P16 immunostaining and HPV testing in histological specimens from the uterine cervix

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Summary

Background: The cellular tumor suppressor protein p16^{INK4a} (p16) has been identified as a biomarker for transforming human papilloma virus (HPV) infections. P16 is a cyclin-dependent kinase inhibitor that regulates the cell cycle and cell proliferation by inhibiting cell cycle G1 progression. *Purpose of the study:* To confirm the role of p16 as biomarker for transforming HPV infections and possible clinical applications in histological samples from the uterine cervix. *Materials and Methods:* The subject of this study included 56 biopsies of the cervical canal collected from January 2012 to September 2012 in the Institute of Pathology of the University of Sassari. The search for HPV immunohistochemistry was performed with the monoclonal antibody DAKO 1:25, while for the detection of p16 was used CINtecTM p16 (INK4a) histology kit. *Results:* In 56 biopsies performed in women aged between 23 and 69 years, the authors highlighted, by histological analysis, 24 cases of low-grade squamous intraepithelial lesion (LSIL) - cervical intraepithelial neoplasia (CIN1) and 31 cases of high-grade squamous intraepithelial lesion (HSIL) - CIN2/3); 15 CIN2, 14 CIN3, and two cervical squamous cell carcinoma in situ (SCIS). One case was an infiltrating squamous cell carcinoma (ISC). In 24 CIN1, there was a 16.67% positivity for p16 and an equal percentage occurred for HPV. In 15 cases of CIN2 the percentage of positivity for p16 was considerably increased (73.33%), unlike the search for HPV which had a positivity rate of 20%. Finally, in 14 cases of CIN3, and in three carcinomas, the positivity for p16 was equal to 100%, however the search for HPV positivity was between 0% and 7.14%. *Conclusions:* These results demonstrated that p16 was a highly sensitive marker of cervical dysplasia. The authors have shown that p16 overexpression increased with the severity of cytological abnormalities and that had a greater ability to identify the viral infection compared to the classical immunohistochemical staining for HPV.

Key words: Cervical cancer; Human papilloma virus (HPV); P16^{INK4a}; Immunohistochemistry.

Introduction

P16^{INK4a} (p16), a protein that plays a role in tumor suppression, is a cyclin which has a kinase inhibitory function, whose overexpression has been reported in dysplastic and neoplastic epithelial lesions of the uterine cervix [1]. The overexpression of p16 is indirectly induced by the viral oncoprotein E7 as a consequence of the retinoblastoma protein (pRb) deregulation [2].

Cell replication is, in fact, controlled by means of a complex mechanism involving different regulatory pathways within the cell. One of these is the location of the pRb, which controls cell proliferation. Under normal conditions, Rb binds to the transcription factor E2F, which has the effect of blocking the transcription of genes that promote the proliferation and progression of the cell cycle, but also the p16 gene coding for the inhibitor of cyclin-dependent kinase. Therefore the binding of E2F by pRB is one of the control mechanisms to avoid that the cells continue to replicate and proliferate.

In the course of infection with human papilloma virus (HPV), one of the proteins expressed by the virus inside the cell is the oncogenic protein E7. Its oncogenic activity consists of preventing the function of pRb, which does not bind to transcription factor E2F, and consequently this leads to the transcription of certain genes, in particular the transcription of the p16 gene, which encodes for the protein p16 functional inside the cell. As a result of HPV infection, there will be a final induction of cell proliferation [3].

Usually, p16 is expressed at very low concentrations in healthy cells, whereas it is strongly overexpressed in cervical-cancer cell lines in which pRb has been functionally inactivated by the high-risk HPV E7 oncoprotein [4]. The current literature supports using p16 immunostaining as a surrogate marker for the presence of cervical intraepithelial neoplasia 2/3 (CIN2/3) in cervical biopsy specimens to distinguish CIN2/3 from their mimics, such as immature metaplasia or therapy changes.

The objective of this study was to evaluate the expression of p16 protein in the various types of dysplastic cervical lesions, both low and high grade, with the aim to evaluate the different overexpression of this protein in various types of lesions, in order to permit a judgment of prognosis, especially in low-grade lesions. The aim was to be able to program appropriate protocols for monitoring and personalized follow-ups.

