Silvia Helena Rabelo dos Santos

ASSOCIAÇÃO DOS TIPOS E VARIANTES DE HPV COM O DIAGNÓSTICO HISTOLÓGICO EM MULHERES COM ANORMALIDADES EM CÉLULAS GLANDULARES DO COLO UTERINO

TESE DE DOUTORADO

ORIENTADOR: Prof. Dr. Luiz Carlos Zeferino
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Tese de Doutorado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do Título de Doutor em Tocoginecologia, área de Ciências Biomédicas.

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Dedico esta tese...

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F. Shaar

Estrutura da Tese

Esta tese está sendo apresentada no formato alternativo de disponibilização de dissertações de mestrado e teses de doutorado na UNICAMP e de acordo com o disposto em *""Normas, procedimentos e orientações para publicação de dissertações e teses da Faculdade de Ciências Médicas"* (2004).

Inclui uma introdução sobre o tema, os objetivos da tese, dois artigos originais submetidos às revistas *Human Pathology e International Jornal of Cancer* -- com a descrição dos métodos e resultados obtidos – e, por fim, uma discussão geral e os anexos. Nos anexos foram incluídos os modelos das cartas às pacientes, ao médico do posto de saúde, o consentimento informado e a ficha para coleta de dados.

As etapas e experimentos necessários ao desenvolvimento desta pesquisa foram realizados nos seguintes locais:

Laboratório de Citopatologia – CAISM/UNICAMP

Ambulatório de Patologia do Trato Genital Inferior – CAISM/UNICAMP

Laboratório de Procedimentos Especializados – CAISM/UNICAMP

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Sumário

Símbolos, Siglas e Abreviaturas

AGC	Células Glandulares Atípicas
AGC-SOE	Células glandulares atípicas sem outras especificações
AGC-NEO,	Células glandulares atípicas provavelmente neoplásicas
AIS	Adenocarcinoma *in situ*
AGUS	Células glandulares atípicas de significado indeterminado
ASCUS	Células escamosas atípicas de significado indeterminado
CAISM	Centro de Atenção Integral à Saúde da Mulher
CH II	Captura de híbridos tipo dois
DNA	Ácido desoxirribonucléico
HPV	Papilomavírus humano
HSIL	Lesão intra-epitelial escamosa de alto grau
LSIL	Lesão intra-epitelial escamosa de baixo grau
NIC	Neoplasia intra-epitelial cervical
IC	Intervalo de confiança
OR	*Odds ratio*
PCR	Reação em Cadeia da Polimerase
PTGI	Patologia do Trato Genital Inferior
UNICAMP	Universidade Estadual de Campinas

Resumo

Objetivo: Analisar a associação do tipo de HPV e das variantes dos HPV 16 e 18 com lesão neoplásica do colo uterino em mulheres cujo exame citológico apresenta anormalidades em células glandulares endocervicais.

Métodos: Este estudo de corte transversal analítico incluiu 160 mulheres com citologia cervical de rastreamento, sendo 93 mulheres com *células glandulares atípicas sem outras especificações* (AGC-SOE), 18 com c*élulas glandulares atípicas provavelmente neoplásicas* (AGC-NEO), 35 com por AGC associado a lesões intra-epitelias escamosas de alto grau (HSIL) *e* 14 com adenocarcinoma *in situ* (AIS). No primeiro atendimento foi colhido material cervicovaginal para pesquisa de DNA de HPV, segundo esfregaço cervical, e foi realizada colposcopia em todas as mulheres. Biópsias e/ou conizações foram realizadas em 129 pacientes. Trinta e uma mulheres encaminhadas por diagnóstico citológico de AGC-SOE com colposcopia normal e segunda citologia com resultado negativo foram seguidas a cada quatro meses com exames citológicos e colposcópicos. Todas as mulheres incluídas foram submetidas a ecografia pélvica. A pesquisa de DNA-HPV foi feita por PCR, utilizando os *primers* PGMY09 e PGMY11, e a genotipagem foi realizada através de hibridização reversa em pontos. A determinação das variantes de HPV 16 e HPV 18 foi realizada através de sequenciamento.

Resultados: Dos 129 casos com avaliação histológica, 75 (58%%) mostraram diagnósticos neoplásicos (intra-epiteliais e invasivos). A maioria dos diagnósticos neoplásicos foi categorizada

como de origem escamosa (77%). A prevalência total de HPV foi de 43%. O HPV 16 foi o mais prevalente e esteve significantemente associado a neoplasias escamosas (NIC 2 ou lesão mais grave) e neoplasias glandulares (AIS ou lesão mais grave). O HPV 18 foi o segundo tipo mais prevalente e esteve significativamente associado a neoplasias glandulares. Maior diversidade na distribuição dos tipos foi observada nos diagnósticos histológicos de NIC 2 e NIC 3. As neoplasias glandulares foram exclusivamente relacionadas aos tipos 16 e 18. O estudo de variantes incluiu 24 casos HPV 16 positivos e 6 casos HPV 18 positivos. Variantes Européias e Não Européias (Ásia-Americanas) foram detectadas, respectivamente, em 62% e 38% dos casos HPV 16 positivos. Variantes Européias de HPV 18 também foram mais prevalentes (3/6). Neoplasias glandulares foram mais prevalentes e significativamente associadas a Variantes Ásia-Americas de HPV 16. Variantes Européias de HPV 16 foram mais prevalentes em neoplasias escamosas. Neoplasias glandulares também foram associadas aos HPV 18, mas a análise de suas variantes não foi conclusiva, devido ao pequeno número de casos.

Conclusão: Os HPV estão associados ao tipo histológico de neoplasia cervical detectada em mulheres com anormalidades em células glandulares endocervicais, contudo a identificação dos tipos não é suficiente para explicar o padrão histológico, uma vez que o HPV 16 pode estar associado com neoplasias escamosas e glandulares. A associação do HPV 16 com neoplasias escamosas e glandulares é explicada por suas variantes. Variantes Ásia-Americanas associam-se às neoplasias glandulares, enquanto variantes européias associam-se às neoplasias escamosas.

Summary

Objective: To analyze the association between the different genotypes of oncogenic human papillomavirus (HPV) and HPV 16 and HPV 18 variants with histological diagnosis in women referred for glandular endocervical abnormalities in their cervical smear.

Methods: This cross sectional study included a series of 160 women. Atypical Glandular Cells (AGC) not otherwise specification (NOS), AGC favor neoplastic (FN) were the only diagnosis in 93 and 18 women respectively. Thirty five patients had both AGC and high grade squamous intraepithelial lesion (HSIL). Fourteen women were diagnosed as adenocarcinoma in situ (AIS). All women were subjected a collection of sample for HPV-DNA testing and second cervical smear and underwent a colposcopic examination. Biopsies or conizations were done in 129 due to colposcopically abnormal area or second abnormal cervical smear result. In 31 women, referred due to AGC-NOS at screening cervical smear with adequate and normal colposcopy and second cervical smear results, follow up each 4 months with new cytology and colposcopy were done and the final diagnosis was considered as non-neoplastic. All women had pelvic ultrasound examination. The HPV-DNA testing was done by PCR using the set of PGMY09 e PGMY11 with genotyping by linear array hybridization. The molecular variants of HPV 16 and 18 were tested using PCR sequencing.

Results: Among 129 women with histologic evaluation, 75 (58%) revealed neoplastic diagnoses (intraepithelial and invasive); of them the majority (77%) were of squamous cell origin. The overall

prevalence of HPV was 43%. HPV 16 was the most prevalent genotype and was significantly associated with squamous neoplasias (CIN 2 or worse) and glandular neoplasias (AIS or worse). HPV 18 was the second most prevalent and was significantly associated with glandular neoplasias. Major diversity on HPV distribution was observed in Cervical Intraepithelial Neoplasia (CIN) 2 and CIN 3 histologic diagnosis. The variants study included 24 cases HPV 16 positives and 6 cases HPV 18 positives. European Variants and Asian-American Variants were detected in 62% and 38% of HPV positives cases. European variants of HPV 18 also were more prevalent (3/6). Asian-American HPV 16 variants were significantly associated with glandular neoplasia and European variants were more prevalent in squamous neoplasias. Glandular neoplasias also were associated with HPV18 but in this study the analysis of its variants was not conclusive.

Conclusion: The HPV genotypes are associated with histological type of the cervical neoplasia, but HPV genotypes is not enough to explain at whole, since the HPV 16 were associated with squamous and glandular neoplasia. The association of HPV 16 with squamous or glandular neoplasia is explained by its variants. Squamous neoplasias were nearly related to HPV 16 European variants and glandular neoplasias were related to HPV 16 Asian-American.

1. Introdução

O câncer de colo uterino é um importante problema de saúde pública, principalmente em países em desenvolvimento como o Brasil. As taxas de incidência e mortalidade ajustadas por idade, segundo a Agência Internacional de Pesquisa sobre o Câncer (IARC, 2002) são de 23,4/100.000 e 10,2/100.000. Ë ainda o segundo tipo de câncer da mulher mais incidente no Brasil e no mundo, embora seja uma doença prevenível (Bosch et al., 2002, INCA, 2005).

Considerando os tipos histológicos de câncer cervical, os carcinomas escamosos são mais freqüentes, representando de 75% a 85% do total. Adenocarcinomas ocorrem entre 11% e 25% dos casos e carcinomas adenoescamosos são detectados em cerca de 2% a 3% dos casos (Smith et al., 2000; FIGO, 2003)

Existe definitivamente uma relativa diminuição na incidência de carcinomas escamosos em países onde o rastreamento através de esfregaço cervical está bem organizado e estabelecido. Estudos epidemiológicos, porém, têm indicado um aumento na taxa de adenocarcinomas e carcinomas adenoescamosos. Este aumento, demonstrado em vários países entre 1973 e 1996, tem sido observado em mulheres entre 25 e 49 anos e reflete o pequeno benefício do rastreamento citológico realizado nas décadas de 70 e 80 na prevenção dos adenocarcinomas (Smith et al., 2000; Vizcaino et al., 2000; Franco et al., 2001; Liu et al., 2001, Schoolland et al., 2002; Smith e Padilha, 2002).

Entre as possíveis razões para esta reduzida efetividade do rastreamento para adenocarcinomas, comparada à de carcinomas escamosos, estão a possibilidade de progressão

mais rápida para a forma invasiva da doença e a sensibilidade insuficiente do exame citológico para detecção de lesões precursoras de neoplasias glandulares, seja por erros de amostragens ou de interpretação (Ruba et al., 2004). Em média, adenocarcinomas *in situ* progridem para a forma invasiva em 13 anos, enquanto que o tempo de progressão para carcinomas escamosos é de 17,9 anos (Plaxe e Saltzstein, 1999). Contudo, 13 anos é tempo suficiente para que a neoplasia glandular seja detectada ainda em sua forma intra-epitelial, desde que o exame citológico seja efetivo e que as mulheres realizem os controles periódicos rigorosamente (Ruba et al., 2004).

Os erros de amostragem são justificados pelo fato de que adenocarcinomas e suas lesões precursoras derivam-se do epitélio glandular e, mais freqüentemente, localizarem-se no canal cervical, além da junção escamo-colunar. Como a zona de transformação migra para dentro do canal em mulheres após a menopausa, a detecção precoce por amostragem citológica endocervical é mais limitante. Outro fator importante é que o fundo das glândulas freqüentemente é mais envolvido que a superfície, o que também pode afetar as amostragens citológica e histológica. Assim, a ausência de células representativas da lesão, ou mesmo a paucicelularidade, é um problema diagnóstico a ser considerado (Demay, 1996, Smith et al., 2000).

