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Avaliação da expressão de biomarcadores em lesões intraepiteliais precursoras do carcinoma escamoso do colo uterino

Karen Olivia Bazzo

Caxias do Sul, 2016.

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Tese apresentada ao Programa de Pós-graduação em Biotecnologia da Universidade de Caxias do Sul, visando a obtenção de grau de Doutor em Biotecnologia

Orientador:

Prof. Dr. Fábio F. Pasqualotto

Co-Orientadora:

Prof. Dra. Alessandra Eifler Guerra Godoy

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KAREN OLIVIA BAZZO

AVALIAÇÃO DA EXPRESSÃO DE BIOMARCADORES EM LESÕES INTRAEPITELIAIS PRECURSORAS DO CARCINOMA ESCAMOSO DO COLO UTERINO.

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TESE APROVADA EM 26 DE AGOSTO DE 2016

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Orientador: Prof. Dr. Fábio F. Pasqualotto

_____

Co-orientadora: Profa. Dra. Alessandra Eifler Guerra Godoy

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Profa. Dra. Marcia Silveira Graudenz

_____

Prof. Dr. Renato Luis Rombaldi

_____

Prof. Dra. Eleonora Bedin Pasqualotto

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ÍNDICE

Resumo

Diversas alterações de expressão proteica decorrentes do desenvolvimento canceroso cervical e dependentes do papilomavírus humano (HPV) vêm sendo apontadas na literatura, dentre elas destacam-se proteínas diretamente influenciadas pela ação de oncoproteínas do HPV, como: transglutaminase 2 (TG2) e p53 e proteínas com expressão modificada relacionada a alterações de controle de ciclo celular, como: $p16^{INK4a}$, ki-67 e bcl-2. Neste estudo, objetivou-se analisar níveis expressionais de TG2, $p16^{INK4a}$, p53, Ki-67 e bcl-2 em amostras de lesões escamosas intraepiteliais de baixo (LEIBG) e alto grau (LEIAG), precursoras do carcinoma escamoso de colo uterino e relacionar esta expressão a diferentes tipos virais do HPV. Foram utilizadas amostras histológicas obtidas por biópsia dirigida do colo uterino de pacientes, previamente confirmadas quanto à presença do HPV-DNA por reação em cadeia da polimerase (PCR) e tipagem viral. A quantificação da expressão ocorreu por meio da técnica de imunohistoquímica. Observou-se em LEIBG a elevada expressão de TG2 ($p<0.05$) e diminuída expressão de $p16^{INK4a}$ ($p<0.05$) enquanto que em LEIAG, esta proporção inverteu-se (Pearson value = -0.269). A expressão de p53 demonstrou-se variável em relação aos graus de lesão. As expressões de Ki-67 e bcl-2 variaram conforme os níveis teciduais das biópsias coletadas. A proteína $p16^{INK4a}$ apresentou sua expressão associada a HPV considerados de alto risco oncogênico ($p<0.001$), TG2 teve sua expressão associada ao HPV tipo 11 ($p<0.05$). Não foram evidenciadas associações significantes entre a expressão de p53, Ki-67 e bcl-2 e os tipos virais. Conclui-se que a expressão dos marcadores estudados foi influenciada pelo processo canceroso no colo uterino e pela infecção do HPV.

Abstract

Changes in protein expression resulting from cervical cancer development and dependent on human papillomavirus (HPV) have been indicated in the literature, among them there are proteins directly influenced by HPV oncoproteins, such as transglutaminase 2 (TG2), p53; and proteins with modified expression related to cell cycle control changes, such as p16 ^{INK4a} a, ki-67 and bcl-2. This study aimed to analyze expressionais levels of TG2, p16^{INK4a}, p53, ki-67 and bcl-2 in High-grade (HSIL) and low-grade (LSIL) squamous intraepithelial lesion and relate this expression to different viral types of HPV. Tissue samples obtained by biopsy were used directed cervical patients, previously genotyped HPV-DNA. Expression quantification were performed by immunohistochemical technique. It was observed in LSIL the overexpression of TG2 ($p<0.05$) and decreased expression of p16^{INK4a} ($p<0.05$) although in HSIL, the proportion was reversed (Pearson value = -0.269). P53 expression was variable in relation to the degree of injury. The expression of ki-67 and bcl-2 levels varied depending beyond the basal layer. p16^{INK4a} presented its expression associated with high-risk types of HPV ($p<0.001$) and TG2 expression was associated with HPV 11 ($p<0.05$). No significant associations were found between the viral types and the ki-67, p53 and bcl-2 expression. In conclusion, the expression of the marker is affected by cancerous process and by HPV infection, and expression analysis of these markers may reveal new biomarkers associated with cervical cancer process.

1. INTRODUÇÃO

O câncer de colo uterino representa atualmente um problema de saúde pública mundial, e sua etiologia é relacionada ao papilomavírus humano (HPV), o qual induz com profundas modificações intracelulares no epitélio cervical, que desencadeiam lesões intraepiteliais, sendo estas classificadas em lesões escamosas intraepiteliais de baixo (LEIBG) e alto grau (LEIAG) (Olson et al., 2016; Cheikh et al., 2016).

A frequência de LEIBG e LEIAG vem crescendo ao longo dos anos, assim como a prevalência mundial de HPV-DNA que atingiu cifras de 10% da população, afirmativas que identificam uma maior compreensão das alterações celulares resultantes destas lesões pode acrescer conhecimentos importantes para definição de conduta clínica (Saslow et al., 2012; de Sanjose et al., 2007).

Existem diversas alterações celulares ocasionadas pelo HPV, dentre estas, destacam-se modificações de expressão proteica que ocorrem principalmente em proteínas controladoras do ciclo celular, sendo estas consideradas biomarcadoras do processo canceroso. Portanto, tornam-se relevantes estudos que avaliem a variação de expressão de diferentes biomarcadores da carcinogênese cervical (Cheikh et al., 2016).

Dentre os biomarcadores destacam-se proteínas diretamente influenciadas pela ação de oncoproteínas do HPV, como: transglutaminase 2 (TG2) e p53; e proteínas com expressão modificada relacionada a alterações de controle de ciclo celular, como: $p16^{INK4a}$, ki-67 e bcl-2 (Koeneman et al., 2015).

2. REVISÃO BIBLIOGRÁFICA GERAL

2.1 O vírus HPV

O HPV é um vírus DNA da Família do *Papillomaviridae*, não envelopado, com 72 capsômeros. Possui capsídeo com 55nm de diâmetro e apresenta-se de forma icosaédrica. Seu genoma é circular, composto por dupla fita de DNA com comprimento de 7.900 Kb e massa de 5.000 KDa. Contem nove janelas de leitura (*open reading frame* – ORF), nas quais se posicionam tanto os genes de leitura precoce (*early*) E1, E2, E4, E5, E6 e E7, quanto de leitura tardia (*late*), L1 e L2. Há também uma região não codificadora (*large control region* – LCR) que controla os demais genes (Conway and Meyers 2009). Cada um dos genes virais atua transcrevendo proteínas de funções diferentes, conforme Quadro 1(Garland et al., 2007; Faridi et al., 2011; Tsikouras et al., 2016).

Atualmente diversos tipos virais do HPV já foram identificados, sendo classificados em dois grupos, os considerados de baixo risco oncogênico, os quais estão frequentemente associados às LEIBG e a condilomas acuminados que são os tipos virais 6, 11, 40, 42, 43, 54, 61, 70, 72, 81; e de alto risco oncogênico, por estarem frequentemente associados às LEIAG e às neoplasias invasoras que são os tipos virais 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 e 82 (Tsikouras et al., 2016).

A infecção pelo HPV está relacionada a lesões benignas proliferativas, pré-neoplásicas e neoplásicas. As lesões benignas incluem verrugas comuns nas regiões plantar ou genital (condiloma acuminado) e lesão na cavidade bucal, orofaringe e conjuntival. As lesões malignas associadas ao HPV abrangem neoplasias de células escamosas genitais e de outras regiões como por exemplo nas vias respiratórias e orais. (Garland et al., 2007).

QUADRO 1. Descrição das funções específicas de cada gene do HPV

Gene	Função do gene viral
E1	Atua na replicação episssomal do vírus
E2	Regula negativamente as funções das proteínas E6 e E7
E4	Produz a proteína secundária do capsídeo viral
E5	Induz a proliferação da célula infectada pelo vírus
E6	Afeta diretamente a proteína p53 da célula hospedeira via ubiquitina e mantêm o comprimento da telomerase acima do seu ponto crítico, protegendo a célula contra a apoptose.
E7	Inativa a proteína pRB da célula hospedeira, impedindo o bloqueio do ciclo Célula
L1	Sintetiza a proteína principal do capsídeo viral.
L2	Expressa a proteína secundária do capsídeo viral

(Tsikouras et al., 2016; Garland et al., 2007; Faridi et al., 2011)

2.2 A infecção do vírus HPV

O HPV atua principalmente em células do tecido escamoso nas quais, o gene L1 desempenha uma função determinante para a entrada do vírus no meio intracelular. A internalização viral ocorre primeiramente em uma fase lenta que dura de 2 a 4 horas, mediado pelo gene L2 é fundamental. Após a internalização, ocorre o transporte intranuclear, processo também fundamentado pelo gene L2 que interage com os microtúbulos celulares. (Moody and Laimins, 2010; Laniosz et al., 2008; Lopukhov et al., 2016).

A produção de proteínas virais do HPV pela célula é controlada por diferentes promotores, há diferenças relevantes na expressão das proteínas E6 e E7 entre os HPV classificados de baixo e de alto risco oncogênico. Nos casos do HPV de alto risco

oncogênico, há somente um promotor que estimula a síntese de E6 e E7. Já no grupo de baixo risco oncogênico, ocorre a influência genética de dois promotores diferentes para a transcrição de E6 e E7 (Korzeniewski et al., 2011; Lopukhov et al., 2016).

A replicação viral ocorre no decorrer da infecção inicial dos queratinócitos basais, após isto, a seguinte fase se define como manutenção do genoma. Nestas células infectadas, o DNA viral é conservado em diversas cópias de plasmídio estável. A replicação viral é sincronizada com o ciclo celular, o que assegura uma infecção latente. (Conway and Meyers, 2009; Korzeniewski et al., 2011; Lopukhov et al., 2016).

Estudos epidemiológicos têm demonstrado que o HPV está presente em virtualmente 100% das mulheres com câncer do colo uterino (Murphy et al., 2003). Por outro lado, grande parte das mulheres que tem diagnóstico de infecção pelo HPV, frequentemente curam a infecção de modo espontâneo e apenas uma minoria desenvolve infecção persistente e evolui para lesões pré-neoplásicas (Eluf Neto et al., 1998). Isso leva a crer que, sob ponto de vista do comportamento biológico, trata-se de uma doença, com duas patogenias, uma delas “infectante” e a outra “transformante” (Schiffman, 1995; Hang et al., 2016).

2.3 Epidemiologia

Diferentes dados epidemiológicos são evidenciados na literatura, esta variação é resultante de distintos níveis de desenvolvimento dos países estudados e diferentes perfis de população pesquisada. Em uma metanálise sobre esta frequência foi observada uma prevalência de 10% de HPV-DNA na população mundial (de Sanjose et al., 2007).

O HPV é um vírus muito prevalente e as verrugas cutâneas são afecções virais muito frequentes, com incidência estimada de 7% a 10% na população européia e de 1%

na população americana. Nos imunodeprimidos e nos receptores de transplante renal., esse número aumenta de 50 a 100 vezes, chegando a mais de 90% após 15 anos do transplante. As verrugas podem ocorrer em qualquer idade e a sua incidência aumenta durante a idade escolar, com pico na adolescência e nos adultos jovens (Kilkenny and Marks 2007; De Flora e Maestra, 2015).

Um estudo realizado na Tanzânia com 3.603 mulheres da população urbana e rural revelou a prevalência do HPV em 20,1% da população, sendo sua detecção maior em mulheres soropositivas (46,7%) (Dartell et al., 2012). Em outra pesquisa realizada na Nova Guiné em mulheres com câncer cervical, a detecção do HPV foi evidenciada em 100% da população estudada (Tabone et al., 2012).

No Brasil, em uma pesquisa desenvolvida nas cidades de São Paulo, Porto Alegre e Campinas, demonstrou prevalência da infecção genital por HPV de alto risco oncogênico em 17,8% da população estudada (2.300 mulheres de 15-65 anos). Os autores observaram a prevalência do HPV em 27,1% (abaixo dos 25 anos), 21,3% (25-34 anos), 12,1% (35-44 anos), 12% (45-54 anos) e de 13,9% (55-65 anos). As mulheres com maior número de parceiros sexuais durante a vida apresentaram maior frequência da prevalência do HPV (Rama et al., 2008).

No Rio Grande do Sul, Entiauspe et al., (2010) evidenciaram a prevalência do HPV de 66,3%, sendo que destas, 60% foram correlacionadas a coinfecção pelo vírus HIV. Oliveira et al., (2013) relataram uma prevalência do HPV de 18,2% em pacientes de Unidades Básicas de Saúde e de um Hospital Universitário do Sul do Brasil, os genótipos mais frequentes 16 e 58.

2.4 Diagnóstico

O diagnóstico de LEIBG e LEIAG pode ser realizado por diferentes métodos como: exame de Papanicolau e análise histopatológica de tecido obtido por biópsia do colo uterino orientada por colposcopia. Estudos indicam que a análise histopatológica apresenta maior sensibilidade e especificidade em relação ao exame citopatológico (Patel et al., 2012; Tsikouras et al., 2016).

O exame de Papanicolau é realizado anualmente como teste de triagem para lesões possivelmente ocasionadas pelo HPV, porém, a sua crítica sensibilidade indica a necessidade de exames complementares como a pesquisa biomarcadores de infecção viral (Martins et al., 2005; Tsikouras et al., 2016).

O diagnóstico da infecção pelo HPV é realizado através de técnicas biomoleculares que detectam o HPV-DNA como: reação em cadeia da polimerase (PCR), captura híbrida e hibridização *in situ*. Estudos epidemiológicos realizados em amostras colhidas com o sistema DNA-CITOLIQ (DIGENE - Brasil) e que demonstraram resultados negativos no exame de Papanicolau quanto à presença de lesões, quando submetidos aos testes do HPV-DNA resultaram em positivos, evidenciando assim a elevada sensibilidade das técnicas biomoleculares (Utagawa et al., 2004; Tsikouras et al., 2016).

2.4.1 Vacinas

Atualmente estão disponíveis dois tipos de vacinas para HPV, a Bivalente e a Quadrivalente. A Bivalente contempla HPV-DNA considerados de alto risco oncogênico, prevenindo infecções de dois tipos virais (tipos 16 e 18) que estão associados à maioria dos tumores do colo de útero. Apesar do grande número de HPV-DNA identificados, a maioria das infecções genitais são causadas por apenas quatro

deles, que estão presentes na vacina quadrivalente (tipos 6, 11, 16 e 18) (Grabiel et al., 2013; Tsikouras et al., 2016).

2.5 Transglutaminase tipo 2

A TG2 vem sendo apontada como fator que interfere na infecção pelo vírus HPV, estando envolvida em uma grande variedade de funções como: morte celular, rearranjo do citoesqueleto e estabilização extracelular. A sua ativação alterada vêm sendo associada a diversas patologias (Facchiano et al., 2006; Falasca et al., 2008; Huang et al., 2015).

A TG2 também está associada à patogênese viral do HPV. Estudos indicaram que TG2 com sua atividade catalítica pode incorporar uma poliamina na proteína E7 do HPV do tipo 18, e assim inibir sua ligação à proteína retinoblastoma 1 (pRb) no citosol e núcleo (Lu et al., 2001; Jeon and Kim, 2006; Min et al., 2016).

A TG2 também tem a capacidade de se translocar do citosol para o núcleo, incorporando poliaminas da proteína do retinoblastoma (Rb) protegendo assim a degradação via caspases (Boehm et al., 2002; Milakovic et al., 2004; Huang et al., 2015). Adicionalmente, foi evidenciado em células cancerosas a elevação do processo de *splicing* de TG2, indicando uma correlação desta proteína com as alterações no processo intracelular diferencial do câncer (Phatak et al., 2011).

As LEIBG apresentam nível elevado da enzima, enquanto que nas LEIAG estes níveis estão diminuídos. Esta enzima é referida como um fenômeno precoce não restrito aos HPV classificados como alto risco oncogênico (Del Nonno et al., 2011; Min et al., 2016).

2.6 $p16^{INK4a}$

A proteína $p16^{INK4a}$ tem função regulatória no ciclo celular, tendo sua expressão controlada em células normais. A $p16^{INK4a}$ atua como supressor de tumores inibindo as quinases ciclo-dependente (CDK) 4 a 6, estas enzimas CDK fosforilam o retinoblastoma (Rb), o qual esta usualmente ligado com E2F, que regula a entrada da célula na fase S do ciclo celular. Na infecção pelo HPV, a proteína E7 rompe e ligação de Rb com E2F resultando em um aumento expressional de $p16^{INK4a}$. Sendo assim, autores observaram um aumento expressional em LEIAG e uma diminuição em LEIBG (Sano et al., 1998; Hang et al., 2016).

2.7 p53

A proteína chamada p53 é codificada pelo gene TP53, *tumor protein p53.* Esta proteína regula diversos genes em resposta a situações de estresse celular, interferindo assim no controle do ciclo celular, apoptose, reparo de DNA e modificações no metabolismo. (Parrales e Iwakuma, 2015; Williams e Schumacher, 2016).

Sob condições normais a proteína p53 atua em situações “emergenciais” dentro da célula, como em condições de hipóxia ou em danos de DNA resultantes de radiação, luz ultravioleta ou agentes mutagênicos. Através da ativação de p53, genes de reparo do DNA são induzidos e a célula interrompe seu ciclo celular. Quando o reparo celular não é possível, a célula lesada é induzida a apoptose via p53. A inatividade de p53 causa descontrole de ciclo celular de células em proliferação, o que leva a uma instabilidade genética e acúmulo de mutações em oncogenes (Carrilho et al., 2003; Katiyar et al., 2005; Min et al., 2016).

HPV de alto risco codificam as oncoproteínas E6 e E7. A oncoproteína E6 atua sobre p53 resultando na perda do processo de apoptose enquanto que E7 atua sobre o supressor de tumor pRb (Huibregtse et al., 1991; Scheffner et al., 1991; Min et al., 2016). E6 liga-se a p53 estimulando a sua degradação, ação que é dependente de ATP e envolve o sistema de protease ubiquitina-dependente, processo este, que promove novos mecanismos de ação em proteínas oncogênicas do HPV (Scheffner et al., 1990; Martinez-Zapien et al., 2016; Zhou et al., 2015).

Baseado no exposto, pode-se observar que a progressão tumoral exerce influência na expressão de p53, indicando assim sua possível utilização como biomarcador em processos tumorais dependentes do HPV.

2.8 Ki-67

A ki-67 é uma proteína presente no núcleo de células em crescimento, sendo expressa em todas as fases do ciclo celular, estando esta expressão associada a proliferação celular (Beishline and Azizkhan-Clifford, 2015). O mecanismo de controle transcricional não está bem estabelecido, mas foi evidenciado uma influência de Sp1 (*specificity protein 1*) no controle de transcrição de ki-67, isso porque a Sp1 liga-se à região promotora do gene codificador de ki-67. A Sp1 é um fator de transcrição com função ampla, incluindo processos como diferenciação celular, crescimento celular e apoptose (Tian et al., 2011).

Diversos estudos relacionaram a expressão de ki-67 a diferentes tipos de câncer, dentre eles o câncer cervical, sendo que a sua expressão esteve associada a um mau prognóstico para o paciente (Vasilescu et al., 2009). A ki-67 como marcador, pode ser utilizado para verificar o aumento de atividade proliferativa correlacionada ao HPV

(Reyes and Cooper 2014). Assim sendo, diversos estudos investigam a associação de ki-67 com diferentes marcadores no processo canceroso cervical (Vasilescu et al., 2009; Kisser and Zechmeister-Koss, 2015; Peres et al., 2016).

2.9 bcl-2

A proteína bcl-2 é encontrada na membrana mitocondrial externa e possui função relacionada à sobrevivência celular. A inibição da apoptose via bcl-2 ocorre através do bloqueio da liberação da proteína citocromo C da mitocôndria, isso após o estímulo apoptótico, impedindo assim a ação das enzimas responsáveis pela morte celular programada, denominadas de caspases (Zheng et al., 2015).

A expressão do gene codificador de bcl-2 vem sendo apontada como um fator que confere resistência à morte celular programada em uma variedade de tumores. A inativação do gene inibidor do ciclo celular, p16MTS1, parece estar envolvida na carcinogênese dependente do HPV, isso devido a ação das oncoproteínas E6 e E7, as quais influenciam diretamente $p16^{INK4a}$ e indiretamente as funções da bcl-2 (Namazie et al., 2002; Kim et al., 2013).

Quando analisada a expressão em LEIBG e LEIAG, foi evidenciado que a bcl-2 pode ser utilizada como marcador do processo, indicando que a infecção por HPV pode ser responsável pela elevada expressão de blc-2 (Grace et al., 2003). Foi evidenciado também, o aumento da expressão de bcl-2 em LEIAG, indicando assim a associação entre bcl-2 e a progressão tumoral cervical (Brychtova et al., 2000).

2.10 Análise de biomarcadores no progresso do câncer cervical

A Sociedade Americana de Patologia Clínica, definiu novas formas de triagem para a detecção de câncer cervical, dentre as definições da Diretriz, está a aferição de expressão de proteínas envolvidas na carcinogênese pelo HPV (Saslow et al., 2012).

Baseado no exposto, observa-se uma variedade de proteínas que tem a sua expressão influenciada pelo processo canceroso e/ou pela infecção do HPV. A análise de expressão de marcadores em associação ou isoladamente demonstra dados importantes para elucidação de mecanismos que podem auxiliar na definição de biomarcadores ou na indentificação de possíveis alvos terapêuticos. Desta maneira, torna-se relevante estudos que analisem a expressão de diversos marcadores a fim de compreender mecanismos associados à carcinogênese cervical.

2.10.1 Técnicas Quantitativas

Atualmente dispõe-se de diversas metodologias para a quantificação de expressão proteica, como a imunohistoquímica (IMQ) (Torres et al., 1995), na qual objetiva-se avaliar a expressão da proteína através de ligação de anticorpos específicos para a proteína investigada. A análise ocorre através da visualização de coloração diferencial em lâminas com cortes de tecidos fixados e corados proporcionalmente à intensidade de expressão proteica (Santos et al., 1999; Laurinavicius et al., 2016).

A quantificação da expressão proteica através de IMQ é realizada de diferentes formas e os métodos utilizados até o momento resumem-se a dois grupos. No primeiro grupo está o método binário simples, que apenas classifica os casos em positivo ou negativo. No segundo grupo estão os métodos semi-quantitativos, que utilizam escalas,

buscando agrupar casos dentro daqueles classificados como positivos. São utilizadas algumas designações tais como: negativo/marcação fraca/marcação forte; negativo/marcação esporádica/marcação focal/marcação difusa; escala de cruzes e escala por números, variando de 0 a 3 (Thal et al., 1995; Nieh et al., 2005; Laurinavicius et al., 2016).

Estes métodos mostraram-se subjetivos e apresentaram grande variabilidade intra e interobservador. Apesar destas características indesejáveis, os métodos semi-quantitativos buscam diferenciar subgrupos dentro daqueles casos classificados como "positivos" (Matos et al., 2006; Kok et al., 2010; Malik et al., 2011).

Na tentativa que diminuir a variação existente na literatura, propomos neste trabalho padronizar a quantificação de expressão proteica através de método computacional, e assim compará-los com os métodos previamente utilizados, objetivando assim estabelecer uma metodologia com sensibilidade e especificidade suficientemente adequados para amostras histológicas de colo uterino obtidas por biópsias.

OBJETIVO GERAL

Avaliar a expressão de TG2, p16INK4a, P53, ki-67 e bcl-2 em lesões escamosas intraepiteliais cervicais de baixo (LEIBG) e alto grau (LEIAG) associadas a genótipos virais do HPV.

OBJETIVOS ESPECÍFICOS:

- Revisar aspectos relacionados à infecção pelo HPV e proteínas marcadoras deste processo;
- Verificar eficácia de método quantitativo computacional para expressão proteica em material histológico obtido por biópsias de colo uterino;
- Verificar a expressão isolada e combinada de p53, ki-67 e bcl-2 em LEIBG, LEIAG associadas a diferentes tipos virais do HPV;
- Avaliar a expressão de TG2 em LEIBG, LEIAG associadas a tipos virais do HPV;
- Avaliar e correlacionar a expressão de TG2 e $p16^{INK4a}$ em LEIBG, LEIAG associadas a tipos virais do HPV.

3. RESULTADOS E DISCUSSÃO

Os resultados prévios da presente tese serão apresentados em forma de capítulos.

- *Capítulo 1:*

Artigo de Revisão: HPV pathogenesis and biomarkers of viral progression and cervical cancer: A literature review

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- *Capítulo 2:*

Artigo Original: Application of computational method for quantitative analysis of protein expression of $p16^{INK4a}$ in cervical biopsies through immunohistochemistry.

Publicado no Periódico: International Journal of Engineering Science Invention. ISSN (Online): 2319 – 6734, ISSN (Print): 2319 – 6726. www.ijesi.org Volume 3 Issue 11 ǁ November 2014 ǁ PP.09-13.

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- *Capítulo 3:*

Artigo Original: Expression analysis of P53, Ki-67 and bcl-2 in pre-malignant lesions of the cervix.

Publicado no Periódico: Open Journal of Obstetrics and Gynecology, 4, 462-469. http://dx.doi.org/10.4236/ojog.2014.48067

- *Capítulo 4:*

Artigo Original: Expression Analysis of Transglutaminase 2 in Pre-Malignant Lesions of the Cervix.

Submetido no Periódico: Applied Cancer Research

- *Capítulo 5:*

Artigo Original: Analysis of Transglutaminase 2 and $p16^{INK4a}$ expression in premalignant cervical lesions

Submetido no Periódico: British Journal of Cancer

Capítulo 1

HPV AND BIOMARKERS OF VIRAL PROGRESSION AND CERVICAL CANCER: A LITERATURE REVIEW

Karen Olivia Bazzo[1,2]

Cidade Universitária de Caxias do Sul

Rua Francisco Getúlio Vargas, 1130 - CEP 95070-560 Caxias do Sul - RS Brasil

Telefone/Telefax: (54) 3218-2100

E-mail: karenbazzo@gmail.com

CPF: 834.319.180-34

Alessandra Eifler Guerra Godoy [3,4]

Cidade Universitária de Caxias do Sul

Rua Francisco Getúlio Vargas, 1130 - CEP 95070-560 Caxias do Sul - RS Brasil

Telefone/Telefax: (54) 3218-2100

E-mail: aeggodoy@gmail.com

CPF: 679.746.780-20

Fábio Firmbach Pasqualotto[5,6]

Cidade Universitária de Caxias do Sul

Rua Francisco Getúlio Vargas, 1130 - CEP 95070-560 Caxias do Sul - RS Brasil

Telefone/Telefax: (54) 3218-2100

E-mail: fabio@conception-rs.com.br

CPF: 618.469.700-59

ABSTRACT

The critical sensitivity and specificity of the screening system for detection of HPV and cervical cancer indicates a necessity to study new biomarkers of early viral infection. This study aimed to review the literature evidence of novel biomarkers of viral progression and cervical cancer. To conduct this review, the electronic databases Medline, SciELO, Pubmed, in Portuguese and English, referring to biomarkers of viral progression and HPV pathogenesis were consulted, using the keywords: Uterine Cervical Neoplasms, HPV, Biological Markers. The viral infectious and carcinogenic action of HPV among the biomarkers of tumor progression described in the literature was observed, with highlights to microRNAs (miRNA) Transglutaminase Type 2 (TG2), Ki-67, $p16^{INK4a}$ and Dynamin2. Based on scientific evidence, it was concluded that it is possible to improve the process by feasible techniques of high predictive value, thus highlighting the need for further studies to prove the biomarker effectiveness for subsequent use in the national screening system.

Key-words: Uterine Cervical Neoplasms, HPV, Biological Markers

Introduction

The infection for HPV is becoming more incident and prevalent in population, it is known that HPV infects the organism in two different ways, inflammatory and carcinogen, however we are seeing new details and markers from both processes, besides, the critical sensitivity and specificity of the screening system for detection of HPV and cervical cancer indicates the need to study new early biomarkers of viral infection (Conway et al., 2009; Zur, 2002; Garland et al., 2007; Faridi et al., 2011).

In this study we aimed to review, in a narrative way, current data about HPV infection and the disclosure of new early biomarkers for HPV.

To initiate the study the following question was formulated: what has the scientific production been presenting about the thematic of HPV pathogenesis? For this review, the data bases of Medline, SciELO, and Pubmed were consulted, in Portuguese and English, referring to biomarkers of viral progression and HPV pathogenesis, using the describers: HPV, biomarkers, cancer.

1. Literature review

The HPV virus

The virion of human papillomavirus (HPV) is a DNA virus, from the *Papillomaviridae* family, not enveloped, with 72 capsomers. It has capsid with 55nm of diameter and is presented in an icosahedral form. Its genome is circular, with a double-stranded DNA measuring 7,900 Kb and mass of 5,000 KDa. It contains nine open reading frames - ORF, in which are placed not only the early reading genes E1, E2, E4, E5, E6 and E7, but also those of late reading, L1 and L2. There is also a non coding region (large control region - LCR) that control the other genes[1].

Each one of those viral genes works transcribing proteins of different functions, according to Table 1(Zur, 2002; Garland et al., 2007; Faridi et al., 2011).

Currently, different viral kinds of HPV have already been identified, and those are split in two groups, being considered of low carcinogen risk, which are frequently associated to intraepithelial lesions of low-grade and to accumulated condylomata that are the viral subtypes of HPV: 6, 11, 40, 42, 43, 54, 61, 70, 72, 81: and the group of high-level oncogenic risk, for being frequently associated to high-level of intraepithelial neoplasm and the invasive neoplasms are the viral subtypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 (Crawford etal., 2011).

HPV infection is related to proliferative lesions, malignant or benign. The benign lesions include common warts, plantar or genital (condyloma acuminata) and oral lesions and oropharynx in the conjunctiva. The malignant lesions associated to HPV include squamous-cell cancers of the respiratory and oral tract (Crawford etal., 2011).

Table 1. Description of the specific functions of each HPV gene

HPV gene	**Function**
E1	Operates in the episomal replication of the virus
E2	Negatively regulates the functions of proteins E6 and E7
E4	Produces the protein secondary viral capsid
E5	Induces proliferation of the virus infected cell
E6	Directly affects the p53 of the host cell ubiquitin and keeps the telomerase length above its critical point, protecting cells against apoptosis.
E7	Inactivates the pRB protein of the host cell, preventing cycle lock Cell
L1	Synthesizes the main protein of the viral capsid.

