Combined Analysis of HPV DNA and p16^{INK4a} Expression to Predict Prognosis in ASCUS and LSIL Pap Smears

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ABSTRACT

Human papillomavirus (HPV) is known to play an important etiological role in the genesis of cervical cancer, but only a very small proportion of infected women develop invasive cervical cancer. The purpose of cervical cancer prevention is early diagnosis of its precursors. The molecular detection of HPV DNA as a diagnostic test to cervical carcinogenesis gave a low positive predictive value as compared to the use of biomarkers. p16^{INK4a} has been proposed as putative surrogate biomarkers that would allow identification of dysplastic cervical epithelia. Serial consecutive cervical smears were test for high-risk HPV, stained with immunocytochemistry for p16^{INK4a} and followed-up for 36 months. The aim of the study was to evaluate the immunohistochemical expression of p16^{INK4a} as a marker of progression risk in low-grade dysplastic lesions of the cervix uteri. In the present series, significant p16 overexpression was observed in the group that progressed from low to high-grade squamous intraepithelial lesion when compared with the group that did not progress. In conclusion, overexpression of p16^{INK4a} acts as potential biomarkers for cervical cancer progression from premalignant lesions.

Keywords: *cervical intraepithelial neoplasia, cervical cancer, human papillomavirus, p16^{INK4a}*

Introduction

Epidemiological and molecular studies over the past two decades have demonstrated that high-risk human papillomavirus (HR-HPV) types are etiologically related to the progression to cervical cancer. Although more that 85 types of HPV have been detected in the genital mucosa, in the majority of HPV-infected individuals, the virus is eliminated. A substantial proportion of HPV lesion regresses spontaneously over 6–18 months period. Several studies have shown that viral persistence is necessary for cervical intraepithelial neoplasia (CIN) lesions to progress or in fact be maintained.

Although HPV testing has been successfully used and proposed for triaging to colposcopy those patients with minor cytologic abnormalities, its positive predictive value (PPV) is suboptimal and a substantial proportion of patients are still referred unnecessarily to colposcopy. In the ALTS study[1] the PPV for CIN3 of a positive HR-HPV test in a patient with and atypical squamous cells of undetermined significance (ASCUS) Pap was only 10%.

Identifying other molecular events associated with progression from low (L)- to high (H)-grade squamous intraepilelial lesions (SIL) is a crucial area of research, as it may further improve selection of HPV-positive patients really worthy of assessment and treatment.

The use of modulators involved in the cell cycle as biomarkers of HR-HPV infected cells may be an important tool in the future to identifying those smears containing HSIL of patients that might progress and develop to cervical carcinoma.

The p16^{INK4} is a tumour suppressor protein that inhibits the function of cdk4 and cdk6, which in turn regulate the G1 checkpoint. CDK/cyclin-D phosphorylate the retinoblastoma protein (pRb), resulting in a conformational change, with the release of E2F from Rb. Thus, inactivation of either p16 or Rb function allows the cell to enter the S phase after only a brief pause as the G1 checkpoint. In addition, the E6 HPV oncoprotein has the ability to bind p53, resulting in its degradation, and the E7 gene product inactivates the pRb pathway.

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Pathogenic activity of HR-HPV indicated by p16 expressions on smears could be a strategy to identify patients at major risk to develop cervical lesions.

The p16^{INK4a} immunostaining has been suggested as a tool for triaging women with low-grade or borderline cytology; p16 could be particularly interesting among women with LSIL cytology, where triage by HPV is inefficient. Several studies[2,3] reported a differential expression of p16 in HSIL, LSIL and normal cervical epithelial cells.

The p16^{INK4a} has been shown to be associated with HPV-infected high grade lesions but its PPV and sensitivity in prospective follow-up for relevant outcomes (> CIN2) has yet to be determined.

In our previous report[4] we assessed the accuracy of p16 and HR-HPV testing in identifying high-grade cervical lesions in 283 cervical samples (ThinPrep) on a consecutive series of women referred to colposcopy for abnormal cytology (≥ASCUS). In this follow-up study we analyzed the role of immunocytochemical expression of p16 in HPV infected women as prognostic markers of the progression of SIL.