Os erros de interpretação referem-se ao fato de que lesões precursoras para adenocarcinomas são consideravelmente menos definidas, e também são muito menos freqüentes. Em média, 0,5% dos esfregaços cervicais mostram alguma anormalidade relacionada a células glandulares atípicas, enquanto que 2% ou mais mostram anormalidades relacionadas a lesões precursoras escamosas. Conseqüentemente, a correlação entre os diagnósticos citológico e histológico é pobre (Zeferino et al., 2000; Mitchell et al., 2003; Verdiani et al., 2003; Covell et al., 2004; Derchain et al., 2004; Syrjanen, 2004).

É importante destacar que mais recentemente tem sido dado maior valor à presença e avaliação das células endocervicais nos esfregaços citológicos. A avaliação da adequabilidade da amostra como parte do resultado do exame citológico e a criação da categoria diagnóstica AGUS (*Células Glandulares Atípicas de Significado Indeterminado*) pelo Sistema de Bethesda, em 1988, significaram uma ação para atenuar estas dificuldades do diagnóstico citológico. A obrigatoriedade da amostragem do canal endocervical e da zona de transformação foi um fato importante para a melhoria da qualidade do rastreamento cervical na década de 90 (Lee e Flynn, 2000). Resultados positivos já foram relatados por Mitchell et al., (2003) que demonstraram que a partir de 1994 houve uma diminuição no risco de adenocarcinoma cervical associada com resultado negativo de citologia cervical com representação endocervical.

O diagnóstico de AGUS, diferente do ASCUS, mostrou-se associado a lesões clinicamente mais significantes, o que impunha que se adotasse conduta mais imediata para a avaliação diagnóstica definitiva. Diversos estudos mostraram que AGUS revelava de 17% a 80% de lesões escamosas e até 30% de lesões glandulares (Wright et al., 2002; Levine et al., 2003).

Assim, o melhor conhecimento adquirido sobre o significado das células glandulares atípicas a partir do Sistema de Bethesda de 1988, levou à introdução de mudanças, em 2001, em sua segunda revisão. O diagnóstico de AGUS foi ajustado para o diagnóstico de células glandulares atípicas (AGC), com denominação de “sem outras especificações” (SOE) ou “provavelmente neoplásicas” (NEO). O diagnóstico de AGC (NEO) está associado a maior prevalência de lesões de alto grau e adenocarcinoma (Wright et al., 2002, Levine et al., 2003).

Ainda como resultado do melhor conhecimento das anormalidades de células glandulares, em 2001 foi introduzido o diagnóstico citológico de adenocarcinoma *in situ*, que tem sido confirmado pela histopatologia em 48% a 69% dos casos, e cerca de 40% destas mulheres apresentam adenocarcinoma invasivo inicial ou bem diferenciado. Este fato é explicado pela

semelhança de critérios citomorfológicos entre estas lesões (Cangiarella e Chieeng, 2003; Simsir et al., 2003; Chhieng et al., 2004, Derchain et al., 2004).

No Centro de Atenção Integral à Saúde da Mulher (CAISM) Departamento de Tocoginecologia da Universidade Estadual de Campinas (Unicamp), há uma linha de pesquisa sobre anormalidades em células glandulares. Verdiani et al., (2003) detectaram uma prevalência de 0,2% (443) de diagnósticos de AGC entre 217.245 lâminas avaliadas pelo Laboratório de Citopatologia. Nesse estudo, os autores detectaram lesões cervicais pré-neoplásicas e neoplásicas em 62,2% dos casos submetidos à avaliação histológica e concluíram que a repetição da citologia e a colposcopia permitiram selecionar as mulheres que se beneficiariam com a avaliação histológica.

Torres et al. (2005) realizaram um estudo analisando, entre 27 critérios citomorfológicos, quais seriam os de maior valor para indicar diagnósticos neoplásicos e verdadeiramente glandulares. Estes autores que indicaram o aumento da relação núcleo-citoplasmática e a presença de disqueratinócitos foram fortemente associados com neoplasias intra-epitelial e invasiva, e os critérios diferenciais para neoplasias glandulares foram citoplasma escasso, membranas nucleares irregulares e presença de nucléolos.

Oliveira et al. (2004) analisaram a positividade do DNA-HPV e os resultados de uma segunda citologia em mulheres incluídas por citologia de rastreamento sugestiva de AGC. Considerando os resultados da segunda citologia, foi detectado DNA-HPV em 87% das mulheres com HSIL, em 100% das mulheres com AIS e em 11% das mulheres com resultado negativo na segunda citologia.

Derchain et al. (2004) detectaram uma prevalência de 38% em mulheres com diagnóstico citológico de AGC e AIS; contudo a prevalência de HPV foi respectivamente de 96%, 75% e 85% em mulheres com diagnósticos histológicos de NIC 2 ou NIC 3, AIS e carcinomas invasivos,

incluindo carcinomas escamosos e adenocarcinomas. Outros estudos têm encontrado resultados semelhantes utilizando captura híbrida II ou reação em cadeia da polimerase (PCR) (Ronnet et al., 1999; Krane et al., 2004).

Contudo, faltam informações sobre a associação dos tipos específicos de HPV presentes nos esfregaços com diagnóstico de AGC ou AIS com o diagnóstico histológico final.

Dos mais de 40 tipos de HPV encontrados no trato genital, cerca de 15 (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82) têm sido encontrados no carcinoma cervical invasivo, com risco estimado que varia de 50 até mais de 100, tanto para risco relativo quanto para *razão de* chances *(odds ratio).* Os tipos mais freqüentes em pacientes com carcinoma do colo uterino são representados por HPV 16, 18, 45, 31, 33, 52, 58 e 35, espectro muito semelhante ao encontrado na população feminina em geral (Franco et al., 2001; Munõz et al., 2003; Franceschi, 2005). A rigor, em adição aos 15 tipos de alto risco, os tipos 26, 53 e 66 devem ser considerados como provavelmente oncogênicos (Munõz et al., 2003).

Há uma diversidade em relação à distribuição viral entre países ou mesmo entre regiões de um mesmo país (Franco et al., 1999; Andersson et al., 2005; Brestovac et al., 2005; Chatuverdi et al., 2005). No Brasil, o tipo 16 é predominante em todas as regiões, contudo existe variação em relação à distribuição dos demais tipos (Rabelo-Santos et al., 2003).

Os carcinomas escamosos são principalmente relacionados ao HPV 16, pois cerca de 50% das neoplasias escamosas são HPV 16 positivas. Adenocarcinomas estão associados aos HPV 16 e HPV 18 (Tenti et al., 1996; Pirog et al., 2000; Altekruse et al., 2003). Pirog et al. (2000) analisaram uma série de resultados de diversos estudos, relacionados aos tipos de HPV presentes em adenocarcinomas, e mostraram que as prevalências dos tipos 16 e 18 são semelhantes.

A distribuição diferente dos HPV 16 e HPV 18 em neoplasias escamosas e glandulares poderia ser explicada por diferenças oncogênicas entre os tipos do HPV, e destaca um possível papel do HPV na definição do tipo histológico do carcinoma do colo uterino.

Atualmente, com o avanço do conhecimento genético dos HPV, diferenças intratipos têm sido descritas, que correspondem a pequenas variações na seqüência de nucleotídeos de vírus do mesmo tipo em relação a um protótipo já descrito (Ong et al., 1993; Yamada et al., 1997). As variantes dos HPV são mais conhecidas para os tipos 16 e 18, o que é justificável por serem os dois tipos mais prevalentes. Amostras de carcinoma do colo uterino de diferentes regiões do mundo têm mostrado que as variantes têm distribuição geográfica distinta. A denominação destas variantes baseia-se no padrão de diferenças nucleotídicas e na região onde é mais prevalente. Variantes de HPV 16 distribuem-se em quatro grandes classes, designadas Européias, Ásia-Americanas, Asiáticas e Africanas. A distribuição de variantes relacionadas ao HPV 18 mostra similaridades à distribuição geográfica dos HPV 16, sendo que para este vírus, três classes são definidas: Européias, Ásia-Amerindianas e Africanas (Ong et al., 1993; Yamada et al., 1997; Villa et al., 2000; De Boer et al., 2004; 2005).

Um estudo mundial acerca da distribuição das variantes de HPV 16 indicou as Variantes Européias como mais freqüentes em todos os continentes, com uma prevalência variando entre 60% no Sudeste da Ásia a 93% na América do Norte. Variantes Asiáticas constituem 26% dos espécimes oriundos do Sudeste da Ásia, mas são raras ou ausentes em outros continentes. Variantes Ásia-Americanas ocorrem somente na Américas Central e do Sul (20% dos espécimes) e Europa (14% dos espécimes, todos detectados na Espanha). Variantes Africanas constituem 92% dos espécimes obtidos na África (Yamada et al., 1997).

Há evidências de que variantes intratipos de HPV podem conferir riscos diferentes para os carcinomas cervicais. As variantes não européias de HPV 16 foram observadas mais comumente em espécimes de adenocarcinomas (Lizano et al., 1997; Burk et al., 2003).

Em resumo, o diagnóstico de anormalidades de células glandulares é pouco freqüente, porém clinicamente significante, porque freqüentemente revela lesões escamosas ou glandulares. O rastreamento do câncer do colo uterino não tem sido efetivo na redução da incidência e mortalidade por adenocarcinoma, como tem sido para o carcinoma escamoso. Os tipos específicos de HPV e variantes de HPV 16 e HPV 18 podem associar-se diferentemente a neoplasias escamosas e glandulares, sendo que o HPV 16 está presente no carcinoma escamoso e no adenocarcinoma. Portanto, é possível que além dos tipos específicos de HPV, as variantes intratipos também estejam associadas a determinado tipo histológico, a partir de células de reserva infectadas por este vírus.

Diante do exposto, o presente estudo foi desenhado para avançar no conhecimento do papel do HPV na determinação do tipo histológico do carcinoma do colo uterino e de suas lesões precursoras, a partir dos tipos virais e das variações intratipos, com enfoque maior para os HPV 16 e 18. Considerando que as anormalidades precoces das células glandulares são pouco conhecidas, mas representam importância clínica, optou-se por avaliar mulheres com o diagnóstico de células glandulares atípicas e adenocarcinoma *in situ* no esfregaço citológico realizado no rastreamento do câncer do colo uterino.

Os resultados estão sendo apresentados na forma de dois artigos, o primeiro analisando a prevalência e associação dos tipos específicos de acordo com os diagnósticos citológico e histológico. O segundo analisou a prevalência e a associação das classes de variantes de HPV 16 e HPV 18 com o diagnóstico histológico.

2. Objetivos

2.1 Objetivo geral

Analisar a associação do tipo de HPV, e das variantes dos HPV 16 e 18, com neoplasia escamosa ou glandular do colo uterino detectada em mulheres cujo exame citológico apresenta anormalidades em células glandulares.

2.2 Objetivos específicos

- Artigo 1

1 - Analisar a associação entre tipos específicos de HPV oncogênicos e o diagnóstico histológico em mulheres encaminhadas por diagnóstico citológico de células glandulares atípicas (AGC) e adenocarcinomas *in situ* (AIS), associado ou não à lesão intra-epitelial escamosa de alto grau (HSIL).