L2	Expresses the viral capsid secondary protein

(Zur, 2002; Garland et al., 2007; Faridi et al., 2011)

The HPV virus infection

The HPV virus is connected to the cells through proto cutaneous receptors highly prevalent in cell membranes (Roden et al., 1994; Moody et al., 2010). The HPV acts mostly on the squamous tissue cells, the L1 gene contains a major determinant for the entry of the virus in the intracellular. Some studies support the hypothesis that heparan sulfate proteoglycan is essential for early interactions between the HPV virus and epithelial cells, whereas administration of heparinase inhibited viral inclusion (Joyce et al., 1999; Giroglou et al., 2001).

A viral inclusion is not substantially detected within 24 hours after capsid binding , the viral internalization occurs primarily in a slow phase which lasts from 2 to 4 hours after binding to the cell membrane. It is known that the L2 gene is determinant for this stage, being it structurally modified by the Furin protein; in the presence of these protein inhibitors the virus loses its capacity of cellular invasion (Culp et al., 2004; Richards et al., 2006).

The pathways of endocytosis and intracellular transport are extensively studied, it has become clear that these pathways directly depend on the viral genotype, and the role of a protein called Claritin in the endocytic process was clarified as an important step in viruses like HPV - 16. It is known that Claritin inhibitors block the viral infection. In contrast, several studies have shown that HPV 31 does not have the same endocytosis pathway, this viral genotype has its process dependent of caveolae, but research failed to block infection by inhibiting viral caveolae (Laniosz et al., 2008; Bousarghin et al., 2003).

Traffic to the late endosome is dependent on L1, intranuclear transport currently is not well elucidated and it is known that this process is founded on L2 gene by interacting with the cellular microtubules. Complexes based on the nuclear domains promote transcription of the

viral genome, the reorganization of these domains is observed in cervical wounds, these are produced by the L2 gene (Maul, 1998; Florin et al., 2002; Florin et al., 2002; Darshan et al., 2004).

Several promoters shown to be related in the generation of Messenger RNA (mRNA) from HPV that infects the genital tract. There are significant differences in the expression of E6 and E7 proteins from HPV classified as being of low and high oncogenic risk. In cases of high-risk HPV, there is only one promoter which stimulates the synthesis of E6 and E7. In the low-risk group, there is a genetic influence of two different promoters for transcription of E6 and E7 (Korzeniewski et al., 2011).

The noncoding region of HPV has been identified as having important elements for the initial expression of viral genes; however this same region appears to be important in the viral latency process (Korzeniewski et al., 2011).

Viral replication occurs in three possible models. The first model happens during the initial infection of basal keratinocytes, with the amplification of 50 to 100 viral copies[1], the next stage is defined as genome maintenance in these infected cells, viral DNA is conserved in multiple copies of stable plasmid. Viral replication is synchronized with cell cycle, which ensures a latent infection[1, 18]. The last type of viral replication occurs in a vegetative way in the already differentiated epithelial cells. At this point it is possible to detect HPV late gene expression and release of new virions. The regulation genome at this stage is not well understood and can be influenced or not by the cell controlling factors (Conway et al., 2009; Korzeniewski et al., 2011).

Epidemiology

HPV is a very prevalent virus; warts are very frequent viral diseases, with an estimated incidence of 7% to 10% in the European population and 1% in the U.S. population (Hengee, 2004). In immunocompromised and patients recipients of renal transplant, this number increases 50 to 100 times, reaching 90% after 15 years of transplantation (Lindelof et al., 2000). Warts can occur at any age and the incidence increases during school age, peaking in youth and young adults.

A recent study conducted in Tanzania with 3603 women from urban and rural population of the country revealed the prevalence of HPV in 20.1% of the population, its detection being higher in HIV-positive women (46.7%) (Kilkenny et al., 1996; Dartell et al., 2012). In the presence of cervical cancer, HPV detection was observed in 100% of the population in New Guinea(Tabone et al., 2012).

A study conducted in the cities of São Paulo, Porto Alegre and Campinas showed prevalence of genital HPV infection of high risk in 17.8% of the studied population (2,300 women 15-65 years). It was observed a rate of infection of 27.1% (below 25 years), 21.3% (25-34), 12.1% (35-44), 12.0% (45-54 years) and 13.9% (55-65 years). Subjects with the highest number of sexual partners during lifetime had higher frequency of infection (Rama et al., 2008).

The HPV virus prevalence evaluated at the Federal University of Rio Grande was 66.3%, 60% of those were related to HIV virus coinfection (Entiauspe et al., 2010).

Diagnostic

The current diagnosis of HPV infection is made after detection of wounds caused by HPV with Pap test and histopathological analysis. Studies indicate that histopathological analysis of biopsy demonstrates 100% accurancy (Souza et al., 2001; Patel et al., 2012).

Epidemiological studies carried out on samples collected using the DNA-CITOLIQ system (DIGENE - Brazil) showed cases with negative results in the cytological screening, , which were positive in HPV-DNA tests, and thus evidenced the high sensitivity of biomolecular techniques (Utagawa et al., 2004).

The Pap smear test is held annually as a screening test for possible injuries caused by HPV virus, but, with a critical sensitivity, indicates the need for new biomarkers of early viral infection (Souza et al., 2010).

The use of biomarkers in cervical cytology and histology has been demonstrating high predictive values, several biomarkers for the detection of cervical diseases have been indentified, many of them involved in the cell cycle regulation, transduction cascades, DNA replication and cell proliferation (Malinowski, 2005a; 2007b).

Recently, Patel et al (2012) demonstrated that the high methylation of candidate genes particularly of HPV 16 is associated with chances of abnormal cervical cytology results.

The role of biomarkers in screening tests improvement

The screening by cervical cytology has been reducing morbidity and mortality from cervical cancer, but its limitations in terms of sensitivity and specificity indicate the need for improvement in the screening method (Bulet et al., 2008) . Recently, Andrade (2012) points in his review, limitations in the process of cervical cancer diagnosis in Brazil, among these stands the lack of standardization in cytological reading and low specificity of colposcopy, corroborating the need for new tools in the diagnosis of cervical cancer.

This new need for early diagnosis was confirmed by Whilock et. al. (2011), which demonstrated the ineffectiveness of liquid cytology as a screening test for cervix cancer, thus concluding the need for new scientific evidence for progression biomarkers.

The Pap smear screening has been proved as a limiting exam on the screening process for cervical cancer, and its sensitivity has been decreased due to the determination of atypical squamous cells of undetermined significance, elucidating again the need for accessory diagnostic parameters to the traditional screening method in order to contribute for the sensitivity and specificity levels of the procedure (Briet et al., 2010).

The use of biomarkers of dysplastic progression is reported as a facilitator for abnormal cell detection together with Pap smear cytopathological screening. Recent publications demonstrate the predictive power of several biomarkers in screening tests, showing high levels of protein expression in high-grade precursor wounds (Brown et al., 2012).

Recently, Alonso et al. (2012) demonstrated through semi-quantification by immunohistochemical $p16^{INK4a}$ a significant relationship between expressional levels of the protein and development of malignancies in the genital tract caused by HPV, evidencing important diagnostic tools in the progression of viral infection.

Zagels et al. (2010) investigated, using proteomics technique, the cervical fluid in order to identify biomarkers for pathologies of the genital system, and elucidated in his study the correlation of the technique with the disclosure of new biomarkers for various diseases.

Therefore, a clear need for new biomarkers with sensitivity and specificity complementary to this screening system is identified, in order to corroborate the already performed procedure in the female population and improve the early detection of cervical cancer. Based on this need, following are potential biomarkers demonstrated in recent studies of the literature.

Biomarkers and screening

- **Ki-67**

Ki-67 is a protein located in the nucleus and nucleolus expressed during G1, S, G2 and M phases. Its cellular function is not evidenced in literature; however it has its protein expression related to cell proliferation (Endl et al., 2000). Because HPV induces high rates of cell proliferation, there is a described correlation between the expressional levels of Ki-67 and the progression of infectious HPV in dysplasia and neoplasia (Keating et al., 2001).

Recently associations were identified of expressional levels of p16 ^{INK4a} and Ki-67 in patients diagnosed with HPV, reaching high positive and negative predictive values, thus it can be used as an adjunct to the gynecological routine (Ziemke et al., 2012).

- **p16^{INK4a}**

The p16^{INK4a} protein has a regulatory role in the cell cycle, and its expression is highly controlled by normal cells. p16INK4a acts as a tumor suppressor by inhibiting cycle-dependent kinase 4 and 6, which phosphorylate the retinoblastoma (Rb) which is usually bonded to E2F which regulates the entry of the cell in stage S of the cell cycle. On HPV infection, the E7 protein breaks the binding of Rb with E2F, resulting in an expressional increase of p16INK4a (Khleif et al., 1996; Sano et al., 1998).

Godoy et al.,(2008) have recently demonstrated a correlation between the expressional levels of p16^{INK4a} and cervical wounds caused by HPV, indicating p16INK4a as a biomarker for neoplastic lesions caused by HPV.

- **p16^{INK4a} /Ki-67**

Recently, a study in Germany with women over thirty years old demonstrated a new screening model for HPV using the marking for the p16^{INK4a} and ki-67 proteins simultaneously,

the efficiency of this type of screening was verified as an important factor in the progression of viral infection and cancer pathogenesis (Petry et al., 2011).

Donà et al. (2012) analyzed cervical smears from 140 women, the samples went through ki-67 and $p16^{INK4a}$ quantification process simultaneously via CINtec® PLUS, the results evidenced in this study corroborate previous data, showing that the immunohistochemistry for these markers may have an important diagnostic value, and the positivity for these biomarkers is highly related to the presence of high-grade premalignant lesions.

Other immunohistochemistry kits with simultaneous action for $p16^{INK4a}$ and Ki-67 have been proven effective in the early diagnosis of cervical cancer, the use of ProEx™ immunohistochemistry demonstrated results with sufficiently effective accuracy, besides also highlighting the need for repetition in only one third of the samples (Walts et al., 2009).

- **miR-100**

MicroRNAs (miRNA) are RNA molecules that act by inhibiting messenger RNA (mRNA) in several biological processes, including proliferation, differentiation, development, metabolism and cell death. Studies show that miRNA is often super expressed in malignancies and its role was evidenced in the emergence and development of tumors. As a potential tumor suppressor, the reduction of miR-100 expression has been evidenced in some types of tumors, such as low level bladder cancers, oral., ovarian and liver cancer. However, the expression and function of miR-100 in cervical cancer is still poorly studied (Catto et al., 2009; Henson et al., 2009; Yang et al., 2008; Thorgeirsson, 2011).

Li et al. (2011) investigated different miRNAs through microarray and as a result, the miRNAs are separated into groups according to their expression level, miR-100 showed to be the miRNA with lower expression based on the associations described above. The same study investigated their relationship in cervical cancer by analyzing 125 cases of cervical cancer, cervical intraepithelial neoplasm, normal tissues and also evaluating their role in cell culture.

This study showed a negative proportional relationship between miR-100 levels and the development of cervical cancer, and its lower expression was identified in high-grade lesions. In cell culture, the inhibitor of miR-100 increased apoptosis and decreased cell proliferation as well as accelerated G2/M phase in HaCaT cells, whereas the increase of miR-100 had the reverse effect, miRNA increased apoptosis and decreased malignant cell proliferation.

The miR-100 targets are not known, some associations are identified in other types of tumor, for example, in ovarian tumor it was observed that overexpression of miR-100 suppressed the mTOR (mammalian target of rafamicina), which acts in cell cycle progression, death, and size (Nagaraja et al., 2010).

Another molecule strongly associated with miR-100 inhibitory action is PKL1, protein in the regulation of cell mitosis, being the checkpoint of the M/G2 stage; its alteration can cause chromosomal instability. Its expression levels demonstrate to be high in several types of tumors (Schmit et al., 2009; Nappi et al., 2009; Feng et al., 2009), including cervical cancer (Gao et al., 2006). Studies have shown that depletion caused by interference RNAs results in inhibition of cell proliferation and increased apoptosis.

Recently it was demonstrated an inverse relationship between the expression of miR-100 and PKL1 contributing to nasopharyngeal cancer progression (Shi et al., 2010). This correlation was observed only in high-grade lesions and cervical tumors. However, more studies are needed to evaluate the expressional levels of those proteins.

- **Transglutaminase type 2:**

The transglutaminase enzyme type 2 (TG2) has been identified as an influencing factor in HPV infection (Jeon et al., 2006). TG2 is involved in a wide range of functions, such as cell death, cytoskeleton rearrangement and extracellular stabilization. Its altered activation has been

associated with several pathologies (Fesus et al., 2002; Facchiano et al., 2006; Falasca et al., 2005a 2008b).

TG2 is also associated with viral pathogenesis; several proteins involved in the infection process demonstrate to be modified by the TG2 protein (Amendola et al., 2001; Lu et al., 2001; Jeon et al., 2006). A recent study indicated that TG2, with its catalytic activity, can incorporate a polyamine in the E7 protein of HPV18, thus inhibiting its binding to pRb protein in cytosol and nucleus. TG2 also has the ability to translocate from the cytosol to the nucleus incorporating polyamines of retinoblastoma protein (Rb) thereby protecting the degradation by caspases (Boehm et al., 2002; Milakovic et al., 2004).

Del Nonno et. al. (2011) demonstrated that TG2 can incorporate E7 oncoprotein from HPV18 virus, thereby participating in the cellular response to the virus. Low-grade lesions have high levels of the enzyme, while in high-grade lesions those levels are diminished. This enzyme is now referred to as an early phenomenon not restricted to high-risk genotypes.

• **Dynamin2**

Dynamin2 represents one of the proteins from the family of GTP-binding proteins that is associated with microtubules and is involved in endocytosis processes of cell motility and membrane changes. Dynamin binds to proteins that bind to actin and other cytoskeletal proteins (Durieux et al., 2011).

Lee et. al. (2012) recently investigated the correlation between the expressional levels of dynamin2 and the emergence of cervical tumors in HPV-infected patients; there were results of association between marker and cancer progression, thus demonstrating a new possible early marker for infectious and cancerous lesion by HPV.

Final Thoughts

Continuous improvement of the screening process is a global need, the institution of new biomarkers of HPV infection progression and its consequent cervical neoplasm indicate, with proven effectiveness, the improvement of sensitivity and specificity of the screening process.

Based on the scientific evidence cited in this review, it is concluded that it is possible to improve the program through feasible and high predictive techniques, highlighting the importance and need for new studies to prove the full effectiveness of biomarkers for later use in the national screening system.

Contributors and Contributions:

Alessandra Eifler Guerra Godoy - Contribution: Writing and literary research

Fábio Firmbach Pasqualotto - Contribution: Writing and literary research

REFERENCES:

Alonso I, Felix A, Torné A, Fusté V, del Pino M, Castillo P et al (2012). Human papillomavirus as a favorable prognostic biomarker in squamous cell carcinomas of the vagina. Gynecol Oncol., 125(1):194-9.

Amendola A, Rodolfo C, Caro A, Ciccosanti F, Falasca L, Piacentini M (2001). “Tissue” Transglutaminase Expression in HIV-Infected Cells. Ann N Y Acad Sci., 946(1):108-20.

Boehm JE, Singh U, Combs C, Antonyak MA, Cerione RA (2002).Tissue transglutaminase protects against apoptosis by modifying the tumor suppressor protein p110 Rb. J Biol Chem., 277(23):20127-30.

Boulet GAV, Horvath CAJ, Berghmans S, Bogers J (2008). Human papillomavirus in cervical cancer screening: important role as biomarker. Canc Epid Biomarkers & Prevention., 17(4):810-7.

Bousarghin L, Touze A, Sizaret PY, Coursaget P (2003). Human papillomavirus types 16, 31, and 58 use different endocytosis pathways to enter cells. J Virol., 77(6):3846.

Briët MC, Berger THD, van Ballegooijen M, Boon ME, Rebolj M (2010). Effects of Streamlining Cervical Cancer Screening the Dutch Way: Consequences of Changes in the Dutch KOPAC-Based Followv-up Protocol and Consensus-Based Limitation of Equivocal Cytology. Acta Cytol.,54(6):1095-100.

Brown CA, Bogers J, Sahebali S, Depuydt CE, De Prins F, Malinowski DP (2012). Role of Protein Biomarkers in the Detection of High-Grade Disease in Cervical Cancer Screening Programs. J Oncol.

Catto JWF, Miah S, Owen HC, Bryant H, Myers K, Dudziec E et al (2009). Distinct microRNA alterations characterize high-and low-grade bladder cancer. Cancer Res.,69(21):8472.

Conway M, Meyers C (2009). Replication and assembly of human papillomaviruses. J Dent Res.,88(4):307-17.

Crawford R, Grignon AL., Kitson S, Winder D, Ball S, Vaughan K, et al (2011). High prevalence of HPV in non-cervical sites of women with abnormal cervical cytology. BMC cancer., 11(1):473.

Culp TD, Christensen ND (2004). Kinetics of in vitro adsorption and entry of papillomavirus virions. Virology.,319(1):152-61.

Darshan MS, Lucchi J, Harding E, Moroianu J (2004). The L2 minor capsid protein of human papillomavirus type 16 interacts with a network of nuclear import receptors. J Virol.,78(22):12179.

Dartell M, Rasch V, Kahesa C, Mwaiselage J, Ngoma T, Junge J et al (2012). Human Papillomavirus Prevalence and Type Distribution in 3603 HIV-Positive and HIV-Negative Women in the General Population of Tanzania. Sex Transm Dis.,39(3):201-8.

de Andrade JM (2012). Limitações para o sucesso do rastreamento do câncer de colo no Brasil. Rev Bras Ginecol Obstet.,14049(34):900.

Del Nonno F, Pisani G, Visca P, Signore F, Grillo LR, Baiocchini A et al (2011). Role and predictive strength of transglutaminase type 2 expression in premalignant lesions of the cervix. Mod Pathol.,24(6):855-65.

Donà MG, Vocaturo A, Giuliani M, Ronchetti L, Rollo F, Pescarmona E et al (2012). p16/Ki-67 dual staining in cervico-vaginal cytology: Correlation with histology, Human Papillomavirus detection and genotyping in women undergoing colposcopy. Gynecol Oncol., 126(2):198-202.

Durieux AC, Prudhon B, Guicheney P, Bitoun M (2010). Dynamin 2 and human diseases. J Mol Med.,88(4):339-50.

Endl E, Gerdes J (2000). The Ki-67 protein: fascinating forms and an unknown function. Exp Cell Res.,257(2):231-7.

Entiauspe LG, Teixeira LO, Mendoza-Sassi RA, Gonçalves CV, Gonçalves P, Martinez AMB (2010). Papilomavírus humano: prevalência e genótipos encontrados em mulheres HIV positivas e negativas, em um centro de referência no extremo Sul do Brasil. Rev Soc Bras Med Trop.,43(3):260-3.

Facchiano F, Facchiano A, Facchiano AM (2006). The role of transglutaminase-2 and its substrates in human diseases. Front Biosci.,11(1758):73.

Falasca L, Farrace MG, Rinaldi A, Tuosto L, Melino G, Piacentini M (2008). Transglutaminase type II is involved in the pathogenesis of endotoxic shock. J Immunol.,180(4):2616.

Falasca L, Iadevaia V, Ciccosanti F, Melino G, Serafino A, Piacentini M. Transglutaminase type II is a key element in the regulation of the anti-inflammatory response elicited by apoptotic cell engulfment. J Immunol. 2005;174(11):7330.

Faridi R, Zahra A, Khan K, Idrees M (2011). Oncogenic potential of Human Papillomavirus (HPV) and its relation with cervical cancer. Virol J.,8(1):269.

Feng YB, Lin DC, Shi ZZ, Wang XC, Shen XM, Zhang Y et al (2009). Overexpression of PLK1 is associated with poor survival by inhibiting apoptosis via enhancement of survivin level in esophageal squamous cell carcinoma. Int J Cancer Suppl.,124(3):578-88.

Fesus L, Piacentini M (2002). Transglutaminase 2: an enigmatic enzyme with diverse functions. Trends Biochem Sci.,27(10):534-9.

Florin L, Schäfer F, Sotlar K, Streeck RE, Sapp M (2002). Reorganization of nuclear domain 10 induced by papillomavirus capsid protein l2. Virology.,295(1):97-107.

Gao Q, Huang X, Tang D, Cao Y, Chen G, Lu Y et al (2006). Influence of chk1 and plk1 silencing on radiation-or cisplatin-induced cytotoxicity in human malignant cells. Apoptosis., 11(10):1789-800.

Garland S, Hernandex-Avila M, Wheeler C. Cervical Cancer and HPV Vaccination. N Eng J Med. 2007;356:1915-27.

Giroglou T, Florin L, Schafer F, Streeck RE, Sapp M (2001). Human papillomavirus infection requires cell surface heparan sulfate. J Virol.,75(3):1565.

Godoy A, Mandelli J, Oliveira F, Calegari S, Moura L, Serafini E (2008). p16INK4 expression in precursor lesions of squamous cell cervical cancer related to the presence of HPV-DNA. Braz J Med Biol Res.,41(7):583-8.

Hengge UR (2004) [Papillomavirus diseases]. Hautarzt.,55(9):841-54.

Henson BJ, Bhattacharjee S, O'Dee DM, Feingold E, Gollin SM (2009). Decreased expression of miR-125b and miR-100 in oral cancer cells contributes to malignancy. Genes Chromosomes Cancer.,48(7):569-82.

Jeon JH, Cho SY, Kim CW, Shin DM, Jang GY, Lee HJ et al (2006). Alteration of Rb binding to HPV 18 E7 modified by transglutaminase 2 with different type of polyamines. Front Biosci.,11(6):1540-8.

Jeon JH, Kim IG (2006). Role of protein modifications mediated by transglutaminase 2 in human viral diseases. Front Biosci.,11:221-31.

Joyce JG, Tung JS, Przysiecki CT, Cook JC, Lehman ED, Sands JA et al (1999). The L1 major capsid protein of human papillomavirus type 11 recombinant virus-like particles interacts with heparin and cell-surface glycosaminoglycans on human keratinocytes. J Biol Chem.,274(9):5810.

Keating JT, Cviko A, Riethdorf S, Riethdorf L, Quade BJ, Sun D et al (2001). Ki-67, cyclin E, and p16 INK4 are complimentary surrogate biomarkers for human papilloma virus-related cervical neoplasia. Am J Surg Pathol.,25(7):884.

Khleif SN, DeGregori J, Yee CL, Otterson GA, Kaye FJ, Nevins JR, Howley PM (1996). Inhibition of cyclin D-CDK4/CDK6 activity is associated with an E2F-mediated induction of cyclin kinase inhibitor activity. Proc Natl Acad Sci U S A.,93(9):4350.

Kilkenny M, Marks R (1996). The descriptive epidemiology of warts in the community. Australas J Dermatol.,37(2):80-6.

Korzeniewski N, Spardy N, Duensing A, Duensing S (2011). Genomic instability and cancer: Lessons learned from human papillomaviruses. Cancer Lett.,305(2):113-22.

Laniosz V, Holthusen KA, Meneses PI (2008). Bovine papillomavirus type 1: from clathrin to caveolin. J Virol.,82(13):6288.

Lee Y-Y, Song SY, Do I-G, Kim T-J, Kim B-G, Lee J-W et al (2012). Dynamin 2 expression as a biomarker in grading of cervical intraepithelial neoplasia. Eur J Obstet Gynecol Reprod Biol., 164(2):180-4.

Li BH, Zhou JS, Ye F, Cheng XD, Zhou CY, Lu WG et al (2011). Reduced miR-100 expression in cervical cancer and precursors and its carcinogenic effect through targeting PLK1 protein. Eur J Cancer.,47(14):2166-74.

Lindelöf B, Sigurgeirsson B, Gäbel H, Stern R (2000). Incidence of skin cancer in 5356 patients following organ transplantation. Br J Dermatol.,143(3):513-9.

Lu W, Strohecker A, Ou J (2001). Post-translational modification of the hepatitis C virus core protein by tissue transglutaminase. J Biol Chem., 276(51):47993.

Malinowski DP (2005). Molecular diagnostic assays for cervical neoplasia: emerging markers for the detection of high-grade cervical disease. Biotechniques.,5:17-24.

Malinowski DP (2007). Multiple biomarkers in molecular oncology. I. Molecular diagnostics applications in cervical cancer detection. Expert Rev Mol Diagn.,7(2):117-31.

Maul GG (1998). Nuclear domain 10, the site of DNA virus transcription and replication. Bioessays.,20(8):660-7.

Milakovic T, Tucholski J, McCoy E, Johnson GVW (2004). Intracellular localization and activity state of tissue transglutaminase differentially impacts cell death. J Biol Chem.,279(10):8715.

Moody CA, Laimins LA (2010). Human papillomavirus oncoproteins: pathways to transformation. Nat Rev Cancer.,10(8):550-60.

Nagaraja AK, Creighton CJ, Yu Z, Zhu H, Gunaratne PH, Reid JG et al (2010). A link between mir-100 and FRAP1/mTOR in clear cell ovarian cancer. Mol Endocrinol.,24(2):447-63.

Nappi TC, Salerno P, Zitzelsberger H, Carlomagno F, Salvatore G, Santoro M (2009). Identification of Polo-like kinase 1 as a potential therapeutic target in anaplastic thyroid carcinoma. Cancer Res.,69(5):1916.

Patel DA, Rozek LS, Colacino JA, Van Zomeren-Dohm A, Ruffin MT, Unger ER et al (2012). Patterns of cellular and HPV 16 methylation as biomarkers for cervical neoplasiaJ Virol Methods.,184(1-2):84-92

Petry KU, Schmidt D, Scherbring S, Luyten A, Reinecke-Lüthge A, Bergeron C et al (2011). Triaging Pap cytology negative, HPV positive cervical cancer screening results with p16/Ki-67 Dual-stained cytology. Gynecol Oncol.,121(3):505-9.

Rama CH, Roteli-Martins CM, Derchain SFM, Longatto-Filho A, Gontijo RC, Sarian LOZ et al (2008). Prevalência do HPV em mulheres rastreadas para o câncer cervical. Rev Saude Publica.,42(1):123-30.

Richards RM, Lowy DR, Schiller JT, Day PM (2006). Cleavage of the papillomavirus minor capsid protein, L2, at a furin consensus site is necessary for infection. Proc Natl Acad Sci U S A.,103(5):1522.

Roden R, Kirnbauer R, Jenson AB, Lowy DR, Schiller JT(1994). Interaction of papillomaviruses with the cell surface. J Virol.,68(11):7260.

Sano T, Oyama T, Kashiwabara K, Fukuda T, Nakajima T (1998). Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions. Am J Pathol.,153(6):1741.

Schelhaas M, Ewers H, Rajamäki ML, Day PM, Schiller JT, Helenius A (2008). Human papillomavirus type 16 entry: retrograde cell surface transport along actin-rich protrusions. PLoS Pathog.,4(9):e1000148.

Schmit TL, Zhong W, Nihal M, Ahmad N (2009). Polo-like kinase 1 (Plk1) in non-melanoma skin cancers. Cell Cycle.,8(17):2697-702

Shi W, Alajez NM, Bastianutto C, Hui ABY, Mocanu JD, Ito E et al (2010). Significance of Plk1 regulation by miR-100 in human nasopharyngeal cancer. Int J Cancer Suppl., 126(9):2036-48.

Souza N, Melo V, Castro L (2001). Diagnóstico da infecção pelo HPV em lesões do colo do útero em mulheres HIV+: acuidade da histopatologia. Rev Bras Ginecol Obstet.,23 (6): 355-361.

Tabone T, Garland SM, Mola G, O'Connor M, Danielewski J, Tabrizi SN (2012). Prevalence of human papillomavirus genotypes in women with cervical cancer in Papua New Guinea. Int J Gynaecol Obstet.,117(1):30-2.

Thorgeirsson SS (2011). The almighty MYC: Orchestrating the micro-RNA universe to generate aggressive liver cancer. J Hepatol.

Utagawa ML, Pereira SMM, Longatto Filho A, Martins CR, Aguiar LS, Pittoli JÉ et al (2004). Citologia de base líquida associada à captura de híbridos para HPV-DNA pode otimizar a

qualidade diagnóstica do método de Papanicolaou?; Could liquid based cytology associated with hybrid capture II assay for HPV-DNA optimize the cytological diagnosis? Rev Inst Adolfo Lutz.,63(1):100-3.

Walts AE, Bose S (2009). p16, Ki-67, and BD ProEx™C immunostaining: a practical approach for diagnosis of cervical intraepithelial neoplasia. Hum Pathol.,40(7):957-64.

Whitlock EP, Vesco KK, Eder M, Lin JS, Senger CA, Burda BU (2011). Liquid-based cytology and human papillomavirus testing to screen for cervical cancer: a systematic review for the US Preventive Services Task Force. Ann Intern Med.,155(10):687.

Yang H, Kong W, He L, Zhao JJ, O'Donnell JD, Wang J et al (2008). MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN. Cancer Res.,68(2):425.

Zegels G, Van Raemdonck GAA, Tjalma WAA, Van Ostade XWM (2010). Use of cervicovaginal fluid for the identification of biomarkers for pathologies of the female genital tract. Proteome Sci.,8(1):63.

Ziemke P, Marquardt K (2012). Immunocytochemistry of p16 (INK4a) and Ki-67 as adjunctive method for routine gynecological cytology of mild and moderate dysplasia.Pathologe.,4.

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

Capítulo 2

APPLICATION OF COMPUTATIONAL METHOD FOR QUANTITATIVE ANALYSIS OF PROTEIN EXPRESSION OF $p16^{INK4a}$ IN CERVICAL BIOPSIES THROUGH IMMUNOHISTOCHEMISTRY

Karen Bazzo[1,2], Alessandra Eifler Guerra Godoy[3,4], Fábio Firmbach Pasqualotto [3,5,6]

[1] PhD Student in Biotechnology at the University of Caxias do Sul

[2] Professor at the Fátima Education School

[3] Professor at the University of Caxias do Sul

[4] Pathologist of the Laboratory Diagnose

[5] Coordinating professor of the urology and nephrology unit of the Medicine School of the University of Caxias do Sul,

[6] Full professor of the Associação Instituto Sapientae.

Institution where the study was conducted: Institute of Biotechnology – University of Caxias do Sul.

