Ki-67 AND p16^{INK4A} IMMUNOEXPRESSION STRONGLY CORRELATES WITH INCREASING GRADE OF CIN AND HPV INFECTION.

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INTRODUCTION

The early detection of precursors of invasive cervical cancer represents an important advance in many developed countries to reduce significantly the morbidity and mortality of this kind of cancer. For this reason, it is basic to improve the histopathologic interpretation of cervical biopsies, which still shows considerable interobserver variations despite well-defined criteria.

Ki-67 immunoquantitative features have been correlated with the grade of dysplasia in cervical epithelium, while p16 accumulation has been related to the presence of high grade intraepithelial lesions (HSIL) and detection of human papillomavirus (HPV). Being immunohistochemistry techniques widely available and feasible, the aim of this study was to assess the value of these markers in assisting CIN grading and HPV infection, which has been well-established as a necessary cause of cervical cancer.

PATIENTS AND TISSUES

A total of 124 formalin-fixed, paraffin-embedded samples, collected from 109 women, were reviewed by two pathologists in order to obtain the consensus histologic diagnoses. Our series included 7 normal cases, 8 HPV-suspicious, 41 CIN I, 31 CIN II, 35 CIN III and 2 invasive cervical squamous-cell carcinomas.

METHODS

Immunohistochemistry

Immunohistochemical analysis was performed by ABC immunoperoxidase staining, using mouse monoclonal antibodies Ki-67 (DakoCytomation, Denmark A/S) and p16^{INK4A} (Biocare Medical, Concord, CA). Briefly, 4µm thick sections were treated with 0.1M citrate buffer, pH 6.0, for antigen retrieval, followed by endogenous peroxidase blocking, incubation with horse serum and incubation with unlabeled primary antibodies. Specifically bound antibodies are then visualized by incubation with a biotinylated secondary horse anti-mouse antibody (Vector Laboratories Inc., Burlingame, CA) (1:100 dilution) and a preformed avidin-biotin complex (Vectastain ABC PK4000 ST, Vector Laboratories) was applied at 1:100. Finally, diaminobenzidine (0.05%) was used as the final chromogen and Harris-modified hematoxylin as counterstain. Positive controls -that were cases known to have positive staining- and negative controls -in which the primary antibody was omitted- were included in each experiment.

Results were reviewed by two pathologists. Ki-67 was classified in four categories according to the extension of nuclear staining within the epithelial thickness. Based on previous reports, Ki-67 staining was defined as positive when a cluster of at least two strongly stained epithelial nuclei were present. Results were also reported in a semiquantitative fashion as when staining of cells was found in the upper 1/3 of the epithelium (3+), in the middle 1/3-2/3 (2+) or in the lower 1/3 (1+) (figure 1). Ki-67 0 category defined negative cases in which only staining of parabassal cells of the squamous epithelium was observed and served as internal control. Nuclear or nuclear and cytoplasmic diffuse strong staining was considered positive for p16, focal staining or absence of staining was considered negative (figure 2).

HPV detection and typing

HPV detection and typing was performed using a commercial PCR-based assay (Clinical arrays HPV, Genomica). Briefly, five 5µm thick sections adjacent to a hematoxylin-eosin (H&E) section were used for each case. Areas of interest were selected for scrapping in which HPV associated changes were identified. A negative control -a blank without tissue- was included in each series of cases. DNA was extracted from the microdissected tissue using a proteinase K solution at 56°C during 3 h. The enzyme was inactivated at 70°C for 10min and the DNA purified using the columns provided and eluted in 50µL. PCR amplifications were carried out with 5µL of DNA and PCR mix provided with the kit. This mix included the MY09/MY11 consensus primers, which target a 450bp fragment within the HPV L1 ORF, biotin with which the amplified products are labeled and a pair of primers that allow the amplification of a 892bp fragment of the human CFTR gene to serve as a DNA quality control. Finally, the mix also includes a plasmid to serve as a control for the presence of PCR inhibitors. This plasmid contains an insert flanked by the same pair of CFTR primers that gives a 1202bp fragment. PCR was carried out using the following cycling profile: 94°C 9min; 94°C 30s, 55°C 1min, 72°C 1.5min (45 cycles); and 72°C 8min. Amplified products were hybridized onto low density microarrays that contain fixed probes of 35 types of HPV in triplicate as well as probes to detect the CFTR gene fragment and the internal control plasmid. Detection was carried out using streptavidin-peroxidase and analysis of the results was performed with the provided reader. Only signals in triplicate were considered true positive results. Negative samples without signal from the CFTR gene were considered not assessable.