2 – Analisar a distribuição dos tipos de HPV em neoplasias escamosas e glandulares detectadas no seguimento de mulheres encaminhadas por diagnóstico citológico de células glandulares atípicas (AGC) e adenocarcinomas *in situ* (AIS), associado ou não à lesão intra-epitelial escamosa de alto grau (HSIL).

- Artigo 2

1. Determinar a prevalência de variantes de HPV 16 e HPV 18 em mulheres com diagnostico citológico de células glandulares atípicas (AGC) e adenocarcinomas *in situ* (AIS), associado ou não à lesão intra-epitelial escamosa de alto grau (HSIL).
2. Analisar a distribuição das variantes de HPV 16 e HPV 18 em neoplasias escamosas e glandulares em mulheres com diagnostico citológico de células glandulares atípicas (AGC) e adenocarcinomas *in situ* (AIS), associado ou não à lesão intra-epitelial escamosa de alto grau (HSIL).

3. Publicações

Artigo 1

Specific types of Human Papillomavirus and histological findings in women referred for atypical glandular cells or adenocarcinoma in situ in cervical smears

Silvia Helena Rabelo-Santos, Sophie Françoise Mauricette Derchain, Luísa Lina Villa, Luis Otávio Zanatta Sarian, Maria Cristina do Amaral Westin, Maria Cecília Costa, Janet Kornegay, Luiz Carlos Zeferino

Artigo a ser submetido à Human Pathology

Artigo 2

Variants of Human Papillomavirus16 and 18: histological findings in women referred for atypical glandular cells or adenocarcinoma in situ at cervical smear

Silvia Helena Rabelo-Santos, Luísa Lina Villa, Sophie Françoise Mauricette Derchain, Silvaneide Ferreira, Luis Otávio Zanatta Sarian, Liliana Aparecida Lucci Ângelo-Andrade, Maria Cristina do Amaral Westin, Luiz Carlos Zeferino

Artigo submetido ao International Journal of Cancer

O artigo *Specific types of Human Papillomavirus and histological findings in women referred for atypical glandular cells or adenocarcinoma in situ in cervical smears* está sendo avaliado por um dos autores (Janet Kornegay) e será submetido à revista *Human Pathology* assim que esta avaliação for concluída.

Specific genotypes of Human Papillomavirus and histological findings in women referred for atypical glandular cells or adenocarcinoma in situ in cervical smear

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Abstract

This study evaluated the association between the different genotypes of oncogenic human papillomavirus (HPV) and histological diagnosis in women with atypical glandular cells (AGC) or adenocarcinoma in situ (AIS) diagnosed by cervical smear testing. Of the 160 women included, 111 were diagnosed with AGC, 35 had both AGC and HSIL, while 14 women had AIS, in one case associated with HSIL. All women underwent colposcopic examination and biopsy was performed in 129/160. Thirty-one women were considered negative for neoplasia and scheduled for follow-up. All specimens were tested for 27 HPV genotypes by PCR-reverse line blot using the PGMY09/PGMY11 L1 consensus primers. Histological diagnosis was either cervical intraepithelial neoplasia (CIN) or invasive carcinoma in 75 (58%) women, and negative for neoplasia in 54 (42%). The overall prevalence of HPV was 43%. HPV 16 was the most prevalent form, followed by HPV 18. HPV 16 was significantly associated with squamous and glandular neoplasia and HPV 18 with glandular neoplasia. CIN 2 or 3 revealed 11 different HPV genotypes, while invasive glandular or invasive carcinoma revealed predominantly HPV 16 and HPV 18. HPV genotypes are associated with the histological type of cervical neoplasia, but this is not the full explanation. HPV 16 was associated with squamous neoplasia but it was also strongly associated with adenocarcinoma, showing an association even higher than that attributed to HPV 18. Most neoplastic diagnoses related to HPV 18 were adenocarcinomas. CIN 2 or 3 were associated with a great variety of HPV genotypes.

Key words: AGC, AIS, human papillomavirus, PCR, cervical carcinoma

Introduction

Glandular cell abnormalities represent a class of challenging diagnoses in gynecological cytology. The interpretation of cytological findings may be responsible for both the over- and under-diagnosis of glandular lesions, and for low inter-observer reproducibility [1]. Atypical glandular cells (AGC) may be attributable to benign conditions; however, frequently they may result from high-grade squamous precursor lesions and glandular neoplasias, including not only cervical adenocarcinoma but also endometrial and extra-uterine adenocarcinomas [2-6].

Women with AGC not otherwise specified (NOS) at cervical smear presented a rate of neoplasia ranging from 9% to 41%. Women with AGC-favor neoplasia (AGC-FN) presented a higher rate of neoplasia, ranging from 7% to 96% [7,8]. Cervical adenocarcinoma in situ (AIS) is nowadays classified in a different category. Studies have shown that women with AIS following cervical smear testing will be diagnosed with preinvasive or invasive disease at histological examination because both entities share similar features [4,9,10].

Cervical adenocarcinomas are highly associated with HPV 16 and HPV 18, although HPV 18 is more frequently associated with adenocarcinoma [11-13]. The finding of glandular hyperplasia, less severe than AIS, may appear as AGC in the cervical smear, and has shown lower HPV detection rates than AIS or cervical adenocarcinoma [14,15].

In fact, the role of specific HPV genotypes in cervical carcinogenesis has not yet been fully established in cases in which the first cytological abnormalities detected are AGC or AIS. Therefore, this study was designed to analyze the association between specific HPV genotypes and histological diagnosis in women with glandular abnormalities detected in cervical smear.

Methods

Patients

A series of 160 women referred to the colposcopy clinic because of glandular abnormalities detected during their cervical cancer screening smears between March 2002 and March 2005 were included in this study. The Institutional Review Board approved the study protocol and all participants gave their written informed consent prior to admission to the study.

Before patients were selected, those cervical smears identified as containing glandular abnormalities were revised by the same cytopathologist and cytologist in accordance with The 2001 Bethesda System (TBS) [16] and the final cytological diagnosis was established by consensus. AGC-NOS alone was diagnosed in 93 women, while in 18 women, AGC-FN alone was diagnosed. Thirty five patients had both AGC (19 NOS and 16 FN) and high grade squamous intraepithelial lesion (HSIL). Fourteen women were diagnosed with adenocarcinoma in situ, and in one of these cases, it was associated with HSIL.

Procedures

Women were considered eligible for the study if they met all of the following requirements: a) age between 18 and 60 years; b) having an intact uterus (i.e. no previous surgical procedure of the cervix or corpus); c) no history of abnormal cervical smear tests in the past year; d) were not under treatment for genital condyloma (external or cervical); e) had abstained from sexual intercourse during the three days prior to the consultation; and f) had no confirmed or clinically suspected immunossuppression (HIV, corticosteroids, chemotherapy or any other chronic diseases that might compromise the immune system). After signing the Informed Consent, women were subjected to a thorough pelvic examination, consisting first of the collection of another cervical smear, and then of a cervical sample that was maintained in 1.0ml of Universal Collection

Medium (*Digene Corporation*) for DNA HPV testing and colposcopy. All women underwent pelvic ultrasound examination.

Specimens were taken during directed punch biopsy, cervical conization (cold knife or loop electrosurgical excision procedures), uterine curettage or hysterectomy, according to the cytologic and colposcopic abnormalities. When a woman underwent more than one histological examination, the most severe diagnosis was considered. Of the 160 women admitted to the study, 129 underwent histological examination (Figure 1). The remaining 31 women in the study presented a satisfactory negative colposcopic evaluation, a negative second cervical smear, and normal pelvic ultrasound examination, and the final diagnosis was therefore considered negative for neoplasia. These 31 women were scheduled to return for follow-up visits every 4 months, and the duration of follow-up ranged from 4 to 18 months at the time of data collection for this analysis.

Sample processing and DNA extraction

Aliquots of 200ul of Universal Collection Medium (*Digene Corporation*) were taken for polymerase chain reaction (PCR) testing and were centrifuged for 10 min at 13.000 X g. The supernatants were immediately removed and stored as split cellular pellets at -80^{o}C prior to nucleic acid extraction and HPV detection. The cellular pellets were resuspendeds in 200ul of digestion solution (1mM Tris, 200ug of proteinase K per ml, 0.5% SDS) and digested at 55^{o}C for 2 hours. The digestion was followed by 5-minute incubation at 95^{o}C to inactivate the proteinase K. After that, 200ul phenol:chloroform:isoamyl alcohol (25:24:1), commercialized by Invitrogen Life Technologies, were added and shaken carefully before centrifugation for 10 min at 5.000 X g. The water phase was taken and transferred to a new tube and 1/10 of the volume of 3M Na AC ph 5.2 was added. The water phase was taken and once again transferred to a new tube, and 2.5

volumes of ice-cold 95ºC ethanol were added. Finally, they were centrifuged at 15000 X g for 15 min and after the DNA pellet was dried, it was dissolved in 100ul TE (1X). Nucleic acids were stored at -80ºC prior to HPV detection.

HPV DNA testing

HPV DNA genotyping was carried out using a reverse line-blot hybridization assay (LBA) which involved the hybridization of a 450-nt PCR amplicon generated by the PGMY primer set to a nylon strip containing immobilized probes [17,18]. The strip contained two levels of β-Globin control probes, 18 high risk HPV (HR-HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59, 68, 73, 82, 83) probes and 9 low-risk (LR-HPV probes 6, 11, 40, 42, 53, 54, 57, 66, 84) (Figure 2). PCR reagents, probe strips and developing reagents were kindly supplied by Roche Molecular Systems, Inc. (Pleasanton, CA). The 100ul amplification mixture contained 4mM $MgCl_2$, 50mM KCl, 7.5 units of Ampli*Taq* Gold DNA polymerase (Perkin-Elmer, Foster City, Calif.), 200uM each of dATP, dCTP, dGTP, 600uM of dUTP, 100 pmol of each biotinylated PGMY09/PGMY11 primer pool and 2.5 pmol each of 5' biotinylated β-Globin primers, GH20 and PCO4. The following ultrasensitive amplification profile was used: Activation of Ampli*Taq* Gold for 9 min at 95ºC, denaturation for 1 min at 95ºC, annealing for 1 min at 55ºC, extension at 72ºC for 1 min for a total of 40 cycles, followed by a 5-min terminal extension step at 72ºC. Agarose gel electrophoresis was done to identify specimens positive for HPV-DNA before strip development. Amplicons were denatured in 0.4 N NaOH and 40uL of denatured product was reacted in 3 ml of hybridization buffer with a reverse line-blot containing HPV genotypes and β-Globin probe at two concentrations immobilized on nylon strips. Positive hybridization was detected by streptavidin-horseradish peroxidase mediated color precipitation on the membrane at the probe line.

Histopathology

The specimens were reviewed according to the WHO criteria [19] and classified as: 1) *Neoplastic Diagnosis* - defined as any neoplastic lesions, including CIN 1, CIN 2, CIN 3, invasive squamous carcinoma, in situ adenocarcinoma, invasive cervical adenocarcinoma, and endometrial adenocarcinoma. 2) *No Neoplastic Diagnosis* - including cervicitis, squamous metaplasia, tubal metaplasia, tunnel cluster hyperplasia, microglandular hyperplasia and polyps. All histological analyses were carried out at the Laboratory of Pathology and diagnosed by the same pathologist, who was unaware of the cytology diagnoses.