Corresponding author: Karen Olivia Bazzo

Address: Cidade Universitária de Caxias do Sul

Rua Francisco Getúlio Vargas, 1130 - CEP 95070-560 Caxias do Sul - RS Brasil

Phone/Fax: +55 54 32182100

E-mail: karenbazzo@gmail.com

Current e-mails of the study's coauthors:

Alessandra Eifler Guerra Godoy - E-mail: aeggodoy@gmail.com

Fábio Firmbach Pasqualotto - E-mail: fabio@conception-rs.com.br

ABSTRACT

OBJECTIVE: The purpose of this study was to standardize the quantification of protein expression of $p16^{INK4a}$ in cervical biopsies using computational method, and to compare it with methods used previously, as well as with HPV viral types, aiming to establish an efficient methodology for cervical biopsies. METHODS: Fifty-eight cases of Pap slides and cervical biopsies were selected. Then, immunohistochemistry for $p16^{INK4a}$ in cervical biopsies and quantitative analysis using the software Image J were performed. The final interpretation was performed using the Richart system, German Scoring System, and a qualitative method. Finally, a genotyping for HPV was performed. RESULTS: A significant association between quantification by Image J software and Richart system, German Scoring System, and qualitative method ($p<0.05$, $p<0.001$) was observed. When comparing the results obtained with high and low risk viral types, and negative samples, a proportional and significant association ($p<0.001$) was also observed; when comparing the results with the viral types separately, a significant correlation between genotypes 16, 18, 33, 39, 52, 58, and 73 ($p<0.05$, $p<0.001$) was reported. CONCLUSIONS: A proportional correlation of $p16^{INK4a}$ quantification and Richart, German Scoring System, and qualitative method scores, as well as with different viral genotypes was observed. Currently, the evaluation systems show deficiencies due to diagnostic inter- or intrapersonal variations. From the data obtained in the present study, we conclude that the quantification of $p16^{INK4a}$ in cervical biopsies by Image J software shows significant results for improving diagnostic techniques of cervical cancer, which may help in effectively choosing a clinical treatment without inter- or intrapersonal variations.

Keywords: Human papillomavirus, diagnosis, neoplasms, histology, p16 genes.

INTRODUCTION

Currently, we have several methods for measuring protein expression. A widely used method is immunohistochemistry [1], whose purpose is to evaluate protein expression by the binding of antibodies specific of the protein studied. The analysis occurs by visualization of differential staining on slides with tissue sections fixed and stained proportionally to the intensity of protein expression [2].

Immunohistochemistry is a tool used for investigating biomarkers, in especial for studying their relationship with cancer, such as cervical cancer [3]. Recently, the American Society of Clinical Pathology found, with relevant scientific evidences, new screening methods for detecting cervical cancer, among the definitions of the Guideline, is the quantification of expression of proteins involved in HPV carcinogenesis [4].

Several biomarkers for the detection of cervical diseases have been identified, many of them involved in cell cycle regulation, signal transduction cascades, DNA replication, and cell proliferation [5, 6]. Some studies have shown that the expression of p16INK4 is markedly influenced in carcinomas of the cervix due to functional inactivation of pRb by HPV oncoprotein E7 [7, 8].

Therefore, it is important to standardize the evaluation methods of biomarker expression such as the p16INK4; however, there is considerable diversity in measurements due to the great variability of methods used to grade the staining observed on immunohistochemistry slides [9]. Different authors randomly establish cutoff points for ranking the cases, which makes negative cases by some authors be considered positive by others and vice versa [10, 11].

The methods used so far in the literature can be summed up in two groups. The first group includes the simple binary method, which ranks the cases either as positive or negative. The second group includes semiquantitative methods, which use scales and seek to group cases among those classified as positive. Some designations used are negative/weak marking/strong marking; negative/sporadic marking/focal marking/diffuse marking; cross scale and number scale, ranging from 0 to 3 [9, 12]. These methods have been shown to be subjective and to have high intra- and interobserver variability. Despite these undesirable characteristics, semiquantitative methods seek to differentiate subgroups within those cases classified as "positive" [13].

In an attempt to decrease the variation found in the literature, we propose in this work to standardize the quantification of protein expression in cervix biopsies via computational method, and thus compare them with methods used previously, in order to establish a methodology with sensitivity and specificity sufficiently suitable for cervix biopsies. In addition, we aimed to correlate protein quantification by the proposed method with viral and histopathological classifications in order to detect associations corroborating data published in the literature and so determine the quality of the proposed method.

MATERIALS AND METHODS

The sample size was defined by statistical calculation, and was set to a security level of 95% and a sampling error of 5%. Furthermore, the sample size was verified in studies with patients diagnosed with cervical cancer [14-16]. Based on the above, we selected 58 slides of cervical biopsies, embedded in paraffin and stained with HE method. The samples were separated into three groups according to the classification

proposed by Richart and Barron in 1968 [17], which provides criteria for identifying cervical intraepithelial neoplasia (CIN) according to the degree of histological lesion of the cervical epithelium, while the samples of this study were divided into control group, a group comprising patients with HPV/CIN1, and group comprising patients with CIN2/CIN3.

The immunohistochemistry process by EnVision® system was performed by using the Cintec® $p16^{INK4a}$ histology kit (Dako Cytomation, Glostrup, Denmark), according to the manufacturer's instructions, with primary $p16^{INK4a}$ mouse anti-human antibody, clone E6H4. The 3-µm cuts performed on silanized slides were counterstained with Mayer's hematoxylin.

Each case was reviewed separately by two pathologists, who classified the samples of the present study according to different methods. After classification by both professionals, the data was checked and a mean of the determinations was established.

The samples were classified according to the semiquantitative method with the cross scale of the German semiquantitative scoring system, which correlates with the expression levels from 0 to 3 (0, 1+, 2+, 3+) [10, 13, 17], with rating "0" being used in cases without immunostaining for $p16^{INK4a}$, "1+" for weak immunostaining, "2+" for moderate immunostaining, and "3+" for strong immunostaining.

From the biopsies collected, genotyping for HPV was performed by using the polymerase-chain-reaction (PCR) method to identify the viral type.

The scanned images were transferred to a computer, and the intensity of staining was determined using the NIH ImageJ 1.36b software (National Institutes of Health, Maryland, USA).

Image J is a public-domain image-analyzing software on Java platform inspired by NIH Image software for Apple's Macintosh. Therefore, it can be run in different operating environments provided they have a suitable Java virtual machine. The repertoire of the software's functions can be extended by different off-the-shelf plug-ins, available on the Internet [2].

The NIH ImageJ 1.36b software was downloaded from NIH's website (http://rsb.info.nih.gov/ij); color selection and classification of positively stained points was done by using a distribution diagram for the colors red, green and blue (RGB), which shows the changes in intensity and color saturation. This distribution provides information on pixel quantity of the analyzed image [18]. This was obtained through the threshold_colour plug-in, in which the color range of interest was defined.

The software turns white those areas that meet this standard and black the remaining areas (Figure 1) [18, 19].To verify the effectiveness of protein expression quantification by using the NIH Image J 1.36b software, we quantified the immunostaining of cervical biopsies and associate these with different types of semiquantitative methods previously established in the literature [9, 20].

For statistical comparison, we used one-way ANOVA test for nonparametric data and Tukey's post-test, with $p < 0,05$ being considered significant.

RESULTS

To evaluate the effectiveness of quantification by using the Image J software, the values obtained with the pathological classification of lesion types described by Richart's (1990) standardized system were compared [21]. A significant relationship can be observed between the immunostaining values obtained for p16INK4 and

classification as CIN1, CIN2, CIN3, and negative, with and statistically significant differences having been evidenced when comparing negative samples and samples classified as CIN3 ($p<0.001$) and samples classified as CIN1 and negative samples ($p<0.05$) (Figure 2a).

When comparing the immunostaining quantification and the German semiquantitative score, which correlates the expression levels with a scale between 0 and 3 (0, 1+, 2+, 3+), the results obtained were also proportional with regard to the German score, showing significant differences among all the groups ($p<0.001$) (Figure 2b).

In order to get more evaluative parameters, we compared quantified immunostaining with subjective rating as "negative", "weakly positive" (WEAK+), and "strongly positive" (STRONG+). As a result, a significant relationship ($p<0.001$) between immunostaining and immunohistochemistry classification was observed (Figure 2c).

To explore associations between $p16^{INK4a}$ levels and HPV viral types, we analyzed expression levels by Image J software and compared them with viral Types identified in samples (Figure 2e). We also segmented the types into two groups, one high-risk group (HPV HR) (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) and one group of types with potentially high-risk genotypes (HPV PHR) (HPV 26, 53, 66, 67, 68, 70, 73, and 82), and HPV-negative samples[22] (Figure 2d).

Significant associations ($p<0.001$) between the p16INK4 expression levels and high-risk types were observed, as already evidenced in the literature (Figure 2d) [23]. When we consider the rate of protein expression and relate it with the different viral

types observed in the sample, we can analyze the immunostaining of $p16^{INK4a}$ in types 16, 18, 33, 52, 58, and 73 ($p<0.001$) (Figure 2e), corroborating previous data found in the literature [23, 24].

DISCUSSION

The quantitative or semiquantitative assessment of immunohistochemistry slides varies inter- and intrapersonally; the study of Galloway et al. (2011) confirmed this interpretation variation among histopathologists and trainees from the same area; it was considered that these professionals are not free from error, and that they tend to overestimate the quantification of immunohistochemistry [25]. Thus, it is crucial to develop methods that aim to decrease this variation in order to increase the accuracy and exactness of immunohistochemistry.

Other authors have demonstrated the application of computational methods in the final interpretation of biopsy slides of cancerous tissue [25, 26]; Image J software has been used by some authors [27, 28]; however, to this day, there are no studies that evolve using the Image J software in biopsies of cervical cancer precursor lesions.

In the present study, we compared the quantification of slides of cervical biopsies immunostained for p16INK4 by using Image J software, and compared the results with the main quantification and semiquantification methods established in the literature. The first method analyzed was the classification by the Richart et al. system (1990) [21], which is well established in clinical practice and has high significance levels [29]. Despite its high applicability, Raab et al. (2006) showed that there is an error rate of 9.52% between the correlation of cytological and histological analysis [30], thus indicating the need for improving the technique. With the computational method

used in this study, a correlation between the data obtained and the classification by the Richart et al., system (Figure 2a) was observed, indicating that the higher the Richart et al., score the higher the expression of $p16^{INK4a}$ ($p<0.05$, $p<0.001$), corroborating data found in the literature [31].

To verify the effectiveness of the proposed method, we compared the data obtained with the German score, in which grading of the immunostaining is given by number of crosses; in the presence of intense immunostaining, a greater number of crosses is assigned (0, 1+, 2+, 3+). In this analysis, an association between the levels obtained from a quantitative technique and the semiquantitative technique can be clearly verified ($p<0.05$, $p<0.001$) (Figure 2b), showing once again that the results in the present study corroborate data previously published[32], and demonstrating for the first time the utilization of a quantitative technique with great effectiveness when compared to other methods.

To increase the significance of the method proposed in the present study, we compared results obtained with the qualitative classification of immunohistochemistry used by some authors [33]; an association between the techniques can also be observed ($p<0.001$) (Figure 2c), indicating that the quantification proposed has a correlation with different standardized techniques in the literature .

Several studies show a correlation between expression levels of $p16^{INK4a}$ and different viral types of HPV [34, 35]; to investigate if this association is also present when u sing the proposed quantitative method, we compared data obtained with HPV HR (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) and HPV PHR (HPV 26, 53, 66, 67, 68, 70, 73, and 82) [22]; the expression was significantly higher in the HPV HR group when compared to biopsies negative for HPV ($p<0.001$) (Figure 2d), thus

corroborating previously published data [36, 37]; the same was observed in Figure 2e, which shows $p16^{INK4a}$ expression segmented by viral type.

From the data obtained in this study, we conclude that quantification of $p16^{INK4a}$ in cervical biopsies by using Image J software has significant results for improving diagnostic techniques of cervical cancer and therefore might help determining an effective clinical treatment without inter- or intra-personal variations. Correlation of the quantification by the proposed technique with viral types indicates its practical and reliable applicability.

More studies are needed to confirm specificity and sensitivity levels of the proposed method, which would provide the basis for further studies.

REFERENCES

1. Torres, L.F.B., L.d. Noronha, and J.E.Q. Telles, *A importância da imunohistoquimica no diagnóstico anátomo-patológico em hospital geral: análise de 885 casos.* J. bras. patol, 1995. **31**(2): p. 65-71.
2. Matos, L.L., et al., *Immunohistochemistry quantification by a digital computer-assisted method compared to semiquantitative analysis.* Clinics, 2006. **61**(5): p. 417-24.
3. Gadducci, A., M.E. Guerrieri, and C. Greco, *Tissue biomarkers as prognostic variables of cervical cancer.* Crit Rev Oncol Hematol, 2013. **86**(2): p. 104-29.
4. Saslow, D., et al., *American Cancer Society, American Society for Colposcopy and Cervical Pathology, and American Society for Clinical Pathology screening guidelines for the prevention and early detection of cervical cancer.* Crit Rev Oncol Hematol, 2012. **137**(4): p. 516-542.
5. Malinowski, D.P., *Molecular diagnostic assays for cervical neoplasia: emerging markers for the detection of high-grade cervical disease.* Biotechniques, 2005. **5**: p. 17-24.
6. Malinowski, D.P., *Multiple biomarkers in molecular oncology. I. Molecular diagnostics applications in cervical cancer detection.* Expert Rev Mol Diagn, 2007. **7**(2): p. 117-131.
7. Walts, A.E. and S. Bose, *p16, Ki-67, and BD ProEx™C immunostaining: a practical approach for diagnosis of cervical intraepithelial neoplasia.* Hum Pathol, 2009. **40**(7): p. 957-964.
8. Van Niekerk, D., et al., *p16 and MIB1 improve the sensitivity and specificity of the diagnosis of high grade squamous intraepithelial lesions: Methodological*

issues in a report of 447 biopsies with consensus diagnosis and HPV HCII testing. Gynecol Oncol, 2007. **107**(1, Supplement): p. S233-S240.

9. Thal., D.R., M. Horn, and W. Schlote, *Selective quantitative analysis of the intensity of immunohistochemical reactions.* Acta Histochem, 1995. **97**(2): p. 203-211.
10. Kok, L.F., et al., *Comparing the scoring mechanisms of p16 INK4a immunohistochemistry based on independent nucleic stains and independent cytoplasmic stains in distinguishing between endocervical and endometrial adenocarcinomas in a tissue microarray study.* Arch Gynecol Obstet, 2010. **281**(2): p. 293-300.
11. Yang, H., et al., *MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN.* Cancer research, 2008. **68**(2): p. 425-433.
12. Nieh, S., et al., *Is* $p16^{INK4a}$ *expression more useful than human papillomavirus test to determine the outcome of atypical squamous cells of undetermined significance-categorized Pap smear? A comparative analysis using abnormal cervical smears with follow-up biopsies.* Gynecol Oncol, 2005. **97**(1): p. 35-40.
13. Malik, S.M., et al., *Assessment of immunohistochemistry for p16INK4 and high-risk HPV-DNA by in situ hybridization in esophageal squamous cell carcinoma.* Int J Surg Pathol, 2011. **19**(1): p. 31-34.
14. Tosun, G., et al., *Immunohisto-chemical expressions of p16 and p53 proteins in cervical intraepithelial neoplasia and in benign cervical tissue.* Eur J Gynaecol Oncol, 2010. **31**(6): p. 627-631.

15. Gurrola-Díaz, C.M., et al., p16^{INK4a} *immunohistochemistry improves the reproducibility of the histological diagnosis of cervical intraepithelial neoplasia in cone biopsies.* Gynecologic Oncology, 2008. **111**(1): p. 120.

16. Rajkumar, T., et al., *Identification and validation of genes involved in cervical tumourigenesis.* BMC cancer, 2011. **11**(1): p. 80.

17. Barron, B.A. and R.M. Richart, *A statistical model of the natural history of cervical carcinoma based on a prospective study of 557 cases.* J Natl Cancer Inst, 1968. **41**(6): p. 1343-1353.

18. Wu, J.Z., et al., *The experiment research of edge detection in digital image [J]*. Control & Automation, 2004. **5**.

19. Abràmoff, M.D., P.J. Magalhães, and S.J. Ram, *Image processing with ImageJ.* Biophot international., 2004. **11**(7): p. 36-42.

20. Matos, L.L., et al., *Immunohistochemistry quantification by a digital computer-assisted method compared to semiquantitative analysis.* Clinics, 2006. **61**(5): p. 417-424.

21. Richart, R.M., *A modified terminology for cervical intraepithelial neoplasia.* Obstet Gynecol, 1990. **75**(1): p. 131-3.

22. Bouvard, V., et al., *A review of human carcinogens--Part B: biological agents.* Lancet Oncol, 2009. **10**(4): p. 321.

23. Guo, M., et al., *Evaluation of p16 immunostaining to predict high-grade cervical intraepithelial neoplasia in women with Pap results of atypical squamous cells of undetermined significance.* Diagn Cytopathol, 2010. **39**(7): p. 482-488.

24. Masumoto, N., et al., *P16 overexpression and human papillomavirus infection in small cell carcinoma of the uterine cervix.* Hum Pathol, 2003. **34**(8): p. 778-783.

25. Samsi, S., A.K. Krishnamurthy, and M.N. Gurcan, *An efficient computational framework for the analysis of whole slide images: Application to follicular lymphoma immunohistochemistry.* J Comput Sci, 2012.

26. Fuchs, T., et al., *Computational pathology analysis of tissue microarrays predicts survival of renal clear cell carcinoma patients.* Med Image Comput Comput Assist Interv, 2008: p. 1-8.

27. Silva, R.B., et al., *Morphological and immunological changes in the skin of autopsied women with AIDS.* Virchows Archiv, 2012: p. 1-7.

28. Vijayan, L., D. Bansal., and S. Basu Ray, *Nimodipine down-regulates CGRP expression in the rat trigeminal nucleus caudalis.* Indian J Exp Biol, 2012. **50**(5): p. 320.

29. Joste, N., *Overview of the cytology laboratory: specimen processing through diagnosis.* Obstet Gynecol Clin North Am, 2008. **35**(4): p. 549-63.

30. Raab, S.S., et al., *Improving Papanicolaou test quality and reducing medical errors by using Toyota production system methods.* Am J Obstet Gynecol, 2006. **194**(1): p. 57-64.

31. van Bogaert, L.J., $p16^{INK4a}$ *immunocytochemistry/immunohistochemistry: need for scoring uniformization to be clinically useful in gynecological pathology.* Ann Diagn Pathol, 2012. **16**(5): p. 422-6.

32. Wang, J.L., et al., *Predictive significance of the alterations of* $p16^{INK4a}$*, p14ARF, p53, and proliferating cell nuclear antigen expression in the progression of cervical cancer.* Clinical cancer research, 2004. **10**(7): p. 2407-2414.

33. George, E., N.L. Polissar, and M. Wick, *Immunohistochemical evaluation of* $p16^{INK4a}$*, E-cadherin, and cyclin D1 expression in melanoma and Spitz tumors.* American journal of clinical pathology, 2010. **133**(3): p. 370-379.

34. Guo, M., et al., *Cervista HPV HR and HPV16/18 Assays in Head and Neck FNA Specimens, a Valid Option of HPV Testing, Compared with HPV In Situ Hybridization/p16 Immunostaining Assays in Tissue Specimens from Patients with Oropharyngeal Carcinoma.* Journal of the American Society of Cytopathology, 2012. **1**(1): p. S71-S71.

35. Yıldız, I.Z., et al., *Efficiency of immunohistochemical p16 expression and HPV typing in cervical squamous intraepithelial lesion grading and review of the p16 literature.* Pathol Res Pract, 2007. **203**(6): p. 445-449.

36. Pinto, A.P., C.P. Crum, and M.S. Hirsch, *Molecular markers of early cervical neoplasia.* Diagnostic Histopathology, 2010. **16**(10): p. 445-454.

37. Ozaki, S., Y. Zen, and M. Inoue, *Biomarker expression in cervical intraepithelial neoplasia: potential progression predictive factors for low-grade lesions.* Human Pathology, 2011. **42**(7): p. 1007-1012.

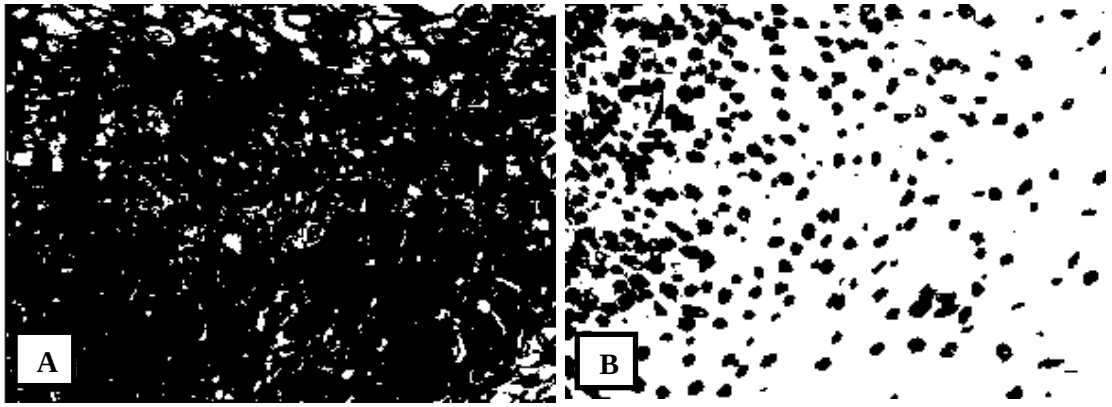

Figure 1. Image representation after definition and implementation of the positivity interval with the threshold color plugin of the Image J software. Illustration A shows the analysis of a cervical biopsy with $p16^{INK4}$ staining with a value of "3+", while illustration B shows the analysis of a cervical biopsy with negative staining for $p16^{INK4}$.

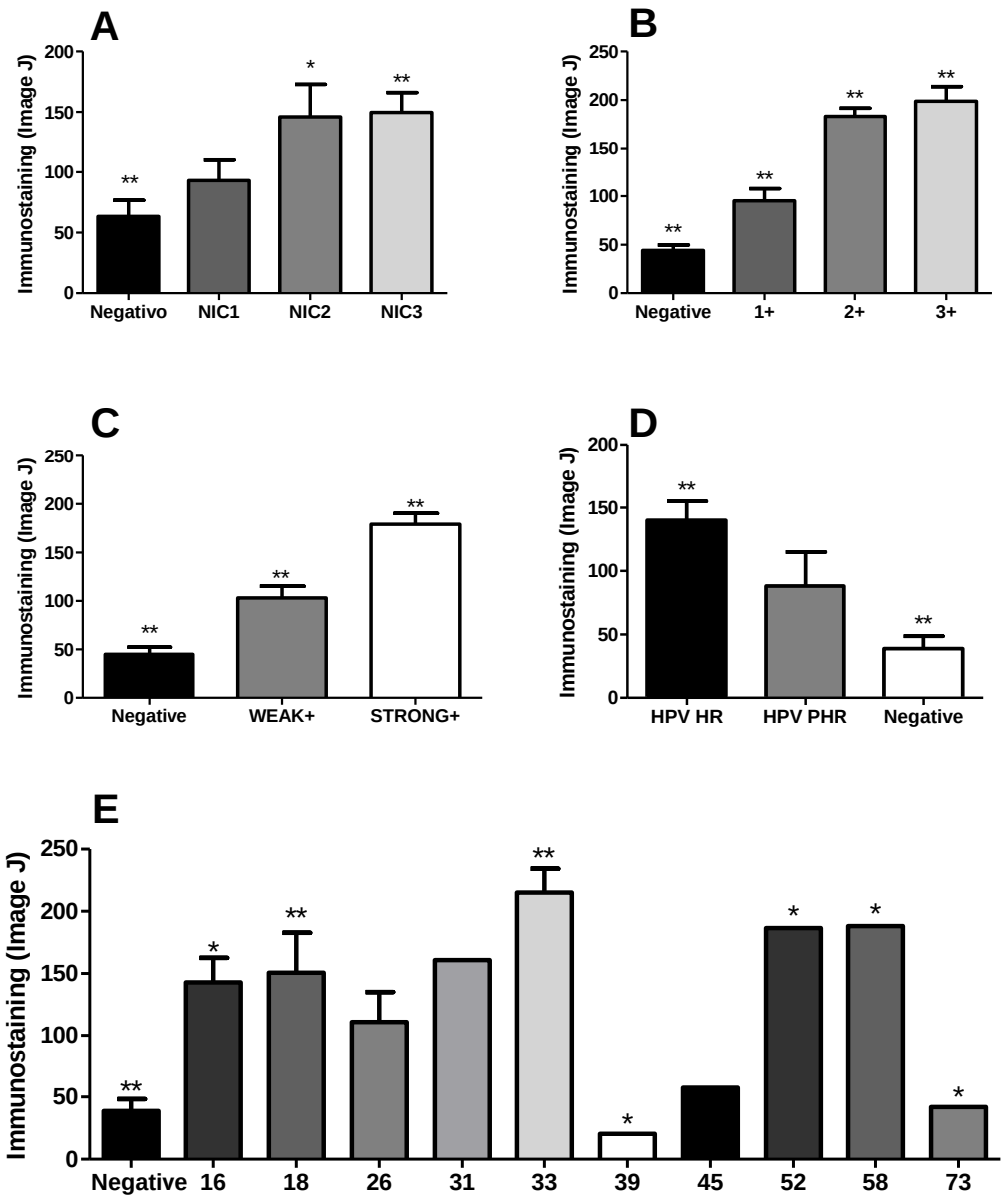

Figure 2. Comparison between quantification of $p16^{INK4a}$ in cervical biopsies and Richart system (A), German scoring system (B), qualitative method (C), HPV HR (high risk) , HPV PHR (potentially high-risk genotypes), and negative cervical biopsies (D), HPV type (E). *p<0.05, **p<0.001.

Capítulo 3

EXPRESSION ANALYSIS OF P53, Ki-67 and bcl-2 IN PRE-MALIGNANT LESIONS OF THE CERVIX

Alessandra Eifler Guerra Godoy, Karen Olivia Bazzo, Luciane Bertuol De Moura, Eduardo Pretto Serafini

ABSTRACT

Objectives. The present study has tried to analyze the individual and combined expression of the three markers - p53, Ki-67 and bcl-2 - in HPV associated pre-malignant lesions of the cervix.

Methods. Thirty-four cases of high-grade lesions, 21 low-grade cases and 18 normal cases were histologically assessed. HPV-DNA presence and the viral types were determined through PCR and RFLP, respectively. Marker expression was performed by immuno-histochemistry.

Results. With regards to viral types, HPV with high oncogenic potential represented the majority, with just one case reporting low oncogenic potential for viral infection (HPV 6). HPV was the most frequent and could be associated with the high-grade lesion group, however, no significant associations were found between the viral ones and the marker expression. When the expression location, beyond the basal layer, was taken into account, the three markers proved to be good indicators of the lesion grade. The markers combined expression: p53(-), ki-67(+), bcl-2(-) and p53(-), Ki-67(-), bcl-2(-) was statistically significant when associated with the lesion grade.

Conclusions. The results suggest that the p53, Ki-67 and bcl-2 markers can contribute to differentiate the lesion grades, whether in its isolate form or in combined form.

KEY WORDS: P53, Ki-67, bcl-2, pre-malignant lesions of the cervix, HPV

INTRODUCTION

Cervical cancer is one of the most frequent neoplasias in women, with approximately 530 new cases worldwide per year; cervical cancer is the third most common kind of cancer among women, being responsible for 274 thousand women's deaths per year [1]. It is the third most frequent tumor in the female population, after breast and colorectal cancer, and the fourth most common cause of female death by cancer in Brazil, with 15,590 new cases expected in 2014. [2] Epidemiological and experimental evidence indicate that certain kinds of HPV are directly involved in cervical cancer pathogenicity.

Although it is present in about 100% of cervical carcinoma, isolatedly, HPV cannot be considered solely responsible for the malignant transformation [3]. It is believed that HPV as an initiator factor, as the progression of the HPV infection into cancer also requires a number of other co-factors to be taken into consideration: environmental factors, such as physical and chemical carcinogens, and host restricted factors, such as immune rsponse, genetic susceptibility, among others [4- 7].

Taking into consideration that most viral infections, including high risk HPV, recede spontaneously and that just a small fraction of infected women will progress into cervical cancer, it is necessary to identify early in which, among the infected patients, it will really evolve into cancer [5,8-9].

The cell protein p53 suppresses the transcription of some cells. However, its greatest tumor suppressor effect is due to the genes transcriptional activation that maintains genomic stability [10-13]. Under normal conditions, protein p53 acts as an *emergency brake* in hypoxia or DNA lesion situations due to irradiation, ultra-violet light or mutagenic agents. Through p53 action, repair genes are induced and the cell cycle is interrupted in G1 phase. When repair becomes impossible, the injured cell is induced to cell death by p53 (apoptosis) [13-14]. The inactivity or loss of p53 causes loss of control in cell proliferation which results in genetic instability and accumulation of oncogenic mutations responsible for the development of malignant tumors [15-16].

The P53 gene can also be inactivated through other mechanisms besides somatic and hereditary mutations. The HPV E6 transformed protein can bind with p53 protein and induce its degradation. The affinity of the viral onco-proteins with p53 protein varies according to the HPV type. The binding affinity of the E6 low risk genital HPV onco-protein is lower than the high oncogenic risk HPV [15, 17]. The importance of binding between E6-p53 in cervical cancer pathogenicity has been supported by recent molecular studies [17-25].

Ki-67 is characterized as a cell proliferation marker in phases G1, S, M and G2 of the cell cycle, able to identify a nuclear protein present in the interphase proliferative cells, as well as in the mitotic cells, thus, it has been widely used in the growth stage investigation and in several tumor kinetic activities, such as breast cancer, colon and rectum cancer, and lymphomas [26-27].

The bcl-2 protein is characterized for being an intracellular membrane protein that does not allow cell death (apoptosis) in several situations. An inadequate bcl-2

expression might extend the life-time of the damaged cells, thus increasing significantly the probability of malignant transformation. In addition, the bcl-2 super-expression can interrupt the arrest in the G1 stage of the cell cycle, mediated by p53, therefore inhibiting apoptosis [28-30].

Therefore, this study tried to verify the p53, ki-67 and bcl-2 marker expression in cervical pre-malignant lesions, HPV associated, as well as relate these marker expression with viral types and with the lesion grade, trying to propose a combined analysis of apoptosis and cell proliferation markers for cervical pre-malignant lesions.

MATERIAL AND METHODS

This retrospective study has been approved by the University of Caxias do Sul Research and Ethics Committee with no conflicts of interest.

Seventy-three cases of cervix material biopsy were used. These cases were in the archives of the Diagnose Laboratório de Patologia e Citologia, from January 2003 to December 2004.

The study groups were divided into: 18 cases with normal histology (control-group), 21 cases with low-grade intraepithelial squamous lesions (LSIL), and 34 cases with high-grade intraepithelial squamous lesions (HSIL).

