Human papillomavirus genotyping and p16^{INK4a} expression in cervical intraepithelial neoplasia of adolescents

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Adolescents have high rates of human papillomavirus (HPV) infection, and persistent high-risk HPV infection can lead to the development of cervical cancer. The cyclin-dependent kinase inhibitor, p16^{INK4a} is overexpressed in cervical intraepithelial neoplasia (CIN), probably due to a persistent and integrated HPV infection. This study investigated p16^{INK4a} expression, grades of CIN, and high-risk HPV infection in adolescent cervical biopsies. Biopsies were immunohistochemically stained for p16^{INK4a}. The presence of wide-spectrum, low-risk, or high-risk HPV was determined by amplifying DNA extracted from the cervical biopsies. Biopsies were classified as cervicitis, 15 cases; CIN 1, 48 cases; CIN 2, 46 cases, and CIN 3, 52 cases. The distribution of p16^{INK4a} staining was graded as patchy, diffuse basal, and diffuse full thickness. Pearson's χ^2 tests analyzed the relationships between p16^{INK4a} staining, HPV infection, and CIN. Biopsies of cervicitis were negative for HPV and for p16^{INK4a} expression. High-risk HPV 16, 18, and 31 increased from 18% in CIN 1 to 66% in CIN 2/3 ($P < 0.001$). In CIN 1, p16^{INK4a} was positive in 44% of biopsies with 35% showing patchy, 7% diffuse basal, and one case (2%) showing diffuse full thickness staining. In CIN 2/3, p16^{INK4a} was positive in 97% of biopsies with 23% showing patchy, 21% diffuse basal, and 53% diffuse full thickness staining. The difference in the proportions of biopsies showing patchy p16^{INK4a} staining in CIN 1 and diffuse full thickness staining in CIN 2/3 was significant ($P < 0.001$). In CIN 1, 61% of high-risk HPV-positive biopsies were p16^{INK4a} negative, while all high-risk HPV-positive CIN 2/3 biopsies were p16^{INK4a} positive. Diffuse, full thickness p16^{INK4a} expression discriminated low-grade from high-grade CIN and appears to be a marker of persistent high-risk HPV infection.

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Early sexual activity with multiple partners places many female adolescents at high risk for the future development of cervical cancer. The prevalence of human papillomavirus (HPV) infection in adolescents ranges from 19 to 46%, but the cervical intraepithelial neoplasia (CIN) in adolescents are predominantly low-grade and in the great majority of cases they resolve spontaneously. Only 0.12–3% of adolescents known to be infected with high-risk

HPV develop high-grade CIN, and cervical carcinoma is extremely rare.^{1–5} Woodman *et al*⁴ reported that adolescents infected with HPV 16, 18, and 31 had a higher risk of developing high-grade CIN than persons infected with other oncogenic HPV types. Moscicki *et al*⁶ observed that persistently positive high-risk HPV testing precedes the development of high-grade CIN.

High-risk HPV DNA can be detected in almost all high-grade CIN and cervical cancers.^{7,8} From a screening study of 7932 women aged 21–30 years, Clavel *et al*⁹ reported that 23% had high-risk HPV infections. For the majority of women, the infections are transient and last 8–10 months.^{9–11} It was the repeated positive high-risk HPV test results, reflecting persistent infection that predicted a likelihood of having a high-grade CIN.^{3,9}

In susceptible adolescents, either repeated exposure or an inability to suppress immunologically an infection may promote the oncogenic potential of

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the virus.⁶ The cyclin-dependent kinase (CDK) inhibitor p16^{INK4a} overexpression in high-grade CIN and cervical cancer appears to be related to viral integration during the neoplastic transformation of cervical cells.^{12–16} The purpose of this study was to observe in CIN of adolescents from high-risk clinics in Mississippi the distribution of HPV types 16, 18, 31, 33, 35, 45, 52, and 58 that are considered oncogenic by the International Agency for Research on Cancer (IARC).¹⁷ The relationship of HPV typing with p16^{INK4a} expression in different grades of CIN was evaluated in order to assess the ability of p16^{INK4a} immunostaining to identify transforming oncogenic viral infection and an increased risk of developing cervical cancer.