Data Analysis

The prevalence rates of HPV genotypes were described according to referral cytology diagnosis and histology result. The association between HPV genotype and some cytological and histological categories was calculated using odds ratios (OR) with 95% confidence interval (95% CI). For statistical purposes, high risk genotypes were first analyzed separately. In a second step, high risk HPV genotypes were analyzed according to phylogenetic trees and were classified as group A, group C and group D [20]. Low risk genotypes were grouped together for the purpose of analysis. Cases shown to be HPV-DNA positive according to consensus primer but which were negative for specific low or high risk genotypes were classified as untyped. All statistical analysis was carried out using the SAS program, version 8.0.

Results

Of the 129 women who were evaluated histologically, 54 (42%) were diagnosed as negative for neoplasia. Of the 75 women who were diagnosed with neoplasia, 77% were of

squamous cell origin and 23% were of glandular cell origin. Neoplasia was identified histologically in 21% of women with AGC-NOS, and in 85% of women with AGC-NOS associated with HSIL. In the case of AGC-FN, these rates were, respectively, 50% and 100%. In cases of cytological AIS, 93% of women had a diagnosis of neoplasia. Among the diagnoses of squamous neoplasia, CIN 3 was the most frequent lesion (31/75), and invasive squamous cell carcinoma (SCC) (5%) was the most severe lesion. Biopsy-confirmed AIS was detected in five women. In two of these cases, it was associated with CIN 3 and in one case it was associated with microinvasive squamous carcinoma. Invasive adenocarcinoma (AC) was diagnosed in 12 women, endocervical carcinoma in 8 (in one case it was associated with CIN 3), endometrial carcinoma in 2, and multiple primary synergic adenocarcinomas (endocervix, endometrium, fallopian tube and ovary) in another 2 women (data not shown).

Beta-globin was not detected in three cases (histologically diagnosed as cervicitis, CIN 3 and multiple adenocarcinomas); therefore 157 women were included in the HPV analysis. HPV genotype detection and cytology results are shown in Table 1. Total overall detection rate of HPV was 43% (68/157), and among HPV-positive cases, 91% (62/68) were positive for high risk genotypes. Only two cases showed low risk HPV genotypes and four cases of HPV positive results (by agarose gel analysis) were untyped. HPV 16 was the most common genotype in all cytological categories. In 20 instances, this genotype was the only infection, while in 6 there were multiple infections, two of which were associated with HPV 18. HPV 18 was the second most common genotype, occurring in 7 specimens; in 5 as a single virus, and in 2 associated with other genotypes as multiple infections. HPV 52 and 58 occurred respectively in six and five cases, followed by HPV 53 and 31, which occurred alone in three specimens each.

Analyzing the distribution of HPV and the cytological results, it can be seen that AGC associated with HSIL showed a higher prevalence of HPV compared with AGC alone. The cytological diagnosis of AGC (NOS or FN) with HSIL was more strongly associated with high risk HPV than AGC (NOS or FN) alone. All HPV types and groups showed high odds ratio values (Table 2).

The distribution of HPV genotypes and histological diagnoses are shown in Table 3. Two cases were diagnosed as endometrial adenocarcinomas and were excluded from this analysis. The positivity rates of HPV were very similar in women who did not undergo biopsy (negative colposcopy, negative second cervical smear, and normal pelvic ultrasound), women who had a negative histological diagnosis for neoplasia, and women with a histological diagnosis of CIN1. In women with CIN 2 or worse, the prevalence of HPV was, respectively, 91% in cases of CIN2 and CIN 3, 100% in SCC, 100% in AIS and 66% in cases of endocervical adenocarcinoma. Most of the cases of HPV 16 were associated with squamous neoplasia, while most of the cases of HPV 18 were associated with glandular neoplasia. Squamous neoplasia showed a greater diversity of HPV genotype, while glandular neoplasia showed positivity only for HPV 16 and HPV 18. Only one case diagnosed as CIN 3 showed positivity for HPV 18; however, this specimen was co-infected with HPV type 84. In both of the two cases that were positive for both HPV 16 and HPV 18, histology revealed microinvasive squamous carcinomas.

HPV 16 and Group A sequences were strongly associated with squamous neoplasia. HPV 53 and Group D sequences were also associated with this neoplasia to a lesser extent. Multiple infections, including infections with HPV types 16 and 18 and those related to HPV 16 and other types, showed a significant association with squamous neoplasia. However, the presence of HPV16 may be the largest risk factor rather than multiple infections with additional

HPV types. On the other hand, HPV 18 in single and Group C sequences failed to show a statistically significant association with squamous neoplasia. Only when HPV 18 was associated with another genotype (84), did the OR value indicate a significant association. The magnitude of the association of HPV 16 with glandular neoplasia was higher than that of HPV 18(Table 4).

Discussion

The overall prevalence of HPV detected in the sample of women enrolled to this study was 43%, of which 91% were high risk genotypes. When ACG, NOS or FN, was associated with HSIL, the prevalence rate was around 90%, similar to rates observed for HSIL alone [21]. Krane et al. [3] detected high risk HPV using PCR in 32% of 108 women referred for AGC. In other populations, the detection rate of high-risk HPV in women referred for AGC varied from 28% to 38% using high risk probe (probe B) of hybrid capture II (HCII) [4,14,22]

Regarding the association between histological result and HPV detection in women referred due to glandular abnormalities, our data were similar to results published from other studies. Using the HCII technique, Ronnet et al. [14] detected high-risk HPV DNA in 92% of women with biopsy-confirmed HSIL, 100% with AIS, and 56% with LSIL. Derchain et al [4] detected high-risk HPV DNA in 96% of women with biopsy-confirmed CIN 2 or CIN 3, and in 83% with AIS or invasive cervical adenocarcinoma. Krane et al. [3] detected high risk HPV using PCR in 82%, 100% and 80%, respectively, of biopsy-confirmed AIS, high grade squamous lesion, and invasive adenocarcinoma. However, these authors did not identify specific HPV genotypes.

In our study, HPV 16 was the most prevalent genotype in women with cytological glandular abnormalities, while HPV 18 was the second most prevalent. HPV 16 is associated with squamous neoplasia but, as this study showed, it was also strongly associated with in situ or

invasive adenocarcinoma. HPV 16 has been shown to be the most prevalent genotype, irrespective of geographical location, in women with normal, premalignant or cancerous lesions [23-26]. Nevertheless, considering only women who were positive for HPV 18, the majority of diagnoses of neoplasia consisted of adenocarcinoma. This data is in agreement with previously published studies [11, 15].

HPV 16 was the most prevalent genotype detected in cases of CIN2 or CIN3; however, 10 other different HPV genotypes were also detected in these cases. The second most prevalent HPV genotype associated with CIN2 or CIN3 was HPV 58, rather than HPV 18. In fact, only 1/35 cases with CIN2 or CIN3 was HPV 18-positive. Other studies have similarly reported HPV 58 to be the second most prevalent genotype in women with cervical squamous intraepithelial lesions [24]. In a cohort study conducted in Brazil to analyze the acquisition and clearance of cervical HPV infection, Franco et al. [27] reported HPV 58 to be the third most prevalent genotype. In this same study, Franco et al. reported HPV 53 to be the second most frequent genotype, adding to the information from some other studies that reported HPV 53 to be relatively common, including in Brazil. The oncogenic risk of HPV53 has not yet been fully established. Munõz et al. [23] considered that this HPV genotype was probably oncogenic; however, others have classified it as being non-oncogenic [25,28]. In this study comprised of women specifically referred for glandular cell abnormality during cytological Pap screening and subsequent review, we did observe a statistically significant association of HPV 53 and also of Group D HPV with CIN 2 or higher grade squamous neoplasia.

The distribution of HPV genotypes in SCC varied slightly more than in cases of adenocarcinoma (AC), HPV 16 being the most prevalent in both carcinomas [11-13,29]. In our study, all cases of HPV-positive adenocarcinoma were related to genotypes 16 and 18. Although

HPV 18 played a prominent role in the development of adenocarcinoma, HPV 16 has been shown to have a similar or higher prevalence than HPV 18 in adenocarcinomas, and there are two possible hypotheses that may explain this fact. The first hypothesis is that HPV 16 is the most prevalent genotype in the general population, and secondly, it may also be involved in the carcinogenic process of glandular neoplasia [12,29]. It has been suggested that intratype variants of HPV 16 may confer differential risk for adenocarcinomas [13]. Furthermore, cofactors associated with the development of SCC and AC could be distinct, with cigarette smoking and multigravidity considered as risk factors for SCC, and the long-term use of oral contraceptives as a risk factor for cervical AC [30].

The overall prevalence rate of HPV observed in women with invasive adenocarcinoma was 66%. This lower rate could be explained by the insufficient content of episomal viral particles in exfoliated cells [31,32], or by insufficient sampling of the endocervical glandular lesion. Pirog et al. [12] have also speculated that the glandular epithelium does not support productive viral infection, and HPV DNA in endocervical neoplasm is usually present in the integrated form. In addition, considering both glandular and squamous neoplasia, loss of portions of the viral genome during integration may result in deletion of the viral genome containing the sequences targeted in the PCR reaction. In such cases, the detection of HPV DNA in the assay will depend on the presence of intact episomal HPV copies [12].

AGC and AIS in cervical smears may not represent the actual lesion [8] but they could represent immature cells with disordered cell proliferation. In fact, immature reserve cell hyperplasia, and immature metaplastic cells show similar morphological features, and differentiation between them is not easy [33]. Taking into account that HPV is the causal factor for

cervical cancer [11], HPV may be responsible for the early disorders manifested during the stage of reserve cell hyperplasia.

Considering factors related to the HPV genotypes and the reserve cell ability to develop into different epithelial cell types, we may hypothesize that the carcinogenic process could follow either of two routes: one towards squamous neoplasia, and the other towards glandular neoplasia. Specifically, if HPV 18 is present, the preferential route may be towards glandular neoplasia. If HPV 16 is present, either route could be taken, which suggests that other factors could be involved in this process, probably related to HPV 16 variants [13]. The other high risk HPV genotypes preferentially follow the route towards squamous neoplasia.

