

Oncogenic human papillomaviruses and ploidy in cervical lesions

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Abstract

Aim—To compare ploidy measurements obtained on tissue sections of selected low and high grade squamous intraepithelial lesions containing oncogenic HPV (types 16, 18 or 33) detected by in situ hybridisation (ISH) or PCR.

Methods—DNA ploidy was assessed by image cytometry after Feulgen staining of contiguous serial sections of eight lesions exhibiting atypical squamous cells or squamous atypia and 53 low and 63 high grade squamous intraepithelial lesions in which HPV had been detected by ISH or PCR.

Results—Aneuploidy was strongly associated with the presence of oncogenic HPV, being detected in 50% of lesions with squamous atypia and 75.5% of the low and 95.2% of the high grade squamous intraepithelial lesions. The multiploid profile was highly associated with high grade lesions and with the pattern of HPV DNA integration.

Conclusions—The presence of aneuploidy is strongly suggestive of the presence of oncogenic HPV types. Combining the detection of HPV by ISH and PCR with DNA image cytometry may provide the pathologist and the physician with important prognostic information about low grade lesions, especially when these lesions have a multiploid DNA profile and contain oncogenic HPV.

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There is increasing evidence, from molecular biology studies,¹ epidemiological investigations^{2,3} and clinical observations,⁴ that human papillomavirus (HPV) is an aetiological agent in cervical carcinogenesis. More than 70 HPV types have been identified to date. HPV types 6, 11, 42, 43, and 44 are regarded as low risk, and types 16, 18, 33, and several others as high risk.⁵

Detection of oncogenic HPV types has been proposed as a method of evaluating the prognosis of low grade squamous intraepithelial lesions, such as condylomas and grade I cervical intraepithelial neoplasia (CIN I)^{6,7} or to detect high grade squamous intraepithelial lesions (including CIN II and III) in women with minor smear abnormalities.⁸ However, Downey *et al*⁹ showed that a histological diagnosis of low grade cervical disease was a better

long term predictor of disease progression than HPV type 16 positivity.

Thus, the prognostic significance of detecting high risk HPV DNA in low grade squamous intraepithelial lesions is still a matter of debate. The presence of high risk HPV does not necessarily lead to carcinogenesis. The possibility of regression of lesions containing HPV has been well established and has been estimated by Syrjänen¹⁰ to occur in 40% of patients. The role(s) of cofactors (immunosuppression, pregnancy, tobacco, microtraumas, nutritional and hormonal factors, number of sexual partners, and infections) in the progression of these lesions cannot be ignored.¹¹ The persistence of high risk HPV in dysplastic cervical lesions has also been found to be associated with progressive disease.¹²

DNA can be analysed using a variety of techniques, including flow cytometry,¹³⁻¹⁵ computerised digital imaging and image cytometry after Feulgen staining.¹⁶⁻²⁵ The DNA ploidy profile seems to be a useful indicator of prognosis in CIN. In a retrospective study of 100 women with all grades of CIN, Fu *et al*²² reported that, of 64 lesions that regressed, 85% were euploid and 15% were aneuploid. By contrast, of 58 lesions that persisted, 95% were aneuploid. All eight lesions that progressed to invasive cancer were aneuploid. Bibbo *et al*¹⁶ also showed that CIN lesions with a diploid profile were more likely to return to normal than those exhibiting an aneuploid pattern.

Nevertheless, while reports of ploidy or HPV detection in cervical lesions are numerous, few authors have used both approaches in parallel.¹⁷⁻¹⁹

The aim of the present study was to compare ploidy measurements obtained on tissue sections of selected low and high grade squamous intraepithelial lesions containing oncogenic HPV (types 16, 18 or 33) detected by in situ hybridisation (ISH) or PCR. DNA ploidy was assessed by image cytometry after Feulgen staining of contiguous serial sections of the same lesions in which HPV had been detected by ISH or PCR. This approach may provide more information as to the nature and evolution of low grade squamous intraepithelial lesions.