Subjects, Material and Methods

Initially we assessed p16^{INK4a} immunostaining and HR-HPV in 283 patients consecutively referred to colposcopy for cytologic evidence of LSIL or ASCUS within the Florence (Italy) District screening programme for cervical cancer.

HPV and p16 testing were performed in the whole series prior to colposcopy assessment: cervical material was collected using ThinPrep® (Cytic Corp., Boxborough, MA), allowing for multiple slide preparation and residual fluid. Laboratory operators performing the testing were blinded to the colposcopy assessment outcome. The patients without CIN2 or more were invited regularly for follow-up cytology; if cytology was ASCUS or more colposcopy was performed.

The 238 out of 252 patients were followed for 36 month period and then stratified according the cytology results and final outcome. Final outcome was defined according to colposcopy-directed biopsy result (<CIN2 or >CIN2) and was assumed to be negative in the presence of negative colposcopy, indicating no biopsy. Observed differences were tested by the Chi-square test, with statistical significance set at $p < 0.05$.

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HPV Testing

From each specimen, 2 mL of residual ThinPrep fluid was used, and DNA extraction was carried out using a QIAmp DNA Mini Kit (Qiagen Corporation, Venlo, the Netherlands) according to the manufacturer's protocol. Polymerase chain reaction (PCR) analysis was performed according to a previously described protocol[5] using primers for the *E6/E7* region of HR HPV types (HPV 16, 18, 31, 33, 35, 45, 52 and 58). For a quality control of DNA extraction, the primer set PC04 and Gh20 was employed to amplify a 268-base pair (bp) fragment of the human beta globin gene in all specimens. In each PCR reaction, negative and positive controls were introduced.

p16^{INK4a} Testing

From each specimen, 2 mL of residual ThinPrep fluid was used for a cytospin preparation; after cytocentrifugation (5 min at 1000 rpm), slides were air dried for 10 minutes, then treated with spray fixation reagent, containing polyethylene glycol, and immunostained within 24 hours. Before they were immunostained, all spray-fixed specimens were incubated in 50% volume/volume alcohol, followed by one washing step in deionized water. For immunostaining, CINtec^{tm} p16 Cytology kit (Dako Cytomation, now Dako A S, Glostrup, Denmark) was used, according to the manufacter's protocol. In brief, smears were treated with 3% hydrogen peroxide and then submitted for epitope retrieval at 95–99 °C for 40(±1) minutes; after cooling, the p16^{INK4a} antibodies were applied for 30 (±1) minutes and then a reagent for observation and substrate-chromogen solutions were added. Hematoxylin was used as counter stain. The methodology differs from other studies on p16[6,7], but we used the same monoclonal antibody and believe that the results are comparable. The choice of cytospin preparation was essentially aimed at a more efficient use of the residual ThinPrep fluid. Before the study, we made a comparison of p16^{INK4a} testing on cytospin and ThinPrep preparations on limited numbers of negative and positive samples (data not shown), and we observed no differences.

Slides were read by two investigators blinded to final outcome, and a minimum of 500 cells in different fields were analyzed. A negative result was defined if no cells immunoreactive to the p16^{INK4a} antibody were in evidence. Slides showing positive staining for p16 were categorized on the basis of the percentage of positive cells as: <5%, 5–10% or >10%. The cellular staining site was also evaluated and categorized as 1) nuclear, 2) cytoplasmic, or 3) nuclear plus cytoplasmic.

Results

At recruitment[4] we assessed the accuracy of p16 and HR-HPV testing in identifying high-grade cervical lesions in 283 cervical samples (ThinPrep) on a consecutive series of women referred to colposcopy for abnormal cytology (≥ASCUS). The results were compared with colposcopy and biopsy findings. HPV positivity rate was 44.2% among <CIN1, and 89.2% among ≥CIN2 patients (Chi-square for trend $<10^{-6}$). The sensitivity, specificity, and PPV of HPV testing for ≥CIN2 were 89.2% (25 of 28), 47.8 (122of 255) and 15.8% (28 of 158), respectively. P16 positivity rate was 25.3% among <CIN1, 57.4% among CIN1 and 88.0% among ≥CIN2 patients (square for trend $<10^{-6}$). Sensitiv-