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Table 1 – Distribution of HPV genotypes according to referral cytology diagnosis

HPV type	AGC-NOS	AGC-NOS/HSIL	AGC-FN	AGC-FN/HSIL	AIS	Total
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
16	*2(12%)*	*4 (22%)*	*2 (34%)*	*4 (25%)*	*8 (73%)*	*20 (29%)*
18		*1 (5%)*	*1(17%)*	*1 (6%)*	*2 (18%)*	*5 (7%)*
52	*3(18 %)*	*2 (11%)*	*-*	*1(6%)*	*-*	*6 (9%)*
58	*-*	*1 (5%)*	*-*	*4 (25%)*	*-*	*5 (7%)*
53	*1(6 %)*	*2 (11%)*	*-*	*-*	*-*	*3 (4%)*
31		*2 (11%)*	*-*	*-*	*1 (9%)*	*3 (4%)*
45	*1(6%)*	*-*	*-*	*1(6%)*	*-*	*2 (3%)*
56	*-*	*1 (5%)*		*-*	*-*	*1 (2%)*
67	*1(6%)*	*-*	*-*	*-*		*1 (2%)*
35	*-*	*1(5%)*	*-*	*-*	*-*	*1 (2%)*
33	*-*	*-*	*1(17%)*	*-*	*-*	*1 (2%)*
Only HPV16 plus 18	*-*	*-*	*1(17%)*	*1(6%)*	*-*	*2(3%)*
HPV16 plus others	*1(6%)*	*1(5%)*	*-*	*2(12%)*	*-*	*4(6%)*
HPV18 plus others	*1(6%)*	*-*		*1(6%)*	*-*	*2(3%)*
Other types	*3(18%)*	*2(11%)*	*1(17%)*	*-*		*6(9%)*
Untyped	*4 (23%)*	*-*	*-*	*-*	*-*	*4(6%)*
Low risk	*-*	*1 (5%)*	*-*	*1 (12%)*	*-*	*2 (3%)*
Total HPV-positive	17 (100%)	18 (100%)	6 (100%)	16 (100%)	11 (100%)	68 (100%)
HPV-positives among total cases	17 (18%)	18 (95%)	6 (37%)	16 (100%)	11 (79%)	68 (43%)
Total HPV-negative	75 (82%)	1 (5%)	10 (63%)	-	3 (21%)	89 (57%)
Total cases	92	19	16	16	14	157

Abbreviations: AGC – Atypical Glandular Cells, NOS - not otherwise specified, FN - favor neoplasia), HSIL – high grade squamous intraepithelial lesion, AIS adenocarcinoma in situ

Table 2 – Association of HPV genotypes and HPV groups according to AGC-HSIL and AGC cytology diagnosis

HPV	Referral diagnosis		
	AGC/HSIL	AGC	
	n	n	OR (CI 95%)
16 alone	8	4	170.0 (16.9 to 1709.4)
Group A HPV	20	9	188.8 (22.6 to 1577.9)
18 alone	2	1	170.0 (7.6 to 3797.3)
Group C HPV	3	2	127.5 (8.9 to 1827.1)
53 alone	2	1	170.0 (7.6 to 3797.6
Group D HPV	3	1	255.0 (12.7 to 5129.4)
HPV-Negative	1	85	Reference

Abbreviations: AGC – Atypical Glandular Cells, NOS - not otherwise specified, FN - favor neoplasia), HSIL – high grade intraepithelial squamous lesions. AGC – Including the sub-classifications NOS and FN. Group A including the HPV types 16, 58, 31, 52, 35, 67; 33, Group C including HPV types 18 and 45; Group D including HPV types 53 and 56.

Table 3 –Distribution of HPV genotypes according to referral histology diagnosis

HPV type	Negative for Neoplasia		CIN 1	CIN2/CIN3	SCC	AIS	AC
	colposcopy cytology	biopsy					
	n %	n %	n %	n %	n %	n %	n %
16	-	1 (10%)	1 (33%)	10 (29%)	1 (25%)	3* (60%)	4 (66%)
18	-	2 (20%)	-	-	-	2** (40%)	1 (17%)
52	-	1 (10%)	-	4 (11%)	1 (25%)	-	-
58	-	-	-	5 (14%)	-	-	-
53	1(20%)		-	2 (5%)	-	-	-
31	-	-	-	3 (8%)	-	-	-
56	-	1(10%)	-	-	-	-	-
45	-	1(10%)	-	1 (3%)	-	-	-
67	-	-	1 (33%)		-	-	-
35	-	-	-	1 (3%)	-	-	-
33	-	-	-	1 (3%)	-	-	-
Only HPV16 plus 18	-	-	-	-	2(50%)	-	-
HPV16 plus others	-	-	-	4(11%)	-	-	-
HPV18 plus others	-	-	-	1(3%)	-	-	1**(17%)
Other HPV types	2(40%)	1(10%)	-	3(8%)	-	-	-
Untyped	2(40%)	2 (20%)	-	-	-	-	-
Low risk		1 (10%)	1 (33%)	-	-	-	-
Total HPV-positive	5(100%)	10(100%)	3 (100%)	35 (100%)	4 (100%)	5(100%)	6 (100%)
HPV-positives among total cases	5 (19%)	10 (23%)	3 (21%)	35 (91%)	4 (100%)	5 (100%)	6 (66%)
Total HPV-negative	26(81%)	43(77%)	11 (79%)	4 (9%)	-	-	3 (34%)
Total cases	31	53	14	39	4	5	9

* Two cases with associated squamous neoplasia. **One case with associated squamous neoplasia. Abbreviations: WN – without neoplasia including cervicitis and cases without biopsy, SCC – Invasive squamous cells carcinomas, AC – Invasive adenocarcinomas, AIS – adenocarcinoma in situ, CIN – cervical intraepithelial neoplasia. Two cases of endometrial adenocarcinoma were excluded.

Table 4. Association of HPV genotypes and HPV groups according to histological diagnosis

HPV Types and Groups	OR (CI 95%) comparing different histological categories
	Squamous origin CIN2 or worse *vs* negative for neoplasia
HPV 16[1]	189.8 (19.4 to 1858.6)
Group A[1]	418.5 (47.9-4201.5)
HPV 18	3.1 (0.1 to 74.5)
Group C [1]	8.6 (0.6-116.5.)
53[1]	34.5 (2.6-466.2)
Group D[1]	17.3 (1.9-196.3)
Only HPV16 plus 18[1]	77.2 (3.2-1861.7)
HPV16 plus others[1]	139.0 (6.4-3004.6)
HPV18 plus others[1]	46.3 (1.6-1307.0)
Other HPV types[1]	17.3 (2.6-114.3)
	Glandular origin - AIS or worse *vs* negative for neoplasia
HPV 16[1]	161.0 (14.7 to 1762.4)
HPV 18[1]	34.5 (4.1 to 290.4)
HPV18 plus others[1]	46.0(5.9-358.6)

[1] Reference for OR was HPV-negative

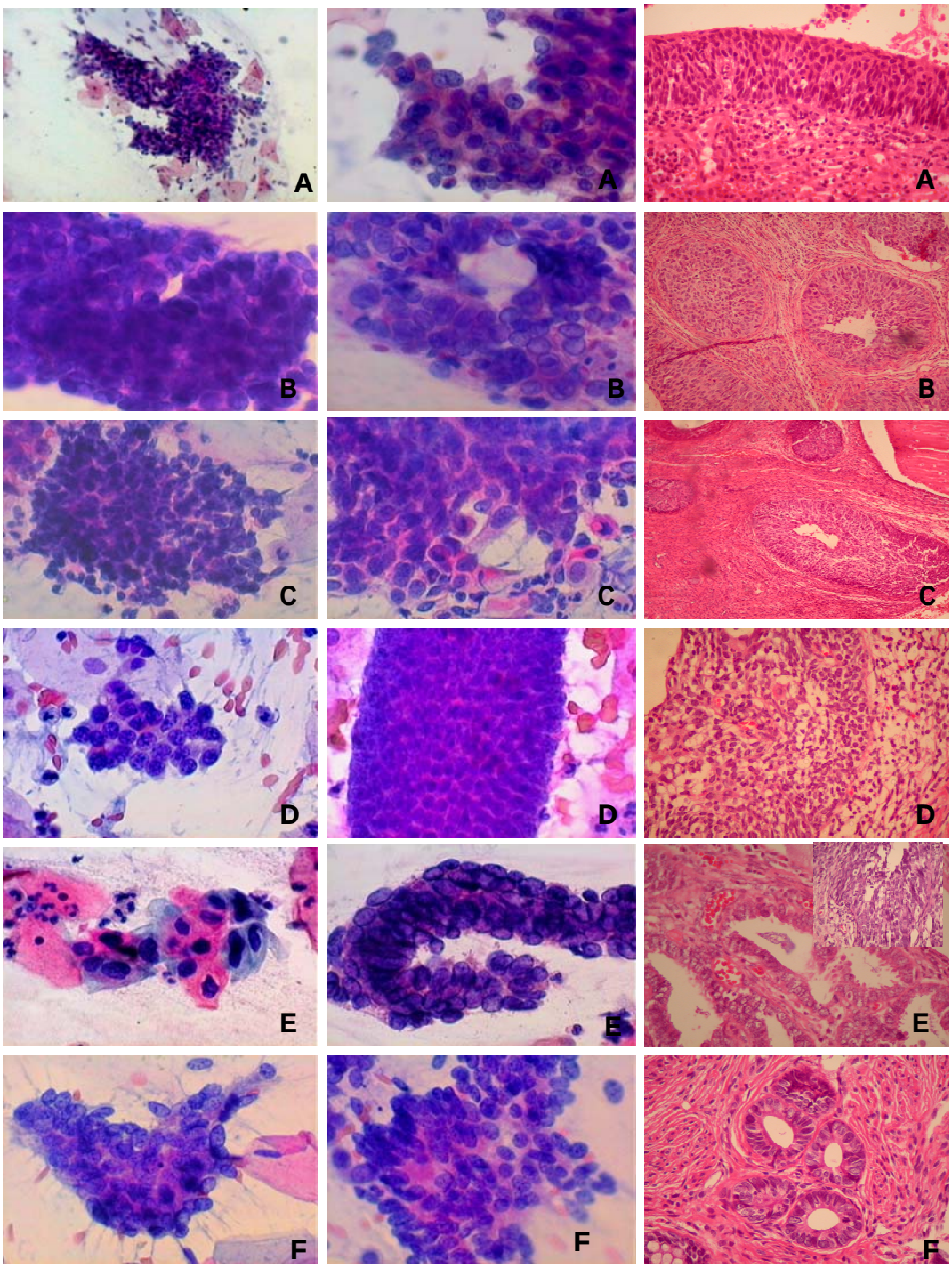

Figure 1- Cytologic and histologic correlation
A: AGC-NOS in cytology; histology CIN3: B: AGC-FN in cytology; histology CIN3 C: AGCFN/HSIL in cytology; histology: CIN3 D: AGC- FN in cytology; histology: microinvasive squamous carcinoma, E: AIS and HSIL in cytology; histology: AIS and microinvasive carcinoma, F: AIS in cytology; histology: invasive adenocarcinoma

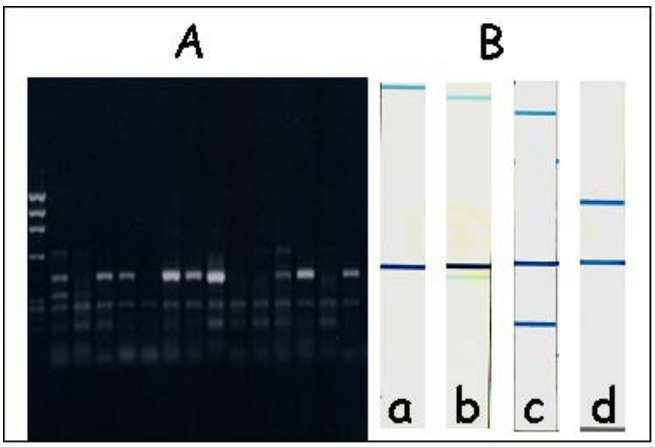

Figure 2-. HPV DNA reverse line-blot hybridization genotyping. PCR using biotinylated L1 consensus primers and the evaluation of products by reverse line-blot hybridization using genotyping strips that contain immobilized probes for a range of HPV types (Roche Molecular Systems, Inc.). (A) Agarose gelelectrophoresis to identify specimens positive for HPV DNA. (B) Example of line blot results. Strip “a” corresponds to HPV16; strip “b” HPV18; strip “c” HPV31 and 53; strip “d” HPV58.