Methods

The study population comprised 115 patients with biopsy (n = 124) proven high risk HPV (types 16, 18 or 33) infection. Eight specimens exhibited squamous atypia (mean patient age 30 years), and 53 were low grade (flat condylomas and CIN I; mean patient age 29 years) and

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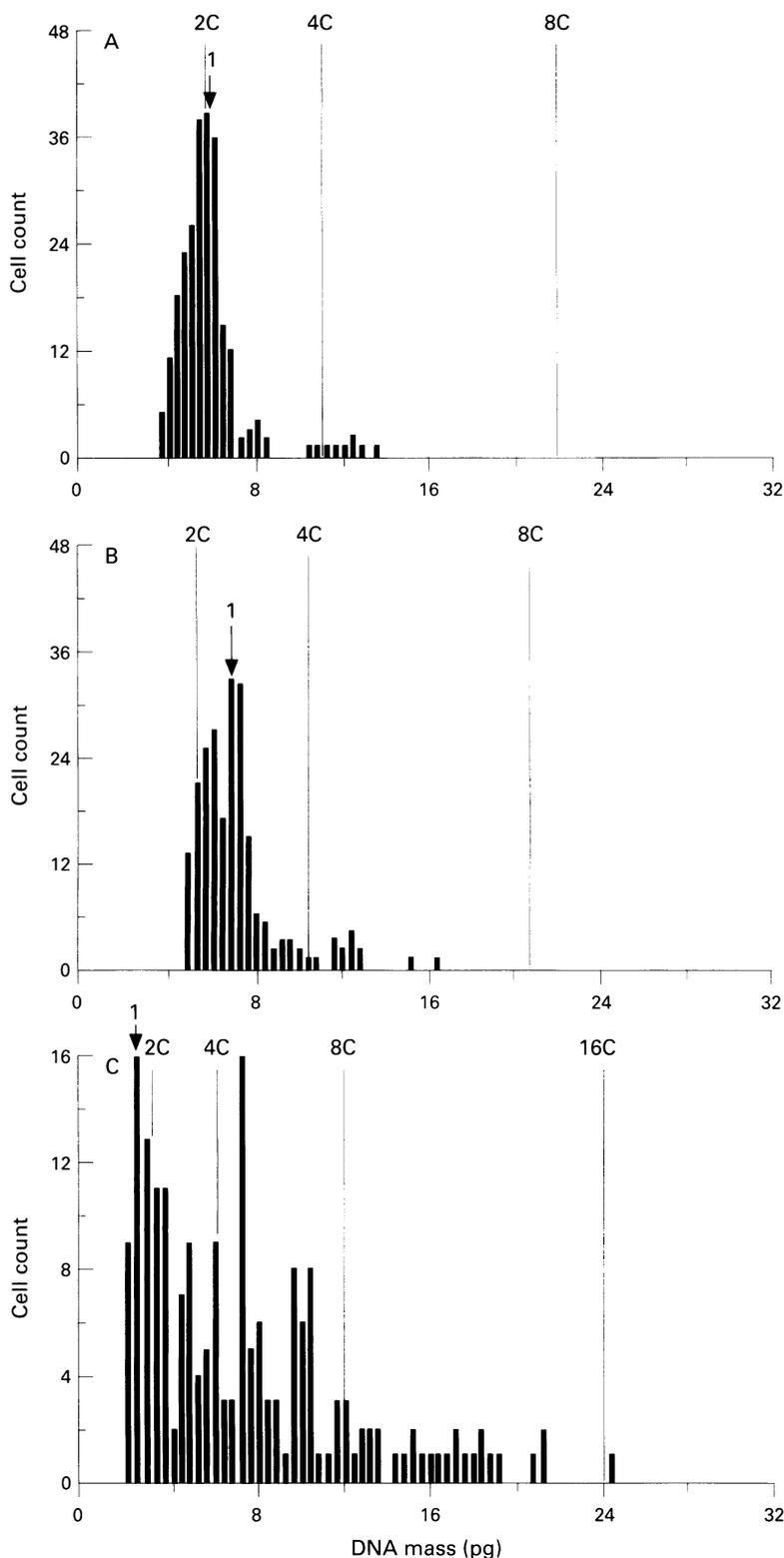


Figure 1 DNA quantification on 6 μ m sections. (A) Diploid profile; (B) single peak aneuploid profile; (C) multiploid profile (arrows indicate main cell population peaks).