The biopsies were submitted to paraffin removal and human DNA extraction by using the methodology suggested by Banerjee et al [31]. They were then sent to generic PCR in order to identify the HPV-DNA using PGMY 09/11(HPV) and GH20/PCO4 (human β-globin) initiators. Viral typing was carried out in 64% of the samples positive

for HPV by using RFLP technique, according to the methodology proposed by Bernard et al. (1994) [32].

The markers (p53, Ki-67 and bcl-2) expression was analyzed by means of immunohystochemistry (LSAB-HRP+). The reading criteria were: presence or absence of expression, and the location of the expression in the different epithelial layers. The cases that presented nuclear brown coloration for p53 and Ki-67 or cytoplasmic brown coloration for bcl-2, were classified as positive. For all cases, positive and negative coloration controls were used. Monoclonal anti-bodies used for the reaction were: 1) Anti-human p53 Protein, clone DO-7, dilution 1:50, DAKO®, 2) Anti-human Ki-67, clone Ki-S5, dilution 1:50, DAKO® e 3) Anti-human bcl-2 Oncoprotein, clone 124, dilution 1:80, DAKO® .

The data were analyzed and processed by the SPSS program, version 12.0. For statistical analysis both the non-parametric tests of Pearson's Chi-square x^2, and Fisher's exact test, were used. $P < 0,05$ values were considered statistically significant.

RESULTS

In the 73 cases studied, protein p53 presented expression in 28 (38.4%) and absence of expression in 45 cases (61.6%).

Out of the 28 cases that presented protein p53 expression, 10 cases (35.7%) were LSIL distributed in the following way: 5 cases (50%) in the basal layer, 1 case (10 %) in the intermediate layer, 1 case (10%) in the surface layer, 1 case (10%) in the intermediate and basal layers and 2 cases (20%) in all layers. Twelve cases (42.8%)

were HSIL, distributed as follows: 4 cases (24%) in the basal and intermediate layers and 5 cases (41.7%) in all layers. Six cases (21.4%) were from the normal group (control), with expression in the basal layer only.

Ki-67 marker showed expression in 53 cases (72.6%) and absence of expression in 16 cases (21.9%). Four cases could not be assessed due to lack of biopsy material along the study.

From the 53 cases that showed expression for Ki-67, 19 cases (35.8%) were HSIL, distributed as follows: 12 cases (63.1%) in the basal layer, 2 cases (10.5%) in the surface layer, 3 cases (15.8%) in the basal an intermediate layers and 2 cases (10.5%) in all layers. Twenty-three cases (43.4%) were HSIL distributed as follows: 3 cases (13.4%) in the basal layer, 1 case (4.35%) in the surface layer, 7 cases (30.4%) in the basal and intermediate layers, 11 cases (48%) in all layers, and one case (4.35%) in the intermediate and surface layers. Eleven cases (21%) were from the normal group (control), with (82%) in the basal layer and 2 cases (18%) in the basal and intermediate layers.

Marker bcl-2 showed expression in 38 cases (52.1%) and absence of expression in 35 cases (47.9%). From the 38 cases that showed expression for protein bcl-2, 11 cases (29%) were HSIL distributed as follows: 10 cases (91%) in the basal layer, 1 case (9%) in all layers. Sixteen cases (42%) were HSIL distributed as follows: 8 cases (50%) in the basal layer, 6 cases (37.5%) in the basal and intermediate layers and 2 cases (12.5%) in all layers.

Table 1. Shows the expression of the three markers and the lesion grade, when the epithelium stratification into upper and basal layers is taken into account.

Table 1 – Expression of the markers and lesion grade

Marker	Expression Location	Normal			LSIL		HSIL	
	Location	**n**	**N**	**%**	**n**	**%**	**n**	**%**
P53*	Basal Layer	15	6	40	5	33.3	4	26.7
	Upper Layers	13	0	0	5	38.5	8	61.5
Ki-67**	Basal Layer	24	9	37.5	12	50	3	12.5
	Upper Layers	29	2	6.9	7	24.1	20	69***
bcl-2****	Basal layer	29	11	37.9	10	34.5	8	27.6
	Upper Layers	9	0	0	1	11.1	8	88.9*****

* Normal x LSIL: p=0.027; ** Normal x LSIL x HSIL: p=0.001; *** Expression location x LSIL : p=0.001; **** Normal x LSIL x HSIL: p=0.004; ***** Expression location x HSIL: p=0.001

The combined expressions of the markers, in the format of an immuno-histochemical panel considering the epithelium stratification in the basal layer and in upper layers with the lesions grade, is represented in Table 2.

Table 2. Immuno-histochemical panel considering the location of the marker expression and the lesion grade.

Markers	Group I (normal)	Group II (LSIL)	Group III (HSIL)
p53+Ki67+ bcl-2+	0	0	2
p53+Ki67+ bcl-2-	0	3	2
p53+Ki67- bcl-2+	0	0	1
p53+Ki67- bcl-2-	0	2	1
p53-Ki67+ bcl-2+	0	1	4
***p53-Ki67+ bcl-2-**	2	3	14
p53-Ki67- bcl-2+	0	0	2
****p53-Ki67- bcl-2-**	16	12	6
Total	18	21	32

-: absence of expression, or expression restricted to the basal layer; +: expression beyond the basal layer; *Normal x LSIL x HSIL: p=0.013; **Normal x LSIL x HSIL: p=0.001

Combinations p53(-)Ki67(+) bcl-2(-) (n=19) and p53(-) Ki-67(-) bcl-2(-) (n=34) were the most frequent among the groups. Combinations p53(-) Ki-67(+) bcl-2(-) presented significant difference among the others, as well as with the lesions grade (p=0.013). Combination p53(-)Ki67(-)bcl-2(-) also showed significant difference among the others, as well as association with the lesions grade (p=0.001).

HPV-DNA presence was not found in the control group. However, different kinds of HPV were found in the lesion groups. HPV 16 was the predominant viral type (65.7%). The remaining viral types represented just a small portion (34.3%). The presence of multiple infections was not considered significant within the groups, since just one case of HPV 26 and HPV 18 association was observed. According to the

oncogenic potential of the virus, just one sample showed low oncogenic potential (HPV6), the others presented high oncogenic potential HPV (HPV16, 18, 26, 31, 33, 39, 45, 52 and 58).

The association between the type of virus and the lesion grade was significant only for HPV 16 (p=0.001). The association between the other viral types and the lesions was not significant, such as the association of the viral types and the expression of the markers.

Any positive association between the viral types and the markers used in this study has been found.

DISCUSSION

The epithelium division into two levels (basal layer and surface layers) allowed to associate the marker expression with the lesion grade. The criteria used for the expression reading (presence or absence of expression and location on the epithelium layers) enabled a new approach through a differentiated, fast and accurate analysis. Studies have shown, in their findings, the importance of the epithelium stratification when assessing intraepithelial lesions. Other authors have used quantitative analysis of the expression by using distinct classification systems [33-34]. These parameters have not been considered, especially for presenting great inter-observational variability in slide reading, making the assessment subjective, in addition to being laborious and needing a longer period of study.

The individual expression of the three markers (p53, Ki-67 and bcl-2) has shown to be a good indicator for the lesion grade as the expression passes through the basal layer towards the surface layers.

Although the immuno-histochemical results for p53 protein detection in the cervix are still contradictory [35-36], the present study shows a p53 expression beyond the basal layer in the LSIL and HSIL groups, and in the latter the most frequent expression reached all layers. The location beyond the basal layer can be related to loss of epithelium maturation in high grade dysplasia as well as to a process of loss of normal proliferative cells regulation [37-38].

The p53 expression, restricted to the basal layer in normal epithelium and in LSIL, also found by Kurvinen et al. (1996) [39], might be representative of cells in proliferation.

The viral types of high oncogenic potential have not shown p53 expression beyond the basal layer, in accordance with the Kurvinen et al. (1996) [39] [24] findings that found low or absence of p53 expression in 53% of the lesions associated with HPV 16 and 18 types, and according to the hypothesis that the E6 viral oncoprotein from the viral types of high oncogenic potential have more affinity with p53, are capable of inducing fast protein degradation, thus decreasing its expression levels [17, 37].

Marker Ki-67 has been studied as an important tumor marker. Its expression is related to proliferative action, lesion grade and HPV in several studies [40-43]. In this study, the normal group presented expressions in the basal layer with more frequence. Only one case presented expression as far as the intermediate layer, which corroborates

Carrilho et al., study (2003) [33] who found Ki-67 expression restricted to the basal layer in normal epithelia.

LSIL and HSIL groups had expression beyond the basal layer, for the LSIL group the most frequent expression reached the basal layer only, and for the HSIL, the most frequent expression reached all the epithelium layers.

The findings have shown that as the Ki-67 expression crosses the basal layer, there is a relation with the presence of lesion (LSIL and HSIL), especially with more serious lesions (HSIL).

There was a strong association between HSIL and the expression location in the epithelium layers, found to be in accordance with the association found between Ki-67 positivity and the levels of dysplasia observed in Keating et al. studies (2001) [44], whose highest proliferative activity measured by ki-67, was found in high grade squamous lesions.

The bcl-2 intense expression in normal epithelia, restricted to the basal layer, was also shown by Kurvinan et al. (1996) [45]. Hockenbery et al. (1991) [46] suggested the existence of a basal layer natural protection system against apoptosis in order to explain the finding. However, as the epithelial cells present dysplastic alterations, bcl-2 expression expands to the upper layers of the epithelium, especially in HSIL group, proving bcl-2 marker to be a good indicator for lesion grade.

Few studies assess the marker expression in cervix pré-malignant lesions in a combined way. Queiroz et al., (2006) [47] assessed the expression of p16, cyclin D1, p53 and Ki-67 proteins, and found greater lesion progression correlation for p16 and Ki-67 than for p53 and cyclin D1. Taking into account the expression results for the three

markers p53, ki-67 and bcl-2 in the present study, the combination p53(-) Ki-67(+) bcl-2(-) has shown a significant difference among the others, as it could also be associated to the lesions grade, suggesting that the p53 and bcl-2 protein absence of expression, plus ki-67 super-expression beyond the basal layer, indicates high-grade lesion. Similarly, the combination p53,(-) ki-67(-) bcl-2(-) showed difference among the others, as it could also be associated to the lesion grade. However, it suggests that the absence of p-53, ki-67, bcl-2 Te expressions in the upper layers, indicates the opposite: control-group and low grade lesion.

As expected, different types of HPV have been found in the lesion groups, being HPV 16 the predominant viral type. Data found in the literature refer to HPV 16 as being the most frequent one, both in the intra-epithelial squamous lesions and in invasive squamous carcinomas [6, 36, 48-49]. Although association has been found between HPV 16 and the lesion grade, especially in HSIL, the association among the other viral types and the lesion grade has not been significant, probably because of the low frequency of the other viral types in this study.

The absence of association among the types in question and the marker expression, both in individual form and combined form, has probably occurred due to the limited number of viral types, therefore, demanding a larger number of samples.

The search for biological markers has been the focus of many research centers, and several molecules involved in cell growth control have been studied singly. Considering that few studies have assessed several markers simultaneously, the findings in this study have shown that p-53, ki-67 and bcl-2 markers, when assessed in

combination, can contribute for differentiating the cervix pre-malignant lesion grade, working as an auxiliary tool in lesion prognosis

REFERENCES

[1] World Health Organization (2008). International Agency for Research on Cancer. Globocan. http://globocan.iarc.fr

[2] Instituto Nacional do Câncer – INCA. (2014). Câncer de Colo do Útero. HTTP: // www.inca.igov.br

[3] Kitahara, S., Chan, R. C., Nichols, W. S., Silva, E. G. (2012). Deceiving high-grade cervical dysplasias identified as human papillomavirus non-16 and non-18 types by Invader human papillomavirus assays. *Annals of Diagnostic Pathology*, 16, 100-106. doi: http://dx.doi.org/10.1016/j.anndiagpath.2011.09.003

[4] Marur, S., D'Souza, G., Westra, W. H., Forastiere, A. A. (2010). HPV-associated head and neck cancer: a virus-related cancer epidemic. *The Lancet Oncology*, 11, 781-789. doi: http://dx.doi.org/10.1016/S1470-2045(10)70017-6

[5] Park, K. J. (2011). Neoplastic Lesions of the Cervix. *Surgical Pathology Clinics,* 4, 17-86. doi: http://dx.doi.org/10.1016/j.path.2010.12.006

[6] Thomas, M. K., Pitot, H. C., Liem, A., Lambert, P. F. (2011). Dominant role of HPV16 E7 in anal carcinogenesis. *Virology*, 421, 114-118. doi: http://dx.doi.org/10.1016/j.virol.2011.09.018

[7] Vidal., L., Gillison, M. L. (2008). Human Papillomavirus in HNSCC: Recognition of a Distinct Disease Type. *Hematology/Oncology Clinics of North America,* 22, 1125-1142. doi: http://dx.doi.org/10.1016/j.hoc.2008.08.006

[8] Priebe, A. M. (2013). 2012 Cervical Cancer Screening Guidelines and the Future Role of HPV Testing. *Clinical obstetrics and gynecology*, 56, 44-50.

[9] von Knebel Doeberitz, M. (2002). New markers for cervical dysplasia to visualise the genomic chaos created by aberrant oncogenic papillomavirus infections. *European Journal of Cancer*, 38, 2229-2242. doi: http://dx.doi.org/10.1016/S0959-8049(02)00462-8

[10] Carrilho, C., Gouveia, P., Cantel, M., Alberto, M., Buane, L., David, L. (2003). Characterization of Human Papillomavirus Infection, P53 and Ki-67 Expression in Cervix Cancer of Mozambican Women. *Pathology - Research and Practice*, 199, 303-311. doi: http://dx.doi.org/10.1078/0344-0338-00422

[11] Katiyar, S., Hedau, S., Jain, N., Kar, P., Khuroo, M. S., Mohanta, J., Das, B. C. et al (2005). p53 gene mutation and human papillomavirus (HPV) infection in esophageal carcinoma from three different endemic geographic regions of India. *Cancer Letters*, 218, 69-79. doi: http://dx.doi.org/10.1016/j.canlet.2004.09.003

[12] Levine, A. J. (1997). P53 the cellular gatekeeper for growth and devision. *Cell*. 88, 323.

[13] Cotran, R. S., Kumar, V., Collins, T. (1999). Patologia Básica. 5.ed. Rio de Janeiro: Guanabara Koogan.

[14] Wang, Z., Sturgis, E. M., Zhang, Y., Huang, Z., Zhou, Q., Wei, Q., Li, G. (2012). Combined p53-related genetic variants together with HPV infection increase oral cancer risk. *International Journal of Cancer*, 131, E251-E258.

[15] Habbous, S., Pang, V., Eng, L., Xu, W., Kurtz, G., Liu, F.-F., Liu, G. et al (2012). p53 Arg72Pro Polymorphism, HPV Status and Initiation, Progression, and Development of Cervical Cancer: A Systematic Review and Meta-Analysis. *Clinical cancer research*, 18, 6407-6415.

[16] Gross, G.E., Barrasso, R. (1999). Infecção por papilomavírus humano: atlas clínico do HPV. Porto Alegre: Artes Médicas.

[17] Storey, A., Thomas, M., Kalita, Harwood, C., Gardiol, D., Mantovani, F., et al.,(1998). Role of a p53 polymorphism in the development of human papillomavirus associated cancer. *Nature*, 393,229-234.

[18] Zehbe, I., Voglino, G., Wilander, E., Genta, F., Tommasino, M. (1999). Codon 72 polymorphism of p53 and it is association with cervical cancer. *Lancet*, 354, 218-219.

[19] Dokianakis, D.N., Spandidos, D.A. (2000). p53 codon72 polymorphism as a risk factor in the development of HPV: associated cervical cancer. *Molecular Cell Biology Research Communications*, 3,111-114.

[20] Makni, H., Franco,E.L., Kaiano, J., Villa, L.L., Labrecque, S., Dudley, R., et al., (2000). P53 polymorphism in codon 72 and risk of human papillomavirus – induced cervical cancer: effect of interlaboratory variation. *International Journal of Cancer*, 87, 528-533.

[21] Andersson, S., Rylander, E., Strand, A., Salsstrom, J., Willander, E. (2001). The significance of p 53 codon 72 polymorphism for the development of cervical adenocarcinomas. *British Journal of Cancer,* 85, 1153-1156. doi: 10.1054/bjoc.2001.2085

[22] Klug, S.J., Wilmotte, R., Santos, C., Almonte, M., Herrero, R., Guerrero, I.; et al., (2001). Tp53 polymorphism, HPV infections, and risk of cervical câncer. *Cancer Epidemiology, Biomarkers and Prevention,* 10,1009-1012.

[23] Zehbe, I., Voglino, G., Wilander, E., Delius, Marongiu, A., Edler, L., et at. (2001). p53 codon 72 polymorphism and various human papillomavirus 16 E6 genotypes are risk factors for cervical cancer development. *Cancer Research,* 61,608-611.

[24] Saranath, D., Khan, Z., Tandle, A.T., Dedhia, P., Sharma, B., Contractor, R., et al., (2002). HPV 16/18 prevalence in cervical lesions / cancer and p53 genotypes in cervical cancer patients from India. *Gynecologic Oncology,* 86,157 -162. http://dx.doi.org/10.1006/gyno.2002.6735

[25] Ojeda, J.M., Ampuero, S., Rojas, P., Prado, R., Allende, J.E., Barton, S.A.; et al (2003). p53 codon 72 polymorphism and risk of cervical cancer. *Biological Research,* 36, 279-283.

[26] Silva-Filho, A. L., Traiman, P., Triginelli, S. A., Reis, F. M., Pedrosa, M. S., Miranda, D., Cunha-Melo, J. R. et al (2004). Expression of p53, Ki-67, and CD31 in the vaginal margins of radical hysterectomy in patients with stage IB carcinoma of the cervix. *Gynecologic Oncology,* 95, 646-654. doi: http://dx.doi.org/10.1016/j.ygyno.2004.07.059

[27] Ungureanu, C., Teleman, S., Socolov, D., Anton, G., Mihailovici, M. (2010). Evaluation of $p16^{INK4a}$ and Ki-67 proteins expression in cervical intraepithelial neoplasia and their correlation with HPV-HR infection. *Revista medico-chirurgicală a Societăţii de Medici şi Naturalişti din Iaşi,* 114, 823.

[28] D'Angelo, E., Espinosa, I., Ali, R., Gilks, C. B., Rijn, M. v. d., Lee, C.-H., Prat, J. (2011). Uterine leiomyosarcomas: Tumor size, mitotic index, and biomarkers Ki67, and Bcl-2 identify two groups with different prognosis. *Gynecologic Oncology,* 121, 328-333. doi: http://dx.doi.org/10.1016/j.ygyno.2011.01.022

[29] Subapriya, R., Kumaraguruparan, R., Nagini, S. (2006). Expression of PCNA, cytokeratin, Bcl-2 and p53 during chemoprevention of hamster buccal pouch carcinogenesis by ethanolic neem (Azadirachta indica) leaf extract. *Clinical Biochemistry,* 39, 1080-1087. doi: http://dx.doi.org/10.1016/j.clinbiochem.2006.06.013

[30] Zyada, M. M., Grawish, M. E., Elsabaa, H. M. (2009). Predictive value of cyclooxygenase 2 and Bcl-2 for cervical lymph node metastasis in mucoepidermoid carcinoma. *Annals of Diagnostic Pathology*, 13, 313-321. doi: http://dx.doi.org/10.1016/j.anndiagpath.2009.06.003

[31] Banerjee, S. K., Makdisi, W. F., Weston, A. P., Mitchell, S. M., Campbell, D. R. (1995). Microwave-based DNA extraction from paraffin-embedded tissue for PCR amplification. *Biotechniques*, 18, 768-70.

[32] Bernard, H.U., Manos S.Y., Ong M.M., Villa C.K., Delius L.L., et al., (1994). Identification and assessment of known and novel human papillomaviruses by polymerase chain reaction amplification, restriction fragment length polymorphisms,

nucleotide sequence, and phylogenetic algorithms. *Journal of Infecttious Diseases*, 170, 1077-1085. doi: 10.1093/infdis/170.5.1077

[33] Carrilho, C., Gouveia, P., Cantel, M., Alberto, M., Buane, L., David, L. (2003). Characterization of Human Papillomavirus infection, p53 and Ki-67 expression in cervix cancer of Mozambican women. *Pathology- Research and Practice*, 199,303-311. http://dx.doi.org/10.1078/0344-0338-00422

[34] Kedzia, W., Schimidt, M., Frankowski, A., Spaczynski, M.(2002). Immmunohistochemical assay of p53, cyclin D1, c-erbB2, EGFr and Ki-67 proteins in HPV-positive and HPV negative cervical cancers. *Folia Histochemica et Cytobiologia*, 40, 37 – 41.

[35] Barea, J.A., Pardini, M.I.M.C., Gushiken, T. (2004). Extração de DNA de materiais de arquivo e fontes escassas: revisão e apontamentos sobre as possíveis aplicações. *NewsLab*, 63, 96-108.

[36] Zehbe, I., Wilander, E. (1997). Human papillomavirus infection and invasive cervical neoplasia: a study of prevalence and morphology. *Journal of Pathology*, 181,270-275. doi: 10.1002/(sici)1096-9896(199703)181:3<270::aid-path767>3.0.co;2-r

[37] Gadducci, A., Guerrieri, M. E., Greco, C. (2012). Tissue biomarkers as prognostic variables of cervical cancer. *Critical Reviews in Oncology/Hematology*. 86, 104–129. doi: http://dx.doi.org/10.1016/j.critrevonc.2012.09.003

[38] Tosun, G., Sendag, F., Zeybek, B., Cosan, T. M., Guven, C., Zekiogly, O., Bilgin, O. (2010). Immunohisto-chemical expressions of p16 and p53 proteins in cervical

intraepithelial neoplasia and in benign cervical tissue. *Eur J Gynaecol Oncol,* 31, 627-631.

[39] Kurvinen, K., Syrjanen, K., Syrjanen S. (1996). P53 and bcl-2 Proteins as prognostic markers in Human Papillomavirus: associated cervical lesions. *Journal of Clinical Oncology,* 14,2120-2130.

[40] Martin, C. M., O'Leary, J. J. (2011). Histology of cervical intraepithelial neoplasia and the role of biomarkers. *Best Practice & Research Clinical Obstetrics & Gynaecology,* 25, 605-615. doi: http://dx.doi.org/10.1016/j.bpobgyn.2011.04.005

[41] Pavlakis, K., Vrekoussis, T., Tsipoura, A., Tsenga, A., Arapantoni-Dadioti, P., Gavresea, T., Barbounis, V. et al (2012). Her2 negative luminal breast carcinoma and Ki-67 evaluation. *The Breast,* 21, 529-533. doi: http://dx.doi.org/10.1016/j.breast.2012.04.004

[42] Walts, A. E., Bose, S. (2009). p16, Ki-67, and BD ProEx™C immunostaining: a practical approach for diagnosis of cervical intraepithelial neoplasia. *Hum Pathol,* 40, 957-964. doi: http://dx.doi.org/10.1016/j.humpath.2008.12.005

[43] Wang, W.-C., Wu, T.-T., Chandan, V. S., Lohse, C. M., Zhang, L. (2011). Ki-67 and ProExC are useful immunohistochemical markers in esophageal squamous intraepithelial neoplasia. *Human Pathology,* 42, 1430-1437. doi: http://dx.doi.org/10.1016/j.humpath.2010.12.009

[44] Keating, J.T., Cviko, A., Riethford, S., Riethdord, L., Quade, B., Sun, D., et al., (2001). Ki-67, cyclin E, and p16INK4 are complimentary surrogate biomarkers for

human papilloma virus: related cervical neoplasia. *American Journal of Surgical Pathology*, 25, 884-891. doi: 10.1097/00000478-200107000-00006

[45] Kurvinen, K., Syrjanen, K., Syrjanen S. (1996). P53 and bcl-2 Proteins as prognostic markers in Human Papillomavirus: associated cervical lesions. *Journal of Clinical Oncology*, 14,2120-2130.

[46] Hockenbery, D.M., Zutter, M. W., Naham, M., Korsmeyer, S.J. (1991). Bcl-2 protein is topographically restricted in tissues characterized by apoptotic cell death. *Proceedings of the National Academy of Sciences of the United States of America*, 88,6961-5.

[47] Queiroz, C., Silva, T. C., Alves, V. A., Villa, L. L., Costa, M. C., Travassos, A. G., de Freitas, L. A. R. et al (2006). Comparative study of the expression of cellular cycle proteins in cervical intraepithelial lesions. *Pathology-Research and Practice*, 202, 731-737. http://dx.doi.org/10.1016/j.prp.2006.07.003

[48] Eaton, S., Wiktor, P., Thirstrup, D., Lake, D., Nagaraj, V. J. (2011). Efficacy of TRAIL treatment against HPV16 infected cervical cancer cells undergoing senescence following siRNA knockdown of E6/E7 genes. *Biochemical and Biophysical Research Communications*, 405, 1-6. doi: http://dx.doi.org/10.1016/j.bbrc.2010.12.056

[49] Guo, M., Dhillon, J., Khanna, A., Katz, R., Gong, Y., Staerkel, G. (2012). Cervista HPV HR and HPV16/18 Assays in Head and Neck FNA Specimens, a Valid Option of HPV Testing, Compared with HPV In Situ Hybridization/p16 Immunostaining Assays in Tissue Specimens from Patients with Oropharyngeal Carcinoma. *Journal of the American Society of Cytopathology*, 1, S71-S71.

Capítulo 4

Expression Analysis of Transglutaminase 2 in Pre-Malignant Lesions of the Cervix

Karen Bazzo1,2 , Alessandra Eifler Guerra Godoy3 , Fábio Firmbach Pasqualotto4

1 phD student in biotechnology at UCS, RS, Brazil.

2 Professor at Faculdade da Serra Gaúcha, RS, Brazil.

3 phD, Pathologist, Professor at the Medical School of UCS. Director of IPCEM

4 phD, Urologist, Professor at the Medical school of UCS.

This study objective to evaluate transglutaminase 2 (TG2) expression in Low-grade and high-grade squamous intraepithelial lesion (LSIL and HSIL) and relate it to different HPV viral types. Were included 146 patients with LSIL or HSIL, genotyping and TG2 immunohistochemistry analysis were performed. Patients with normal and LSIL are locally associated with TG2 expression levels >50% ($p<0.05$), and patients with HSIL are associated with low TG2 expression ($p<0.05$). The analysis of the HPV types showed a significant association for HPV11 ($p=0.031$). Our data suggest that TG2 may represent a biomarker of lesions associated with HPV.

Key-words: Human Papillomavirus, Uterine Cervical Neoplasms, Transglutaminase 2

Introduction

Cervical cancer is the fourth most common cancer among women, accounting for the death of 265.000 women each year worldwide (1). It is characterized by uncontrolled replication of the organ epithelium, compromising the underlying tissue

(stroma) and may invade adjacent or distant structures and organs. This type of cancer is related to human papillomavirus (HPV), which is responsible for the appearance of cancer precursor lesions. The mechanism involved in the appearance of cancer is related to a persistent infection by a large number of HPV types, which depends on the HPV type and site of infection, as well as on host factors that regulate the virus persistency, regression, and latency (2-4).

Several proteins are important in the HPV infection process. Two HPV proteins are relevant to the cervical cancer mechanism. One of them is E7, which is associated with pRb (retinoblastoma tumor suppressor protein) and other members of the protein family. This protein disrupts the association between the pRb and E2 factor family. The other protein is E6, which is associated with p53 preventing growth arrest or apoptosis in response to E7-mediated cell cycle entry in the upper epithelial layers, which might otherwise occur through the activation of the ADPribosylation factor pathway (5, 6).

Type 2 transglutaminase (TG2) has been proposed as a cellular-interfering factor in HPV infections (2, 7). TG2 belongs to the transglutaminase (TG) iso-enzyme family, which is related with multiple functions (8). TG2 participates in signal transduction by activating and hydrolyzing the guanidine triphosphate (GTP) enzyme. In normal tissues, TG2-GTP binding is proportional to the TG2 expression level. Furthermore, TG2 acts in the regulation of extracellular matrix (ECM) stability and in the reconstruction of the intracellular cytoskeleton. Interestingly, TG2 is also an active participant in promoting the mobility of malignant cells, and in inducing the chemo-resistance of cancer cells (9).

Few studies have shown a relationship between the HPV infection and TG2 activity and expression. Apparently, the catalytic activity of TG2 can incorporate polyamine into HPV18 E7, thereby inhibiting its binding to pRb. Moreover, TG2 can

translocate from the cytosol into the nucleus incorporating polyamines into the retinoblastoma protein (Rb) to protect it from caspase-mediated degradation, suggesting that TG2 interacts with pRB both in the cytosol and in the nucleus (10-13).

Currently, the medical management of low-grade squamous intraepithelial lesions (LSIL) is variable (14, 15). Biomarkers could then contribute in evidencing indicators of more serious injuries or for their resolution. Therefore, this study had the objective to evaluate TG2 expression in low and high-grade precursor lesions and relate it to different viral HPV types.

MATERIALS AND METHODS

Patients and Tissues Samples

This retrospective study has been approved by the Review Board of our Institution on the advice number 183 756 and all patients have given written informed consent. Women seen at the Lower Genital Tract Pathology Service of the Central Outpatient Clinic (UCS) and at Diagnosis Laboratory were included in this study. This outpatient clinic is a secondary referral unit for women sent by primary health care units in the region due to Pap smear abnormal results.

The study included patients with abnormal conventional Pap smear and subsequent cervical biopsy under colposcopic guidance on suspicion of: viral infection, changed colposcopic examination or molecular biology examination positive for HPV-DNA.

Cervicovaginal samples were obtained from all patients included in the study and were frozen at –20°C in TE buffer for molecular biology studies. Next, according to a

flowchart prepared by the outpatient department of the Lower Genital Tract Pathology service, the patients were submitted to a colposcopy and to a guided biopsy when a lesion was identified.

A transversal analysis of 146 formalin-fixed cervical biopsies were performed, obtained from women who underwent biopsy, between 2003 and 2008. The study included patients sexually active, non-pregnant patients who were all but negative for other sexually transmitted diseases. The women were aged between 20 and 50 years old (average age of 35 years old).

Cytopathological and Histopathological analysis

Histopathological analyses were processed by the paraffin embedding method with 3µm thick histological sections, using staining with hematoxylin-eosin (HE), with a subsequent analysis by optical microscopy, performed by a pathologist. The cytological examinations were reviewed and classified according to the Bethesda classification criteria (16).

The classification was oriented as follows: "negative" when no significant cytological changes were observed; "Low-grade squamous intraepithelial lesion (LSIL)" when cellular changes consistent with mild dysplasia/CIN 1 and HPV infection were present; "high-grade squamous intraepithelial lesion (HSIL)" when cellular changes consistent with moderate dysplasia/CIN 2 and sharp/CIN 3 were observed.