Variants of Human Papillomavirus16 and 18: histological findings in women referred for atypical glandular cells or adenocarcinoma in situ in cervical smear

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Short title: Human Papillomavirus Variants and histological findings

Keywords: human papillomavirus, type 16, type 18, variants, cervical neoplasia, adenocarcinoma

Abbreviations used: HPV, human papillomavirus; SCC, squamous cell carcinoma; AC, adenocarcinoma ; E, European; As, Asian; AA, Asian-American; Af, African; ASAI, Asia Amerindian; AGC, atypical glandular cells; AIS adenocarcinoma in situ; HSIL, high grade squamous intraepithelial lesion, PCR, polymerase chain reaction; WHO, World Health

Organization; CIN cervical intraepithelial neoplasia; HE, hematoxilin and eosin; LCR, long control region; OR, odds ratio; CI, confidence interval.

Journal category: Infectious Causes of Cancer

Novelty :

Women with glandular abnormalities detected during cervical smears may have squamous or glandular neoplasia. This study shows that the Asian-American variants of HPV 16 are associated with histological findings of adenocarcinoma in women with glandular abnormalities in cervical smears, while European variants are associated with findings of squamous neoplasia.

Impact

The analysis of molecular variants adds important information to currently available knowledge on the association between HPV and cervical carcinogenesis related to the definition of histological types.

Abstract

HPV genotypes cannot fully explain the histological diagnosis of women with glandular abnormalities detected by cervical smear. Thus, this study was designed to analyze the distribution of HPV 16 and HPV 18 variants in women referred due to atypical glandular cells (AGC) and adenocarcinoma in situ (AIS) in their cervical smears, and its association with histological results. Twenty-four women with HPV 16 and six with HPV 18, selected from 160 women with cervical smears suggestive of glandular abnormalities, were included. Histological results showed cervicitis (1 case), squamous neoplasia (18 cases), glandular neoplasia (7 cases), and glandular neoplasia associated with a squamous component (4 cases). Among the twenty-four cases presenting HPV 16, the European variant was detected in 15 (62%) and the Asian-American in 9 (38%). Among the 15 cases associated with the European variant, 14 presented squamous neoplasia and only one invasive adenocarcinoma. Asian-American HPV 16 variants were significantly associated with histological diagnosis of glandular neoplasia alone, OR 9.3 (1.4-.60.2), or associated with squamous neoplasia, OR 18.7 (1.5-232.3). Adenocarcinomas were detected in 4/6 HPV 18-positive cases. Of these, two cases had the European variant, one the Asian Amerindian variant and one the African variant. The association of HPV 16 with squamous or glandular neoplasia is explained by its variants. In this study, squamous neoplasia was related to the European variant of HPV 16, while glandular neoplasia was related to the Asian-American variant. Glandular neoplasia is associated with HPV 18, but the results of our analysis of its variants were inconclusive.

Introduction

Persistent infection with oncogenic human papillomavirus (HPV) is strongly associated with cervical squamous or glandular cancer and its precursor lesions[1, 2, 3, 4]. HPV types 16 and 18 are the most prevalent genotypes in all cases of cervical cancer; however the distribution of the various HPV genotypes differs according to histologic diagnostic. HPV 16 can be present in squamous cell carcinoma (SCC) and adenocarcinoma (AC) of the cervix, whereas HPV 18 plays a prominent role in the development of AC [2, 5, 6.]

Variations in DNA sequences between HPV 16 and HPV 18 have been found in various geographic locations and ethnic groups[7-9]. For HPV 16, the long control region (LCR) from a worldwide collection of cervical samples demonstrated that HPV-16 sequence variants should be grouped within branches based on the nucleotide difference profiles[10,11]. These variant branches are referred to as European (E), Asian (As), Asian-American (AA) and African (Af), and form the distinct phylogenetic clusters distributed within the continents[8, 10]. The sequence considered to be the reference for HPV 16, referred to as the prototype, belongs to the European branch [12]. Each cluster presents a prototype and some variation. The European (E) variants are detected in all regions of the world except Africa. The Asian variant (As) is found in Southeast Asia. The Asian-American (AA) variants are found principally in Central and South America and Spain, and in addition have been identified in two specimens from Southeast Asia. The African variants (Af1 and Af2) are found mainly in Africa [8].

The intra-type diversity of HPV 18 has striking similarities to that of HPV 16, and HPV 18 has been classified in three variant branches: European (E), African (Af) and Asia Amerindian (ASAI). The HPV-18 reference clone derives from a sample from a patient in northeastern Brazil [11].

There are indications that variants of the same HPV type differ biologically and etiologically [6,9,13-15]. Such differences may contribute to the disparities found throughout the world in the incidence of cervical cancer associated with the same HPV type. However, this question still requires substantial research before conclusions can be drawn[9, 16,17].

There is little information about the distribution of HPV 16 and 18 variants in Brazil. Studies have shown that infections with non-European branch variants of HPV 16 and HPV 18 have a tendency to be more persistent and are more associated with pre-invasive lesions [9, 18]. Women with glandular endocervical abnormalities in their cervical smear can reveal squamous or glandular neoplasia [4,19,20]. This association could be related to HPV types and variants. Therefore, this study was designed to analyze the association of HPV 16 and HPV 18 variants with histological diagnosis in women referred due to atypical glandular cells (AGC) and adenocarcinoma in situ (AIS) in their cervical smears.

Patients and methods

Study subjects

Twenty-six women with HPV 16 and seven with HPV 18 were selected from 160 women with cervical smear screening results suggestive of glandular abnormalities. Twenty-four women with HPV 16 and six with HPV 18 had samples available for sequencing and then were included in the study. The women were attended at the Colposcopy Clinic of the State University of Campinas, Brazil between March 2002 and March 2005. The average age of patients with squamous neoplasia, glandular neoplasia or glandular neoplasia associated with squamous neoplasia was 39, 49 and 43 years, respectively. All cervical smears were examined by two observers who established the cytological diagnosis by consensus: AGC not otherwise specified (NOS), AGC favor neoplasia (FN), AGC associated with high grade squamous intraepithelial

lesion (HSIL) and AIS. Eligible women were informed of the study protocol. Women were considered eligible if they met all of the following requirements: a) age between 18 and 60 years; b) had an intact uterus (i.e. no previous surgical procedure of the cervix or corpus); c) had no history of abnormal cervical smear in the past year; d) were not under treatment for genital condyloma (external or cervical); e) had abstained from sexual intercourse during the three days prior to the consultation; and f) had no confirmed or clinically suspected immunossuppression (HIV, corticosteroids, chemotherapy or other chronic diseases that might compromise the immune system). After signing the Informed Consent, women were subjected to a thorough pelvic examination, consisting of collection of a second cervical smear followed by collection of a sample for polymerase chain reaction (PCR), colposcopy, directed punch biopsy or cervical conization according to the cytologic and/or colposcopic abnormalities present. All women underwent pelvic ultrasound examination.

All histology was performed at the same pathology laboratory by the same pathologist, who was unaware of the cytological diagnoses. Specimens for pathological analyses were stained with hematoxilin and eosin (HE) and were reviewed according to the WHO [21] criteria. Lesions were classified as: (1). Non neoplastic diagnoses including cervicitis, squamous metaplasia, tubal metaplasia, tunnel cluster hyperplasia, microglandular hyperplasia and polyps or (2) neoplastic diagnoses including: cervical intraepithelial neoplasia (CIN) of squamous origin, grade 1, 2 or 3, invasive SCC, in situ adenocarcinoma (AIS) associated or not with squamous lesion, invasive adenocarcinoma associated or not with SCC.

HPV DNA detection and genotyping

HPV detection and typing were performed using Roche line blot assays (reagents kindly provided by Roche Molecular Systems, Inc., Pleasanton, CA). This assay involved the

hybridization of a 450 nt PCR amplicon generated by the PGMY primer set to a nylon strip containing immobilized probes[22,23]. Twenty-four samples positive for HPV 16 and six for HPV 18 were used for variant analysis.

Molecular variant analysis

HPV 16-positive samples were classified into phylogenetic branches based on the nucleotide sequence of the E6 and the MY09/11 region of L1 [8,9]. HPV 18-positive samples were classified into phylogenetic branches on the basis of variation of the long control region (LCR) [9,11]. The molecular variants of HPV 16 and 18 were tested using a PCR sequencing method (9). A 364 bp segment (nt 7478-7841) or a 321 bp segment (7462—7825) within the HPV-16 nucleotide or HPV-18 LCR, respectively, was amplified as previously described [24,25]. The PCR products were cloned using the TOPOTA cloning vector (Invitrogen) and transformed into *E. coli* strain XL1-Blue. Recombinant plasmid DNA was isolated from positive clones and sequenced by automated DNA sequencing in an ABI 3100 sequencing machine. Variants were grouped into geographical branches based on the nucleotide difference profiles: European (E), Asian (As), Asian-American (AA) and African (Af) in the case of HPV-16; and based on the different LCRs: European (E), African (Af) and Asia Amerindian (ASAI) in the case of HPV-18.

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Data Analysis

All statistical analysis was carried out using the SAS software, version 8.0. Odds ratios (OR) with 95% confidence interval (95%CI) were used to evaluate the associations between HPV variants and histological results.

Results

Results are shown in Table 1. Of the 24 women with HPV 16, 15 with cervical intra-epithelial neoplasia (1 CIN1; 3 CIN2; 11 CIN3), two with squamous invasive neoplasia, 5 were found to have glandular lesions, while 2 had glandular lesions associated with squamous neoplasia. With respect to HPV 18, one woman presented only cervicitis, while another presented CIN3, two had AIS (in one case AIS was the only diagnosis while in the other it was associated with CIN3), and there were two cases of invasive adenocarcinoma (one alone and the other associated with CIN 3).

The most prevalent HPV 16 variant was the European branch (15/24), of which the European B-12 variant was identified in 7 samples, prototype in 7 samples and G-10 variants in 1 sample. The prevalence of the Asian-American variant was 38% (9/24). The European variant of HPV 16 was associated with squamous invasive or preinvasive neoplasias in 14 out of 15 cases. Asian-American HPV 16 variants were significantly associated with glandular neoplasia alone, OR 9.3 (1.4-60.2), or associated with squamous neoplasia, OR 18.7 (1.5-232.3) (Table 2).

The most frequent HPV 18 variant was also the European branch (3 cases). Histologically, the European variants were related to one case of cervicitis, one AIS and one invasive adenocarcinoma associated with CIN 3. Two cases were classified as the Asian-Amerindian variant, one of them associated with adenocarcinoma in situ and with CIN 3, and the other with CIN 3. One case that was classified as the African variant was diagnosed as invasive adenocarcinoma. Among the glandular neoplasia cases, two out of four had HPV 18 European variants.

Discussion

The main finding of this study was the significant association of the Asian-American variant of HPV 16 with cervical adenocarcinoma. The prevalence of the Asian-American and European variants was, respectively, 38% and 62%. Nevertheless, six out of nine (67%) cases of the Asian-American variant revealed adenocarcinoma associated or not with squamous neoplasia in the histological diagnosis, while only one out of 15 (7%) of the European variant revealed squamous neoplasia, resulting in a very high odds ratio. These results are in accordance with those of other studies that showed Asian-American and European variants as more prevalent in glandular and squamous neoplasia, respectively [6,13,26,27].