63 high grade (32 CIN II and 31 CIN III; mean patient age 34 years) squamous intraepithelial lesions. Both low (except squamous atypia) and high grade squamous intraepithelial lesions displayed koilocytosis, which is evocative of HPV infection. HPV DNA was detected by ISH or PCR, or both.

Sixteen serial sections (3–6 μ m) of each biopsy specimen were used as follows: the first section was for conventional diagnosis, three for ISH, one for Feulgen staining and DNA

quantification, 10 for PCR, and the last to confirm that the entire series was still within the initial lesion. When this lesion was not found or was of a different grade, the case was excluded from the study.

Oncogenic HPV was detected by ISH using digoxigenin labelled probes applied to formalin fixed, paraffin wax embedded tissue sections, as described previously.²⁶ Positive controls for ISH and PCR comprised CaSki cells (HPV 16), HeLa cells (HPV 18) and CKEJ cells (HPV 33); negative controls comprised the pBR 322 plasmid and water. Although Southern blotting is the reference technique for the detection of integrated HPV DNA, this procedure is cumbersome and cannot be used in routine analyses. Therefore, we applied the relevant criteria of Cooper *et al*²⁷ to assess viral integration by ISH in tissue sections of the same biopsy sample: a diffuse signal throughout the nucleus corresponds to episomal HPV DNA and a punctate signal to integrated HPV DNA.

In some specimens whose histological patterns were suggestive of HPV infection but were negative by ISH, HPV DNA was extracted from contiguous serial sections and subjected to PCR, using the AMPLICIS kit (CIS Biointernational, Gif-sur-Yvette, France) which amplifies the E6 and E7 regions of HPV types 16 and 18.²⁸

DNA QUANTIFICATION

DNA was quantified by image cytometry using the CAS 200 image analysis system (Becton Dickinson, Leiden, The Netherlands) with the ploidy measurement software set at the "tissue section" option.²⁹ One contiguous 6 μ m paraffin wax section of each biopsy specimen was analysed. The wax was dissolved and tissue sections were rehydrated, hydrolysed for 60 minutes (5 N HCl) and stained with Feulgen stain.

The calibration slides were those recommended by the CAS system—that is, imprints of fresh rat liver. For the ploidy measurement software, at least 20 calibration cells were required to proceed to the quantification section. The cells were chosen by the computer and the user could accept or reject each cell. The coefficient of variation for the calibration histograms was always less than 3%.

Other reference cells to control for diploidy were chosen systematically outside the lesion but within the same tissue section (40 normal fibroblasts or normal cervical epithelial cells). To quantify DNA in the cervical cell nuclei, at least 200 cells were evaluated where possible.

The results are given with the DNA index (DI) which represents the ratio of the DNA content of the analysed nuclei to the DNA content of the reference cells in G₀/G₁ phase. By definition, the DI of a diploid population is 1 (fig 1A). The tetraploid pattern (DI = 2) was grouped with the diploid cases. When the index was different from 1 or 2, the cell was regarded as aneuploid. In this study, we observed two aneuploid profiles: a single aneuploid peak (fig 1B) or several aneuploid peaks (multiploid profile; fig 1C).

Table 1 The various types of HPV found in lesions with squamous atypica, and low and high grade squamous intraepithelial lesions with diploid, single peak aneuploid and multiploid profiles. Results are expressed as n (%)