Material and methods

Case Selection

This study was approved by the Institutional Review Board of the University of Mississippi Medical Center. The study material consisted of formalin-fixed, paraffin-embedded blocks of cervical biopsies from 161 adolescents accessioned at the Department of Pathology, the University of Mississippi Medical Center between 1992 and 2002. The age of adolescents ranged from 12 to 19 years old, with a median age of 18 years old. None of the

patients had been treated for cervical abnormalities prior to the biopsy. The specimen included 15 cases of within normal limits/cervicitis, 48 cases of CIN 1, 46 cases of CIN 2, and 52 cases of CIN 3.

HPV DNA Detection and Genotyping

DNA was extracted using the microwave method.¹⁸ Wide-spectrum HPV DNA was amplified by PCR using general primers GP5 + /GP6 + within the L1 open-reading frame.¹⁹ Specimens negative for wide-spectrum HPV DNA were tested for β -globin using PCR amplification with primers GH20 and PC04.²⁰ If both wide-spectrum HPV DNA and β -globin were negative, the samples were considered unsatisfactory and eliminated from the study. The specimens positive for wide-spectrum HPV DNA were genotyped by PCR for HPV types 16, 18, 31, 33, 35, 45, 52, and 58 using type-specific primers^{21–23} (Table 1). A total of 35 PCR cycles were performed as follows: denaturation at 94°C for 1 min, annealing for 1 min at temperatures indicated in Table 1, extension at 72°C for 1.5 min. The positive controls for HPV genotyping were plasmids containing corresponding HPV DNA (plasmids containing HPV types 16, 18, 35, and 52 were from ATCC, Manassas, VA; HPV 31 was from Dr Wayne Lancaster, Wayne State University; HPV 33 was from Dr Gerard Orth, Pasteur Institute, France; HPV 45 was from Dr E-M de

Table 1 Consensus and type-specific primers for HPV DNA amplification

Primers	Primer sequences	Location	Annealing temperature (°C)	References
GP5+ GP6+	+5'-TTTGTACTGTGGTAGATAC TAC-3' -5'-GAAAAATAAACTGTAAATCATATTC-3'	L1	40	19
16.1 16.2	+5'-ACCGAAACCGTTAGTATAAAAGC-3' -5'-ATAACTGTGGTAACTTCTGGGTC-3'	50–73 149–126	55	21
18.1 18.2	+5'-CGGTCGGGACCGAAAACGGTG-3' -5'-CGTGTGGATCCTCAAAGCGCGCC-3'	50–70 130–106	55	21
31.1 31.2	+5'-AGAAAGACCTCGGAAATTG-3' -5'-TACCTCTGTTCTGTTAAC-3'	125–143 233–215	54	22
33.1 33.2	+5'-CTACAGTGCCTGGAATGCAAAAAACC-3' -5'-CGGGACCTCCAACACGCCGCAC-3'	190–215 536–515	65	22
35.1 35.2	+5'-ACAAGAATTACAGCGGAG-3' -5'-TAACTGTTTGTTCATTGT-3'	211–215 397–379	50	22
45.1 45.2	+5'-ACCAGGCACGGCAAGAAAGACT-3' -5'-CGTGGGCTGGTAGTTGTGC-3'	530–551 748–728	62	This study
52.1 52.2	+5'-CACAGTGTAGCTAACGCACGGCC-3' -5'-GTTGCTTTGTCTCCACGCATGACG-3'	79–101 572–549	65	23
58.1 58.2	+5'-GGCTACTGCAGGACTATGTTCC-3' -5'-CCTCTCATGGCGTTGTTACAGG-3'	95–116 581–560	65	23
pU-1M pU-31B pU-2R	+5'-TGTCAAAAACCGTTGTGTCC-3' +5'-TGCTAATTCGGTGCTACCTG-3' -5'-GAGCTGTGCGCTTAATTGCTC-3'	E6–E7	55	24

Villiers, German Cancer Research Center, Germany; HPV 58 was from Dr Toshihiko Matsuura, National Institute of Infectious Diseases, Japan). The replicated plasmid DNA for types 16, 18, 31, 33, 35, 52, and 58 were confirmed by PCR using consensus high-risk HPV primers (HPVpU-1M/pU-2R) followed by restriction enzyme digestion.²⁴ The primers of HPV type 45 were designed using GeneTools (Version 1.1, Edmonton, Alberta, Canada). The PCR product of HPV 45 (218bp) was confirmed by restriction enzyme digestion with *Ava*I (67:151). Distilled water instead of template DNA was used as negative controls for each set of PCR reactions.