Materials and Methods

Fifty-six biopsies of the cervical canal were collected from January to September 2012 and sent for diagnosis and phenotypic characterization to the Institute of Pathology, University of Sassari, and the Institutional Review Board approved the study.

Revised manuscript accepted for publication September 20, 2013

The samples, which were fixed in 10% formalin and embedded in paraffin sections, underwent microtomic sections of four µ, stained with hematoxylin, and some sections were prepared on slides pretreated with polylysine for performing immunohistochemistry.

The search for HPV immunohistochemistry was performed with the monoclonal antibody DAKO 1:25, while for the detection of p16 was used CINtecTM p16 (INK4a) histology kit.

The slides were rehydrated through descending scale of alcohol (96%, 70%, and 50%) for about two minutes. Subsequently, the slides were completely covered with citrate buffer; brought for five minutes to boiling pressure, and then left to cool in the respective chambers at room temperature with citrate buffer for 20 minutes.

During the process of staining, the slides were placed in a buffer bath in citrate for five minutes, allowed to dry, and coated from solution. The coverslip was applied and were then left to incubate at room temperature for five minutes. After removal of the coverslip, the monoclonal antibody was applied on each slide and subsequently the slides were left to incubate at room temperature for about 30 minutes. The next step consisted in washing buffer for five minutes, adding a drop of the reagent (biotin-avidin complex enzyme), and the coverslip was placed to incubate 30 minutes at room temperature.

The slides were then washed in buffer for five minutes, then a drop (50 µl) of the chromogen solution was added and after the addition of the coverslip, were placed to incubate at room temperature for ten minutes; then following a washing in distilled water for one minute and counterstained with hematoxylin, mounted on the slide, and analyzed microscopically.

The immunohistochemical positivity was of nuclear type and the cases were classified positive even in the presence of a single positive nucleus.

The authors assigned a positive evaluation, if the sample showed a continuous staining of cells of the basal and parabasal layers of the squamous cervical epithelium, with or without staining of cells of superficial cell layers.

The authors assigned a negative evaluation, if the sample showed a negative staining reaction in the squamous epithelium or staining of isolated cells or in small groups, however, less than 25% of the cells.

Two pathologists, without knowledge of the tissue biopsy and HPV results, independently reviewed the p16 immunostaining results. In 54/56 (96.43%) of the cases, the pathologists agreed on the p16 immunostaining results; in the remaining cases, consensus was reached after review.

Results

The results obtained from histology and immunohistochemistry are summarized in Table 1.

Immunohistochemical analysis totally (Table 2) showed 32/56 cases positive for p16 (57.14%) and 24/56 (42.86%) negative cases, including eight with focal positivity, while only eight (14.29%) were positive for HPV immunoistochemical staining and 48 (85.71%) were negative.

Analyzing the distribution in the different degrees of lesions (CIN1/CIN2/CIN3). The results were the following (Table 3):

– CIN1: 24 cases examined, four (16.67%) were positive for p16 and 20 (83.33%) were negative, including six with focal positivity, while four (16.67%) were positive for HPV and 20 (83.33%) were negative.

Table 1. — *Total results*.

Case	Age	Diagnosis	p16	HPV (Immunohistochemistry)
1	33	CIN1	Negative	Negative
2	38	CIN1	Negative	Negative
3	44	CIN1	Negative	Negative
4	42	CIN1	Negative	Negative
5	28	CIN1	Negative	Negative
6	26	CIN1	Negative	Negative
7	32	CIN1	Negative	Negative
8	26	CIN1	Positive	Negative
9	27	CIN1	Negative	Negative
10	40	CIN1	Focal	Positive
11	24	CIN1	Positive	Negative
12	39	CIN1	Focal	Positive
13	52	CIN1	Focal	Negative
14	47	CIN1	Negative	Negative
15	38	CIN1	Negative	Negative
16	23	CIN1	Focal	Negative
17	31	CIN1	Positive	Positive
18	33	CIN1	Focal	Positive
19	30	CIN1	Negative	Negative
20	44	CIN1	Positive	Negative
21	20	CIN1	Focal	Negative
22	32	CIN1	Negative	Negative
23	65	CIN1	Negative	Negative
24	45	CIN1	Negative	Negative
25	27	CIN2	Negative	Negative
26	25	CIN2	Focal	Positive
27	30	CIN2	Focal	Negative
28	26	CIN2	Positive	Negative
29	47	CIN2	Positive	Negative
30	35	CIN2	Positive	Negative
31	35	CIN2	Positive	Negative
32	53	CIN2	Positive	Negative
33	33	CIN2	Positive	Negative
34	30	CIN2	Positive	Negative
35	44	CIN2	Positive	Negative
36	33	CIN2	Negative	Negative
37	40	CIN2	Positive	Negative
38	37	CIN2	Positive	Positive
39	33	CIN2	Positive	Positive
40	32	CIN3	Positive	Negative
41	32	CIN3	Positive	Negative
42	24	CIN3	Positive	Negative
43	45	CIN3	Positive	Positive
44	42	CIN3	Positive	Negative
45	32	CIN3	Positive	Negative
46	56	CIN3	Positive	Negative
47	43	CIN3	Positive	Positive
48	28	CIN3	Positive	Negative
49	40	CIN3	Positive	Negative
50	44	CIN3	Positive	Negative
51	54	CIN3	Positive	Negative
51	30	CIN3	Positive	Negative
53	44	CIN3	Positive	Negative
54	42	SCIS	Positive	Negative
55	69	ISC	Positive	Negative
56	38	SCIS	Positive	Negative