HPV type	Squamous atypia			Low grade squamous intraepithelial lesions			High grade intraepithelial lesions			Total (%)		
	Diploid	Aneuploid	Total	Diploid	Aneuploid	Total	Diploid	Aneuploid	Total			
		(single peak)		Single peak	Multiploid		Single peak	Multiploid				
16	3 (60.0)	2 (40.0)	5 (62.5)	6 (20.7)	14 (48.3)	9 (31)	29 (54.7)	2 (4.5)	9 (20.5)	33 (75.0)	44 (69.8)	78 (63.0)
18	0	1 (100)	1 (12.5)	5 (55.5)	2 (22.2)	2 (22.2)	9 (17.0)	0	3 (50.0)	3 (50.0)	6 (9.5)	16 (12.9)
16 and 18	1 (100)	0	1 (12.5)	1 (33.3)	1 (33.3)	1 (33.3)	3 (5.7)	0	2 (50.0)	2 (50.0)	4 (6.4)	8 (6.4)
33	0	1 (100)	1 (12.5)	1 (8.3)	4 (33.3)	7 (58.3)	12 (22.6)	1 (11.0)	4 (44.5)	4 (44.5)	9 (14.3)	22 (17.7)
Total	4 (50.0)	4 (50.0)	8	13 (24.5)	21 (39.6)	19 (35.9)	53	3 (4.8)	18 (28.6)	42 (66.7)	63	124

Table 2 The number of integrated and episomal forms of HPV in the various lesions. Results are expressed as n (%)

Lesion	ISH			PCR (total)	Total
	Integrated	Episomal	Total		
Squamous atypia	0	4 (100)	4 (50.0)	4 (50.0)	8
LSIL	28 (66.7)	14 (33.3)	42 (79.3)	11 (20.7)	53
HSIL	42 (91.3)*	4 (8.7)	46 (73.0)	17 (27.0)	63
Total	70 (76.0)	22 (24.0)	92	32	124

* $\chi^2 = 11.70$; 1 DF; $p < 0.001$.

LSIL = low grade squamous intraepithelial lesion; HSIL = high grade squamous intraepithelial lesion.

STATISTICAL ANALYSIS

In bivariate analyses, the association between: HPV type and ploidy, the grading of the cervical lesions and ploidy and then the integration pattern, and lastly, the integration pattern and multiploidy, were examined using the χ^2 test or a corrected χ^2 test (Yates's correction for small samples). For statistical analyses, squamous atypia were regarded as low grade lesions.

Results

In all cervical specimens, the predominant oncogenic HPV was type 16 (63%) (table 1). Of the 124 specimens examined, 20 were diploid and 104 aneuploid: 43 exhibited a single aneuploid peak, whereas 61 had multiploid profiles. There was no significant correlation between the HPV type and aneuploidy ($\chi_c^2 = 2.87$; 3 DF; NS) (table 1). The frequencies of aneuploidy (single peak and multiploidy) and multiploidy as a function of the grade of the cervical lesion increased significantly ($\chi^2 = 12.23$; 1 DF; $p < 0.001$ and $\chi^2 = 15.64$; 1 DF; $p < 0.001$, respectively; table 1).

LESIONS EXHIBITING SQUAMOUS ATYPIA

HPV was detected in eight patients, predominantly type 16 ($n = 5$) and one case each of the other oncogenic types (table 1). HPV DNA was detected by ISH in four cases and by PCR in the remainder (table 2). Integrated HPV DNA was not detected by ISH in these lesions. These lesions had single peak aneuploid and diploid profiles (fig 1B).

Table 3 Comparative results of ploidy measurements of HPV DNA according to the method of detection. Results are expressed as n (%)

Lesion	Aneuploid			Diploid			Total
	ISH	PCR	Total	ISH	PCR	Total	
Squamous atypia	3 (75.0)	1 (25.0)	4 (50.0)	1 (25.0)	3 (75.0)	4 (50.0)	8
LSIL	34 (85.0)	6 (15.0)	40 (75.5)	8 (61.5)	5 (38.5)	13 (24.5)	53
HSIL	45 (75.0)	15 (25.0)	60 (95.2)	1 (33.3)	2 (66.7)	3 (4.8)	63
Total	82 (78.8)	22 (21.2)	104 (83.9)	10 (50.0)	10 (50.0)	20 (16.1)	124

LSIL = low grade squamous intraepithelial lesion; HSIL = high grade squamous intraepithelial lesion.