Specimens positive for wide-spectrum HPV but negative for HPV type-specific PCR were further tested using consensus high-risk HPV primers (HPVpU-1M/pU-2R) and consensus low-risk HPV primers (HPV31-b/pU-2R) for HPV 6 and 11 followed by restriction enzyme digestion.²⁴ All of the amplification products and enzyme digestion products were electrophoresed in 2% agarose gels and were visualized by ethidium bromide.

Immunohistochemistry

All p16^{INK4a} immunostaining was performed manually over a period of 10 working days. Antigen retrieval was performed in 0.01M citrate buffer, pH 6.0, in a high-pressure cooker for 5 min. Mouse anti-human p16^{INK4a} monoclonal antibody (clone 6H12, Novocastra, UK) was used at a dilution of 1:60. Primary antibody was applied for 1h followed by 30 min incubation with biotinylated anti-mouse IgG and 30 min incubation with avidin-biotin-peroxidase complex (ABC, Vector Laboratories, Burlingame, CA, USA). Color was developed by incubating with 3,3'-diaminobenzidine for 5 min. Finally, the sections were counter-stained lightly with hematoxylin. A p16^{INK4a}-positive CIN 3 cervical biopsy was used as a positive control. The negative control, consisted of the same section in which diluent without primary antibody, was applied.

Nuclear p16^{INK4a} staining in dysplastic cells with or without cytoplasmic staining was interpreted as a positive reaction. The staining pattern of p16^{INK4a} was classified as follows:

- Negative, if there were no positive cells or <1% of cells were positive.
- Patchy, if focally aggregated cells involving <25% of an area of epithelium were positive (Figure 1).
- Diffuse basal, if positive cells lay in continuity with each other in the lower half of a broad area of epithelium (Figure 2).
- Diffuse full thickness, if positive cells lay in continuity with each other and involved the full thickness of a broad area of epithelium (Figure 3).

Figure 1 Patchy pattern of p16^{INK4a} immunostaining: focal aggregates of p16^{INK4a} positive cells involve <25% of an area of epithelium.

Figure 2 Diffuse basal pattern of p16^{INK4a} immunostaining: positive cells lie in continuity with each other in the lower half of a broad area of epithelium.

Figure 3 Diffuse full thickness pattern of p16^{INK4a} immunostaining: positive cells lie in continuity with each other and involved the full thickness of a broad area of epithelium.

Statistical Analysis

Using the software package SPSS (version 11.5, Chicago, IL, USA), the Pearson χ^2 test was performed to compare the significance of the positive rates of wide-spectrum HPV, high-risk HPV types or p16^{INK4a}

expression in each grade of CIN. A *P*-value of <0.05 was considered statistically significant.

Results

A total of 15 cases of cervical biopsies diagnosed as within normal limits/cervicitis were negative for HPV DNA. One of these 15 cases was negative for β -globin. In the CIN biopsies that were negative for wide-spectrum HPV, three cases of CIN 1 and one case of CIN 3 were also negative for β -globin. These five cases were eliminated from the study.

Wide-spectrum HPV was found in 87% of CIN 1, 91% of CIN 2, and 96% of CIN 3, the differences were not significant (*P*=0.941) (Figure 4). The combined positivity of all high-risk HPV types increased from 40% in CIN 1 to 72% in CIN 2 and 75% in CIN 3 with the difference between CIN 1 and CIN 2 and 3 together (CIN 2/3) being highly significant (*P*<0.001) (Figure 4). Of wide-spectrum HPV-positive and high-risk HPV-negative cases, low-risk HPV 6/11 were positive in nine cases of CIN 1 (20%) and one case of CIN 3 (2%) (Figure 4).