SCIS: squamous cell carcinoma in situ. ISC: infiltrating squamous cell carcinoma.

– CIN2: 15 cases examined, 11 (73.33%) were positive for p16 and four (26.67%) negative, two of which had a focal positivity, while three (20%) were positive for HPV, and 12 (80%) negative.

Table 2. — *Distribution of positive and negative p16 and HPV (IIC) on total cases*.

Total	p16 positive	p16 negative	HPV (IIC) positive	HPV (IIC) negative
56	32 (57.14%)	24-8 Focal- (42.86%)	8 (14.29%)	48 (85.71%)

IIC: immunohistochemical evaluation.

Table 3. — *Distribution of positive and negative p16 and HPV (IIC) according to CIN*.

Total	p16 positive	p16 negative	HPV (IIC) positive	HPV (IIC) negative
24 CIN1	4 (16.67%)	20-6 Focal- (83.33%)	4 (16.67%)	20 (83.33%)
15 CIN2	11 (73.33%)	4-2 Focal- (26.67%)	3 (20%)	12 (80%)
14 CIN3	14 (100%)	0	1 (7.14%)	13 (92.86%)

IIC: immunohistochemical evaluation.

Table 4. — *Distribution of positive and negative p16 and HPV (IIC) according to CIN*.

Groupe	Cases	p16 positive	p16 negative	HPV (IIC) positive	HPV (IIC) negative
LSIL	24	4 (16.67%)	20-6 Focal- (83.33%)	4 (16.67%)	20 (83.33%)
HSIL	31	27 (87.10%)	4-2 Focal- (12.90%)	4 (12.90%)	27 (87.10%)

IIC: immunohistochemical evaluation.

– CIN3: 14 cases examined, all (100%) were positive for p16, one (7.14%) was positive for HPV and 13 (92.86%) negative.

Both two cases of cervical squamous cell carcinoma in situ (SCIS) and one case of infiltrating squamous cell carcinoma (ISC) were positive for p16 and all were negative for HPV.

Table 4 shows the distribution of positive and negative p16 and HPV according to Bethesda classification low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL). In particular, histological analysis indicated 24 cases of low-grade lesions (LSIL-CIN1) and 31 cases of high-grade lesion (HSIL) of which 15 CIN2, 14 CIN3 and two ISSC. One case was an ISC. HSIL (CIN2 + CIN3 + ISSC): examining how a single group the high-grade lesions the authors reported that of the 31 cases, 27 (87.10%) were positive for p16 and four (12.90%) negative (two with focal positivity), while four (12.90%) were positive for HPV and 27 (87.10%) negative. LSIL and HSIL were positive for p16 in 16.67% and 87.10%, respectively (negative 83.33% and 12.90%, respectively) and positive for HPV (four cases, 16.67% and 12.90%, respectively).

Discussion

P16 is a cyclin-dependent kinase inhibitor, the expression of which is negatively controlled by the RB1 gene product. In differentiated epithelial cells, p16 is expressed in levels typically not evaluated by immunohistochemistry. In the course of infection with HPV, one of the proteins expressed by the virus inside the cell is the oncogenic protein E7. Its oncogenic activity consists of preventing the function of pRb, which does not bind to transcription factor E2F [1].