LOW GRADE INTRAEPITHELIAL LESIONS (FLAT CONDYLOMAS AND CIN I)

Again, the predominant oncogenic HPV was type 16 (54.7%) (table 1). HPV DNA was detected by ISH in the majority of cases (79.3%) and the integrated form in 66.7% of these. HPV DNA was detected by PCR in the remaining 11 (20.7%) cases (table 2).

Aneuploidy profiles were recorded for 40 cases (75.5%): 21 (39.6%) exhibited a single aneuploid peak on the histogram, whereas 19 (35.9%) had a multiploid profile (table 1).

HIGH GRADE INTRAEPITHELIAL LESIONS (CIN II AND III)

HPV type 16 was detected in 69.8% of cases, whereas types 18, 33, and 16 and 18 were found, respectively, in 9.5, 14.3 and 6.4% of cases (table 1). HPV DNA was detected by ISH in 46 (73.0%) of the 63 patients; integrated DNA was found in 42 (91.3%). HPV DNA was detected by PCR in 17 (27.0%) patients (table 2). The integrated form was found significantly more frequently in high grade lesions ($\chi^2 = 11.70$; 1 DF; $p < 0.001$).

Aneuploid profiles were observed in 60 (95.2%) cases: 18 (28.6%) exhibited a single aneuploid peak, whereas 42 (66.7%) has a multiploid profile (table 1); the latter profile was present in 28 (90.3%) of the 31 CIN III lesions.

Low grade lesions were also observed in 10 sections containing CIN III lesions with a multiploid profile. These low grade lesions harboured the same HPV type and were multiploid. These results were excluded from the analysis.

A comparison of ploidy as a function of the method of detecting HPV DNA is shown in table 3. Aneuploidy was strongly associated with cervical lesions containing oncogenic HPV, being detected in 50% of lesions exhibiting squamous atypia, 75.5% of the low grade and 95.2% of the high grade intraepithelial lesions. Aneuploidy was found mainly in

Table 4 Relation between ploidy and HPV integration. Results are expressed as n (%)

Ploidy	ISH		Total
	Integrated	Episomal	
Multiploid	50 (92.6)*	4 (7.4)	54 (58.7)
Aneuploid (single peak)	13 (46.4)	15 (53.6)	28 (30.4)
Diploid	5 (50.0)	5 (50.0)	10 (10.9)
Total	70 (76.1)	22 (23.9)	92

* $\chi^2 = 23.66$; 1 DF; $p < 0.001$.

lesions in which oncogenic HPV were detected by ISH (85% of low grade and 75% of high grade intraepithelial lesions).

Table 4 shows the relation between ploidy and the pattern of HPV DNA integration into the host genome according to the criteria of Cooper *et al.*²⁷

Aneuploidy did not correlate significantly with the pattern of integration ($\chi^2 = 2.09$; 1 DF; $p < 0.001$). By contrast, multiploidy correlated significantly with the integrated form in 92.6% of cases ($\chi^2 = 23.66$; 1 DF; $p < 0.001$).

FOLLOW UP

Of the nine patients who underwent two successive biopsies, six were followed but received no treatment (mean follow up 12 months). One low grade lesion persisted but changed from diploid to a single peak aneuploid profile over 18 months. Two low grade lesions with a single peak aneuploid profile progressed to high grade disease with a multiploid profile over two and 33 months, respectively. One low grade lesion with a multiploid profile progressed to high grade disease with a persistent multiploid profile over eight months. Two CIN II lesions progressed to CIN III with one exhibiting a single aneuploid peak becoming multiploid over two months whereas the other was persistently multiploid. In all of these cases the same HPV type (HPV 16) was detected in successive biopsy specimens.

Discussion

The present results clearly demonstrate a strong association between aneuploidy and the presence of oncogenic HPV. Aneuploidy was detected in 50% of lesions exhibiting squamous atypia, and in 75.5% of low grade and 95.2% of high grade intraepithelial lesions. These findings suggest that aneuploidy is an early event in carcinogenesis, particularly in the presence of oncogenic HPV.