Table 2 shows the distribution of eight types of high-risk HPV in the different grades of CIN. In CIN 1, HPV 52 was the predominant type (13%) followed by HPV 58 (11%), 16 (9%), 31 (7%), and 18, 35, and 45 (2% each). HPV type 33 was not identified. In CIN 2/3, HPV 16 increased significantly to 40% (*P*=0.007). HPV 18 at 12% and HPV 31 at 13% also increased although the number of cases studied did not allow the differences to achieve significance. HPV 52 and 58 remained about the same as in CIN 1. There were 10 cases with negative reactions using HPV type-specific primers

but having positive reactions using high-risk HPV consensus primers. Of these, specific high-risk HPV types were identified in six cases using restriction enzyme digestion. The remaining four cases (three CIN 1 and one CIN 3) that were positive using high-risk HPV consensus primers were classified as unknown high-risk HPV types, because specific HPV could not be identified by restriction enzymes digestion. Coinfection with high-risk HPV was found in 19 cases occurring in 11% of CIN 1, 9% of CIN 2, and 20% of CIN 3 (Table 3). HPV 16 and 31

Figure 4 Distribution of results for wide-spectrum, low-risk, and high-risk HPV in different grades of CIN. There was no significant difference of wide-spectrum HPV DNA between CIN 1 (87%), and CIN 2 (91%), CIN 3 (96%) (*P*=0.941). The difference in the distribution of high-risk HPV between CIN 1 and CIN 2 and 3 together (CIN 2/3) was highly significant (*P*<0.001).

Table 2 Distribution of high-risk HPV types in different grades of CIN in adolescent cervical biopsies

CIN grade	Cases n	High-risk HPV types								
		16	18	31	33	35	45	52	58	Unknown
CIN 1	45	4 (9%)	1 (2%)	3 (7%)	0	1 (2%)	1 (2%)	6 (13%)	5 (11%)	3 (7%)
CIN 2	46	18 (39%)	5 (11%)	5 (11%)	0	2 (4%)	1 (2%)	4 (9%)	2 (4%)	0
CIN 3	51	21 (41%)	7 (14%)	8 (16%)	1 (2%)	1 (2%)	1 (2%)	4 (8%)	4 (8%)	1 (2%)
										Total ^a
										18 (40%)
										33 (72%)
										38 (75%)

Abbreviations: HPV, human papillomavirus; CIN, cervical intraepithelial neoplasia.

The positivity of combined high-risk HPV types between CIN 1 and CIN 2/3 were significantly different (*P*<0.001).

^aCoinfection was observed in five cases of CIN 1, four cases of CIN 2, 10 cases of CIN 3.

Table 3 High-risk HPV coinfection in different grades of CIN in adolescent cervical biopsies

CIN grade	Cases n	Coinfection (No. of cases)	Total
CIN 1	45	16/58 (1); 16/31/58 (1); 31/52 (1); 52/58 (1)	5 (11%)
CIN 2	46	16/18 (1); 16/58 (1); 18/52 (1); 31/35 (1)	4 (9%)
CIN 3	51	16/18 (2); 16/31 (1); 16/45 (1); 16/52 (2); 16/58 (1); 31/52 (1), 31/58 (1); 31/35/58 (1)	10 (20%)

Abbreviations: see Table 2.

Table 4 Pattern of p16^{INK4a} expression in different grades of CIN in adolescent cervical biopsies

CIN grade	Cases n	Positive cases	Patchy	Diffuse basal	Diffuse full thickness
CIN 1	45	20 (44%)	16 (35%)	3 (7%)	1 (2%)
CIN 2	46	43 (93%)	14 (30%)	11 (24%)	18 (39%)
CIN 3	51	51 (100%)	8 (15%)	9 (18%)	34 (67%)

Abbreviations: see Table 2.

The distribution of the staining patterns of p16^{INK4a} between CIN 1 and CIN 2/3 were significantly different ($P < 0.001$).

were the types most commonly involved in coinfection with HPV 16 being found in 11 and HPV 31 in seven of the 19 biopsies.

Table 4 shows the p16^{INK4a} immunohistochemistry results. All 15 biopsies within normal limits/cervicitis were p16^{INK4a} negative. The positivity of p16^{INK4a} in CIN 1 was 44%. This increased to 93% in CIN 2 and 100% in CIN 3, with the difference in p16^{INK4a}-positivity between CIN 1 and CIN 2/3 being significant ($P < 0.001$). The pattern of p16^{INK4a}-positive staining in CIN 1 was predominately patchy, while in CIN 2/3, it was mainly diffuse full thickness. The distribution of the pattern of p16^{INK4a} staining between patchy in CIN 1 and diffuse full thickness in CIN 2/3 was also significantly different ($P < 0.001$).