TABLE 1
HPV TEST AT RECRUITMENT ACCORDING TO THE CYTOLOGICAL DIAGNOSIS DURING THE FOLLOW-UP

	N (%) of HR-HPV negative	N (%) of HR-HPV positive	Total – N (%)
Normal	100 (92.6)	89 (83.2)	189 (87.9%)
ASCUS	3 (2.8)	2 (1.9)	5 (2.3%)
LSIL	3 (2.8)	10 (9.3)	13 (6.04%)
HSIL	2 (1.8)	6 (5.6)	8 (3.7%)
Total	108 (50.2%)	107 (49.8%)	215

HR – high-risk, HPV – human papillomavirus, ASCUS – atypical squamous cells of undetermined significance, LSIL – low-grade squamous intraepilelial lesions, HSIL – high-grade squamous intraepilelial lesions

TABLE 2
P16 AT RECRUITMENT ACCORDING TO THE CYTOLOGICAL DIAGNOSIS DURING THE FOLLOW-UP

	N (%) of p16^{INK4a} negative	N (%) of p16^{INK4a} positive	Total – N (%)
Normal	58 (87.9)	31 (77.5)	89 (84.0)
ASCUS	1 (1.5)	1 (2.5)	2 (1.9)
LSIL	6 (9.1)	4 (10)	10 (9.4)
HSIL	1 (1.5)	4 (10)	5 (4.7)
Total	66 (62.3)	40 (37.7)	106

HR – high-risk, HPV – human papillomavirus, ASCUS – atypical squamous cells of undetermined significance, LSIL – low-grade squamous intraepilelial lesions, HSIL – high-grade squamous intraepilelial lesions

ity for ≥CIN2 was 88% (22 of 25), specificity was 61.2% (79 of 129) and PPV was 30.5% (22 of 72).

The patients with cytological abnormalities but without CIN2/3 lesions were followed-up, for 36 months by cytology and colposcopy was performed if cytology was ASCUS or worse.

Between 252 patients, 215 women have had a follow-up procedure: 108/122 (88%) with a negative result for HR-HPV and 107/130 with a positive result for HR-HPV.

After 36 months (Table1), 8 (7.4%) women with a negative result for HR-HPV at recruitment, still displayed abnormal smears (3 ASCUS, 3 LSIL and 2 HSIL). Final outcome of these cases was: 2 CIN2 (1.8%), 3 CIN1 (2.8%) and 3 Negative (2.8%).

Between women with a positive result for HR-HPV at enrolment (Table1), after 36 months of follow-up, 18 (16.8%) women still displayed abnormal smears (2 ASCUS, 10 LSIL and 6 HSIL). Final outcome of these cases was: 9 CIN2+ (8.4%), 3 CIN1 (2.8%) and 6 Negative (5.6%), ($p < 0.05$).

Considering p16 immunostaing, 66/79 (84%) p16 negative and 40/50 (80%) p16 positive have had follow-up (Table 2). During the follow-up of p16 negative women 3 (3/66=4.5%) CIN2 were founded, while 6 CIN2 (6/40= 15%) were founded in p16 positive women ($p<0.05$).

At the enrolment the number of p16 positive cells did not correlate with the probability of ≥CIN2 (<5% = 36.3%, 5–10% = 27.2%, >10% = 36.3) and was no further considered a relevant variable. No evident differences in pattern or intensity of p16 expression were observed between the specimens with high grade lesions and without high lesions at follow-up.

Discussion

HPV DNA testing appears useful in the triage of equivocal Pap-smears; however studies could not demonstrate a high level of specificity of HPV DNA testing for clinically significant cervical disease. The p16 immunostaining has been suggested as a tool for triaging women with low-grade or borderline cytology; p16 could be particularly interesting among women with LSIL cytology, where triage by HPV is inefficient. Moreover, an obvious problem in using HPV testing as a screening tool is that a sizable proportion of normal women are HPV positive; however a report[8] suggested that about 15% of women in annual screening programme who concurrently have a negative Pap test and a positive oncogenic HPV test will have a subsequent abnormal Pap test within 5 years.