Statistical analyses

Fisher exact tests and exact confidence intervals for odd-ratios were used to assess statistical differences in p16 and Ki-67 detection amongst different grades of lesions, as well as HPV DNA detection and the prevalence of high risk viruses. P-values less than 0.05 were considered statistically significant. All these statistical computations were performed using the R package (R version 2.6.0, © 2007, R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org). Kendall's Tau-c was performed using Spss 12.0.

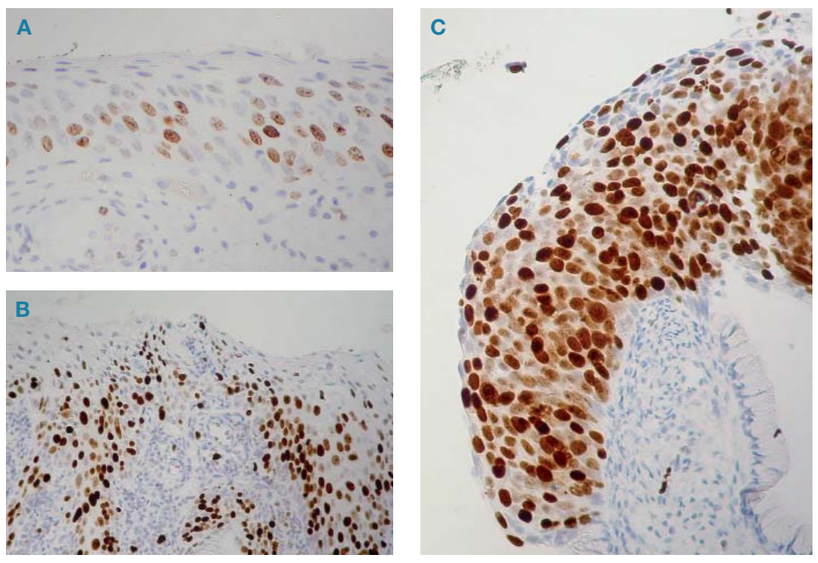

Figure 1. Ki-67 immunostaining. A, CIN I lesion showing a 1+ positivity (100x). B, 2+ positivity on a CIN II lesion (100x). C, 3+ positivity on a CIN III lesion (100x).

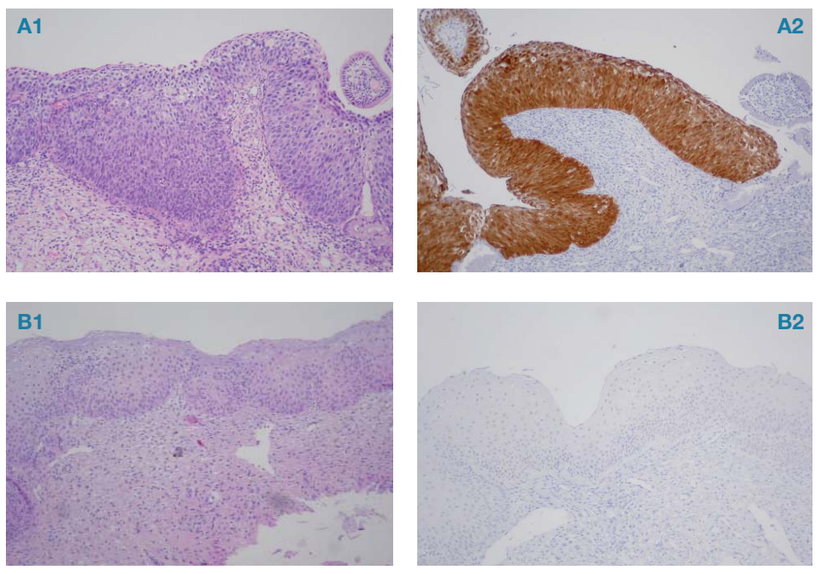

Figure 2. P16 immunostaining. A1, high grade squamous epithelial lesion (H&E, 100x). A2, strong diffuse p16 staining on a HSIL (100x). B1, low grade squamous epithelial lesion (H&E, 100x). B2, absence of p16 staining on a LSIL (100x).

	Ki-67					
	0	*1+*	*2+*	*3+*		
Normal cases	6	1	0	0	**7**	**P16 (-)**
	0	0	0	0	**0**	**P16 (+)**
HPV-suspicious	2	5	1	0	**8**	**P16 (-)**
	0	0	0	0	**0**	**P16 (+)**
CIN I	5	29	6	0	**40**	**P16 (-)**
	0	0	1	0	**1**	**P16 (+)**
CIN II	0	5	6	4	**15**	**P16 (-)**
	0	0	8	8	**16**	**P16 (+)**
CIN III	0	0	1	0	**1**	**P16 (-)**
	0	0	0	34	**34**	**P16 (+)**
Invasive cervical squamous-cell carcinoma	0	0	0	0	**0**	**P16 (-)**
	0	0	0	2	**2**	**P16 (+)**
	13	**40**	**23**	**48**	**124**	

Table 1. Immunohistochemistry results according to the histological diagnosis.