Table 5 shows the relationships between p16^{INK4a} staining patterns and high-risk HPV positivity. In the 18 high-risk HPV-positive CIN 1 biopsies, 11 were p16^{INK4a} negative (61%) and seven were p16^{INK4a} positive with the staining pattern being patchy in five cases (28%) and diffuse basal in two cases (11%). In contrast, all CIN 2/3 biopsies that were high-risk HPV positive were p16^{INK4a} positive and the staining pattern was diffuse full thickness in 38 of the 71 cases (54%).

Discussion

Just as in several large cohort studies on adolescents and young women,^{4,25} we found that HPV 16, 18, and 31 were the most common oncogenic viruses occurring in high-grade CIN of adolescents. We further observed that these high-grade CIN were characterized by a diffuse expression of p16^{INK4a} in the transformed epithelium suggesting that p16^{INK4a} expression particularly in a diffuse pattern may serve as a marker for viral integration and a greater risk of progression to cancer.

Woodman *et al*⁴ studied 1075 British 15–19-year-old adolescents using type-specific PCR for HPV 16, 18, 31, 33, 52, and 58. The distribution of viral types in this cohort was HPV 16 in 27.0%; followed by 18 (15.7%), 33 (8.1%), 31 (7.6%), 58 (6.6%), and 52 (2.5%). Those infected with HPV 16 showed the highest relative risk (RR) of developing high-grade CIN (RR: 8.5). An increased risk was also observed

Table 5 Pattern of p16^{INK4a} immunostaining in grades of CIN in adolescent cervical biopsies that were high-risk HPV positive

CIN grade	p16 ^{INK4a} (+)/high-risk HPV (+)	Patchy	Diffuse basal	Diffuse full thickness
CIN 1	7/18 (39%)	5 (28%)	2 (11%)	0
CIN 2	33/33 (100%)	12 (36%)	9 (27%)	12 (36%)
CIN 3	38/38 (100%)	5 (13%)	7 (18%)	26 (68%)

Abbreviations: see Table 2.

with HPV 18 (RR: 3.3), 31 (RR: 3.5), 52 (RR: 2.3), and 58 (RR: 2.9). In contrast to the relatively high proportion of patients infected, HPV 33 had a minimal relative risk (RR: 0.6) of inducing high-grade CIN. In Denmark, Kjaer *et al*²⁵ evaluated CIN developing in a cohort of 10 758 women 20–29 years old. They found HPV 16 in 48%, 18 in 11%, and 31 in 17% of the high-grade CIN followed by 33 (8%), 45 (6%), 51 (7%), 52 (6%), 58 (6%), and 66 (5%).

Recently, Clifford *et al*²⁶ analyzed published data on HPV distribution in high-grade CIN and cervical cancer from different regions of the world. The pooled prevalence of oncogenic HPV in high-grade CIN was: HPV 16 (45.0%), 18 (7.1%), 31 (8.8%), 33 (7.2%), 35 (4.4%), 45 (2.3%), 52 (5.2%), and 58 (6.9%). This compares with the distribution in cervical cancer of: HPV 16 (54.3%), 18 (12.6%), 31 (4.2%), 33 (4.3%), 35 (1.0%), 45 (4.2%), 52 (2.5%), and 58 (3.0%), in which HPV 16, 18, and 45 were found at a greater frequency than in high-grade CIN. These findings suggest that CIN caused by HPV 16, 18, and 45 may preferentially progress to carcinoma.²⁶

Most CIN 1 regress and infection with oncogenic HPV is thought to be clinically significant only if it is persistent.²⁷ Epidemiological studies have shown that about 10% of women with high-risk HPV infection develop high-grade CIN.^{5,6,16} It is speculated that persistent infection by specific viral types, particularly HPV 16 and 18 has the greatest tendency to cause CIN 2/3.^{3,27} Kjaer *et al*²⁵ demonstrated that the risk for low-grade and high-grade CIN was markedly increased if there were repeat positive tests for HPV, but the most substantial increase in risk was for high-grade CIN if the repeat positive test was for the same HPV type. The odds ratio for developing high-grade CIN in women having repeat positive tests was 192.7 for nonidentical and 813.0 for identical HPV types compared to women tested as negative on two occasions.