HPV Typing

Cervical-vaginal swabs for HPV-DNA detection by the PCR method were collected at the time of the gynecological examination. The determination of the presence of HPV-DNA and viral typing was performed by the polymerase chain reaction method

(PCR) (17).The DNA samples obtained from cervical smears were isolated and purified by using GFX Genomic DNA Purification Kit Blood® kit (Amersham Biosciences, Piscataway, NJ, USA), according to the manufacturer's instructions.

After the DNA extraction the samples were subjected to PCR using a set of generic primers for HPV, PGMY 09/11 (17), capable of amplifying a 450 base pair bases (bp) from the L1 gene of different types of genital HPV. GH20 and PCO4 primers are added to the same PCR (18) They amplify 268 bp of the gene of human β-globin gene serving as internal control to assess the integrity and completeness of DNA from each sample.

The samples were amplified in the first PCR reaction using the degenerate primers GP-E6-3F (GGG WGK KAC TGA AAT CGG T), GP-E6-5B (CTG AGC TGT CAR NTA ATT GCT CA) and GP-E6-6B (TCC TCT GAG TYG YCT AAT TGC TC), and W, A / T; K, G / T; R, A / G; Y, C / T and C, A / C / G / T. These primers amplify one 630 pb region of the E6/E7 region of the 38 most common HPV types. The nested PCR reaction was performed specifically to the types 45, 11, 16, 6, 18, 42, 52, 33, 58, 53, 39, 54, 66, 51, 31, 67, 59, 62, 69, 35 e 68. All procedures were carried out both for the first reaction (PCR) and the second reaction (nested PCR) (19).

TG2 Immunohistochemistry

Immunohistochemistry (IHC) to TG2 occurred according to the manufacturer's instructions. The IHC was carried out in an automated manner in a Autosteiner-Link 48 Dako equipment.

This TG2 IHC assay utilizes a monoclonal mouse antibody (Diagnostic Biosystems, Diluition 1:50) directed against the domain of human TG2. The negative control reagent is a monoclonal rabbit IgG isotype control (DA1E; Cell Signaling Technology,

Danvers, MA). The deparaffinization, rehydration, and target retrieval were performed in the PT Link (Dako PT100). Slides were then processed on the Autostainer Link 48 (Dako AS480) using an automated staining protocol validated for TG2 IHC assay. The IHC staining protocol includes sequential application of a peroxidase-blocking reagent, TG2 primary antibody or negative control reagent, mouse anti-rabbit IgG linker, visualization reagent consisting of secondary antibody molecules and horseradish peroxidase coupled to a polymer backbone, 3,3′-diaminobenzidine tetrahydrochloride (DAB) chromogen reagent with hydrogen peroxide substrate, and a DAB enhancer which modifies the color of the precipitated chromogen. Reagents utilized in addition to TG2 assay components included a wash buffer specially formulated for automated IHC staining, and a hematoxylin counterstain. IHC-stained slides were mounted in a nonaqueous, permanent mounting medium.

TG2 expression by staining patterns is remarkable. Cells showing TG2 expression are shown stained in brown, which could be seen both in the nucleus and in the cytoplasm.

Interpretation and Quantification of the Staining

The extent of immunoreactivity in the samples was assessed by two different pathologists, using the same microscope by using a 40x objective lens with a field diameter of 0.52 mm. The official histopathology classification of the samples was not disclosed before the scoring was performed.

Analysis of protein expression by immunohistochemistry was performed in two ways, the first immunohistochemical results were evaluated considering the overall proportion of positive cells: the samples considered positive for TG2 had their fields

photographed. Positive cancer cells were counted relating to the total number of cancer cells in the sample. Cells with both cytoplasmic and nuclear staining were considered to be positive. TG2 relative quantification was categorized into three different intervals markings: > 50%, ≤50% or negative. This classification was based on a previous study (2).

In the second evaluation mode the images were scanned and transferred to a computer, and the intensity of staining was defined using the NIH Image J 1.36b software (National Institutes of Health, Maryland, USA). Image J is a public-domain image-analyzing software on Java platform inspired by the NIH Image software for Apple's Macintosh. Therefore, it can be run in different operating environments provided they have a suitable Java virtual machine. The repertoire of the software's functions can be extended through different off-the-shelf plug-ins, available on the web (18). The NIH Image J 1.36b software was downloaded from the NIH's website (http://rsb.info.nih.gov/ij). The color selection and classification of positively stained points was done by using a distribution diagram for the colors red, green and blue (RGB), which shows the changes in intensity and color saturation. This distribution provides information on pixel quantity of the analyzed image. This was obtained through the Threshold Colour plug-in, in which the color range of interest was defined. The software colors white the areas that meet this standard and black the remaining areas (20-23).

Statistical analysis

The statistical analysis was performed using the SPSS - Statistical Package for Social Sciences (version 20.0). For comparison of qualitative variables as frequencies and proportions, the $\chi 2$ test was used for independent samples with a possible analysis of adjusted residuals. The ordinal qualitative and quantitative data were compared with the nonparametric Mann-Whitney test with the level of significance set at 5%.

The variable HPV was dichotomized into "high risk" and "low risk". According to the literature (24) the following types were considered high-risk HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 26, 53, 66 , 67, 68, 69, 70, 73 and 82. The low-risk types are classified as follows: 6, 11, 40, 42, 43, 44, 55, 54 and 62. Patients who presented any subtype of high and low risk concomitantly, they were classified as high risk HPV. The TG2 tests were categorized in ≤50%, >50% and 0 (negative).

RESULTS

TG2 and Histopathological Analysis

A total of 146 patients were analyzed. Of these, 114 patients underwent histopathological examination. According to the histopathological analysis, the control group consisted of 38 (26%) cases, LSIL of 44 (30.1%) cases, HSIL of 32 (21.9%) cases. TG2 expression was observed in 93% of the total sample, and in the control group there was an average 74% positivity; in LSIL group immunostaining was 74.7%, and HSIL group there was an average expression of 22.3% (Table 1).

The association between histopathological examination and TG2 was statistically significant ($p<0.05$). Among the histopathological analysis results, which was performed with the $\chi 2$ test complemented by the Adjusted Residual Analysis, at a

significance level of 5%, patients with normal samples and LSIL were locally associated with expression levels of TG2>50 % ($p<0.05$), and patients with HSIL were associated with low TG2 expression ($p<0.05$) (Figure 1A, 1B).

The analyzes of samples by using Image J software show a significant ($p<0.001$) decrease in immunostaining of TG2 in HSIL compared to normal and LSIL samples (Figure 1D). This depicts a correlation between the relative analysis by the pathologist and Image J.

When analyzing the quantification of Image J, compared to relative quantification it is also possible to observe a significant difference ($p<0.001$) (Figure 1G). This demonstrates a relationship between the two analytical methods (Figure 1F).

Table 1. Description of sample groups and its relation to TG2 expression.

			HPV Typing									
Histopathological analysis	**Patients**		**Negative**		**Low Risk**		**High Risk**		**Not Defined**		**TG2 (%)**	**TG2 - Image J**
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**		
Control	38	26.0	9	23.7	1	2.6	16	42.1	12	31.6	74%* (SD* 17.13)	110,9 (SD 87.27)
LSIL	44	30.1	4	9.1	3	6.8	24	54.5	13	29.5	74,7%* (SD 17.55)	80,4 (SD 66.03)
HSIL	32	21.9	3	9.4	3	9.4	16	50	9	28.1	22,3%* (SD 24.12)	5,1 **(SD 11.67)

*$p<0.05$, **$p<0.001$, *Standard Deviation

HPV Distribution in Cervical Lesions

All evaluated patients were tested for HPV diagnosis through PCR, all 146 cases showed amplification of the human ß globin gene (100%), confirming the adequacy of the specimen regarding the quantity and integrity of the genetic material. Patients were classified as high and low risk depending on the type of HPV identified. Patients who had low and high risk viral types simultaneously were classified as high risk. Some patients infected with HPV had the type classified as not defined due to the fact that the HPV type was not identified by any specific PCR technique used.

The evaluated control group consisted of patients without malignant precursor lesions, including cases of squamous metaplasia, endocervicitis and cervicitis. In this group all patients had squamous metaplasia, and 76.3% of these showed HPV infection. It is important to note that even in the presence of HPV, the collected samples showed no related precursor lesions of high or low degree. Among the patients in the control group, 42.1% were infected with high-risk genotypes, 2.6% for low-risk HPV, 31.6% by non-defined types and 23.7% were negative for HPV.

The LSIL cases show the presence of high-risk HPV in 54.5% cases, low-risk HPV in 6.8% cases, not defined HPV in 29.5% cases and negative in 30.1% cases. HSIL cases showed the presence of high-risk HPV in 50% cases, low-risk HPV in 9.4% cases, not defined HPV in 28.1% cases and negative HPV in 9.4% cases. These results demonstrate the highest prevalence of high-risk genotypes in HSIL (Table 1).

TG2 and HPV typing

Regarding the diagnosis of the HPV infection, the presence of the virus was observed in 124 (84.9%) cases, among these, 66 (45.2%) patients were classified as

high risk HPV, 15 (10.3%) low risk and 43 (29.5%) with not defined HPV. A total of 22 patients who did not have HPV infection were excluded (Table 2).

TG2 expression was observed in all groups, with an average of 72.5% for low-risk HPV types, 59% for high-risk types and 60.7% for not defined types (Figure 1C). There was no significant statistical association between the relative quantification of TG2 and different classifications of HPV viral types.

A significant similarity between the expression of not defined types and high-risk types, can be observed, thus demonstrating the possible presence of high-risk HPV in the not defined group.

Table 2. Description of sample groups and the relation to TG2 expression.

HPV typing	**Patients**		**TG2 (%)**		**TG2 Image J**	
	n	**%**	**%**	**SD***	**n**	**SD***
Low-Risk HPV	15	10.3	72.5	17.08	35,99	55.11
High-Risk HPV	66	45.2	59	31.27	51,77	72.24
Not Defined HPV	43	29.5	60,7	29.85	58,55	70.26

*Standard Deviation.

The most frequently identified virus types were: 16 with 29 cases, 6 with 20 cases, 11 with 18 cases, and 41 cases belonged to the not defined group. When individual HPV types were analyzed in relation to TG2 expression levels, a statistical significance ($p = 0.031$) was demonstrated for HPV 11, and borderline for HPV 54 ($p = 0.087$), and HPV 6 ($p = 0.084$); indicating that patients with type 11 had higher values of TG2 compared

to patients without this type. These results may represent a trend as the evaluation counts with a limited number of patients.

When analyzing TG2 expression according to the HPV type, one notices that generally the expression decreases in high-risk types and increases in low-risk types (Figure 1C). Particularly, when the not defined HPV types are observed by relative quantification and Image J, TG2 behaves similarly to the high-risk types. This shows the importance of analyzing other viral types, since it is more likely they become malignant tissues (Figure 1E). Among these cases, two patients with not defined HPV demonstrated TG2 expression that was very similar to patients with high-risk HPV.

Viral types investigated in this study were defined according to their prevalence in cervical lesions, the identified types are the most common high and low risk, however, considering the obtained results, it became relevant to delineate the not defined HPV, owing to the fact that probably among them are prevalent high-risk viral types. When the association between TG2 and viral HPV types was verified trough Image J analysis, no significant association was found (Figure 1E).

The results suggest that TG2 involving the presence of LSIL and non-malignant alterations (Figure 2) of the cervix is significantly associated to low-risk viral types, thus indicating that TG2 variable expression is associated with cancerous changes of the cervix.

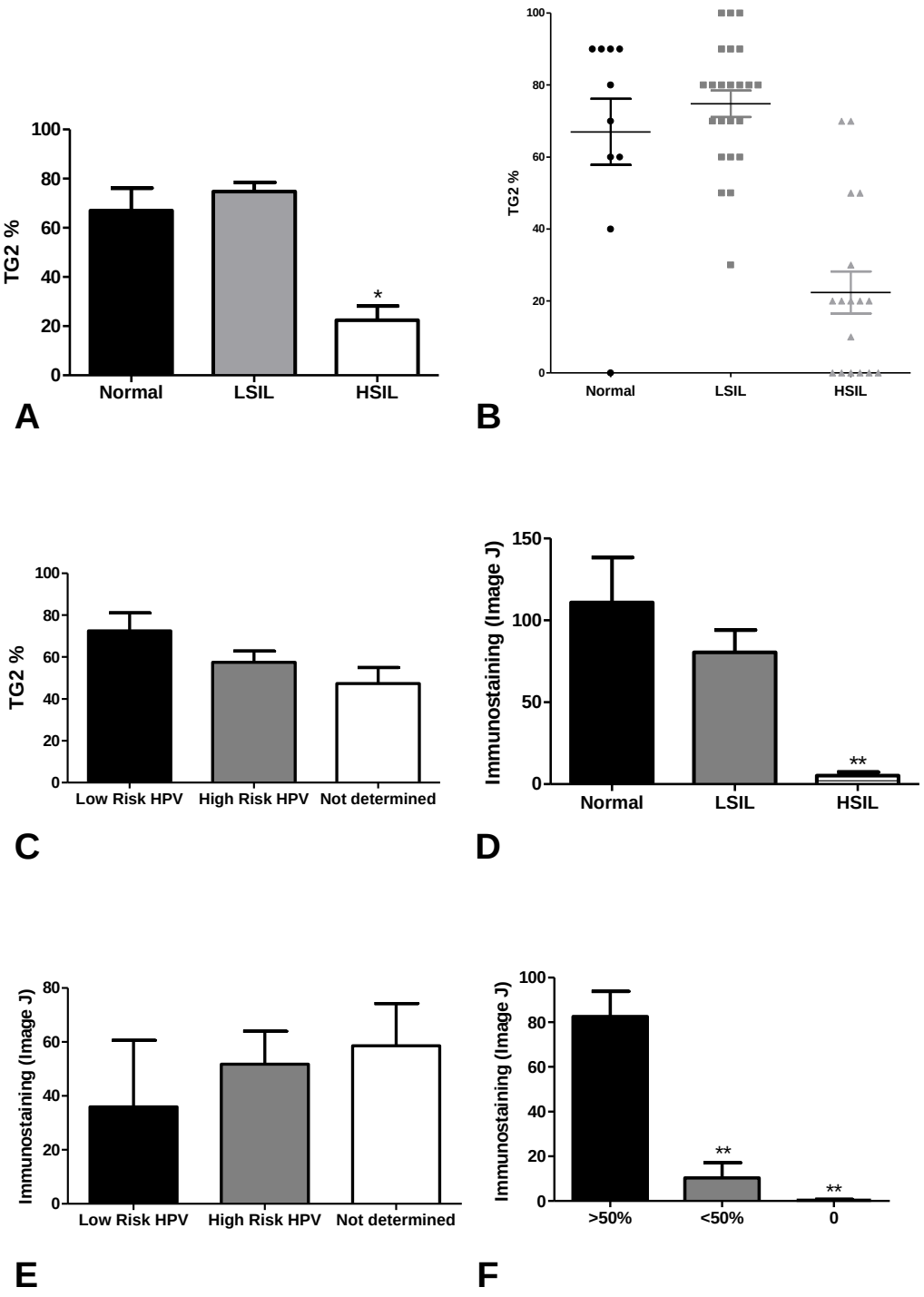

Figure 1. Distribution of the expression levels of the type 2 transglutaminase (TG2) according to the different lesion grades (A and B). Distribution of expression levels of the type 2 transglutaminase (TG2) according HPV type (C). Quantification of TG2 by Image J and its association with histopathological classification (D) and viral HPV

types (E). Relation between categorization of relative quantification and quantification of TG2 by Image J (F) *p<0.05 **p<0.001.

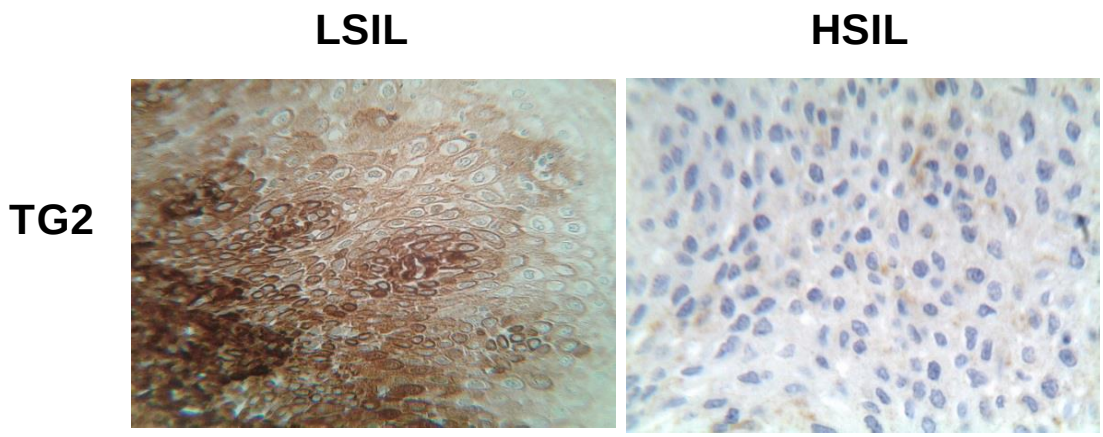

Figure 2. Transglutaminase type 2 (TG2) immunostaining in high (A) and low (B) grade cervical lesion.

DISCUSSION

HPV Epidemiology

Regarding the presence of HPV-DNA, several authors have found different rates depending on the development rate of the country studied. In a meta-analysis including about 150.000 women, after adjusting to the possible extent for study design, age, and HPV-DNA detection assays, the estimated world wide HPV-DNA prevalence was approximately 10%. The highest estimates were found in Africa and Latin America (20–30%), and the lowest in southern Europe and South East Asia (6–7%) (25, 26). According to a systematic review in Brazil, in which the region and the population type was considered, the values for the prevalence of HPV range from 13.7% to 54.3% (27). In a study that investigated the prevalence of HPV infection in Basic Units of Health and patients of a university hospital in southern Brazil, a prevalence of 18.2% was

observed (28), otherwise HPV-DNA is present in 99.7% in patients with cervical intraepithelial neoplasia (29).

Our data showed a frequence of HPV of 84.9%, which corroborates with previously published studies. In this study, the samples were obtained from patients with suspected LSIL or HSIL detected in the routine conventional Pap test in clinical pathology of cervical biopsy under colposcopic guidance on suspicion of: viral infection, changed colposcopic examination or molecular biology examination positive for HPV-DNA. This procedure explains the high prevalence of HPV in the samples.

Among the most common HPV types, genotypes 16, 18, 31, 33, 45, 52 and 58 are responsible for approximately 90% of cervical cancers (30). In Brazil, Oliveira et al. demonstrated that the most frequent types were HPV16 (77.6%), HPV18 (12.3%), HPV31 (8.8%), HPV33 (7.1%) and HPV35 (5.9%) (31). According to other studies, HPV16 is the most prevalent type in all regions of Brazil, HPV 18 is the second most prevalent in North, South and Southeast of Brazil, and 31 and 33 are the next most prevalent in the Northeast and central areas of Brazil, respectively (32). Our data corroborate the information present in the literature, since HPV16 was the most prevalent type in patients, with 29 (19.9%) positive cases.

The presence of HPV in normal samples is also shown in other studies (27, 30, 31), in a study performed in the city of Porto Alegre, the prevalence of HPV was demonstrated in 11.6% of women without LSIL or HSIL (33), which was also shown in this study where the HPV infection was shown in control samples (76.3%), which consisted of patients with squamous metaplasia.

Our study did not investigate the presence of TG2 in patients with biopsies without any alteration or HPV infection owing to the difficulty of obtaining biopsies for healthy patients. Only two studies have investigated TG2 levels in samples such as these, both of which found negative expression in normal tissues, indicating that TG2 expression is solely related to non-malignant changes or LSIL (2, 7).

TG2 and Cervical Lesions

In this study, we have analyzed TG2 expression in the epithelium of human cervix in relation to HPV-induced dysplastic modifications. Our findings indicate that TG2 expression levels in patients with metaplasia (74%) were similar to patients with LSIL (74.7%). Moreover, TG2 expression is significantly related to genotype 11 ($p<0.05$), which is classified as low-risk. Thus, the correlation of TG2 with productive HPV infections and low malignant potential lesions is observed. Furthermore, the results show that TG2 is less detected in HSIL, whereas it is strongly up-regulated in early cervical lesions in HPV infection.

TG2 has been identified as an interfering factor in HPV infection. This protein is involved in a wide range functions, such as cell death, cytoskeletal rearrangement and extracellular stabilization. Its modified activation has been associated with a wide range of pathologies (8, 36-38).

TG2 also is associated with viral pathogenesis, and several proteins involved in the process of infection are modified by TG2 (10, 12). Some studies indicated that TG2 with its catalytic activity can incorporate a polyamine in HPV18 E7 protein, and thus inhibit its binding to pRb protein in the cytosol and nucleus, thereby protecting the caspases degradation pathway (13, 39, 40). In addition, recently, it has been shown that

there is an increase in the splicing process of TG2 in cancer cells, indicating a relation between this molecule with alterations in differential intracellular processing in cancer (41).

It has been demonstrated that LSIL present high level of the enzyme, while in HSIL these levels are decreased. Moreover this enzyme is currently referred as a phenomenon not restricted to high-risk genotypes (2). The present results corroborate previously published data (2, 7), emphasizing that HSIL patients demonstrated a significant statistical association with low TG2 expression ($p<0.05$).

The findings in this study demonstrated for the first time the use of Image J software for quantification of TG2 in cervix samples. The results suggest that the two quantification methods can be applied and corroborate previously published data. In this sense, the individual variation is a significant limitation in the relative quantification. This factor could be decisive in some cases and the problem could be overcome by using the software Image J. This software has been widely used in researches and presents several advantages, such as being a quantitative method, presenting a lower chance of variation in the outcome.

TG2 and HPV Typing

When the relationship between TG2 and the HPV types was analyzed, a significant statistical association to HPV 11 ($p=0.031$) and borderline values for HPV 54 and HPV 6 ($p=0.087$ and $p=0.084$) was found, whereas these viral types are classified as low-risk, the data suggest that TG2 can act as a analytical factor in elucidating the development of cervical cancer particularly associated with HPV11. This is evidenced for the first time in the literature.

Currently, HPV types 6 and 11 are responsible for 90% of all genital warts, which are included in the quadrivalent vaccine for HPV. The infection time of low-risk HPV types is lower compared to high-risk types. The low-grade types generate productive lesions, where certain viral gene products stimulate the cell proliferation, resulting in benign lesions such as warts. These HPV types do not integrate their genome into cellular DNA (42).

TG2 as new LSIL management tool

Further study of HPV-related carcinogenesis processes generated new controlling strategies, providing a more rational approach to diagnosis and treatment of cervical lesions. Understanding that LSIL lesions are not always true cervical cancer precursors has raised expectant management of women with these lesions; however, management approaches are still disadvantaged by the incapacity to better predict who is at risk for HSIL and cancer and who is not; particularly in LSIL treatment (15).

Moreover, the treatment decision of LSIL depends on a number of complex factors related to the patient. New biomarkers that could predict which LSIL are at a highest risk for development of HSIL appear to be on prospect and may make the management of LSIL as clear as present guidelines for HSIL (15). In the present study we evidenced the behavior of an important biomarker in HPV carcinogenesis process, TG2 showed a differential expression in LSIL and HSIL, which can be an indication that this enzyme behaves as a marker, or an important new tool for defining the evolution and treatment of LSIL.

TG2 decreases its expression in HSIL cases. This suggests that the presence of this marker indicates lower chances of cancer development, serving as an early indicator.

The study of TG2 expression in association with other lesion markers is not widely investigated. In a paper in which TG2 was correlated with $p16^{INK4a}$, a regulatory protein of the cell cycle and widely studied in cervical cancer, an inverse correlation was observed between the markers (Pearson: -0.930) (2). This, however, was not observed in another research, which did not show any correlation between the expression of both markers (7). Nevertheless, the association between TG2 and other biomarkers should be investigated in order to optimize the use of this tool associated to other proteins related to cervical cancer, thereby raising its analytical power.

Conclusion

Our data suggest that TG2 may represent a new tool for monitoring and controlling premalignant lesions associated with HPV. More studies are needed to promote the use of this biomarker in clinical practice.

REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al., Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. International Journal of Cancer. 2015;136(5):E359-E86.

2. Del Nonno F, Pisani G, Visca P, Signore F, Grillo LR, Baiocchini A, et al., Role and predictive strength of transglutaminase type 2 expression in premalignant lesions of the cervix. Mod Pathol. 2011;24(6):855-65.

3. Wentzensen N, Schiffman M, Palmer T, Arbyn M. Triage of HPV positive women in cervical cancer screening. J Clin Virol. 2015;28(15):00749-0.

4. Wieringa HW, van der Zee AG, de Vries EG, van Vugt MA. Breaking the DNA damage response to improve cervical cancer treatment. Cancer Treat Rev. 2015;24(15):00227-3.

5. Dyson N, Howley PM, Munger K, Harlow E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science. 1989;243(4893):934-7.

6. Fiedler M, Muller-Holzner E, Viertler HP, Widschwendter A, Laich A, Pfister G, et al., High level HPV-16 E7 oncoprotein expression correlates with reduced pRb-levels in cervical biopsies. Faseb J. 2004;18(10):1120-2.

7. Gupta R, Srinivasan R, Nijhawan R, Suri V. Tissue transglutaminase 2 as a biomarker of cervical intraepithelial neoplasia (CIN) and its relationship to $p16^{INK4a}$ and nuclear factor kappaB expression. Virchows Arch. 2010;456(1):45-51.

8. Fesus L, Piacentini M. Transglutaminase 2: an enigmatic enzyme with diverse functions. Trends Biochem Sci. 2002;27(10):534-9.

9. Begg GE, Carrington L, Stokes PH, Matthews JM, Wouters MA, Husain A, et al., Mechanism of allosteric regulation of transglutaminase 2 by GTP. Proc Natl Acad Sci U S A. 2006;103(52):19683-8.

10. Amendola A, Rodolfo C, Di Caro A, Ciccosanti F, Falasca L, Piacentini M. "Tissue" transglutaminase expression in HIV-infected cells: an enzyme with an antiviral effect? Ann N Y Acad Sci. 2001;946:108-20.

11. Lu W, Strohecker A, Ou Jh JH. Post-translational modification of the hepatitis C virus core protein by tissue transglutaminase. J Biol Chem. 2001;276(51):47993-9.

12. Jeon JH, Kim IG. Role of protein modifications mediated by transglutaminase 2 in human viral diseases. Front Biosci. 2006;11:221-31.

13. Jeon JH, Choi KH, Cho SY, Kim CW, Shin DM, Kwon JC, et al., Transglutaminase 2 inhibits Rb binding of human papillomavirus E7 by incorporating polyamine. Embo J. 2003;22(19):5273-82.

14. Tranbaloc P. [Natural history of precursor lesions of cervical cancer]. Gynecol Obstet Fertil. 2008;36(6):650-5.

15. Cox JT. Management of women with cervical cancer precursor lesions. Obstet Gynecol Clin North Am. 2002;29(4):787-816.

16. Solomon D, Nayar R. The Bethesda System for reporting cervical cytology: definitions, criteria, and explanatory notes: Springer Science & Business Media; 2004.

17. Gravitt P, Peyton C, Alessi T, Wheeler C, Coutlee F, Hildesheim A, et al., Improved amplification of genital human papillomaviruses. Journal of clinical microbiology. 2000;38(1):357-61.

18. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, et al., Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science. 1988;239(4839):487-91.

19. Stoler MH. A brief synopsis of the role of human papillomaviruses in cervical carcinogenesis. Am J Obstet Gynecol. 1996;175(4 Pt 2):1091-8.

20. Schindelin J, Rueden CT, Hiner MC, Eliceiri KW. The ImageJ ecosystem: An open platform for biomedical image analysis. Mol Reprod Dev. 2015;82(7-8):518-29.

21. Wu JZ, Zhang X, Men F, Pengtao Sun Z. The experiment research of edge detection in digital image [J]. Control & Automation. 2004;5.

22. Thal DR, Horn M, Schlote W. Selective quantitative analysis of the intensity of immunohistochemical reactions. Acta Histochem. 1995;97(2):203-11.

23. Matos LL, Stabenow E, Tavares MR, Ferraz AR, Capelozzi VL, Pinhal MA. Immunohistochemistry quantification by a digital computer-assisted method compared to semiquantitative analysis. Clinics. 2006;61(5):417-24.

24. Souho T, Benlemlih M, Bennani B. Human papillomavirus infection and fertility alteration: a systematic review. PLoS One. 2015;10(5).

25. Castellsagué X dSS, Aguado KS et al., HPV and cervical cancer in the 2007 report. Vaccine. 2007;1(25):C1-230.

26. de Sanjose S, Diaz M, Castellsague X, Clifford G, Bruni L, Munoz N, et al., Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis. Lancet Infect Dis. 2007;7(7):453-9.

27. Ayres ARG, Silva GA. Cervical HPV infection in Brazil: systematic review. Revista de Saúde Pública. 2010;44(5):963-74.

28. Oliveira GRd, Vieira VC, Barral MFM, Döwich V, Soares MA, Conçalves CV, et al., Fatores de risco e prevalência da infecção pelo HPV em pacientes de Unidades Básicas de Saúde e de um Hospital Universitário do Sul do Brasil. Rev bras ginecol obstet. 2013;35(5):226-32.

29. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, et al., Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol. 1999;189(1):12-9.

30. Wagner M, Bennetts L, Patel H, Welner S, de Sanjose S, Weiss TW. Global availability of data on HPV genotype-distribution in cervical., vulvar and vaginal disease and genotype-specific prevalence and incidence of HPV infection in females. Infect Agent Cancer. 2015;10(13):015-0008.

31. de Oliveira CM, Fregnani JHTG, Carvalho JP, Longatto-Filho A, Levi JE. Human papillomavirus genotypes distribution in 175 invasive cervical cancer cases from Brazil. BMC cancer. 2013;13(1):357.

32. Rabelo-Santos S, Zeferino L, Villa L, Sobrinho J, Amaral R, Magalhães A. Human papillomavirus prevalence among women with cervical intraepithelial neoplasia III and invasive cervical cancer from Goiânia, Brazil. Memórias do Instituto Oswaldo Cruz. 2003;98(2):181-4.

33. Becker E, Edelweiss MI, Nonnenmacher B, Bozzetti MC. Prevalence and epidemiologic correlates of atypical squamous cells of undetermined significance in women at low risk for cervical cancer. Diagnostic cytopathology. 2001;24(4):276-82.

34. Kurita T. Normal and abnormal epithelial differentiation in the female reproductive tract. Differentiation. 2011;82(3):117-26.

35. Kinjo T, Kamiyama K, Chinen K, Iwamasa T, Kurihara K, Hamada T. Squamous metaplasia induced by transfection of human papillomavirus DNA into cultured adenocarcinoma cells. Mol Pathol. 2003;56(2):97-108.

