Lê Bem

Italiano Compreende Razoavelmente, Fala Razoavelmente, Escreve Razoavelmente, Lê Razoavelmente

Prêmios e títulos

2009 Menção honrosa pela apresentação do trabalho "Estudo comparativo entre expressão intra e extracelular em *Pichia pastoris* para a produção da proteína recombinante L1 do papilomavírus bovino tipo 2, Programa de Pós Graduação em Inovação Terapêutica

Produção bibliográfica

Artigos completos publicados em periódicos

1. **Cordeiro, Marcelo Nazário**, Paolini, Francesca, Massa, Silvia, Curzio, Gianfranca, Illano, Elena, Duarte Silva, Anna Jessica, Franconi, Rosella, Bissa, Massimiliano, Morghen, Carlo de Giuli, de Freitas, Antonio Carlos, Venuti, Aldo
Anti-tumor effects of genetic vaccines against HPV major oncogenes. Human Vaccines & Immunotherapeutics. , v.11, p.41 - 48 - 7, 2015.
2. Freitas AC, Mariz FC, Coimbra EC, **Cordeiro MN**, Jesus ALS
Vaccine Strategies against Human Papillomavirus: A Discussion Focused on Developing Countries. Journal of Clinical & Cellular Immunology. , v.004, p.1 - 6, 2012.

3. Freitas AC, Silva MAR, Jesus ALS, Mariz FC, **Cordeiro MN**, Albuquerque BMF, Batista MVA
Recent insights into Bovine Papillomavirus. Afr J Microbiol Res. , v.5(33), p.6004 - 6012, 2011.
4. Jesus ALS, Mariz FC, Souza HM, **Cordeiro MN**, Coimbra EC, Leitão MCG, Nascimento LM, Stocco RC, Beçak W, Freitas AC
Expression of the bovine papillomavirus type 1, 2 and 4 L1 genes in the yeast Pichia pastoris. Genetics and Molecular Research. , v.11, p.2598 - 2607, 2012.

Trabalhos publicados em anais de eventos (resumo)

1. **Cordeiro MN**, Freitas AC

Design of HPV-16 E5 gene-based expression vectors for genetic immunization against cervical cancer In: XXIII Congresso Brasileiro de Virologia, 2012, Foz do Iguaçu.
XXIII Congresso Brasileiro de Virologia. , 2012.

2. Chagas BS, **Cordeiro MN**, Freitas AC

Evaluation of single-nucleotide polymorphisms (SNPs) in the gene of the interleukin-10-1082A/G (IL-10) among brazilian women with human papillomavirus related cervical cancer In: XXIII Congresso Brasileiro de Virologia, 2012, Foz do Iguaçu.
XXIII Congresso Brasileiro de Virologia. , 2012.

3. **Cordeiro MN,** Freitas, AC

Desenvolvimento de estratégia de imunização genética contra o câncer de colo de útero baseado no gene E5 do papilomavírus humano tipo 16 In: I Jornada de Pós-graduação em Genética, 2011, Recife.
I Jornada de Pós-graduação em Genética. , 2011.

Educação e Popularização de C&T

Curso de curta duração ministrado

1. **Cordeiro MN**, Coimbra EC, Mariz FC

Vacinas Recombinantes contra Papilomaviroses, 2009. (Outro, Curso de curta duração ministrado)

Orientações e Supervisões

Co-orientação concluída

Trabalhos de conclusão de curso de graduação

1. Anna Jéssica Duarte da Silva. **Construção e avaliação de vetor de expressão bacteriana para gene E5 do papilomavírus humano tipo 16**. 2014. Curso (Biomedicina) - Universidade Federal de Pernambuco

Bancas

Participação em banca de trabalhos de conclusão

Graduação

1. **Cordeiro MN**, Chagas BS, Freitas AC
Participação em banca de Ana Karolyna Xavier de Morais. **Participação em banca de Ana Karolyna Xavier de Morais.Padronização de PCR em Tempo Real para Quantificação dos Oncogenes E5, E6 e E7 do HPV**, 2013
(Biomedicina) Universidade Federal de Pernambuco