European variants of HPV 16 have been identified as the most prevalent (76.8%) in Central and South America, and in specimens of invasive cervical carcinoma from Brazil the prevalence was 52.2% [8]. A cohort study conducted in São Paulo, Brazil also showed 54% of European variants in women infected with HPV 16 who had no cervical disease [9]. Another Brazilian study reported 50% of European variants in 34 women infected with HPV 16 with abnormal cervical smears including atypical squamous or glandular cells, cytological alterations suggestive of HPV infection, cervical intraepithelial neoplasia, squamous cell carcinoma, and adenocarcinoma [18]. The prevalence of Asian-American variants detected in our study (38%) was higher than that reported by Villa et al [9] (22%) and Yamada et al (19.7%), but similar to the prevalence detected by Cruz et al [18] (41.2%). If the Asian-American variant of HPV 16 is associated with adenocarcinoma, then patients presenting AGC or AIS should present a higher prevalence of this variant.

Glandular abnormalities in cervical smears pose clinical problems because of the poor correlation between smear result and final histopathology. The most important factor in this diagnosis is the high percentage of cases associated with underlying high-grade disease that included a wide spectrum of pathological findings such as pre-invasive and invasive squamous

and glandular lesions [4,19, 20]. Because HPV types express different oncogenic potential, it is reasonable to hypothesize that intra-type variants could also have differential oncogenicity. In fact, previous studies have shown the same higher oncogenic potential of the Asian-American variant of HPV 16 [9, 13, 26] as reported in our study.

Regarding HPV-18 variants, the European branch was also the more prevalent (50%), followed by the Asian-Amerindian (33%) and African clusters (17%), and these results are in accordance with the only study that also evaluated HPV 18 variants in Brazil [9]. However, the Asian Amerindian variant detected in our study was not observed in the study carried out by Villa et al [9]. Additionally, HPV 18 variants also differ in oncogenic potential. Non–European variants are more frequently persistent and more often associated with high grade lesions and cervical cancer compared to European variants [9, 15].

HPV 18 intra-type variants are differentially associated with adenocarcinomas and SCC of the cervix. The Asia-Amerindian HPV 18 variants were significantly associated with adenocarcinoma and the African HPV variants were exclusively related to SCC [15, 7]. Other studies failed to observe this correlation [6]. Our series showed that the distribution of HPV 18 was related principally to glandular neoplasia. However, we failed to find any significant association between HPV 18 variants and histopathology, probably because of the small sample size.

Populations of mixed ethnicity present HPV-16 and HPV-18 variants deviated from the apparent ethnic composition of the population. Ong et al [11] found that one in six HPV-18 variants from a non-indian population came originally from the American Indian population in Brazil. Nevertheless, the population of Brazil has a greatly mixed ethnicity, with Caucasian, African and minor American Indian genetic components. This fact may reflect biological differences favoring the spread or bottlenecks of variants in the establishment of viral composition [11.]

Cofactors associated with endocervical adenocarcinomas and SCC are distinct and bear more resemblance to those observed in endometrial cancer, pointing towards hormonal involvement [5,28]. Burk et al. [6] suggested that normal endocervical columnar cells invariably express estrogen and progesterone receptors, whereas basal cells in the squamous epithelium are negative for progesterone receptors and infrequently positive for estrogen receptors. Glandular and squamous neoplasia can differ according to hormone responsiveness. The HPV genome contains a number of glucocorticoid responsive elements and estrogen responsive elements that may play an important role in the regulation of the viral gene expression. At least two glucocorticoid responsive elements exist at positions 7477-7491 and 7643-7657 in the LCR region of the HPV 16 genome. Sequence variability in these two regions has been seen between different variants of HPV 16, and all the Asian-American variants differ within 7477-7491 glucocorticoid responsive elements. This may influence hormonal involvement, but the functional significance of these differences remains to be elucidated.

AGC and AIS in cervical smear most probably do not represent the actual lesion [19], but they may also represent immature cells with disordered cell proliferation. In fact, immature reserve cell in hyperplasia and immature metaplastic cells show similar morphological features, and it is not easy to differentiate between them [29]. Taking into account that HPV is the causal factor of cervical cancer [1], HPV may be responsible for the disorders manifested at the early stage of reserve cell hyperplasia.

Considering factors related to the HPV types and the reserve cell ability to develop into different epithelial cell types, we may hypothesize that the carcinogenic process could take two paths, one towards squamous neoplasia and the other towards glandular neoplasia. If HPV 18 is present, the preferential path could be towards glandular neoplasia. When HPV 16 is present, the

variants should be considered. If the Asian-American variant is present, the preferential path may be towards glandular neoplasia; if the European variant is present, the path is towards squamous neoplasia.

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Table 1: Histological findings and HPV 16 and 18 variants distribution

	Non neoplastic	CIN[3]	SCC	AIS[1]	AC[2]	Total
	n	n	n	n	n	n (%)
HPV 16						
AA (B2)		3		3[1]	3	9 (38)
E (prototype)		7				7 (30)
E (B12)		4	2		1	7 (30)
E (G10)		1				1 (2)
Total HPV 16		15	2	3	4	24 (100)
HPV 18						
ASAI (B-18-3)		1		1[2]		2 (33)
E (B-18-2)	1			1	1[2]	3 (50)
Af (T-18-8)					1	1 (17)
Total HPV 18	1	1		2	2	6 (100)
Total	1 (3)	16 (53)	2 (7)	5 (17)	6 (20)	30 (100)

CIN: cervical intre-epithelial neoplasia, SCC: invasive squamous cellcarcinoma, AIS: in situ adenocarcinoma, AC: invasive adenocarcinoma. 1 Two cases associated with squamous neoplasias (2 CIN3 and 1microinvasive carcinoma) 2 One case associated with CIN 3, [3] 15 cases diagnosed as CIN2 or CIN 3.

Table 2: Association of histological diagnosis and HPV 16 variant branches

Histological diagnosis	Variants branches		OR (CI95%)
	Asian-American	European	
Squamous Neoplasia	3	14	reference
Glandular Neoplasia	4	1	9.3 (1.4-60.2)
Glandular neoplasia associated or not with squamous neoplasia	6	3	18.7 (1.5-232.3)

4. Discussão

O diagnóstico citológico de anormalidades em células glandulares, incluindo AGC e AIS, revela com maior freqüência neoplasias cervicais escamosas e glandulares endocervicais. Sabe-se que as neoplasias cervicais escamosas e glandulares endocervicais têm em comum o fato de se originarem de células de reserva e de serem associadas com infecções persistentes por HPV de alto risco oncogênico (Demay, 1996; Andersson et al., 2001; Anderson et al., 2005). A maioria dos carcinomas cervicais origina-se de células de reserva hiperplásicas da endocérvice. Quando, depois de uma infecção por HPV, os ciclos celulares das células de reserva totipotentes são encurtados ou danificados por uma superestimulação hormonal ou toxicidade química, o reparo do DNA viral não é mais possível. As células imortalizadas podem tornar-se pré-malignas e malignas, com diferenciação para carcinomas escamosos ou adenocarcinomas (Dallenbach-Helweg, 1997).

Em condições normais, sem infecção de HPV, o estrogênio induz a diferenciação da célula de reserva para células glandulares com produção de muco, o que pode estar exacerbado em qualquer condição clínica que se caracterize pelo hiper-estrogenismo (Dallenbach-Helweg, 1997). Outros fatores poderiam induzir a diferenciação para epitélio escamoso normal (Demay, 1996). A presença persistente da infecção por HPV do tipo 18, ou variantes não-européias do tipo 16, poderia levar ao desenvolvimento de um adenocarcinoma a partir das células de reserva. Por outro lado, o HPV 16 da classe variante européia e, provavelmente, a maioria dos outros tipos de HPV de alto risco poderiam provocar um desvio deste processo para a neoplasia escamosa,

envolvendo processo metaplásico. O estímulo estrogênico atuaria como co -fator de risco para adenocarcinoma e também para a neoplasia escamosa (Elson et al., 2000).

De fato, cerca de 90% das lesões precursoras escamosas e dos carcinomas escamosos invasivos originam-se de zona de transformação. As células metaplásicas mostram maior susceptibilidade à transformação induzida pela infecção por HPV de alto risco oncogênico, e lesões intra-epiteliais escamosas, com freqüência, derivam de metaplasia imatura, que se desenvolve a partir de uma hiperplasia de células de reserva (Dufloth et al., 2005). Células metaplásicas imaturas possuem morfologia semelhante à das células caracterizadas como AGC e AIS, o que é coerente com o encontro de neoplasia escamosa na avaliação histológica subseqüente (Kurman et al., 1992; Covell et al., 2004).

Critérios citomorfológicos e características histológicas são melhores definidos para adenocarcinoma *in situ;* contudo, o diagnóstico de lesões precursoras menos severas que adenocarcinoma *in situ* ainda é controverso e de baixa reprodutibilidade, e mostra menor positividade para DNA-HPV (Kurman et al., 1992; Covell et al., 2004; Syrjanen, 2004). A ocorrência de hiperplasia atípica de células de reserva, sem metaplasia, é importante no desenvolvimento de adenocarcinomas. Atipias nucleares, em menor ou maior grau de estratificação epitelial, são características importantes no diagnóstico de lesões precursoras glandulares, porém ainda pouco conhecidas (Kurman et al., 1992; Lee et al., 2002).

Com base nos resultados deste estudo, podemos inferir que o papel do HPV na carcinogênese do colo uterino é muito significativo, não apenas como fator necessário para desenvolver a neoplasia do colo do útero, mas também determinando o tipo histológico. Até o presente momento o conhecimento sobre variantes avançou mais para os HPV dos tipos 16 e 18 porque são os mais freqüentes e, portanto, têm maior importância clínica. Também há algum conhecimento em relação aos tipos 45, 52, 58 (Lizano et al., 1997, Chan et al., 2002, Aho et al.,

2004), porém muito limitado em descrever a existência de polimorfismos genéticos, sem caracterizar o papel oncogênico. Para ampliar o conhecimento dos HPV menos prevalentes serão necessários estudos com casuísticas muito grandes. Todavia, pela menor prevalência dos demais tipos, a importância deste conhecimento é mais limitada.

Neste estudo, foi encontrada uma variante ainda não detectada no Brasil. O conhecimento da prevalência destas variantes ainda é limitado e, considerando a alta miscigenação da população brasileira, é muito provável que se encontre todas as variantes até então descritas. A identificação e o conhecimento da prevalência das variantes dos tipos de HPV em populações ou grupos étnicos específicos, possivelmente, poderão servir para orientar estratégias mais apropriadas de prevenção e controle da infecção pelo HPV.

5. Conclusões

5.1. O HPV 16 foi o tipo mais prevalente seguido pelo HPV 18. Considerando os carcinomas invasivos, a quase totalidade dos casos foi associada aos tipos 16 e 18. O HPV 16 foi mais prevalente em neoplasias glandulares do que o HPV 18. Houve maior diversidade de tipos considerando-se os diagnósticos histológicos de NIC 2 e NIC 3, sendo que, nestes casos, o segundo tipo mais prevalente foi o HPV 58.

5.2. O HPV 16, isoladamente, agrupado a outros tipos conforme sua seqüência de nucleotídeos (Grupo A) ou em infecções múltiplas, foi significativamente associado a neoplasias escamosas. Contudo, apenas o HPV 16 isolado foi associado a neoplasias glandulares. O HPV 18 mostrou associação significativa com neoplasias escamosas quando associado a outro tipo em infecção múltipla. O HPV 18 isoladamente ou em infecções múltiplas foi significativamente associado a neoplasias glandulares. O HPV 53, isoladamente, agrupado a outros tipos conforme sua seqüência de nucleotídeos (Grupo D) foi significativamente associado a neoplasias escamosas.