36. Falasca L, Iadevaia V, Ciccosanti F, Melino G, Serafino A, Piacentini M. Transglutaminase type II is a key element in the regulation of the anti-inflammatory response elicited by apoptotic cell engulfment. J Immunol. 2005;174(11):7330-40.

37. Facchiano F, Facchiano A, Facchiano AM. The role of transglutaminase-2 and its substrates in human diseases. Front Biosci. 2006;11:1758-73.

38. Falasca L, Farrace MG, Rinaldi A, Tuosto L, Melino G, Piacentini M. Transglutaminase type II is involved in the pathogenesis of endotoxic shock. J Immunol. 2008;180(4):2616-24.

39. Boehm JE, Singh U, Combs C, Antonyak MA, Cerione RA. Tissue transglutaminase protects against apoptosis by modifying the tumor suppressor protein p110 Rb. J Biol Chem. 2002;277(23):20127-30.

40. Milakovic T, Tucholski J, McCoy E, Johnson GV. Intracellular localization and activity state of tissue transglutaminase differentially impacts cell death. J Biol Chem. 2004;279(10):8715-22.

41. Phatak VM, Croft SM, Rameshaiah Setty SG, Scarpellini A, Hughes DC, Rees R, et al., Expression of transglutaminase-2 isoforms in normal human tissues and cancer cell lines: dysregulation of alternative splicing in cancer. Amino Acids. 2013;44(1):33-44.

42. Minaguchi T, Yoshikawa H. [Molecular mechanism of cervical carcinogenesis]. Gan To Kagaku Ryoho. 2010;37(1):18-22.

Capítulo 5

ANALYSIS OF TRANSGLUTAMINASE 2 AND p16^{INK4a} EXPRESSION IN PREMALIGNANT CERVICAL LESIONS

TRANSGLUTAMINASE 2 AND p16^{INK4a} IN CERVICAL LESIONS

Karen Bazzo1,2* , Alessandra Eifler Guerra Godoy3 , Fábio Firmbach Pasqualotto4

1 phD student in biotechnology at University of Caxias do Sul (UCS) Address Francisco Getúlio Vargas, 1130 Code 95070-560 - Caxias do Sul, RS, Brazil.

2 Professor at Faculdade da Serra Gaúcha, RS, Brazil. (karenbazzo@gmail.com)

3 phD, Pathologist, Professor at the Medical School of UCS . Director of IPCEM Address Francisco Getúlio Vargas, 1130 Code 95070-560 - Caxias do Sul, RS, Brazil. (aeggodoy@gmail.com)

4 phD, Urologist, Professor at the Medical school of UCS. Address Francisco Getúlio Vargas, 1130 Code 95070-560 - Caxias do Sul, RS, Brazil. (fabio@conception-rs.com.br)

**corresponding author*

Structured abstract:

Background: This study evaluates the expression of transglutaminase type 2 (TG2) and $p16^{INK4a}$ (p16) as biomarkers in precursor low-grade and high-grade squamous intraepithelial lesion (LSIL and HSIL). The results are then related to different viral types of HPV. **Methods:** The study included 146 patients with suspected LSIL or HSIL identified in Pap smear. The presence of HPV-DNA and viral typing was determined by polymerase chain reaction (PCR). TG2 and p16 Immunohistochemistry (IHC) was performed according to the manufacturer's instructions and was carried out in an Autosteiner-Link 48 Dako equipment. **Results:** Association between histopathological examination, p16 and TG2 was statistically significant ($p<0.05$). A significant ($p<0.05$) inverse correlation (Pearson value = -0.269) was observed between p16 and TG2. This evidences that p16 expression increases proportionately to an increase in the lesion degree. On the other hand, TG2 expression decreases with the increase of the lesion degree. The analysis of individual HPV types in relation to TG2 expressional levels showed statistical significance for HPV11 ($p = 0.031$). Individually, p16 expression had no statistically significant association for different HPV viral types. **Conclusions:** Our findings suggest that p16 and TG2 show variable expression levels according to the cervical injury degree.

Key-words: Uterine Cervical Neoplasms, TG2, p16.

INTRODUCTION

Cervical cancer is a significant health problem for women globally. This is the fourth most common cancer among women, accounting for the death of 265.000 women

each year (Ferlay *et al.,* 2015). Human papillomavirus (HPV), the most common sexually transmitted infection worldwide, plays a major role in cervical cancer carcinogenesis, with HPV types 16 and 18 together accounting for about 70% of all cervical cancers (Crosbie *et al.,* 2013).

Biomarker status evaluation yields information about cancer projection, treatment response, and vulnerability to targeted therapies (Henry & Hayes, 2012). Several biomarkers have been studied in association with cervical cancer. Among these markers, the tumor suppressor protein $p16^{INK4a}$ (p16) has been considered important for prognostic significance in cervical cancer. Gene p16 is expressed by host cells in response to HPV infection and is not normally expressed in non-dysplastic cells. This protein normally arrests the cell cycle at the G1-S phase checkpoint by inhibiting the Cyclin-dependent kinases enzyme (CDK) that phosphorylates and inactivates retinoblastoma protein (pRb) (Krishnappa *et al.,* 2014; Ngugi *et al.,* 2015).

Another marker in evidence is transglutaminase type 2 (TG2) enzyme, which has been considered a cellular-interfering factor in HPV infection (Del Nonno *et al.,* 2011; Gupta *et al.,* 2010). TG2 participates in signal transduction via activating and hydrolyzing guanidine triphosphate (GTP) enzyme. Several proteins involved in the infection process have shown to be modified by TG2 (Amendola *et al.,* 2001; Jeon & Kim, 2006). Thus, TG2 can be associated with viral pathogenesis. Furthermore, some studies indicated that TG2 could incorporate a polyamine in HPV18 E7 protein due to its catalytic activity. Consequently, TG2 binding to retinoblastoma (pRb) protein in the cytosol and nucleus is inhibited, thereby protecting the degradation pathway caspases (Boehm *et al.,* 2002; Milakovic *et al.,* 2004).

Currently, the medical management of the low-grade squamous intraepithelial lesion (LSIL) is variable (Cox, 2002; Tranbaloc, 2008). Biomarkers could then contribute in evidencing indicators of more serious injuries or for their resolution. Therefore, this study had the objective to evaluate TG2 and p16 expression as biomarkars in LSIL and HSIL, and relate them to different HPV viral types.

MATERIALS AND METHODS

Patients

This retrospective study was approved by the Review Board of our institution and all patients gave written informed consent. Women seen at the Lower Genital Tract Pathology Service of the Central Outpatient Clinic (UCS) and at the Diagnosis Laboratory were included in this study. This outpatient clinic is a secondary referral unit for women sent by primary health care units in the region. Women are referred to this clinic when the routine cytopathology test (Pap smear) presents abnormal conditions.

The study included patients with suspected LSIL or HSIL detected in the routine conventional Pap test in clinical pathology of cervical biopsy under colposcopic guidance on suspicion of: viral infection, changed colposcopic examination or molecular biology examination positive for HPV-DNA. This study respected ethical principles and was approved by the Ethics Committee of Faculdade Nossa Senhora de Fátima (advice number 183 756).

Cervicovaginal secretion was obtained from all patients included in the study and samples were frozen at –20°C in TE buffer for molecular biology studies. Then, patients were submitted to colposcopy and to a guided biopsy when a lesion was identified,

according to a flowchart prepared by the outpatient department for Lower Genital Tract Pathology.

A transversal analysis of 146 formalin-fixed cervical biopsies was performed. The samples were from women who underwent biopsy between 2003 and 2008. The study included patients who were sexually active, not pregnant, and all but negative for other sexually transmitted diseases. The women were aged between 20 and 50 years old (average age of 35 years old).

Histopathological analysis

Histopathological analyses were processed by the paraffin embedding method with 3µm thick histological sections, using staining with hematoxylin-eosin (HE), with a subsequent analysis by optical microscopy, performed by a pathologist. The cytological examinations were reviewed and classified according to the Bethesda classification criteria (Solomon & Nayar, 2004).

The classification was oriented as follows: "negative" when significant cytological changes were observed; "LSIL" when cellular changes consistent with mild dysplasia/ cervical intraepithelial lesion grade (CIN) 1 and HPV infection were present; "HSIL" when cellular changes consistent with moderate dysplasia/CIN 2 and sharp/CIN 3 were observed.

HPV Typing

Cervical-vaginal swabs for HPV-DNA detection by the PCR method were collected at the time of the gynecological examination. The determination of the presence of HPV-DNA and viral typing was performed by the polymerase chain reaction method (PCR) (Gravitt *et al.*, 2000) performed on two sample groups. The DNA samples

obtained from cervical smears were isolated and purified by using GFX Genomic DNA Purification Kit Blood® kit (Amersham Biosciences, Piscataway, NJ, USA), according to manufacturer's instructions.

After DNA extraction the samples were subjected to PCR using a set of generic primers for HPV, PGMY 09/11 (Gravitt *et al.*, 2000), capable of amplifying a 450 base pair bases (bp) from the L1 gene of different types of genital HPV. GH20 and PCO4 primers are added to the same PCR (Saiki *et al.*, 1988). They amplify 268 bp of the gene of human β-globin gene serving as internal control to assess the integrity and completeness of DNA from each sample.

The samples were amplified in the first PCR reaction using degenerate primers GP-E6-3F (GGG WGK KAC TGA AAT CGG T), GP-E6-5B (CTG AGC TGT CAR NTA ATT GCT CA) and GP-E6-6B (TCC TCT GAG TYG YCT AAT TGC TC), and W, A / T; K, G / T; R, A / G; Y, C / T and C, A / C / G / T. These primers amplify one 630pb region of the E6/E7 region of the 38 most common types of HPV. The nested PCR reaction was performed specifically to the types 45, 11, 16, 6, 18, 42, 52, 33, 58, 53, 39, 54, 66, 51, 31, 67, 59, 62, 69, 35 e 68. All procedures were carried out both for the first reaction (PCR) and the second reaction (NESTED-PCR) (Stoler, 1996).

TG2 Immunohistochemistry

Immunohistochemistry (IHC) to TG2 occurred according to the manufacturer's instructions. The IHC was carried out in an automated manner in Autosteiner-Link 48 Dako equipment.

This TG2 and p16 IHC assay utilizes a monoclonal mouse antibody (Diagnostic Biosystems, Diluition 1:50) directed against the domain of human TG2 and Cintec

p16INK4 Histology Kit (Dako Cytomation, Glostrup, Denmark) for p16. The negative control reagent is a monoclonal rabbit IgG isotype control (DA1E; Cell Signaling Technology, Danvers, MA). Deparaffinization, rehydration, and target retrieval was performed in the PT Link (Dako PT100). Slides were then processed on the Autostainer Link 48 (Dako AS480) using an automated staining protocol validated for the TG2 IHC assay and processed in the Cintec $p16^{INK4a}$ kit (Dako, clone E6H4, EnVisionsystem, Denmark), according to manufacturer's instructions for p16 IHC. The IHC staining protocol included sequential application of a peroxidase-blocking reagent, TG2 and p16 primary antibody or negative control reagent, mouse anti-rabbit IgG linker, visualization reagent consisting of secondary antibody molecules and horseradish peroxidase coupled to a polymer backbone, 3,3′-diaminobenzidine tetrahydrochloride (DAB) chromogenic reagent with hydrogen peroxide substrate, and a DAB enhancer which modifies the color of the precipitated chromogen.

The TG2 and p16 expression by IHC staining patterns is remarkable. Cells showing TG2 or p16 expression are shown stained in brown. This staining could be seen both in the nucleus and in the cytoplasm.

Interpretation and Quantification of the Staining

The extent of immunoreactivity in the samples was assessed using the same microscope by using a 40x objective with a field diameter of 0.52 mm. The official histopathology classification of the samples was not disclosed before the scoring was performed.

Immunohistochemical results were evaluated considering the overall proportion of positive cells: the samples considered positive for TG2 and p16 had their fields

photographed. Positive cancer cells were counted and related to the total number of cancer cells in the sample. Cells with both cytoplasmic and nuclear staining were considered positive. TG2 relative quantification was categorized into three different intervals markings being >50%, ≤50% or negative, this classification were based on previous studies (Bergeron *et al.*, 2015; Del Nonno *et al.*, 2011).

1. Statistical analysis

The statistical analysis was performed using the SPSS - Statistical Package for Social Sciences (version 18.0). For comparison of qualitative variables as frequencies and proportions, the $\chi 2$ test was used for independent samples with a possible analysis of adjusted residuals. The ordinal qualitative and quantitative data were compared with the nonparametric Mann-Whitney test with the level of significance set at 5%.

The variable HPV was dichotomized into "high risk" and "low risk". According to the literature (Souho *et al.*, 2015) the following types were considered high-risk HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 26, 53, 66 , 67, 68, 69, 70, 73 and 82. The low-risk types are classified as follows: 6, 11, 40, 42, 43, 44, 55, 54 and 62. Patients who presented any subtype of high or low risk HPV concomitantly were classified as “high risk”. The TG2 tests were categorized in ≤50%, >50 and 0 (negative).

RESULTS

TG2, p16 and Histopathological Analysis

A total of 146 patients were analyzed. Of these, 114 patients underwent histopathological examination. According to histopathological analysis, the control group consisted of 38 (26%) cases, LSIL of 44 (30,1%) cases, and HSIL of 32 (21,9%)

cases. TG2 and p16 expression was observed in 93% and 42.4% of patients evaluated, respectively (Table 1).

The association between histopathological examination, p16 and TG2 was statistically significant ($p<0.05$). There was no p16 expression evidenced in the control samples, and an average TG2 expression of 74% was observed in the same group (Table 1). In patients with LSIL, TG2 expression was maintained, with an average positivity of 74.7%. For p16, an expression of 24.7% was observed. For patients with HSIL, the value was inverted, which results in an increased p16 expression, with an average expression of 75.3% (Figure 1B and 1D) and a decreased TG2 expression, demonstrating 22.3% expression (Figure 1A). This suggests both markers are inversely correlated (Figure 1G).

TG2 results observed using the $\chi 2$ test complemented by the Adjusted Residual Analysis with the significance level set at 5%, showed that patients with normal samples and LSIL are locally associated with TG2 expression levels of >50 % ($p<0.05$), and patients with HSIL are associated with no TG2 expression of TG2 ($p<0.05$) (Figure 1C).

The analysis of the association between p16 and TG2 expression through the Pearson's correlation showed a significant ($p<0.05$) inverse correlation (Pearson value = -0.269). This demonstrates that as the lesion degree increases, p16 expression also increases while TG2 expression decreases significantly (Figure 1G).

HPV Distribution in Cervical Lesions

All evaluated patients were tested for HPV diagnosis through PCR, all 146 cases showed amplification of the human ß globin gene (100%), confirming the adequacy of

the specimen regarding the quantity and integrity of the genetic material. Patients were classified as high and low risk depending on the type of HPV identified. Patients who had low and high risk viral types simultaneously were classified as high risk. Some patients infected with HPV had the type classified as not defined due to the fact that the HPV type was not identified by any specific PCR technique used.

As to HPV-DNA, including the control group, the virus was present in 124 (84.9%) cases. Of these, 66 (45.2%) patients were classified as high risk, 15 (10.3%) patients were considered low risk and 43 (29.5%) patients as HPV not defined. A total of 22 patients did not present amplification of the 450 bp of gene L1, which produces several different types of genital HPV (Table 2).

The evaluated control group consisted of patients without malignant precursor lesions, including cases of squamous metaplasia, endocervicitis and cervicitis. In this group all patients had squamous metaplasia, and 76.3% of these patients showed HPV infection. Even in the samples that presented HPV, there were no related high or low degree precursor lesion. Among the patients in the control group, 42.1% were infected with high-risk genotypes, 2.6% for low-risk HPV, 31.6% by non-defined types and 23.7% were negative for HPV.

The LSIL cases show the presence of high-risk HPV in 54.5% cases, low-risk HPV in 6.8% cases, HPV not defined in 29.5% cases and negative in 30.1% cases. HSIL cases showed the presence of high-risk HPV in 50% cases, low-risk HPV in 9.4% cases, not defined HPV in 28.1% cases and HPV negative in 9.4% cases (Table 1).

TG2, p16 and HPV viral type

TG2 and p16 expression was observed in all groups, with an average of 72.5% and 43.3% for low-risk HPV types, 59% and 37.2% for high-risk types, and 60.7% and 30.3% for the not determined type, respectively (Figure 1C). No statistically significant association was found between TG2 or p16 relative quantification and different HPV viral types.

Statistical significance (p=0.031) was found for HPV 11 and borderline significance for HPV 54 (p = 0.087) and HPV 6 (p=0.084) was found in the individual HPV types analyses in relation to TG2 expression levels. This indicates that type 11 patients had higher TG2 values than other patients (Figure 1E).

The behavior of not determined HPV types was similar to high-risk types in regards to TG2 expression. Hence, the importance of analyzing other viral types is clear, since they are more likely to cause malignant alterations. Among these cases, two patients with not determined HPV demonstrated a TG2 expression that was very similar to patients with high-risk HPV.

Viral types investigated in this study were determined according to their prevalence in cervical lesions. The identified viral types were the most common among the high and low risk types. However, considering the results, it became relevant to identify the specific type of HPV in the not determined cases, as prevalent high-risk viral types can probably be found among them.

The results suggest that TG2 is related to LSIL and non-malignant cervix alterations (Figure 2) and that it is associated in a significant way to the low-risk viral types, thus indicating that TG2 variable expression is associated with cancerous changes of the cervix.

When evaluating the p16 expression individually, no statistically significant association with different HPV viral types was found (Figure 1F).

Changes in TG2 and p16 expression

The results described above show that TG2 and p16 have a specific behavior for each type of injury, and their concentrations are statistically significant ($p<0.05$). Furthermore, TG2 and p16 are inversely proportional in regards to the lesion level (Pearson value= -0.269, figure 1G, figure 2). Thus, the quantification of each of these proteins could be an important factor in the clinical conduct. In this sense, up-regulation of TG2 contributes to LSIL non-malignant changes, and up-regulation of p16 contributes to HSIL. The use of both markers simultaneously can assist in determining the management lesions, particularly in the case of LSIL.

DISCUSSION

TG2 e p16 as biomarkers in premalignant cervical Lesions

In the present study the expression of two important markers, p16 and TG2 was observed. p16 is a cervical cancer marker that has been associated with HPV and its intracellular changes in an ample manner. Several studies indicate p16 as a protein that can assist in the management of LSIL and HSIL (Gajanin *et al.*, 2015; Wang *et al.*, 2015). The study of this marker in clinical practice has been increasingly present in literature. Diagnostic studies have demonstrated that the use of p16 immunohistochemistry improves the reproducibility and diagnostic accuracy of

histopathologic diagnoses. p16 cytology is significantly more sensible for detection of cervical precancer lesions in comparison to conventional Pap tests (Bergeron *et al.*, 2015).

The control of p16 expression is altered during the process of carcinogenesis caused by HPV, a study has shown that high level expression of E7, a HPV protein, triggers oncogenic stress signals and induces epigenetic remodeling of the CDKN2A (Cyclin-Dependent Kinase Inhibitor 2A) locus that results in a substantial overexpression of p16 (McLaughlin-Drubin *et al.*, 2011). This protein is a cyclin dependent kinase inhibitor that acts on the phosphorylation of several cyclins and counteracts the phosphorylation and inactivation of pRB (retinoblastoma protein). In normal somatic cells an overexpression of p16 results in cell cycle arrest and chromatin condensation (Drayton *et al.*, 2004).

Because of the high efficiency of p16 as a biomarker, several studies have associated its expression with other proteins related to cervical cancer (Gustinucci *et al.*, 2016; Ngugi *et al.*, 2015). Only two studies have been found that observe p16 and TG2 expression in LSIL and HSIL samples (Del Nonno *et al.*, 2011; Gupta *et al.*, 2010). TG2 has been identified as an early biomarker of cervix tumor progression. This highlights the important of evaluating the use of markers in order to determine the clinical relevance of changes of expression associated with the premalignant lesions of uterine epithelium.

Recent studies indicate that TG2 possesses multiple catalytic functions and is related to different types of cancer. TG2 is able to catalyze a crosslinking reaction, a deamidation reaction and also shows GTP-binding/hydrolyzing and isopeptidase activities. Thus, TG2 can act on numerous substrates, from proteins to peptides, to small

reactive molecules such as mono- and polyamines, to nucleotides(Agnihotri *et al.,* 2013; Brown, 2013; Eckert *et al.,* 2015; Facchiano *et al.,* 2006).

Few studies elucidate the mechanism of TG2 association to cervix cancerous processes. Interestingly, TG2 could both mediate and inhibit tumor progression. TG2 is frequently down-regulated in primary tumors and during tumor progression and it is up-regulated in secondary and chemo-resistant cancers (Huang *et al.,* 2015). The results in this study demonstrate that patients with HSIL are associated with no TG2 expression ($p<0.05$), corroborating previously published data.

Remarkably, TG2 either stimulates or suppresses apoptosis in tumors in a cell type and localization context-dependent manner. The nuclear TG2 modifies core histones (Ballestar *et al.,* 1996), and polyaminates pRB protein. Moreover, his protein is protected by TG2 from caspase-mediated degradation, thus justifying its up-regulation in secondary and chemo-resistant cancers (Boehm *et al.,* 2002).

TG2 activity is mainly modulated by Ca2+ (Antonyak *et al.,* 2004; Lin *et al.,* 2011). Furthermore, TG2 interaction with fibronectin and collagen can mediate cell-extracellular matrix adhesion via integrin. This process is important in maintaining cell station and regulating the cell. Without Ca2+, however, TG2 can function as GTPase and bind GTP/GDP, modulating numerous cellular activities. At the intracellular level, due to the relatively high GTP/GDP level and low Ca2+ level, TG2 mostly exhibits a weak pro-crosslink activity (Basso *et al.,* 2012; Xu *et al.,* 2006). Functions exerted by TG2 in tumors are mainly dependent on its pro-crosslinking and signaling transduction mediation propensities (Facchiano *et al.,* 2006; Fesus & Piacentini, 2002).

Specifically concerning cervical cancer associated with HPV infection, studies indicate that TG2-catalyzed polyamination can antagonize the oncogenic potential of HPV E7 protein by preventing its interaction with cellular proteins. It is possible, thus, that the high E7 levels in epithelium infected by high-risk HPV may overcome the TG2-mediated modification, which promotes cancer progression. It is important noting that TG2 can function as a host-cell defense system against infection of viruses other than HPV (Jeon *et al.,* 2003). Therefore, the fact that TG2 is increased in cases with LSIL and decreased in HSIL cases is justified, as evidenced in our study, wherein a statistically significant association ($p<0.05$) was observed between pathological examination biopsies and TG2 expression.

TG2 expression present in normal tissues is certainly due to metabolic changes caused by HPV infection, since most of the control samples were positive for HPV-DNA (76.3%). This did not occur in relation to p16, which did not mark the control samples. This corroborates previously published data (Redman *et al.,* 2008; Zhang *et al.,* 2015).

The presence of HPV in normal samples is also shown in other studies (Ayres & Silva, 2010; de Oliveira *et al.,* 2013; Wagner *et al.,* 2015), in a research performed in the south of Brazil, the prevalence of HPV was demonstrated in 11.6% of women free from low or high-grade lesions (Becker *et al.,* 2001),. This has also been shown in this study, in which HPV infection was present in control samples (76.3%). Primary health care units in the region referred patients who presented abnormalities in their usual cytopathology test (Pap smear), thus justifying the high prevalence of HPV in samples. Only two studies investigated TG2 levels in these samples, both of which found negative expression in healthy tissues. This indicated that an increased TG2 expression

is more related to HPV infection or LSIL (Del Nonno *et al.*, 2011; Gupta *et al.*, 2010). This paper consisted of patients with squamous metaplasia, in which is squamous cells replace endocervical glandular epithelial lesions. This is a common, very frequent response to irritating agents and it is not considered a pre-malignant condition (Kurita, 2011).

In a study performed in a culture of adenocarcinoma cells, wherein the HPV was transfected into the culture through the use of plasmids, clear induced squamous metaplasia was observed. One of the HPV transfected cell clones with E2 expression and E6-E7 fusion gene mRNA showed squamous metaplasia, quite clearly, and apoptosis (Kinjo *et al.*, 2003). However, the presence of squamous metaplasia cannot be associated with HPV due to the lack of enough evidence. Clearly, this phenomenon can be caused by several factors.

The presence of TG2 in patients with unaltered biopsies of free from HPV infection was not investigated owing the fact that participants were sent by primary health care units in the region because of some change in the usual cytopathology (Pap smear).

TG2 and p16 inverse correlation in cervical lesions

The expressional behavior of p16 and TG2 in regards to the injury degrees (Figure 1G), demonstrates an inverse proportional correlation (Pearson value = -0.269). The same result was observed by Del Nonno et al. (2011), wherein an inverse correlation between the two markers (Pearson value = -0.930) was observed, indicating our data is in accordance with previously published studies (Del Nonno *et al.*, 2011). However, the same correlation between p16 and TG2 was not evidenced by Gupta et. al.

(2010), although a decreased TG2 expression with increased levels of injury was also shown. In the present study TG2 is considered an additional biomarker for all grades of cervical dysplasia, but especially for low-grade dysplasia.

Even though patients who present atypical squamous cells of undetermined significance (ASC-US) and LSIL on Papanicolaou testing are treated conventionally, just 5.2% to 18.8% of them development to HSIL (Lee & Lee, 2016). The treatment of patients identified to have HSIL (Prendiville, 2003) may be excisional or destructive, the excisional treatment has been preferred as it is as effective as the other methods, (Ghaem-Maghami *et al.*, 2007; Oliveira *et al.*, 2012). Additionally, recent data showed that removal of multiple pieces led to the removal of unnecessary tissues. Moreover, the volume/length excised, which varies substantially, correlates with the pregnancy duration (Kyrgiou *et al.*, 2015) and may result in a preterm delivery.

The need for new therapeutic management strategies especially in relation to LSIL is thus demonstrated. Risk factors associated to excisional treatments justify the incessant search for new tools to assist in determining the most appropriate management for these patients (Zsemlye, 2013). In this study, both TG2 as p16 showed different expressions according to the degree of cervical lesion, indicating that both markers can assist in the lesion progress or resolution.

TG2, p16 and HPV viral types

Although p16 was not related to specific HPV types, studies have shown that this marker can be used in the confirming lesions even in cases In which HPV was not identified (Kalof & Cooper, 2006; Tabrizi *et al.*, 2015). This study evidenced 7 patients with negative PCR for HPV, LSIL or HSIL. Of these, 5 patients showed p16

expression. Is known that HPV does not remain definitively in the female genital tract. This virus presents a variable persistency depending on the viral type, which can be of high or low risk, so much so that it is possible to observe patients without HPV infection but who present HPV-associated lesions. Thus, even uninfected patients may present remaining changes caused by p16 in the epithelium.

A study that compared p16 expression to HPV detection by PCR, in the prediction of HSIL, shown that p16 has better diagnostic values than HPV PCR and may be incorporated in the triage of ASC-US and LSIL to replace HPV PCR (Indarti & Fernando, 2013).

The analysis of the relationship between TG2 and the types of HPV, evidenced a significant statistical association to HPV 11 ($p=0.031$) and borderline values for HPV 54 and HPV 6 ($p=0.087$ and $p=0.084$). Albeit these viral types are classified as low risk, the data suggest that TG2 can act as a biomarker related to the development of cervical cancer particularly associated with HPV11. This is a novel finding as it has not been show in the literature until now.

HPV types 6 and 11 are currently responsible for 90% of all genital warts, which are included in the quadrivalent HPV vaccine. The low-grade types generate productive lesions, where certain viral gene products stimulate cell proliferation, resulting in benign lesions such as warts, these HPV types do not integrate their genome into cellular DNA (Minaguchi & Yoshikawa, 2010).

CONCLUSION

Our findings suggest that p16 and TG2 show variable expression levels according to the cervical injury degree. Also, both TG2 and p16 have an inverse

correlation: p16 expression increases alongside the lesion degree, and TG2 expression decreases as the lesion evolves. Finally, both markers can be used in the monitoring and control of LSIL and HSIL.

Conflict of Interest

The authors declare no conflicts of interest.