	P16 (-)	P16 +	
HPV (-)	53	2	55
HPV (+)	18	51	69
	71	**53**	**124**

Table 2. P16 expression versus presence of HPV.

	P16 (-)	P16 +	
HPV low-risk	6	1	7
HPV high-risk	11	50	61
	17	**51**	**68**

Table 3. P16 expression versus risk of HPV.

RESULTS

Table 1 summarizes the results of the Ki-67 and p16 immunohistochemical analyses.

The distribution of the expression of Ki-67 was the following: 3+ category was observed in 48 cases which included 2 invasive cervical squamous-cell carcinomas, 34 CIN III and 12 CIN II; 2+ category involved 23 cases corresponding to 1 CIN III, 14 CIN II, 7 CIN I and 1 normal case; 1+ was observed in 40 cases which were 5 CIN II, 29 CIN I, 5 HPV-suspicious and 1 normal case. Finally, parabassal expression (category 0) was seen in 13 cases which included 5 CIN I, 2 HPV-suspicious and 6 normal cases.

Kendall's Tau-c showed a highly significant association ($p<0.0001$) between increasing grade of CIN and Ki-67 expression, two ordinal-level variables. Further Chi-squared analysis of the 2 by 2 contingency table proved Ki-67 especially useful in discriminating the presence of CIN ($p=1.004e^{-09}$).

Fifty-three out of 124 cases showed positive p16 staining, as defined above. In relation to the histologic diagnoses the positive results were found in 2 invasive cervical squamous-cell carcinomas, 34 CIN III, 16 CIN II, 1 CIN I. No p16 staining was observed in normal or HPV-suspicious cases. A significant relation ($p=2.2\ e^{-16}$) was found between p16 immunoexpression and the presence of HSIL lesions, with 98% specificity, 98% positive predictive value and 78% negative predictive value.

P16 accumulation was also indicative of the presence of HPV which was detected in 51 out of 53 (96%) p16 positive samples (table 2). Furthermore, 50 out of 51 (98%) of these HPV positive cases contained high-risk HPV (table 3).

DISCUSION

Classification of cervical dysplasia is based upon histologic features, with the primary goal of identifying and grading squamous epithelial lesions at increased risk for progression to invasive carcinoma. Criteria for the histologic diagnosis of these lesions are well established. The non-invasive lesions are microscopically evaluated taking into account the epithelial level involved and the nuclear cytologic abnormality in the cervical squamous epithelium. However, in the routinary cervical biopsies assessment may be especially difficult the distinction between low-grade (CIN I) and benign no-HPV related lesions. In addition may be also difficult in some cases of intermediate dysplastic pattern to decide their classification as low or high grade lesions.

As in previous studies, in our experience the pattern of Ki-67 staining in squamous intraepithelial lesions (SIL) differs significantly from that of normal cervical epithelium, in which positive cells are confined to the parabassal cell layer. In cervical SILs the presence of Ki-67 positive cells is increased from the parabassal areas into the intermediate and superficial layers conferring to this marker a potential value in the distinction between low-grade CIN and normal, metaplastic and reactive cervical epithelia. This observation was confirmed in the present study with the use of Kendall's Tau-c as statistical tool which reveal how samples with increasing grade of CIN had a tendency to contain Ki-67 positive cells in gradually more upper layers of their epithelium ($p<0.0001$).

It is well-established that persistent infection with high-risk HPV types is associated with the subsequent development of high-grade dysplasia and invasive cervical carcinoma. Nevertheless, since many infections are transitory and a substantial proportion of low-grade dysplasias are also associated with infection by high-risk HPV types, another tool becomes necessary to help in the assessment of high grade lesions.

Viral DNA integration characteristic of high-risk HPV types, and closely related to malignant transformation, results in increased expression of the E6 and E7 oncogenes. The E7 binds to and inactivates the host cell's retinoblastoma protein, which leads to an overproduction of p16. As confirmed in the present study, p16 diffuse strong staining is a useful predictor of the presence of high grade dysplasia and high-risk HPV infection. In fact it is remarkable that 52 out of the 53 cases showing p16 positivity were high grade lesions, and 51 also presented high-risk HPV infection.

CONCLUSIONS

The combined use of these two markers, Ki-67 and p16, provides a valuable tool to asses the grade of CIN, to predict the presence of high-risk HPV infection and to verify the diagnosis of equivocal cases.

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