In CIN 1, we did not find any prevailing HPV type, while in CIN 2/3, HPV 16, 18, and 31 become predominant occurring, respectively, in 40, 12, and 13% of cases. Together these three HPV increased from 18% in biopsies of CIN 1 to 65% in biopsies of CIN 2/3 while the proportion of other viral types remained approximately the same. This change is highly significant ($P < 0.001$) and may indicate that

infection with HPV 16, 18, and 31 has a greater tendency to persist compared to other potentially oncogenic viral types.

HPV 33 and 45 have been considered important causes of high-grade CIN and carcinoma in some populations of older women.²⁸ We found very few cases of HPV 33 and 45 in either CIN 1 or CIN 2/3. The relatively high proportion of HPV 18 and 31 in our biopsies compared to the pooled data published by Clifford *et al*²⁶ may reflect a higher prevalence of infection by these viral types in this adolescent population. However, the general prevalence of the different types of oncogenic HPV in Mississippi is not known.

The presence of p16^{INK4a} expression can be used to distinguish CIN from regular and atypical cervical squamous metaplasia in which staining is absent.¹⁶ Our findings additionally indicate that p16^{INK4a} expression may help to separate low-grade from high-grade CIN and to identify cases histologically diagnosed as CIN 1 that may contain persisting oncogenic HPV infections and have a high-risk of progressing to cancer. The expression of p16^{INK4a} without regard to pattern was found in 44% of CIN 1 but increased to 93% of CIN 2 and 100% of CIN 3. These results are virtually identical to the observations by Agoff *et al*¹⁶ in which the 57% of CIN1, 91% CIN 2, and 97% of CIN3 were p16^{INK4a} positive. These investigators also showed that p16^{INK4a} staining intensity increased with the severity of CIN.

In our biopsies, p16^{INK4a} expression in CIN 1 was predominantly patchy, while in CIN 2/3 the staining pattern was mainly diffuse full thickness. Sano *et al*¹³ described diffuse p16^{INK4a} expression in both CIN and cervical cancer infected with high-risk HPV. In their study, all high-grade CIN and 70% of low-grade CIN were positive for oncogenic HPV, and diffuse p16^{INK4a} expression was more closely related to high-risk HPV infection than the severity of CIN. In our study, diffuse patterns of expression were divided into diffuse basal and diffuse full thickness. Diffuse staining, particularly the diffuse full thickness pattern of p16^{INK4a} expression was closely associated with high-risk HPV infection and with high-grade rather than low-grade CIN.

In biopsies of CIN 1, we found that 61% (11/18) of cases that were high-risk HPV positive were 16^{INK4a} negative. That divergence was not found in high-grade CIN in which all high-risk HPV-positive biopsies were 16^{INK4a} positive. This may indicate that HPV infections in the 16^{INK4a}-negative CIN 1 biopsies are transient and that viral DNA is not integrated into the cells. Persistent HPV infection is identified by a patient having more than one positive test for the same high-risk HPV type, but repeated type-specific HPV testing would be too cumbersome to apply in clinical practice and may not distinguish persistent infection from reinfection.³

Expression of p16^{INK4a} may provide a surrogate marker for persistent HPV infection. Recently, we

compared p16^{INK4a} immunostaining with Hybrid Capture high-risk HPV DNA testing using liquid-based cytology and found that 16^{INK4a} immunostaining detected high-grade CIN better than the HPV DNA testing.²⁹ This approach may be particularly valuable for evaluating adolescents with low-grade squamous intraepithelial lesions (LSIL) on Pap smears. High-risk HPV DNA testing as a step in the evaluation of LSIL was not recommended by the ASCUS-LSIL Triage Study, because the results were positive in 85% of patients.³⁰ Adolescents with LSIL may be referred for colposcopy,^{30,31} but our results indicate that in CIN 1 there is considerable variability in the degree to which HPV appears to be integrated. Cytology using 16^{INK4a} immunostaining may be more effective than colposcopy or HPV DNA testing for identifying patients with high-grade CIN.

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