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REFERENCES

Agnihotri N, Kumar S, Mehta K (2013) Tissue transglutaminase as a central mediator in inflammation-induced progression of breast cancer. *Breast Cancer Res* **15**(1)

Amendola A, Rodolfo C, Di Caro A, Ciccosanti F, Falasca L, Piacentini M (2001) "Tissue" transglutaminase expression in HIV-infected cells: an enzyme with an antiviral effect? *Ann N Y Acad Sci* **946:** 108-20

Antonyak MA, Miller AM, Jansen JM, Boehm JE, Balkman CE, Wakshlag JJ, Page RL, Cerione RA (2004) Augmentation of tissue transglutaminase expression and activation

by epidermal growth factor inhibit doxorubicin-induced apoptosis in human breast cancer cells. *J Biol Chem* **279**(40)**:** 41461-7

Ayres ARG, Silva GA (2010) Cervical HPV infection in Brazil: systematic review. *Revista de Saúde Pública* **44**(5)**:** 963-974

Ballestar E, Abad C, Franco L (1996) Core histones are glutaminyl substrates for tissue transglutaminase. *J Biol Chem* **271**(31)**:** 18817-24

Basso M, Berlin J, Xia L, Sleiman SF, Ko B, Haskew-Layton R, Kim E, Antonyak MA, Cerione RA, Iismaa SE, Willis D, Cho S, Ratan RR (2012) Transglutaminase inhibition protects against oxidative stress-induced neuronal death downstream of pathological ERK activation. *J Neurosci* **32**(19)**:** 6561-9

Becker E, Edelweiss MI, Nonnenmacher B, Bozzetti MC (2001) Prevalence and epidemiologic correlates of atypical squamous cells of undetermined significance in women at low risk for cervical cancer. *Diagnostic cytopathology* **24**(4)**:** 276-282

Bergeron C, Ronco G, Reuschenbach M, Wentzensen N, Arbyn M, Stoler M, von Knebel Doeberitz M (2015) The clinical impact of using p16(INK4a) immunochemistry in cervical histopathology and cytology: an update of recent developments. *Int J Cancer* **136**(12)**:** 2741-51

Boehm JE, Singh U, Combs C, Antonyak MA, Cerione RA (2002) Tissue transglutaminase protects against apoptosis by modifying the tumor suppressor protein p110 Rb. *J Biol Chem* **277**(23)**:** 20127-30

Brown KD (2013) Transglutaminase 2 and NF-kappaB: an odd couple that shapes breast cancer phenotype. *Breast Cancer Res Treat* **137**(2)**:** 329-36

Cox JT (2002) Management of women with cervical cancer precursor lesions. *Obstet Gynecol Clin North Am* **29**(4)**:** 787-816

Crosbie EJ, Einstein MH, Franceschi S, Kitchener HC (2013) Human papillomavirus and cervical cancer. *Lancet* **382**(9895)**:** 889-99

de Oliveira CM, Fregnani JHTG, Carvalho JP, Longatto-Filho A, Levi JE (2013) Human papillomavirus genotypes distribution in 175 invasive cervical cancer cases from Brazil. *BMC cancer* **13**(1)**:** 357

Del Nonno F, Pisani G, Visca P, Signore F, Grillo LR, Baiocchini A, Garbuglia AR, Sepe S, Piacentini M, Falasca L (2011) Role and predictive strength of transglutaminase type 2 expression in premalignant lesions of the cervix. *Mod Pathol* **24**(6)**:** 855-65

Drayton S, Brookes S, Rowe J, Peters G (2004) The significance of $p16^{INK4a}$ in cell defenses against transformation. *Cell Cycle* **3**(5)**:** 611-5

Eckert RL, Fisher ML, Grun D, Adhikary G, Xu W, Kerr C (2015) Transglutaminase is a tumor cell and cancer stem cell survival factor. *Mol Carcinog* **54**(10)**:** 947-58

Facchiano F, Facchiano A, Facchiano AM (2006) The role of transglutaminase-2 and its substrates in human diseases. *Front Biosci* **11:** 1758-73

Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F (2015) Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *International Journal of Cancer* **136**(5)**:** E359-E386

Fesus L, Piacentini M (2002) Transglutaminase 2: an enigmatic enzyme with diverse functions. *Trends Biochem Sci* **27**(10)**:** 534-9

Gajanin R, Gajanin Z, Vujkovic Z, Gajanin V, Gojkovic Z, Ljubojevic V (2015) Immunohistochemical expression of $p16^{INK4a}$ in inflammatory, preneoplastic and neoplastic cervical lesions. *Med Pregl* **68**(3-4)**:** 85-92

Ghaem-Maghami S, Sagi S, Majeed G, Soutter WP (2007) Incomplete excision of cervical intraepithelial neoplasia and risk of treatment failure: a meta-analysis. *Lancet Oncol* **8**(11)**:** 985-93

Gravitt P, Peyton C, Alessi T, Wheeler C, Coutlee F, Hildesheim A, Schiffman M, Scott D, Apple R (2000) Improved amplification of genital human papillomaviruses. *Journal of clinical microbiology* **38**(1)**:** 357-361

Gupta R, Srinivasan R, Nijhawan R, Suri V (2010) Tissue transglutaminase 2 as a biomarker of cervical intraepithelial neoplasia (CIN) and its relationship to $p16^{INK4a}$ and nuclear factor kappaB expression. *Virchows Arch* **456**(1)**:** 45-51

Gustinucci D, Giorgi Rossi P, Cesarini E, Broccolini M, Bulletti S, Carlani A, D'Angelo V, D'Amico M R, Di Dato E, Galeazzi P, Malaspina M, Martinelli N, Spita N, Tintori B, Giaimo MD, Passamonti B (2016) Use of Cytology, E6/E7 mRNA, and $p16^{INK4a}$ -Ki-67 to Define the Management of Human Papillomavirus (HPV)-Positive Women in Cervical Cancer Screening. *Am J Clin Pathol* **145**(1)**:** 35-45

Henry NL, Hayes DF (2012) Cancer biomarkers. *Mol Oncol* **6**(2)**:** 140-6

Huang L, Xu AM, Liu W (2015) Transglutaminase 2 in cancer. *Am J Cancer Res* **5**(9)**:** 2756-76

Indarti J, Fernando D (2013) Comparison of $p16^{INK4a}$ immunocytochemistry with the HPV polymerase chain reaction in predicting high grade cervical squamous intraepithelial lesions. *Asian Pac J Cancer Prev* **14**(9)**:** 4989-92

Jeon JH, Choi KH, Cho SY, Kim CW, Shin DM, Kwon JC, Song KY, Park SC, Kim IG (2003) Transglutaminase 2 inhibits Rb binding of human papillomavirus E7 by incorporating polyamine. *Embo J* **22**(19)**:** 5273-82

Jeon JH, Kim IG (2006) Role of protein modifications mediated by transglutaminase 2 in human viral diseases. *Front Biosci* **11:** 221-31

Kalof AN, Cooper K (2006) $p16^{INK4a}$ immunoexpression: surrogate marker of high-risk HPV and high-grade cervical intraepithelial neoplasia. *Adv Anat Pathol* **13**(4)**:** 190-4

Kinjo T, Kamiyama K, Chinen K, Iwamasa T, Kurihara K, Hamada T (2003) Squamous metaplasia induced by transfection of human papillomavirus DNA into cultured adenocarcinoma cells. *Mol Pathol* **56**(2)**:** 97-108

Krishnappa P, Mohamad IB, Lin YJ, Barua A (2014) Expression of P16 in high-risk human papillomavirus related lesions of the uterine cervix in a government hospital., Malaysia. *Diagn Pathol* **9**(202)**:** 014-0202

Kurita T (2011) Normal and abnormal epithelial differentiation in the female reproductive tract. *Differentiation* **82**(3)**:** 117-26

Kyrgiou M, Valasoulis G, Stasinou SM, Founta C, Athanasiou A, Bennett P, Paraskevadis E (2015) Proportion of cervical excision for cervical intraepithelial neoplasia as a predictor of pregnancy outcomes. *Int J Gynaecol Obstet* **128**(2)**:** 141-7

Lee H, Lee EJ (2016) HPV infection and p16 promoter methylation as predictors of ASC-US/LSIL progression. *Cancer Cytopathol* **124**(1)**:** 58-65

Lin CY, Tsai PH, Kandaswami CC, Chang GD, Cheng CH, Huang CJ, Lee PP, Hwang JJ, Lee MT (2011) Role of tissue transglutaminase 2 in the acquisition of a mesenchymal-like phenotype in highly invasive A431 tumor cells. *Mol Cancer* **10**(87)**:** 1476-4598

McLaughlin-Drubin ME, Crum CP, Munger K (2011) Human papillomavirus E7 oncoprotein induces KDM6A and KDM6B histone demethylase expression and causes epigenetic reprogramming. *Proc Natl Acad Sci U S A* **108**(5)**:** 2130-5

Milakovic T, Tucholski J, McCoy E, Johnson GV (2004) Intracellular localization and activity state of tissue transglutaminase differentially impacts cell death. *J Biol Chem* **279**(10)**:** 8715-22

Minaguchi T, Yoshikawa H (2010) [Molecular mechanism of cervical carcinogenesis]. *Gan To Kagaku Ryoho* **37**(1)**:** 18-22

Ngugi CW, Schmidt D, Wanyoro K, Boga H, Wanzala P, Muigai A, Mbithi J, von Knebel Doeberitz M, Reuschenbach M (2015) p16(INK4a)/Ki-67 dual stain cytology for cervical cancer screening in Thika district, Kenya. *Infect Agent Cancer* **10**(25)**:** 015-0020

Oliveira CA, Russomano FB, Gomes Junior SC, Correa Fde M (2012) Risk of persistent high-grade squamous intraepithelial lesion after electrosurgical excisional treatment with positive margins: a meta-analysis. *Sao Paulo Med J* **130**(2)**:** 119-25

Prendiville W (2003) LLETZ: theoretical rationale, practical aspects, clinical experience, optimizing the technique. *Colposcopy: Management Options***:** 75-89

Redman R, Rufforny I, Liu C, Wilkinson EJ, Massoll NA (2008) The utility of p16(INK4a) in discriminating between cervical intraepithelial neoplasia 1 and nonneoplastic equivocal lesions of the cervix. *Arch Pathol Lab Med* **132**(5)**:** 795-9

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**(4839)**:** 487-491

Solomon D, Nayar R (2004) *The Bethesda System for reporting cervical cytology: definitions, criteria, and explanatory notes*: Springer Science & Business Media

Souho T, Benlemlih M, Bennani B (2015) Human papillomavirus infection and fertility alteration: a systematic review. *PLoS One* **10**(5)

Stoler MH (1996) A brief synopsis of the role of human papillomaviruses in cervical carcinogenesis. *Am J Obstet Gynecol* **175**(4 Pt 2)**:** 1091-8

Tabrizi SN, Tan SE, von Knebel Doeberitz C, Tan J, Quinn MA, Garland SM (2015) Evaluation of $p16^{INK4a}$ immunostaining for the detection of high-grade changes in cervical cytology. *Pathology* **47**(4)**:** 314-9

Tranbaloc P (2008) [Natural history of precursor lesions of cervical cancer]. *Gynecol Obstet Fertil* **36**(6)**:** 650-5

Wagner M, Bennetts L, Patel H, Welner S, de Sanjose S, Weiss TW (2015) Global availability of data on HPV genotype-distribution in cervical., vulvar and vaginal disease and genotype-specific prevalence and incidence of HPV infection in females. *Infect Agent Cancer* **10**(13)**:** 015-0008

Wang R, Li X, Qian M, Niu J, You Z (2015) [The natural history of cervical intraepithelial neoplasia I and the clinical significance of p16(INK4a) protein as a marker of progression in cervical intraepithelial neoplasia I]. *Zhonghua Fu Chan Ke Za Zhi* **50**(3)**:** 210-5

Xu L, Begum S, Hearn JD, Hynes RO (2006) GPR56, an atypical G protein-coupled receptor, binds tissue transglutaminase, TG2, and inhibits melanoma tumor growth and metastasis. *Proc Natl Acad Sci U S A* **103**(24)**:** 9023-8

Zhang G, Yang B, Abdul-Karim FW (2015) p16 Immunohistochemistry is useful in confirming high-grade squamous intraepithelial lesions (HSIL) in women with negative HPV testing. *Int J Gynecol Pathol* **34**(2)**:** 180-6

Zsemlye M (2013) LSIL: definition and management. *Obstet Gynecol Clin North Am* **40**(2)**:** 283-9

Table 1. Description of sample groups and the relation to the TG2 and p16 expression.

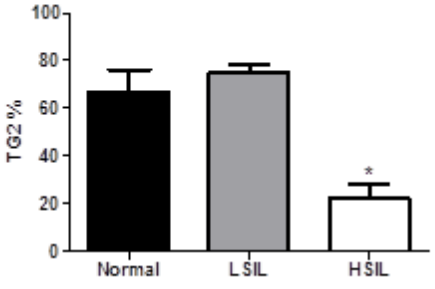
Histopathological analysis	Patients		HPV Typing: Negative		Low Risk		High Risk		Not Defined		TG2	p16
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**		
Control	38	26.0	9	23.7	1	2.6	16	42.1	12	31.6	74%* (SD* 17.13)	0%* (SD0)
LSIL	44	30.1	4	9.1	3	6.8	24	54.5	13	29.5	74,7%* (SD 17.55)	24.4%* (SD3.90)
HSIL	32	21.9	3	9.4	3	9.4	16	50	9	28.1	22,3%* (SD 24.12)	75.3% *(SD25.70)

*p<0.05, *Standard Deviation.

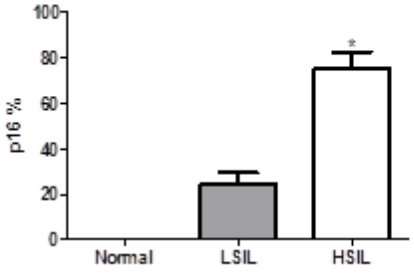
Table 2. Description of HPV viral types and the relation to the TG2 and p16 expression.

HPV typing	Patients		TG2		p16	
	n	**%**	**%**	**SD***	**n**	**SD***
Low-Risk HPV	15	10.3	72.5	17.08	43.33%	45.02
High-Risk HPV	66	45.2	59	31.27	37.29%	37.71
Not Defined HPV	43	29.5	60,7	29.85	30.31%	35.33

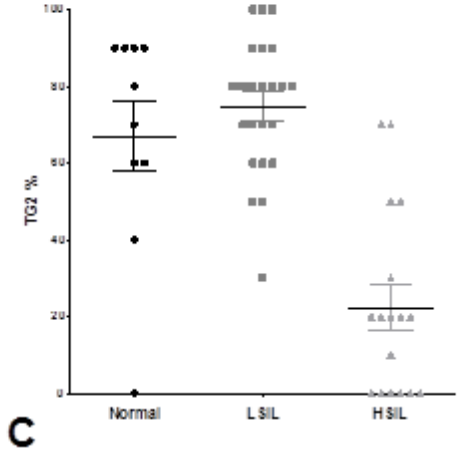
*Standard Deviation.


TG2 %
100
80
60
40
20
0
*
Normal
LSIL
HSIL

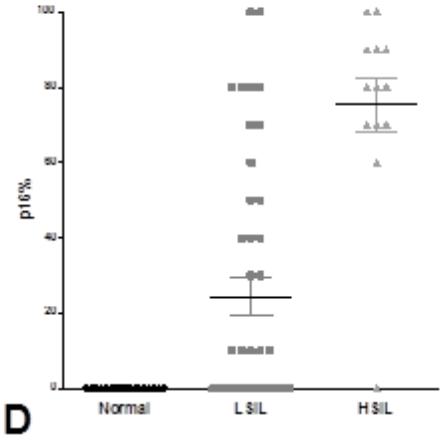
A


p16 %
100
80
60
40
20
0
*
Normal
LSIL
HSIL

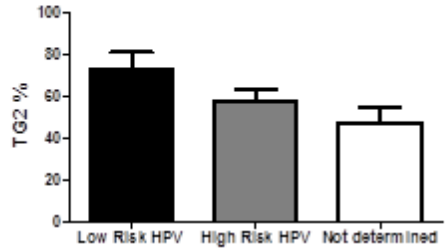
B


TG2 %
Normal
LSIL
HSIL

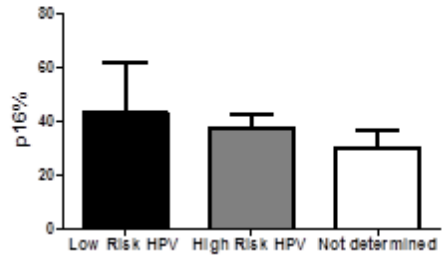
C


p16%
Normal
LSIL
HSIL

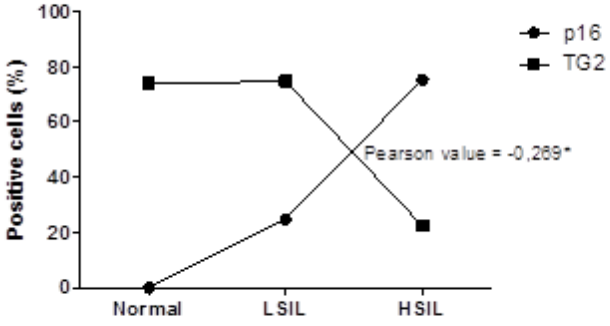
D


TG2 %
100
80
60
40
20
0
Low Risk HPV
High Risk HPV
Not determined

E


p16%
80
60
40
20
0
Low Risk HPV
High Risk HPV
Not determined

F


Positive cells (%)
100
80
60
40
20
0
Normal
LSIL
HSIL
p16
TG2
Pearson value = -0,269*

G

Figure 1. Transglutaminase type 2 (TG2) (A) and p16INK4 (p16) (B) expression related to histopathological analysis represented in column and dispersion graph (C and D). Association between TG2 (E), p16 (F) and HPV viral type classification. The number of p16-positive cells rises with an increasing lesion grade, whereas TG2-stained cells decrease with an advanced lesion grade (G) (Pearson value= -0.269). * $p<0.05$

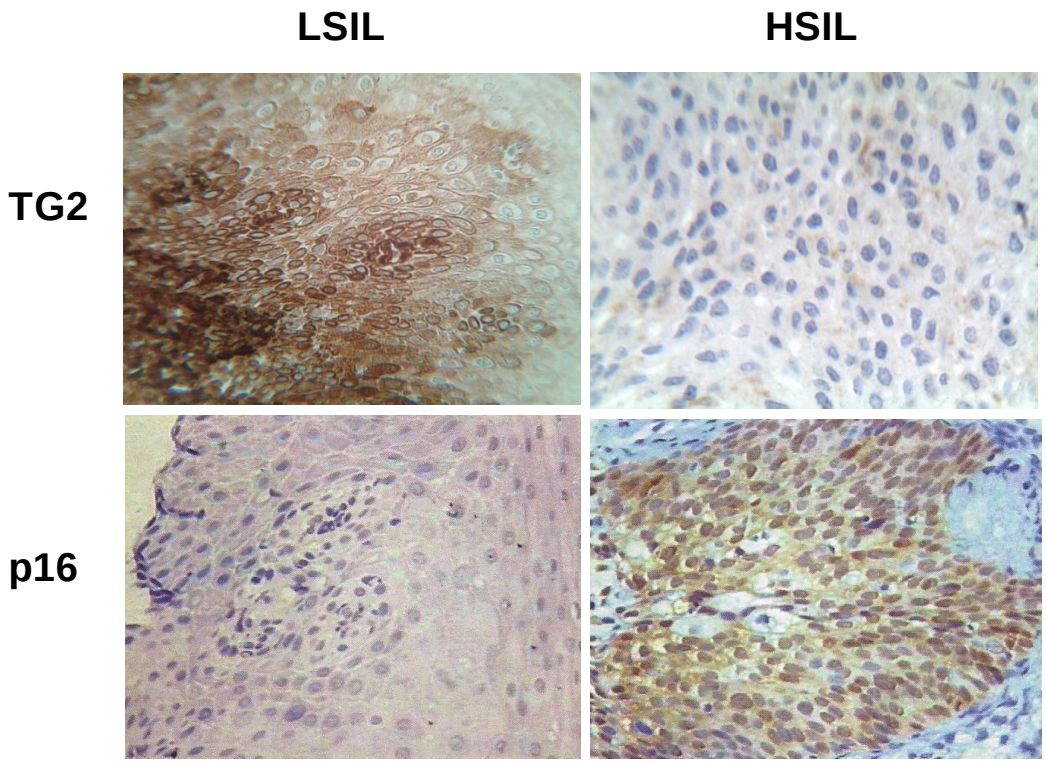


Figure 2. Transglutaminase type 2 (TG2) (A) and $p16^{INK4a}$ (p16) immunostaining in high and low grade cervical.

4. CONCLUSÕES

Baseado nos dados do presente estudo, nota-se a importância da pesquisa de proteínas biomarcadoras do processo canceroso cervical, este tipo de pesquisa auxilia na compreensão das alterações provocadas pelo HPV no epitélio do colo uterino e aponta possíveis biomarcadores que possam auxiliar no diagnóstico e tratamento de lesões intraepiteliais escamosas cervicais.

A quantificação de $p16^{INK4a}$ empregando o método computacional Image J em lesões intraepiteliais do colo uterino demonstrou ser uma ferramenta útil para quantificar sua expressão diminuindo variações intra e interpessoais.

Adicionalmente, a expressão de p53, ki-67 e bcl-2 contribuiu para a diferenciação dos graus de lesão, de maneira isolada ou em combinação como nos seguintes padrões: p53(-), ki-67(+), bcl-2(-) e p53(-), Ki-67(-), bcl-2(-).

A TG2 demonstrou sua expressão alterada conforme graus de lesão: amostras histológicas de pacientes normais e amostras histológicas com LEIBG apresentaram níveis de expressão de TG2 superiores a 50%, já em pacientes com LEIAG evidenciou-se uma expressão menor que 50% da proteína.

A expressão das proteínas TG2 e $p16^{INK4a}$ demonstraram uma correlação negativa (ou inversa), sendo que a expressão de $p16^{INK4a}$ aumentou proporcionalmente ao grau da lesão avaliada e a expressão de TG2 diminuiu proporcionalmente em relação aos graus de lesão.

Baseado nos dados evidenciados conclui-se que a expressão de TG2, $p16^{INK4a}$, p53, bcl-2 e ki-67 variou conforme o grau de lesão escamosa intraepitelial do carcinoma escamoso do colo uterino, sendo esta expressão associada de maneira significante para

diferentes tipos do HPV no caso das proteínas TG2 e $p16^{INK4a}$. Sendo assim, o presente estudo evidencia possíveis biomarcadores proteicos associados à LEIBG e LEIAG.

5. BIBLIOGRAFIA COMPLEMENTAR

Abràmoff, M. D., P. J. Magalhães, et al., (2004). "Image processing with ImageJ." **Biophotonics international** 11(7): 36-42.

Beishline, K. and J. Azizkhan-Clifford (2015). "Sp1 and the 'hallmarks of cancer'." **Febs J** 282(2): 224-258.

Boehm, J. E., U. Singh, et al., (2002). "Tissue transglutaminase protects against apoptosis by modifying the tumor suppressor protein p110 Rb." **Journal of Biological Chemistry** 277(23): 20127-20130.

Brychtova, S., T. Brychta, et al., (2000). "Expression of Bcl-2 in dysplastic and neoplastic cervical lesions in relation to cell proliferation and HPV infection." **Neoplasma** 47(3): 143-147.

Carrilho, C., P. Gouveia, et al., (2003). "Characterization of human papillomavirus infection, P53 and Ki-67 expression in cervix cancer of Mozambican women." **Pathol Res Pract** 199(5): 303-311.

Cheikh, A., El Majjaoui, S., et al., (2016). “Evaluation of the cost of cervical cancer at the National Institute of Oncology, Rabat.” **Pan Afr Med J** 20;23:209. doi: 10.11604/pamj.2016.23.209.7750.

Conway, M. and C. Meyers (2009). "Replication and assembly of human papillomaviruses." **Journal of dental research** 88(4): 307-317.

Dartell, M., V. Rasch, et al., (2012). "Human papillomavirus prevalence and type distribution in 3603 HIV-positive and HIV-negative women in the general population of Tanzania: The PROTECT study." **Sexually transmitted diseases** 39(3): 201.

De Flora, S., La Maestra, S. (2015) ” Epidemiology of cancers of infectious origin and prevention strategies.” **J Prev Med Hyg** 10;56(1):E15-20.

de Sanjose, S., Diaz, M., et al., (2007) “Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis” **Lancet Infect Dis** 7(7):453-9.

Del Nonno, F., G. Pisani, et al., (2011). "Role and predictive strength of transglutaminase type 2 expression in premalignant lesions of the cervix." **Modern Pathology** 24(6): 855-865.

Eluf Neto, J., M. Bibbo, et al., (1998). "Epidemiologia das lesões relacionadas ao HPV." **Bibbo M, Moraes Filho A, organizadores. Lesões relacionadas à infecção por HPV no trato anogenital. Rio de Janeiro: Revinter**: 9-27.

Entiauspe, L. G., L. O. Teixeira, et al., (2010). "Papilomavírus humano: prevalência e genótipos encontrados em mulheres HIV positivas e negativas, em um centro de referência no extremo Sul do Brasil." **Rev Soc Bras Med Trop** 43(3): 260-263.

Facchiano, F., A. Facchiano, et al., (2006). "The role of transglutaminase-2 and its substrates in human diseases." **Front Biosci** 11(1758): 73.

Falasca, L., M. G. Farrace, et al., (2008). "Transglutaminase type II is involved in the pathogenesis of endotoxic shock." **The Journal of Immunology** 180(4): 2616-2624.

Faridi, R., A. Zahra, et al., (2011). "Oncogenic potential of Human Papillomavirus (HPV) and its relation with cervical cancer." **Virol J** 8: 269.

Garland, S., M. Hernandex-Avila, et al., (2007). "Cervical Cancer and HPV Vaccination." **N Eng J Med** 356: 1915-1927.

George, E., N. L. Polissar, et al., (2010). "Immunohistochemical evaluation of $p16^{INK4a}$, E-cadherin, and cyclin D1 expression in melanoma and Spitz tumors." **American journal of clinical pathology** 133(3): 370-379.

Godoy, A. E., J. Mandelli, et al., (2008). "p16INK4 expression in precursor lesions of squamous cell cervical cancer related to the presence of HPV-DNA." **Braz J Med Biol Res** 41(7): 583-588.

Grabiel, M., T. J. Reutzel, et al., (2013). "HPV and HPV Vaccines: The Knowledge Levels, Opinions, and Behavior of Parents." **Journal of Community Health**: 1-7.

Grace, V. M., J. V. Shalini, et al., (2003). "Co-overexpression of p53 and bcl-2 proteins in HPV-induced squamous cell carcinoma of the uterine cervix." **Gynecol Oncol** 91(1): 51-58.

Han, C. P., L. F. Kok, et al., (2009). "Scoring of $p16^{INK4a}$ immunohistochemistry based on independent nuclear staining alone can sufficiently distinguish between endocervical and endometrial adenocarcinomas in a tissue microarray study." **Modern pathology** 22(6): 797-806.

Han, C. P., M. Y. Lee, et al., (2008). "Nuclear Receptor Interaction Protein (NRIP) expression assay using human tissue microarray and immunohistochemistry technology confirming nuclear localization." **Journal of Experimental & Clinical Cancer Research** 27(1): 1-7.

Hang, S., Tiwari, A.F., Ngan, H.Y., et al., (2016) “Extremely stringent activation of p16INK4a prevents immortalization of uterine cervical epithelial cells without human papillomavirus oncogene expression.” **Oncotarget** doi: 10.18632/oncotarget.10120.

Huang, L., Xu, A.M., Liu, W. (2015) “Transglutaminase 2 in cancer.” **Am J Cancer Res** 15;5(9):2756-76.

Huibregtse, J. M., M. Scheffner, et al., (1991). "A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18." **Embo J** 10(13): 4129-4135.

Jensen, J. R. (1996). Introductory digital image processing: a remote sensing perspective, **Prentice-Hall Inc.**

Jeon, J. H. and I. G. Kim (2006). "Role of protein modifications mediated by transglutaminase 2 in human viral diseases." **Front Biosci** 11: 221-231.

Katiyar, S., S. Hedau, et al., (2005). "p53 gene mutation and human papillomavirus (HPV) infection in esophageal carcinoma from three different endemic geographic regions of India." **Cancer Lett** 218(1): 69-79.

Kilkenny, M. and R. Marks (2007). "The descriptive epidemiology of warts in the community." **Australasian journal of dermatology** 37(2): 80-86.

Kim, M. S., Y. Bak, et al., (2013). "Wogonin induces apoptosis by suppressing E6 and E7 expressions and activating intrinsic signaling pathways in HPV-16 cervical cancer cells." **Cell Biol Toxicol** 29(4): 259-272.

Kisser, A. and I. Zechmeister-Koss (2015). "A systematic review of p16/Ki-67 immuno-testing for triage of low grade cervical cytology." **Bjog** 122(1): 64-70.

Koeneman, M.M., Kruitwagen, R.F., et al., (2015). “Natural history of high-grade cervical intraepithelial neoplasia: a review of prognostic biomarkers.” **Expert Rev Mol Diagn** 15(4):527-46. doi: 10.1586/14737159.2015.1012068.

Kok, L. F., M. Y. Lee, et al., (2010). "Comparing the scoring mechanisms of p16 INK4a immunohistochemistry based on independent nucleic stains and independent cytoplasmic stains in distinguishing between endocervical and endometrial adenocarcinomas in a tissue microarray study." **Arch Gynecol Obstet** 281(2): 293-300.

Korzeniewski, N., N. Spardy, et al., (2011). "Genomic instability and cancer: lessons learned from human papillomaviruses." **Cancer letters** 305(2): 113-122.

Laniosz, V., K. A. Holthusen, et al., (2008). "Bovine papillomavirus type 1: from clathrin to caveolin." **Journal of virology** 82(13): 6288-6298.

Laurinavicius, A., Plancoulaine, B., et al., (2016) "Comprehensive Immunohistochemistry: Digital, Analytical and Integrated." **Pathobiology** 83(2-3):156-63. doi: 10.1159/000442389.

Lopukhov, P.D., Briko, N.I., et al., (2016) "Papillomavirus infection: principle characteristics, clinical manifestations, vaccine prophylaxis." **Zh Mikrobiol Epidemiol Immunobiol** (1):71-8.

Lu, W., A. Strohecker, et al., (2001). "Post-translational modification of the hepatitis C virus core protein by tissue transglutaminase." **Journal of Biological Chemistry** 276(51): 47993-47999.

Malik, S. M., D. T. Nevin, et al., (2011). "Assessment of immunohistochemistry for p16INK4 and high-risk HPV DNA by in situ hybridization in esophageal squamous cell carcinoma." **Int J Surg Pathol** 19(1): 31-34.

Martinez-Zapien, D., F. X. Ruiz, et al., (2016). "Structure of the E6/E6AP/p53 complex required for HPV-mediated degradation of p53." **Nature** 529(7587): 541-545.

Martins, L. F. L., Thuler, L. C. S. et al., (2005). "Cobertura do exame de Papanicolaou no Brasil e seus fatores determinantes: uma revisão sistemática da literatura." **Rev Bras Ginecol Obstet** 27(8): 485-492.

Matos, L. L., E. Stabenow, et al., (2006). "Immunohistochemistry quantification by a digital computer-assisted method compared to semiquantitative analysis." **Clinics** 61(5): 417-424.

Maul, G. G. (1998). "Nuclear domain 10, the site of DNA virus transcription and replication." **Bioessays** 20(8): 660-667.

Milakovic, T., J. Tucholski, et al., (2004). "Intracellular localization and activity state of tissue transglutaminase differentially impacts cell death." **Journal of Biological Chemistry** 279(10): 8715-8722.

Min, K.W., Lee, S.H., et al., (2016) "Moonlighting proteins in cancer." **Cancer Lett** 1;370(1):108-16. doi: 10.1016/j.canlet.2015.09.022.

Moody, C. A., L. A. Laimins. (2010) "Human papillomavirus oncoproteins: pathways to transformation." **Nature Reviews Cancer** 10(8): 550-560.

Murphy, N., Ring, M., et al., (2003) "p16INK4 as a Marker for Cervical Dyscaryosis: CIN and cGIN in cervical biopsies and ThinPrep™ Smers." **Journal of Clinical Pathology**, 56: 56 – 63.

Namazie, A., S. Alavi, et al., (2002). "Cyclin D1 amplification and p16(MTS1/CDK4I) deletion correlate with poor prognosis in head and neck tumors." **Laryngoscope** 112(3): 472-481.

Nieh, S., S.-F. Chen, et al., (2005). "Is $p16^{INK4a}$ expression more useful than human papillomavirus test to determine the outcome of atypical squamous cells of undetermined significance-categorized Pap smear? A comparative analysis using abnormal cervical smears with follow-up biopsies." **Gynecol Oncol** 97(1): 35-40.

Oliveira, Gisele Rodrigues de, et al. (2013) "Fatores de risco e prevalência da infecção pelo HPV em pacientes de Unidades Básicas de Saúde e de um Hospital Universitário do Sul do Brasil." **Rev bras ginecol obstet** 35.5: 226-232.

Olson, B., Gribble, B., et al., (2016). "Cervical cancer screening programs and guidelines in low- and middle-income countries." **Int J Gynaecol Obstet**. pii: S0020-7292(16)30155-2. doi: 10.1016/j.ijgo.03.011.