The acquisition of progressive genetic abnormalities, including the development of aneuploidy, has been described for cells immortalised by HPV types 16, 18 or 33.³⁰⁻³² HPV may alter regulation of the cell cycle in infected cells via several mechanisms. The HPV E6 and E7 gene products bind p53 and retinoblastoma tumour suppressor proteins and these specific interactions may play a role in the promotion of increased proliferation. The DNA in actively replicating cells becomes more susceptible to injury than that in resting cells, especially when p53 is defective. There-

fore, it is plausible that the presence of HPV types 16, 18 or 33 induces genetic instability of the host cell genome, ultimately resulting in the development of multiple chromosomal abnormalities and aneuploidy.

We did not find a significant relation between the type of oncogenic HPV and the presence of aneuploidy, although HPV type 18 was less frequently associated with this DNA profile.

If we consider published data on the presence of aneuploidy in all low grade lesions irrespective of the presence of HPV or if HPV DNA was detected, regardless of its type, the frequency of aneuploidy varied from 0%¹⁴ to 57%¹³ in flow cytometry studies. However, this methodology is not very selective and can include normal epithelial cervical and mesenchymal cell DNA. In our study, we used DNA image cytometry after Feulgen staining, which has the advantage of selecting only those epithelial cells in the cervical lesions. Bibbo *et al.*⁶ also applied this methodology and detected aneuploidy in 21 (48.8%) of 43 low grade lesions, but they did not detect HPV in their series. In our investigation, 75.5% of low grade lesions containing HPV DNA were aneuploid.

In extensive studies on large populations using sensitive methods of detecting DNA, such as PCR, the presence of oncogenic HPV was demonstrated in 43–68.6% of low grade lesions.^{6,8} In our investigation aneuploidy was detected in 75.5% of 68.6%—that is, 51% of HPV positive low grade lesions. Therefore, the presence of aneuploidy in low grade lesions is suggestive of the presence of oncogenic HPV. Nevertheless, some authors have described aneuploid lesions containing non-oncogenic HPV types 6 and 11, but the presence of an unidentified associated oncogenic HPV could not be excluded in these cases as they were analysed using ISH alone.¹⁷⁻¹⁹

The two methodologies used to detect HPV DNA in the present study have somewhat different sensitivities, ranging from 20 to 50 viral copies/cell for ISH to one copy/10⁶ cells for PCR. The aneuploid profile was found mainly in lesions with high viral copy numbers using ISH (85% of low and 75% of high grade lesions), but low viral copy numbers, detected by PCR, were also associated with aneuploidy.

The multiploid pattern is particularly interesting. Multiploidy may be associated with a greater proclivity for genetic instability, partly attributable to the integration of viral DNA into the host genome. Most high grade lesions, particularly those containing HPV type 16, probably contain a mixture of integrated and episomal viral forms. However, in our study, a significant correlation was found between the HPV integration pattern observed on ISH, according to the criteria of Cooper *et al.*,²⁷ and multiploidy (92.6% of cases) and not between HPV integration and aneuploidy.

The frequency of the multiploid profile as a function of the grade of the lesion increased significantly from 35.9% in low grade lesions to 90.3% in CIN III lesions. Six patients who were followed, but not treated, underwent two successive biopsies. We observed a progression

in the DNA profile from diploid to a single aneuploid peak and then to a multiploid profile paralleling the evolution from low to high grade CIN. Moreover, in 10 CIN III lesions multiploid profiles, condylomas and CIN I lesions in the same section exhibited the same DNA profile and contained the same HPV type. Therefore, the discovery of a low grade lesion with a multiploid DNA profile and harbouring an oncogenic HPV type suggests that this lesion may progress to cancer or, alternatively, highlights the presence of a high grade lesion elsewhere in the section.

HPV DNA can now be detected in cervical lesions routinely and easily. DNA image cytometry is also widely used to predict of tumour prognosis.³³ In our opinion, combining HPV DNA detection by both ISH and PCR with image cytometry may provide the pathologist and the physician with important prognostic information about low grade lesions, especially when these lesions harbour oncogenic HPV and have a multiploid DNA profile.

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