Some studies [5-7] have investigated the usefulness of the protein p16 as biomarker, especially of high-grade lesions of the uterine cervix HSIL and in situ and ISC and also to assess the ability of progression of low-grade lesions LSIL; CIN1 that, in a certain percentage of cases, may undergo spontaneous regression.

It has been shown, in fact, that there is a significant association between the degree of the cervical lesion and the positivity (also in terms of distribution and intensity) for p16 [8].

There are about 15 types of high-risk HPV, but in any case the effect of the E7 oncoproteins is the same in blocking pRb and lead to overexpression of p16. Since p16 is a cellular protein, it may serve as a biomarker, independent of the type of high-risk HPV, and its overexpression is a direct marker of the oncogenic activity of the virus and the more accurate predictor of cervical cancer.

The use of p16 by immunohistochemistry can be considered a complement of cytology and histology, which allows a better evaluation of women with questionable results and require colposcopy or treatment. The detection of this protein with monoclonal antibodies is a useful parameter both in the interpretation of cytology and because it reduces the variability in the evaluation of suspected cervical biopsies. Regarding the cytological examination, if the outcome is positive for a HSIL there will be a high positive predictive value (PPV), instead for results of lower grade, and therefore for LSIL or atypical squamous cells of undetermined significance (ASC-US), there will be a much lower PPV. For this reason the authors have introduced HPV test in the case of low-grade cytological results. However it has been shown that this test has an important role in identifying the risk lesions and the recurrence of the disease, but HPV test fails in the triage of low-grade lesions. In addition, a single HPV DNA test may confirm if the infection is present in 99% of all cancers of the cervix, but it does not discriminate between chronic and transitory infection. The discrimination between the two types of infection is of fundamental importance, as it is the persistent infection that predisposes to progression of lesions to cervical neoplasia. Consequently, once again the value of p16 was emphasized, especially as a marker of risk of progression of lesions to low-grade dysplasia [9].

Tsoumpou *et al*. [1], in a systematic review and meta-analysis, have reported the overexpression of the protein p16 in a very high proportion of high-grade lesions close to 82% in cases of CIN3, and a range between 38% and 68% in low-grade lesions.

The authors also wanted to evaluate the overexpression of p16 protein in cervical lesions. Immunohistochemical analysis revealed in total, 32 cases positive for p16 (57.14%) and 24 negative cases, including eight with focal positivity (42.86%), while only eight (14.29%) cases positive for HPV and 48 (85.71%) negative for HPV.

The present data confirm the increased ability of p16 to detect viral infection compared to standard immunohistochemical staining for HPV that can now be considered completely useless.

Observing the distribution of the various degrees of injury, the present results are extremely interesting. In fact, of the 24 cases CIN1, four (16.67%) were positive for p16 and 20 (83.33%) were negative, including six with a focal positivity. In cases CIN2 positivity rised to 73.33% (11/15) and even up to 100% in both cases CIN3 (14) and cervical squamous cell carcinoma (two SCIS and one ISC), with a positivity in the overall total of HSIL of 87.10% (27/31).

According the Bethesda classification and identifying only two groups, LSIL and HSIL had a positivity for p16 by 16.67% (4/24) and 87.10% (27/31).

These results demonstrated that the protein p16 was a highly sensitive marker of HPV cervical dysplasia and in particular was able to discriminate between high-grade and low-grade lesions, especially in terms of follow-up.

In fact the authors believe that the relatively frequent negativity of p16 in LSIL is in relation to infection with a strain of non-oncogenic HPV, whereas cases of LSIL positive for p16 indicate possible infection with oncogenic strain. This is in agreement with what is known about the evolutionary history of LSIL, hence about one-third of these lesions should undergo spontaneous regression and are certainly not due to an infection with oncogenic strain.

Positivity for p16 protein allows then to select, in the context of patients with LSIL, those who need a closer follow-up as it is subject to a possible evolution of the disease.

In conclusion, the authors can state that the overexpression of the protein p16 at immunohistochemistry is certainly useful, not so much in the information it can give us in high-grade lesions, but also for the impact it has in the planning of screening program allowing to identify groups of patients at risk already in the LSIL phase, and rationalizing such program in order to obtain more effective prevention and better cost optimization.

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