Nevertheless, HPV testing also identified many transient HPV infections that are not associated with high-grade CIN. Several studies based on molecular markers associated with HPV infection could facilate and optimise diagnosis in a screening setting. It may be possible to detect clinically important disease with risk of progression towards dysplasia and carcinoma, and consequently, improve patient care by combining test results from molecular markers with either cytology or HPV or both.

Several studies[2–4] reported a differential expression of p16 in HSIL, LSIL and normal cervical epithelial cells.

In this study, we evaluated the potential of p16 immunocytochemical expression to predict the course of cytological cervical abnormalities associated with HR-HPV types.

In the present series, significant p16 overexpression was observed in the group that progressed from LSIL to

HSIL when compared with the group that did not progress. To our knowledge, there are a few studies with a prospective follow-up design carry out to evaluate SIL progression and the association of p16 overexpression in cervical specimens in a screening setting for cervical cancer.

Although p16 protein and HPV infection may be detected in low-grade lesions or reactive changes that undergo spontaneous regressions, Wang[9] et al. found that the risk for CIN progression or HPV persistent is higher for women with diffuse staining for p16 protein compared with those without diffuse staining in tissue samples. Negri[10] also found that CIN1 cases with diffused p16 staining had significant higher tendency to progress to a high-grade lesion that p16 protein negative cases.

Conclusion

This study suggest that the combined use of p16 protein and HPV testing may be useful in identifying cervical cells with minor abnormalities and a high risk of progressing to cervical neoplasia, and also defining cases requiring early management, or at least close surveillance. Our study demonstrated that p16 evaluation on ThinPrep samples could be useful as a biomarker for progressive malignancy in HPV-related cytological abnormalities. Patients with ASCUS or LSIL and HR-HPV and p16 positive test were a major risk to develop high grade lesion compared with patients HR-HPV negative or HR-HPV positive but with p16 negative.

These findings suggest that among HPV positive patients, there is a subgroup that may be at increased risk of progression to invasive cancer and should be followed-up more closely. Theoretically, all HR HPV-associated lesions should express p16^{INK4a}. It is unclear why some CIN2 samples that are HPV DNA positive were also p16^{INK4a} negative. We might assume that there are other mechanisms of p16^{INK4a} regulation besides HPV infection, such as promoter methylation, that could occur in cervical cancer. However, according to previous report in the literature, pRb inactivation via the p16/cdk-cyclin/ RB pathway and increase in p16 expression in HPV- transformed cells is an important mechanism for cervical carcinogenesis.

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KOMBINIRANA ANALIZA DNA HPV-A I EKSPRESIJE P16 U PREDVIĐANJU PROGNOZE PAPA-NALAZA ASCUS I LSIL

SAŽETAK

Poznato je da humani papilomavirus (HPV) igra važnu etiološku ulogu u nastanku raka vrata maternice, no samo mali udio inficiranih žena zaista i razvije invazivni rak vrata maternice. Cilj prevencije raka vrata maternice je rana dijagnoza njegovih prekursora. Molekularna detekcija DNK HPV-a kao dijagnostički test za karcinogenezu raka vrata maternice je dala slabo pozitivne prediktivne vrijednosti u usporedbi s upotrebom bioloških biljega. p16^{INK4a} je predložen kao osnovni biljeg koji bi omogućio identifikaciju promjena epitela vrata maternice. Grupa uzastopnih obrisaka vrata maternice je testirana na visokorizične HPV, obojana imunocitokemijski za detekciju p16^{INK4a} i praćena 36 mjeseci. Cilj studije je bio procijeniti imunohistokemijsku ekspresiju p16^{INK4a} kao biljega rizika progresije u oštećenjima niskog stupnja stanica vrata maternice. U toj grupi je zabilježena znatno povećana ekspresija p16 za skupinu koja je prešla iz oštećenja niskog stupnja u oštećenja stanica vrata maternice visokog stupnja u usporedbi sa skupinom kod koje nije došlo do progresije. Zaključili smo da povećana ekspresija p16^{INK4A} djeluje kao mogući biološki biljeg koji ukazuje na progresiju oštećenja vrata maternice u premaligni i maligni stadij.