Parrales, A., Iwakuma, T. (2015). "Targeting Oncogenic Mutant p53 for Cancer Therapy." **Front Oncol** 5(288).

Patel, D. A., L. S. Rozek, et al., (2012). "Patterns of cellular and HPV 16 methylation as biomarkers for cervical neoplasia." **Journal of virological methods**.

Peres, A. L., Paz, E. S. K. M., et al., (2016). "Immunocytochemical study of TOP2A and Ki-67 in cervical smears from women under routine gynecological care." **J Biomed Sci** 23(1): 016-0258.

Phatak, V., Croft, S., et al., (2011). "Expression of transglutaminase-2 isoforms in normal human tissues and cancer cell lines: dysregulation of alternative splicing in cancer." **Amino acids**: 1-12.

Rama, C. H., Roteli-Martins, C. M. et al., (2008). "Prevalência do HPV em mulheres rastreadas para o câncer cervical." **Revista de Saúde Pública** 42(1): 123-130.

Remmele, W., Schicketanz K. H. (1993). "Immunohistochemical determination of estrogen and progesterone receptor content in human breast cancer: Computer-assisted image analysis (QIC score) vs. subjective grading (IRS)." **Pathology-Research and Practice** 189(8): 862-866.

Reyes, M. C., Cooper, K. (2014). "Cervical cancer biopsy reporting: a review." **Indian J Pathol Microbiol** 57(3): 364-368.

Roden, R., Kirnbauer, R. et al., (1994). "Interaction of papillomaviruses with the cell surface." **Journal of virology** 68(11): 7260-7266.

Rodrigues, C. S., Lacerda, M. S. et al., (2015). "Patient Generated Subjective Global Assessment as a prognosis tool in women with gynecologic cancer." **Nutrition** 31(11-12): 1372-1378.

Sano, T., Oyama T., et al., (1998). "Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions." **The American journal of pathology** 153(6): 1741.

Santos, R., Wakamatsu A., et al., (1999). "Procedimentos laboratoriais em imuno-histoquímica e hibridização" in situ." **Manual de Imuno-histoquímica. Sociedade Brasileira de Patologia**: 237-259.

Saslow, D., Solomon D., et al., (2012). "American Cancer Society, American Society for Colposcopy and Cervical Pathology, and American Society for Clinical Pathology screening guidelines for the prevention and early detection of cervical cancer." **Crit Rev Oncol Hematol** 137(4): 516-542.

Scheffner, M., Werness B. A., et al., (1990). "The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53." **Cell** 63(6): 1129-1136.

Scheffner, M., Munger K., et al., (1991). "The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines." **Proc Natl Acad Sci U S A** 88(13): 5523-5527.

Schiffman, M. H. (1995). "New epidemiology of human papillomavirus infection and cervical neoplasia." **Journal of the National Cancer Institute** 87(18): 1345-1347.

Singh, M., Mockler, D., et al., (2012). "Immunocytochemical colocalization of $p16^{INK4a}$ and Ki-67 predicts CIN2/3 and AIS/adenocarcinoma." **Cancer Cytopathology**.

Solomon, D., Davey D., et al., (2002). "The 2001 Bethesda System." **JAMA** 287(16): 2114-2119.

Sotlar, K.; Diemer, D., et al., (2004) “Detection and typing of human papillomavirus by E6 nested multiplex PCR.” **Journal of Clinical Microbiology** 42(7): 3175-3184.

Souho, T., Benlemlih, M., Bennani, B. (2015). “Human papillomavirus infection and fertility alteration: a systematic review.” **PLoS One** 18;10(5):e0126936. doi: 10.1371/journal.pone.0126936.

Tabone, T., Garland, S. M., et al., (2012). "Prevalence of human papillomavirus genotypes in women with cervical cancer in Papua New Guinea." **International Journal of Gynecology & Obstetrics**.

Thal, D. R., Horn M., et al., (1995). "Selective quantitative analysis of the intensity of immunohistochemical reactions." **Acta Histochem** 97(2): 203-211.

Tian, H., Qian G. W., et al., (2011). "A critical role of Sp1 transcription factor in regulating the human Ki-67 gene expression." **Tumour Biol** 32(2): 273-283.

Torres, L. F. B., Noronha, L. et al., (1995). "A importância da imunohistoquímica no diagnóstico anátomo-patológico em hospital geral: análise de 885 casos; Importance of immunohistochemistry in diagnostic pathology in a general hospital: analysis of 885 cases." **J bras patol** 31(2): 65-71.

Tsikouras, P., Zervoudis, S., et al., (2016). “Cervical cancer: screening, diagnosis and staging.” **J Buon** 21(2):320-5.

Utagawa, M. L., Pereira, S. M. M. et al., (2004). "Citologia de base líquida associada à captura de híbridos para DNA-HPV pode otimizar a qualidade diagnóstica do método de Papanicolaou?" **Rev. Inst. Adolfo Lutz** 63(1): 100-103.

Vasilescu, F., M. Ceausu, et al., (2009). "P53, p63 and Ki-67 assessment in HPV-induced cervical neoplasia." **Rom J Morphol Embryol** 50(3): 357-361.

Williams, A. B., B. Schumacher (2016). "p53 in the DNA-Damage-Repair Process." **Cold Spring Harb Perspect Med** 6(5).

Wu, J. Z., X. Zhang, et al., (2004). "The experiment research of edge detection in digital image [J]." **Control & Automation** 5.

Zhang, Q., Zhang Y., et al., (2012). "Reduced expression of Tissue factor pathway inhibitor-2 contributes to apoptosis and angiogenesis in cervical cancer." **Journal of Experimental & Clinical Cancer Research** 31(1): 1.

Zheng, J. H., Viacava Follis, A., et al., (2015). "Discoveries and controversies in BCL-2 protein-mediated apoptosis." **Febs J** 28(10): 13527.

Zhou, R., Wei C., et al., (2015). "The prognostic value of p53 expression for patients with cervical cancer: a meta analysis." **Eur J Obstet Gynecol Reprod Biol** 195: 210-213.

6. ANEXOS

6.1. METODOLOGIA

Foi realizada uma análise retrospectiva, na qual utilizou-se como base, um banco de dados previamente estruturado e amostras referentes a este banco. O banco de dados continha as seguintes informações: nome da paciente, idade, resultado do exame histopatológico, genotipagem para HPV e quantificação relativa de $p16^{INK4a}$ por IMQ. Deste modo, a partir destas amostras histológicas obtidas por biópsias do colo uterino ampliou-se o banco de dados realizando a análise de outros marcadores (TG2, p53, Ki-67, bcl-2), obtendo assim novos dados e correlações.

6.1.2 Caracterização da Amostra

Para o estudo foram utilizadas amostras teciduais obtidas por biópsia dirigidas do colo uterino, de pacientes atendidas no Ambulatório de Patologia do Trato Genital Inferior do Ambulatório Central da Universidade de Caxias do Sul e no Laboratório Diagnose, previamente identificadas quanto à presença do HPV-DNA por PCR com tipagem viral.

Foram coletadas amostras teciduais de 146 pacientes com suspeita de LEIBG e LEIAG, sugeridas pelo exame citopatológico e que foram submetidas a colposcopia. Estas amostras de teciduais de biópsia de colo uterino foram coletadas para a confirmação histopatológica da lesão intraepitelial, assim como, foram submetidas a metodologias moleculares para confirmação e tipagem do HPV-DNA.

Foram selecionadas algumas amostras histológicas obtidas por biópsias do colo uterino para os estudos descritos nos capítulos 2 e 3.

As pacientes incluídas no estudo foram orientadas a assinarem o Termo de Consentimento Livre e Esclarecido, e informadas das posteriores pesquisas a serem realizadas com o objetivo do conhecimento da patogênese do HPV.

De acordo com normas da CNS 196/96 as amostras estão sendo mantidas arquivadas adequadamente em local seco e resguardado, por um período de 20 anos partindo da data da coleta para armazenamento de material biológico humano com fim diagnóstico, configurando assim um Biorrepositório. O presente banco de amostras teve o período de coleta abrangendo os anos de 2003 a 2008, sendo assim em período anterior à resolução CNS Nº 441, de 12 de maio de 2011. O presente estudo foi aprovado pelo Comitê de Ética em Pesquisa da Faculdade Nossa Senhora de Fátima sob parecer número 1442.449 (Anexo 2 e 3).

O banco em questão não foi criado com fins exclusivos de pesquisa, sendo que o material biológico excedente foi utilizado para de diagnóstico assistencial. Conforme a Resolução MS 340 os dados genéticos resultantes de pesquisa associados a um indivíduo não poderão ser divulgados nem ficar acessíveis a terceiros, exceto quando for obtido consentimento do sujeito da pesquisa. Os autores do presente estudo declaram que já foi obtido o consentimento de todos os sujeitos da pesquisa, portanto o presente projeto está de acordo com tal termo de definição da Resolução MS 340.

No presente estudo o "n" amostral foi definido com base em estudos da literatura. Zhang et al. (2012) investigaram a expressão de biomarcadores em 128 de cânceres cervicais (Zhang et al., 2012), Singh et al. (2012) estudaram biomarcadores tumorais através de imunohistoquímica em 122 amostras citopatológicas (Singh et al., 2012), já Rodrigues et al. (2015) avaliaram 146 pacientes (Rodrigues et al., 2015).

Sendo assim, a escolha do “n” amostral demonstrou-se adequada e significante conforme dados previamente publicados.

6.1.3 Exame Histopatológico

As amostras histológicas foram processadas após imersão em parafina, e posteriormente submetidas a cortes histológicos com 3 micras de espessura. Após foi aplicada a técnica de coloração por Hematoxilina-eosina (HE) para posterior análise por microscopia óptica convencional. A seguir, as lâminas foram analisadas por dois médicos patologistas e classificadas em: LEIBG e LEIAG. As LEIBG englobam diversas alterações celulares chamadas de “efeito citopático viral” (coilocitose) e a displasia leve, também chamada de neoplasia intraepitelial cervical 1 (NIC 1), na nomenclatura proposta por Richart. As LEIAG englobam as displasias moderada, severa e o carcinoma “in situ”, ou NIC 2, 3 e carcinoma “in situ”. E, por fim, “HPV”, quando foram detectadas alterações celulares compatíveis com infecção pelo vírus, tais como coilocitose, disceratose, macronucleose, binucleação ou multinucleação e anfofilia. (Solomon et al., 2002).

6.1.4 Biologia Molecular

A determinação da presença do HPV-DNA e tipagem viral foram realizadas pelo método da reação em cadeia da polimerase (PCR) (Gravitt *et al.*, 2000) feitas em dois grupos distintos de amostras. Um deles constituído por amostras histológicas embebidas em parafina e, outro, por amostras citológicas de coleta de secreção cérvico-vaginal conservadas em tampão TE e congeladas em freezer, 20°C negativos.

O material proveniente de blocos de parafina foi submetido a cortes histológicos com 8µm de espessura. A retirada da parafina foi feita de duas formas: química e térmica. Na retirada da parafina pelo método químico, os cortes histológicos foram

acrescidos de 1mL de xilol e incubados a 65°C por 15 minutos. Após este tempo, o material foi centrifugado e lavado em sucessivos banhos de álcool, em concentrações decrescentes, com o objetivo de desparafinizar a amostra. Após a lavagem, o sobrenadante foi desprezado e o "*pellet*" seco em temperatura ambiente.

Na retirada da parafina através de aquecimento, cada amostra, acrescida de 200µL de tampão de digestão, foi levada ao forno de microondas por 4 ciclos de 15 segundos, agitando-se vigorosamente entre cada ciclo. As amostras foram submetidas, então, a centrifugação durante 10 minutos, ao final da qual removeu-se a parafina sobrenadante, adicionando-se 200µL de TEP (6,05g Tris-HCl, 3,72g EDTA, 50µL Triton X-100, 10mL com água destilada).

Após esta etapa, procedeu-se a extração de DNA das amostras. A extração foi através de dois métodos diferentes: utilizando-se o kit *Wizard® Genomic, DNA Purification Kit (Promega Corporation, Madison, Wi, USA*), conforme instruções do fabricante e segundo, o método orgânico.

O método orgânico constituiu-se em: cada amostra foi acrescida 200µL de enzima de digestão e 200µL de TEP e incubada em banho a 50°C por 48 horas, sendo agitada ocasionalmente. Ao fim das primeiras 24 horas de banho, foi acrescentado mais 50µL de TEP. Após o período de incubação, procedeu-se a inativação da enzima de digestão, com elevação da temperatura do banho a 96°C, por 15 minutos. Adicionou-se, então, 200µL de acetato de amônia 6M, pH 8,5. A amostra foi agitada em vortex e após centrifugada. O sobrenadante (que contém DNA) é transferido para um tubo novo e o restante foi desprezado. No novo tubo foi adicionado 600µL de isopropanolol PA frio, misturando com cuidado e incubando em freezer 70°C negativos, por 2 horas. Após a incubação, a amostra foi centrifugada por 15 minutos. A seguir, a fase líquida é cuidadosamente desprezada, adicionando-se 600µL de etanol 70%, misturando por

inversão. A amostra foi então centrifugada por 15 minutos. Este passo, que teve como objetivo lavar o DNA, foi repetido 3 vezes. Por fim, a fase líquida foi desprezada, deixando-se secar o "pellet" sobre papel absorvente. Uma vez seco, o DNA foi ressuspendido em 50µL de tampão TE, pH 7,5 e armazenado em freezer 20°C negativos.

Após a extração do DNA, independente do método empregado, as amostras foram submetidas à PCR, utilizando-se o conjunto de iniciadores genéricos para HPV, PGMY 09/11 (Gravitt et al. 2000), capazes de amplificar 450 pares de bases (pb) do gene L1 de diversos tipos do HPV genitais. Foram adicionados os iniciadores GH20 e PCO4 (Saiki et al. 1988) que amplificam 268 pb do gene da β-globina humana, servindo como controle interno para avaliação da integridade e suficiência de DNA de cada amostra.

A reação em cadeia da polimerase foi realizada em um volume de reação de 20 µL, formada por uma solução equimolar de cada um dos iniciadores genéricos PGMY 09/11 na concentração de 25 µM de cada oligonucleotídeo, iniciadores GH20 e PCO4 (20mM de cada iniciador), 15 mM de 10x PCR Buffer, 4 mM de MgCl2, 100 µM de dCTP, 100 µM de dGTP, 100 µM de dATP, 100 µM de dTTP e 0,2µL de *AmpliTaq Gold® DNA Polymerase* (*Roche, New Jersey,USA*). A amplificação foi realizada em um tubo único, através da utilização de 40 ciclos em termociclador (*PTC – 100 Peltier Thermal Cycler, MJ Research, Waltham, Massachusetts, USA*). Cada ciclo incluiu 1 minuto de desnaturação a 95°C, 1 minuto de anelamento a 55°C e 1 minuto de alongamento da cadeia a 72°C. O primeiro ciclo foi estendido por 13 minutos de desnaturação a 95°C. O último passo de alongamento da cadeia a 72°C foi procedido por 5 minutos, modificado de Gravitt et al. (2000). Foram incluídos controles positivos e negativos na reação. Os controles positivos usados eram compostos por DNA de

plasmídio, contendo genoma completo do HPV, gentilmente cedidos pela Dra. Luisa Lina Villa (Instituto Ludwig para Pesquisa do Câncer – São Paulo). O controle negativo utilizado compreendeu uma mistura da reação, porém sem DNA.

Os *amplicons* foram analisados por eletroforese em gel de agarose 1,5% (1,05g de agarose dissolvido em 75mL de TBE 0,5x), acrescido de 4µL de brometo de etídio a 10mg/mL. O tampão TBE 0,5x foi preparado usando 5,5g de ácido bórico, 10,8g de Tris, 0,925g de EDTA e 2 litros de água destilada. A eletroforese foi conduzida em tampão 0,5x TBE pH 8,3 a aproximadamente 110V constantes. O marcador de peso molecular ϕX174/Hae III (*Invitrogen, Frederick, Mariland, USA)* foi aplicado em cada gel. Os géis foram visualizados e analisados sob luz ultravioleta.

As amostras foram amplificadas na primeira reação de PCR usando os *primers* degenerados GP-E6-3F (GGG WGK KAC TGA AAT CGG T), GP-E6-5B (CTG AGC TGT CAR NTA ATT GCT CA) e GP-E6-6B (TCC TCT GAG TYG YCT AAT TGC TC), sendo W, A/T; K, G/T; R, A/G; Y, C/T e N, A/C/G/T. Estes *primers* amplificam uma região de 630pb da região E6/E7 dos 38 tipos do HPV mais comuns. A reação de NESTED-PCR foi específica e foi realizada para os tipos virais 6/11, 16, 18, 31, 33, 42, 52 e 58. Os primers usados e os tamanhos dos produtos amplificados estão discriminados no Quadro 2. Todo procedimento, tanto a primeira reação (PCR) quanto a segunda reação (NESTED-PCR), ocorreu segundo Sotlar et al. (2004). Os produtos amplificados foram analisados por eletroforese vertical em gel de poliacrilamida 4%, utilizando-se tampão TBE 1X e posterior coloração com nitrato de prata. Como marcador de peso molecular foi utilizado o pGEM (PROMEGA).

Quadro 2: Primers utilizados no PCR “nested”.

Tipo do HPV	*Primer*	Tamanho do fragmento
6/11	TGC AAG AAT GCA CTG ACC AC	334 pb
	TGC ATG TTG TCC AGC AGT GT	
16	CAC AGT TAT GCA CAG AGC TGC	457 pb
	CAT ATA TTC ATG CAA TGT AGG TGT A	
18	CAC TTC ACT GCA AGA CAT AGA	332 pb
	GTT GTG AAA TCG TCG TTT TTC A	
31	GAA ATT GCA TGA ACT AAG CTC G	263 pb
	CAC ATA TAC CTT TGT TTG TCA A	
33	ACT ATA CAC AAC ATT GAA CTA	398 pb
	GTT TTT ACA CGT CAC AGT GCA	
42	CCC AAA GTA GTG GTC CCA GTT A	277 pb
	GAT CTT TCG TAG TGT CGC AGT G	
52	TAA GGC TGC AGT GTG TGC AG	229 pb
	CTA ATA GTT ATT TCA CTT AAT GGT	
58	GTA AAG TGT GCT TAC GAT TGC	**274**
	GTT GTT ACA GGT TAC ACT TGT	

6.1.5 Imunohistoquímica (IMQ)

6.1.5.1 IMQ para p16INK4

No material histopatológico (blocos de parafina com amostras de colo uterino) foi realizada IMQ para detecção da $p16^{INK4a}$, P53, Ki-67 e bcl-2. Foram feitos cortes

histológicos com 4 micras de espessura montados em lâminas de vidro sialinizadas. Os procedimentos de IMQ foram realizados de acordo com as instruções do fabricante do anticorpo. As lâminas sialanizadas foram preparadas em uma bateria composta por um banho de acetona (500ml) por 30 segundos, seguido de um banho de 5 minutos em uma solução composta de 475ml de acetona acrescidos de 25ml de aminopropiltrimetoxisilano 97% (*Sigma-Aldrich, Steinheim, Germany*). Após, foram realizados mais dois banhos em 500ml de acetona por 30 segundos cada, seguidos de secagem em estufa a 40°C.

Os anticorpos utilizados foram: Anti-humano p53 Protein, clone DO-7, diluição 1:50, DAKO®; Anti-humano Ki-67, clone Ki-S5, diluição 1:50, DAKO® e; Anti-humano bcl-2 Oncoproteína, clone 124, diluição 1:80, DAKO® .

Para a metodologia de IMQ da proteína $p16^{INK4a}$, empregou-se o sistema EnVision, utilizando-se *Cintec* $p16^{INK4}$ *Histology Kit* (*Dako Cytomation, Glostrup, Denmark*), conforme instruções do fabricante.

Para a realização da imunohistoquímica seguiu-se as seguintes etapas:

1. desparafinização: remoção da parafina por aquecimento;
2. reidratação: banhos sucessivos com xilol, etanol 95% e etanol 70%;
3. reconhecimento dos epítopos através do uso de solução própria contida no kit;
4. reconhecimento da peroxidase através do uso do reagente próprio presente no kit;
5. aplicação do anticorpo primário ($p16^{INK4}$ rato anti-humano, clone E6H4) ou do reagente para controle negativo;

6. aplicação do DAB;
7. contracoloração com hematoxilina de Mayer.

A IMQ foi realizada no laboratório de Patologia do Centro de Ciências da Saúde da Universidade de Caxias do Sul. Para cada reação foi utilizado um controle negativo na ausência do anticorpo primário.

6.1.5.2 IMQ para TG2

A IMQ para a proteína TG2 foi realizada de maneira automatizadas no equipamento Autosteiner-Link 48 Dako. Este ensaio utilizou um anticorpo monoclonal de rato dirigido contra o domínio de TG2 humano (Diagnostic BioSystem, Dilução 1:50). Foi utilizado como controle negativo o Reativo isotipo IgG monoclonal de coelho (DA1E; Cell Signaling Technology, Danvers, MA). A desparafinação, reidratação, e recuperação foi realizado no PT Link (Dako PT100). A lâminas foram então processadas no equipamento Autostainer Link 48 (Dako AS480) utilizando um protocolo automatizado validado para TG2. O processo de IMQ pelo sistema Autostainer Link 48 (Dako AS480), foi realizado conforme instruções do fabricante do anticorpo para TG2.

6.1.5.3 Quantificação da Imunohistoquímica

A quantificação da IMQ foi realizada empregando-se diferentes métodos. Os resultados foram obtidos através de microscópio óptico (Nikon Eclipse 50i) e câmera digital (DS-5M-L1; Nikon, NY, USA), acoplados. As imagens digitalizadas foram transferidas para um computador.

6.1.5.4 Image J

A intensidade de marcação foi determinada através do programa NIH ImageJ 1.36b (National Institutes of Health, Maryland, EUA). O Image J é um software analisador de imagens em Java de domínio público inspirado no NIH Image para o Apple Macintosh. Sendo assim, pode ser executado em diversos ambientes operacionais desde que os mesmos possuam uma máquina virtual Java apropriada. O repertório de funções do software pode ser ampliado através de diversos *plugins* prontos, disponíveis na internet (Jensen, 1996; Wu et al., 2004).

O software NIH ImageJ 1.36b foi obtido através de download do site NIH (http://rsb.info.nih.gov/ij), a seleção de cores e classificação de pontos corados positivamente foi realizada através do uso de um diagrama de distribuição das cores vermelhas, verdes e azuis (RGB), o qual demonstra as alterações de intensidade e saturação das cores. Esta distribuição fornece informação sobre o número de pixels da imagem analisada (Wu et al., 2004). O *software* torna branca as áreas que satisfazem este padrão e pretas as demais áreas (Figura 1) (Abràmoff et al., 2004; Wu et al., 2004).

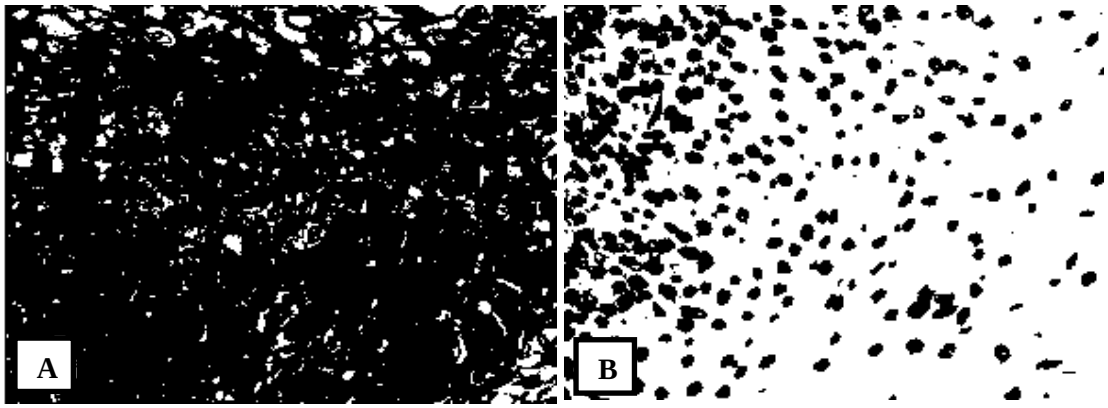


Figura 1. Representação das imagens após definição e aplicação do intervalo de positividade através do *plugin thershold colour* do *software* Image J. A ilustração A

demostra a análise de uma biópsia de colo uterino com marcação para $p16^{INK4a}$ no valor de “3+” e a ilustração B demonstra a análise de uma biópsia de colo uterino com marcação para $p16^{INK4a}$ negativa.

6.1.5.5 Quantificação Relativa

Os resultados da IMQ foram avaliados considerando a proporção de células positiva comparadas ao número total de células no campo. Células com imunomarcação citoplasmática e nuclear foram consideradas positivas. Foram obtidas 4 fotografias dos melhores campos de observação das lâminas no aumento de 400X, cada fotografia foi dividida em 4 quadrantes, e uma contagem manual foi realizada para se obter uma porcentagem de positividade (Godoy et al., 2008).

6.1.5.6 Outros métodos de Quantificação

Cada amostra de colo uterino foi analisada por dois médicos patologistas, em separado, com utilização de vários métodos. Para tal., utilizou-se o método semi-quantitativo com escala de cruzes Sistema de Escore Semi-quantitativo Alemão, o qual correlaciona os níveis de expressão com número de 0 a 3 (0, 1+,2+,3+) (Remmele and Schicketanz 1993; Han et al., 2008; Han et al., 2009; Kok et al., 2010), e um segundo método no qual as amostras foram novamente classificadas subjetivamente em “negativo”, “fracamente positivo” e “fortemente positivo” conforme padronização de estudos da literatura (George et al., 2010).

6.1.6 Análise Estatística

A análise dos dados foi realizada empregando-se o pacote estatístico *SPSS – Statistical Package for Social Science* (versão 18.0). Para comparação de variáveis qualitativas, como frequências e proporções, foi utilizado o teste de qui-quadrado para amostras independentes com possível análise de resíduos ajustados. Para comparação de dados qualitativos ordinais ou dados quantitativos, foi utilizado o teste não-paramétrico de Mann-Whitney (com nível de significância de 5%).

A variável "HPV" foi dicotomizada em "alto risco oncogênico" e "baixo risco oncogênico". De acordo com a literatura (Souho et al., 2015) foram considerados como HPV de alto risco oncogênico os seguintes tipos: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 26, 53, 66, 67, 68, 69, 70, 73 e 82 e classificados como baixo risco oncogênico os tipos: 6, 11, 40, 42, 43, 44, 55, 54 e 62. As pacientes que apresentaram algum DNA viral de alto e baixo risco oncogênico concomitantemente, foram classificados como HPV de alto risco. Para a análise referente as proteínas TG2 e $p16^{INK4a}$ realizou-se a categorização da imunomarcação em ≤50%, >50% e 0 (negativo).

6.2. ANEXO 2

TERMO DE CONFIDENCIALIDADE

Modelo de termo de compromisso do pesquisador para o uso de dados e preservação do material com informações sobre os sujeitos em arquivo - prontuários e material biológico)

Título do projeto: Avaliação de expressão de biomarcadores em Lesões intraepiteliais do carcinoma escamoso do colo uterino: Estudo Retrospectivo

Pesquisador responsável: Karen Bazzo

Demais pesquisadores: Fábio F. Pasqualotto e Alessandra Eifler Guerra Godoy

Instituição de origem do pesquisador: UCS

Área de Conhecimento: Ciências da Saúde

Curso: Doutorado

Telefone para contato: 54 81238989

Local da Coleta de dados: Ambulatório de Patologia do Trato Genital Inferior do Ambulatório Central da Universidade de Caxias do Sul e Laboratório Diagnose

Registro no CEP/UCS:

O(s) pesquisador(es) do projeto acima identificado(s) assume(m) o compromisso de:

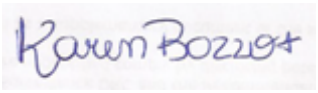
I. Preservar o sigilo e a privacidade dos sujeitos cujos dados (informações e/ou materiais biológicos) serão estudados;

II. Assegurar que as informações e/ou materiais biológicos serão utilizados, única e exclusivamente, para a execução do projeto em questão;

III. Assegurar que os resultados da pesquisa somente serão divulgados de forma anônima, não sendo usadas iniciais ou quaisquer outras indicações que possam identificar o sujeito da pesquisa.

O(s) Pesquisador(es) declara(m) ter conhecimento de que as informações pertinentes às técnicas do projeto de pesquisa somente podem ser acessados por aqueles que assinaram o Termo de Confidencialidade, excetuando-se os casos em que a quebra de confidencialidade é inerente à atividade ou que a informação e/ou documentação já for de domínio público.

Caxias do Sul, ... de ..... de 20..... .



_____

Assinatura Pesquisador

Nome: Karen Bazzo

RG: 1092446689

6.3. ANEXO 3

FACULDADE NOSSA SENHORA DE FÁTIMA/RS ASSOCIAÇÃO CULTURAL E

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação da expressão de biomarcadores em lesões precursoras de carcinoma escamoso do colo uterino

Pesquisador: Karen Olivia Bazzo

Área Temática:

Versão: 1

CAAE: 53365216.6.0000.5523

Instituição Proponente: ASSOCIACAO CULTURAL E CIENTIFICA VIRVI RAMOS

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.442.449

Apresentação do Projeto:

Adequado.

Objetivo da Pesquisa:

Adequado.

Avaliação dos Riscos e Benefícios:

Adequado.

Comentários e Considerações sobre a Pesquisa:

Projeto apresenta o termo de confidencialidade assinado pelo pesquisador responsável.

TCLE foi aplicado previamente, no momento da coleta.

Considerações sobre os Termos de apresentação obrigatória:

Projeto apresenta o termo de confidencialidade assinado pelo pesquisador responsável.

TCLE foi aplicado previamente, no momento da coleta.

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

Não se aplica.

Endereço: Rua Alexandre Fleming, 454
Bairro: Madureira **CEP:** 95.041-520
UF: RS **Município:** CAXIAS DO SUL
Telefone: (54)3535-7300 **Fax:** (54)3535-7300 **E-mail:** cep@fatimaeducacao.com.br

FACULDADE NOSSA SENHORA DE FÁTIMA/RS ASSOCIAÇÃO CULTURAL E

Continuação do Parecer: 1.442.449

Considerações Finais a critério do CEP:

Aprovado.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_658198.pdf	10/02/2016 14:26:22		Aceito
Projeto Detalhado / Brochura Investigador	TESEDOUTORADO.doc	10/02/2016 14:26:04	Karen Olivia Bazzo	Aceito
Folha de Rosto	0339_0001.pdf	10/02/2016 14:23:30	Karen Olivia Bazzo	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não