5.3. As Variantes Européias de HPV 16 foram mais prevalentes que as Variantes Ásia-Americanas. As Variantes Européias de HPV 18 também foram mais prevalentes, seguidas por Variantes Ásia-Amerindianas e Africanas.

5.4. As Variantes Ásia-Americas de HPV 16 foram mais prevalentes e significativamente associadas a neoplasias glandulares. Variantes Européias de HPV 16 foram mais prevalentes em neoplasias escamosas.

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8. Anexos

Anexo 1 - Carta para a paciente

Para Srª _____

Campinas, ____ de _____ de 200___

Prezada senhora,

O resultado do seu exame de prevenção (Papanicolaou) mostrou alterações nas células glandulares. A senhora deve realizar alguns outros exames para saber se tem alguma doença no colo do útero. Estes exames poderão ser realizados em uma consulta de ginecologia a ser realizada no Ambulatório do Trato Genital Inferior e Colposcopia do Centro de Atenção Integral à Saúde da Mulher (CAISM-UNICAMP), localizado à rua Alexandre Flemming, 101, Barão Geraldo, Campinas, São Paulo, CEP 13083-970, Cx Postal 6081. Assim, convidamos a para comparecer neste endereço numa das quintas feiras abaixo relacionadas das 12:00 hs às 13:30 hs. É necessário que a senhora esteja fora do período menstrual para poder realizar exames neste dia, e que TRAGA CÓPIA DO RESULTADO DO EXAME

Dia ____ de _____ de 200___ OU

Dia ____ de _____ de 200___, segundo a sua disposição.

Procurar no CAISM a Secretaria do Ambulatório de Oncologia responsável por marcar as consultas para a patologia cervical, falar com Regina . A senhora será atendida neste mesmo dia pela Drª Eliane Regina Zambeli Mesquita de Oliveira, Luís Otávio Zanatta Sarian ou Drª Sophie Françoise Mauricette Derchain ou Dr. Luiz Carlos Zeferino.

Se não puder comparecer ou precisar modificar o dia da consulta, favor entrar em contato com a Sra Márcia Ávila pelo telefone (0XX19) 3788-9516 das 8 às 12 hs ou das 14 as 16:30 horas.

Atenciosamente,

_____

Drª Luís Otávio Zanatta Sarian

Anexo 2 – Notificação para o profissional do posto.

Prezado (a) colega,

Estamos realizando uma pesquisa referentes às atipias de células epiteliais glandulares anteriormente designadas como AGUS: *células glandulares atípicas e* adenocarcinoma *in situ*. Para tal estamos convidando para o Centro de Atenção Integral à Saúde da Mulher (CAISM) no Ambulatório de Oncologia Ginecológica as mulheres com resultado de exame de prevenção (colpocitologia oncológica) com anormalidades das células glandulares do colo do útero (células endocervicais). Este estudo tem como objetivo analisar os critérios morfológicos (definidos pela revisão do Sistema de Bethesda de 2001) observados no exame citopatológico de mulheres com diagnóstico de anormalidades em células glandulares em associação à presença de tipos específicos de HPV(s) oncogênicos (detectados por PCR) e verificar a possibilidade desta associação ser preditiva do tipo histológico das lesões neoplásicas de colo uterino detectadas no seguimento. Todos estes exames que serão realizados estão indicados para o diagnóstico da lesão do colo do útero quando o exame de prevenção mostra as alterações que foram encontradas neste exame.

Ao voltar ao ambulatório para receber os resultados dos exames as mulheres serão tratadas de acordo com a necessidade. Se desejarem permanecer na Instituição, essas mulheres serão acompanhadas neste serviço. Se houver preferência para ser acompanhada no serviço de origem, será reencaminhada com carta de referência contendo o resultado dos exames e procedimentos aqui realizados. Toda paciente será esclarecida quanto ao seu direito de não participar da pesquisa e de ser atendida no ambulatório sempre que necessário. Em caso de dúvidas ou esclarecimento telefonar para a Dr. Luiz Carlos Zeferino no número (19)37889300.

Atenciosamente,

Luis Otávio Zanatta Sarian

Anexo 3 – Consentimento informado

PESQUISA: Relação entre critérios morfológicos e tipos de HPV detectados por PCR em mulheres com atipias das células glandulares do colo uterino com o diagnóstico histológico.

RESPONSÁVEL: Dr. Luiz Carlos Zeferino

Eu, Sra _____,

idade_____,RG_____,endereço_____

_____,

registro hospitalar _____, atendida no Centro de Atenção Integral à Saúde da Mulher (CAISM) no Ambulatório de Oncologia do Trato Genital Inferior e Colposcopia fui convidada a participar desta pesquisa porque o resultado do meu exame de prevenção (colpocitologia oncológica) mostrou anormalidades das células de dentro do colo do útero (células endocervicais). Sei que responderei a um questionário sobre informações pessoais mantendo meu anonimato (serão avaliadas somente pelo médico que me atendeu) e que as fichas ficarão de posse do(s) Doutore(s) responsáveis pela pesquisa, Dr. Luiz Carlos Zeferino e Drª Sophie Françoise Mauricette Derchain.

Sei que serei submetida à uma investigação que é necessária para esclarecer as alterações encontradas no meu exame preventivo e receber o tratamento que for preciso. Esta investigação consta de novo preventivo, e da mesma forma,será colhida secreção para descobrir se existe um tipo de vírus chamado HPV relacionado ao meu problema. Esta secreção será utilizada para fazer um exame chamado de reação em cadeia da polimerase (PCR). Será realizado também um exame colposcópico, no qual o médico vai olhar o colo do meu útero com lente de aumento, e caso seja encontrada alguma alteração, esta será biopsiada, ou seja, será retirado um pedaço muito pequeno para saber com certeza o que eu tenho. Esta biópsia é feita

no ambulatório, é simples, não dói e o máximo que pode causar é um pequeno sangramento, que logo pára. Caso seja descoberto que eu tenho uma alteração mais grave, ou seja, maior possibilidade de transformar-se em câncer, serei submetida a uma retirada de pedaço maior, feito com anestesia local para que eu não sinta dor. Este procedimento também é simples, normalmente feito no ambulatório, e às vezes pode causar um sangramento maior, porém caso isto ocorra, serei tratada com segurança, pois estarei no hospital com médicos capacitados para resolver o problema.

Todos estes exames estão indicados para o esclarecimento, diagnóstico e tratamento de possíveis lesões de colo de útero que podem estar presentes em casos como o meu. Não serei submetida a exames desnecessários, tudo será feito como normalmente se faz, de acordo com os programas de prevenção do câncer do colo uterino, e tratamento adequado. Após o diagnóstico e tratamento inicial dependendo da indicação médica, deverei retornar em intervalos de mais ou menos 6 meses para acompanhamento e controle de meu problema.

Autorizo o Doutor Luiz Carlos Zeferino a realizar uma cópia dos resultados dos exames de laboratório para que sejam anexados às fichas de pesquisa.

Fui esclarecida quanto ao meu direito de não participar da pesquisa e de ser atendida no ambulatório sempre que necessário. A não aceitação na participação no estudo não implicará na perda dos direitos iniciais rotineiramente oferecidos pelo ambulatório. Em caso de dúvidas ou esclarecimento, tenho o direito de telefonar para a Doutor Luiz Carlos Zeferino número (19) 37889300 ou para o Comitê de Ética da UNICAMP no número (19) 37888836. Sei que não serei paga para participar deste estudo.

Campinas, de de 200

_____

Rúbrica da paciente

_____

Dr.Luiz Carlos Zeferino

Anexo 4 – Ficha pré-codificada para conduta diagnóstica e terapêutica

Ficha I__I__I__I HC I__I__I__I__I__I__I__I

Data da primeira consulta I__I__/__I__/__I__I

Nome completo

Endereço: rua número

Bairro cidade

Telefone: ( )

Telefone para contato: ( ) falar com:

Observação:

1.Citologia oncológica:

1.1.Encaminhamento Data__|__/__|__/__|__

Número I__I__I__I__I__I__I__I__I Código:_____

1.2.Serviço Data__|__/__|__/__|__

Número I__I__I__I__I__I__I__I__I Código: _____

2.Colposcopia Data__|__/__|__/__|__

| 1 | Ausência de imagem | 2 | Epitélio aceto-branco | 3 | Mosaico suspeita

| 4 | Vasos atípicos | 5 | Pontilhado | 6 | Leucoplasia | 7 | Schiller positivo

| 8 | Insuficiente | 9 | não realizado Outros_____

3.Anátomo-patológico:

3.1.Biópsia de colo InãoI IsimI Data__|__/__|__/__|__

Nº Biópsia | __ | __ | __ | __ | __ | Código:_____

_____ _____

3.2.Conização a frio InãoI IsimI Data__|__/__|__/__|__

Nº Biópsia | __ | __ | __ | __ | __ | Código:_____

3.3. MARGENS | | livres | | comprometidas Código_____

3.4. LEEP InãoI IsimI Data __|__/__|__/__|__

Nº Biópsia | __ | __ | __ | __ | __ | Código_____

MARGENS | | livres | | comprometidas Código _____

3.5. I__I HISTERECTOMIA I__I WERTHEIM-MEIGS

Nº Biópsia | __ | __ | __ | __ | __ | Data __|__/__|__/__|__

Código_____

MARGENS | | livres | | comprometidas

Código_____

LINFONODOS I I livres I I Comprometidos

I- Código de Citologia Oncológica:

I.I. Diagnóstico citológico

I1INegativo para Lesões Intra-epiteliais ou Malignidades

I1AI Normal

I1BI Alterações celulares benignas

I3I ASC-US I4I ASC_H I5I HPV I6I NIC I I7I NIC II I8I NIC III

I9I Carcinoma escamoso invasivo

I10I AGC(SOE) │11│AG (NEO.) │12│ AIS │13│Adenocarcinoma invasivo

I14I Outras neoplasia invasivas

I.II. Células presentes

I1I Endocervicais I2I Metaplásicas I3I Endometriais

Outros_____

II. Código do resultado histológico:

I1I Cervicite I2I Metaplasia escamosa madura I3I Metaplasia escamosa imatura

I4I alteração glandular reacional I5I atipia glandular neoplásica sem outras especificações

I6I adenocarcinoma "in situ" I7I Adenocarcinoma invasivo

I8I Carcinoma adenoescamoso invasivo

I9I HPV/Condiloma I10I NIC I I11I NIC II I12I NIC III I13I Carcinoma escamoso microinvasivo I14I Carcinoma escamoso invasivo

III. Testes moleculares

I1I Negativo para DNA de HPV
I2I Positivo para DNA de HPV

III.I Genotipagem

I1I HPV 6	I10I HPV 16	I19I HPV 52
I2I HPV 11	I11I HPV 18	I20I HPV 55
I3I HPV 40	I12I HPV 26	I21I HPV 56
I4I HPV 42	I13I HPV 31	I22I HPV 58
I5I HPV 53	I14IHPV 33	I23I HPV 59
I6I HPV 54	I15I HPV 35	I24I HPV 68
I7I HPV 57	I16I HPV 39	I25I MM4(W13B)
I8I HPV 66	I17I HPV 45	I26I MM7(P291)
I9I HPV MM18(P155)	I18IHPV 51	I27I MM8(P155)

III.I Tipo de variante relacionada aos HPV 16 e 18 detectada: