Expression of p16/Ki-67 in ASC-US/LSIL or Normal Cytology with Presence of Oncogenic HPV DNA

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Abstract. *Aim: To determine if positive dual staining of p16/Ki-67 in cytology samples from women with atypical squamous cells of undetermined significance (ASC-US), low-grade squamous intraepithelial lesion (LSIL) or normal cytological reports with presence of high-risk human papillomavirus (HPV), helps in predicting the risk of developing high-grade cervical lesions during one-year of follow-up after a normal initial colposcopy. Materials and Methods: One-hundred and sixty women with ASC-US, LSIL or otherwise normal cytology, but with the presence of high-risk HPV, were referred to the colposcopy Unit of our Hospital. Cytology and HPV testing were repeated and dual staining of p16/Ki-67 performed on a new cytological specimen, and subsequently patients were colposcopically assessed and prospectively followed-up for one year, after which the colposcopy was repeated. An optional intermediate colposcopical assessment after six months was also offered. Results: Out of 143/160 women with a normal initial colposcopy, 13 were ultimately lost to follow-up. Out of the remaining 130, nine developed histologically verified cervical intraepithelial neoplasia or higher grade (CIN2+) lesions during the one-year follow-up period. Two thirds of them (6/9) were initially p16/Ki-67-positive. Conclusion: Biomarker detection may identify women at higher risk of CIN2+, and these women may benefit from early colposcopic assessment. Women who test negatively for the biomarkers could eventually follow a less aggressive protocol.*

The reduction of cervical cancer incidence has been due to cervical cytology-based screening and triage (1). The finding that human papillomavirus (HPV) infection was necessary for the development of cervical cancer led to the conclusion that high-risk HPV test results could predict the risk of cervical cancer and its precursors (2). Infections with HPV are mostly asymptomatic, and only suggested by abnormal cytology and confirmed by the presence of high-risk HPV during a gynecological examination (3). A large proportion of lesions regress spontaneously; the rest progress due to persistent infection, advancing age, viral type and other unknown factors (4).

In developed countries, the management of women with low-grade lesions, such as atypical squamous cells of undetermined significance (ASC-US), low-grade squamous intraepithelial lesions (LSIL) and positive high-risk HPV tests (5) in the face of an otherwise normal cytology is still an issue. Patients with these reports may experience significant psychological distress, and clinicians are required to repeat tests, which involve high healthcare costs. In this context, it would be of clinical interest to properly identify women with transient HPV infection, and women whose infection may persist and who are more likely to have a future high-grade lesion (6).

As we have learned more about the cellular cycle and the alterations induced by HPV oncoproteins E6 and E7 in cervical cells, it has been found that biomarkers of cell proliferation, such as Ki-67, as well as of proliferation control, such as p16, may be a potential tool for identifying women at risk of developing high-grade precursors and cervical cancer (7). The management of ASC-US, low-grade lesions and positive high-risk HPV testing is based on repeat cytology or colposcopy (8) but due to frequent regression of the precursor lesions, high specificity is required to prevent anxiety and to ensure that any woman referred for a colposcopy is indeed at risk, avoiding unnecessary procedures in other cases and the potential harm associated with unnecessary testing.

Materials and Methods

One-hundred and sixty women with normal cytology, but containing oncogenic HPV, or ASC-US or LSIL cytology were referred to the colposcopy unit of Hospital San Agustín, Avilés, Asturias, Spain

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Key Words: p16/ki-67, oncogenic HPV DNA, low-grade squamous intraepithelial lesion.

from the health area served by the hospital. At the colposcopy unit, cytology and HPV testing were repeated, dual staining of p16/Ki-67 was performed on this new cytological specimen, and subsequently the women were colposcopically assessed and prospectively followed up for one year. The project was approved by the Ethics Committee of the regional reference hospital (approval No. 1/2010) and project funding was obtained from the Research Committee of Hospital San Agustín.

In Spain, preventive gynecological examinations including cervical cancer screening are opportunistic; most are performed annually or biannually. Thus, the cytological reports came from an opportunist triage performed in our population at the primary healthcare centers dependent on our hospital.

At the first colposcopy visit, a new study sample was collected in a solution vial filled with PreservCyt liquid solution (Cytyc Corporation, Marlborough, MA, USA). Interpretation was based on the 2001 Bethesda System (8). The molecular biology test was performed using the same sample specimen used to study viral DNA. The technique employed for diagnosis was Hybrid Capture 2® (HC2) following the methodology of Qiagen (Gaithersburg, MD, USA), which measures the genomic sequence of 13 high-risk viruses. If these genotypes are present in the sample, the result is considered positive for HPV DNA.

From the remaining sample, the biomarker study was conducted. We employed the CINtec® Plus Kit (REF 9531) developed by mtm Laboratories AG, Heidelberg, Germany, following the manufacturer's instructions. The CINtec® Plus Kit is a CE–IVD (European Directives for *In-Vitro* Diagnosis) product, approved for clinical use, which is not yet approved by the US Food and Drug Administration. CINtec® Plus is a dual-stain cytology test. Brown cytoplasmic staining shows overexpression of p16; nuclear red staining shows Ki-67 expression. A result is considered positive when one or more dually stained cervical cells are found. This diagnosis is independent of any cellular morphological abnormalities. Colposcopic terminology follows the criteria approved by the International Federation of Cervical Pathology and Colposcopy, 2002 and 2011 (9). We performed a directed biopsy in any colposcopically suspicious areas. Two biopsies were obtained from the most suspicious areas. On some rare occasions, three biopsies were obtained. If colposcopy was inadequate, an endocervical microcurettage was performed. We used the histological result of the biopsy as diagnostic reference value according to the cervical intraepithelial neoplasia (CIN) system. For statistical purposes, if the cervical biopsy was CIN2 or higher, including high-grade glandular lesions and adenocarcinoma *in situ*, the result was considered positive. Any other less severe or benign lesions were considered negative. All women diagnosed with CIN2+ were treated with loop electrosurgical excision procedures (LEEP). Regardless of the time of diagnosis, they were monitored via colposcopy at 3 months after LEEP, and cytology, viral DNA study and biomarker tests were repeated at this time.

Of the initial 160 women, 141 had their final revision performed at 12 months, at which time cytology, HPV testing, dual immunohistochemical staining and colposcopy were repeated. Of the 19 women with missing data, two women were excluded because of pregnancy, two had undergone hysterectomies, and 15 were lost to follow-up for unknown reasons. Out of the 141 women undergoing final assessment, 123 had also undergone the optional intermediate evaluation, where cytology, immunohistochemical dual staining and colposcopy were repeated, but HPV testing not. If colposcopically suspicious areas which were biopsied at this time yielded a CIN2+ result, the women were obviously treated by means of LEEP and followed-up as stated above.

The study endpoint was the development of CIN2+ lesions by the initially "healthy" population during the follow-up period of one year.

Results

One hundred and sixty women with either an ASCUS or LSIL cytology or a positive high-risk HPV test with otherwise normal cytology were sent to the colposcopy reference Unit of our Hospital from its dependent primary healthcare Centers between May 2000 and June 2013. At our Center, they were all reassessed by means of liquid cytology, HPV testing and dual staining for p16/Ki-67. Following this initial reassessment, 58 had a cytology report of ASC-US, 47 of LSIL, and 55 had a normal cytology but were high-risk HPV-positive. The age range of included women was 17 to 55 years (mean=36 years). All subsequently underwent colposcopy and biopsy of all colposcopically suspicious areas. Following this, 17 (10.6%) were histologically diagnosed with a CIN2+ lesion (CIN 2, CIN 3 or adenocarcinoma *in situ*). They were accordingly treated by means of LEEP, with the exception of one woman diagnosed initially with CIN2 who chose to remain under observation. After six months, the CIN2 lesion was still present in this patient and LEEP was performed at that time. This group of 17 women served as reference for evaluating the diagnostic sensitivity and specificity of the employed screening tests. The remaining 143 women were then prospectively followed-up for one year to ascertain the value of the three employed tests (cytology, HPV, p16/Ki-67 immunohistochemistry) for predicting the appearance of CIN2+ lesions during that period. Out of these 143 women, 13 were ultimately lost to follow-up, which left us with a final pool of 130 initially "healthy" women.

On the initial repeat cytology carried out at the colposcopy unit of our hospital, out of the 160 women studied, seven presented with the cytological diagnosis of high-grade squamous intraepithelial lesion or atypical squamous cell not excluding high-grade squamous intraepithelial lesion (ASC-H) and 153 with ASC-US or LSIL, or normal results. Thus, cytology, when compared to the final histological diagnosis following colposcopy and biopsy, had a sensitivity of 29%, a specificity of 99%, a positive predictive value of 71% and a negative predictive value of 92% for the diagnosis of CIN2+. The HPV-DNA study at the first visit was positive in 94 cases and negative in 66. For the group of 17 patients with known positive histology for high-grade lesions *versus* the rest, the sensitivity and specificity of high-risk HPV-determination were 94% and 45%, respectively, and the positive and negative predictive values were 17% and 98%, respectively.

Of the initial total of 160 women, 45 showed positive dual immunohistochemical staining for p16/Ki-67, and 31% (14/45) of them were initially diagnosed with CIN2+. Of the remaining 115 women testing negative for p16/Ki-67, 2.6% (3/115) were diagnosed with CIN2+. As shown in Table I, the sensitivity of the p16/Ki-67 test for detecting CIN2+ was 82%, and the specificity was 78%.

At six months, six new CIN2+ cases were diagnosed among the group with a normal initial colposcopy, among 123 out of 143 possible candidates with an initial normal colposcopy who underwent intermediate colposcopical assessment. Out of them, three (50%) showed persistently positive dual staining of p16/Ki-67. In all, out of 13 women with persistently positive results for dual staining at six months after an initial negative colposcopy, 23% (3/13) had developed a high-grade lesion during this period.

After one year of follow-up, once the initially treated patients with confirmed CIN2+ lesions were excluded, we had data on 130 women with initially normal findings on colposcopy. During the follow-up period of 12 months, nine of them developed CIN2+ lesions. Of these, seven were detected at the intermediate optional 6-month assessment, and the remaining two at the 12-month one. The sensitivity, specificity, positive and negative predictive values of p16/Ki-67 dual staining for predicting the occurrence of CIN2+ lesions in this population are summarized in Table II.

It is of interest to note that of the small group of women treated with LEEP at the initial visit, all had negative inmunocytological staining during their follow-up. In three women, positive high-risk HPV persisted during the first follow-up consultation after LEEP sugery. The features of the patients with a confirmed histological diagnosis of CIN2+ and at least six months of follow-up after LEEP treatment, together with the results of all tests employed, are summarized in Table III.

Table I. *Data from initial sample. Relationship of the results of p16/Ki-67 dual staining with histological diagnosis of cervical intraepithelial neoplasia grade 2 or higher (CIN2+) (N=160).*

	CIN2+	Not CIN2+	Total
p16/Ki-67-positive	14	31	45
p16/Ki-67-negative	3	112	115
Total	17	143	160
Sensitivity	82.4% (95% CI=61.28-100%)		
Specificity	78.3% (95% CI=71.21-85.45%)		
PPV	31.1% (95% CI=16.47-45.74%)		
NPV	97.4% (95% CI=94.04-100%)		

95% CI: 95% Confidence interval; NPV: negative predictive value; PPV: positive predictive value.

Table II. *Relationship of the results of p16/Ki-67 dual staining with histological diagnosis of cervical intraepithelial neoplasia grade 2 or higher (CIN2+) during 12 months of follow-up in women with an initial negative colposcopy (N=130).*

	CIN2+	Not CIN2+	Total
p16/Ki-67-positive	6	24	30
p16/Ki-67-negative	3	97	100
Total	9	121	130
Sensitivity	66.6% (95% CI=30.31-100%)		
Specificity	80.1% (95% CI=72.64-87.68%)		
PPV	20% (95% CI=4.01-35.98%)		
NPV	97.% (95% CI=93.15-100%)		

95% CI: 95% Confidence interval; NPV: negative predictive value; PPV: positive predictive value.

Discussion

This is a study on a prospective follow-up of a cohort of women with low-grade abnormal cervical findings (ASCUS, LSIL, negative cytology with positive HPV testing) with the aim of assessing the role of dual staining of p16/Ki-67, a recently developed immunocytological staining procedure, in the management of equivocal or low-grade abnormal cytology or presence of high-risk HPV. We compared the predictive value for the development of high-grade lesions during one year of follow-up of immunocytochemical results with the cytological and HPV test results obtained from the same specimen at the initial visit in order to determine whether or not this additional test would facilitate the assessment of risk of high-grade disease within one year among the patients studied.

In keeping with previous work, our study seems to indicate that this new technique might eventually improve the sensitivity of cytology and the specificity of the HPV test in such diagnoses for the detection of CIN2+ (11,12), if confirmed in wider series with a longer follow-up. Until now, the published literature referred to the new immunocytological technique as a triage test in these diagnoses, and in some cases as a prognostic factor in cytological screening (13). We present here a one-year follow-up of enrolled women, with additional examinations in the cases of LEEP. It is interesting to highlight that all women were negative for p16/Ki-67 post-LEEP, and their subsequent colposcopy confirmed the absence of recurrence to that point. However, as has been just remarked, our data are only preliminary due to the relatively scarce number of cases monitored so far.

It is also worth noticing that in women with persistent simultaneous expression of p16 and Ki-67 in any of their cervical cells during follow-up had a 23% risk of developing a high-grade lesion. One of the limitations of our study regarding

Table III. *Follow-up of patients treated by means of loop electrosurgical excision procedure (LEEP) because of histological CIN2+ diagnosis. Follow-up period: 6 months+.*

	Initial					Post-LEEP	
Age, years	Cytology	HPV	p16/Ki-67	p16/Ki-67 at 6 months	Histological diagnosis	p16/Ki-67	HPV
17	LSIL	Pos	Pos	Neg	AIS	Neg	Neg
39	LSIL	Pos	Pos	Neg	CIN2	Neg	Neg
25	LSIL	Pos	Pos	Neg	CIN3	Neg	Neg
33	LSIL	Pos	Pos	Neg	CIN3	Neg	Neg
28	LSIL	Pos	Pos	Neg	CIN3	Neg	Neg
34	LSIL	Pos	Pos	Neg	CIN2	Neg	Neg
42	LSIL	Pos	Neg	Neg	CIN2	Neg	Pos
26	ASC-US	Pos	Pos	Neg	CIN2	Neg	Pos
36	ASC-US	Neg	Neg	Neg	CIN3	Neg	Neg
41	ASC-US	Pos	Pos	Neg	CIN3	Neg	Pos
33	ASC-US	Pos	Pos	Neg	CIN2	Neg	Neg
41	ASC-US	Neg	Neg	Neg	CIN2	Neg	Neg
34	ASC-US	Pos	Pos	Neg	CIN2	Neg	Pos
22	Normal/HPV+	Pos	Pos	Neg	CIN2	Neg	Neg
53	Normal/HPV+	Pos	Pos	Neg	CIN3	Hysterectomy	Hysterectomy
33	Normal/HPV+	Pos	Pos	Neg	CIN2	Neg	Neg
39	Normal/HPV+	Pos	Pos	Neg	CIN2	Neg	Neg
39	LSIL	Pos	Pos	Pos	CIN3	Neg	Neg
47	LSIL	Pos	Pos	Pos	CIN3	Neg	Pos
23	LSIL	Pos	Neg	?*	CIN3	Neg	Pos
35	ASC-US	Pos	Neg	?*	CIN2	NM	NM
44	ASC-US	Pos	Pos	Pos	CIN2	Neg	Neg
37	Normal/ HPV+	Pos	Neg	Pos	CIN3	Neg	Neg
32	Normal/ HPV+	Pos	Pos	Pos	CIN2	Neg	Pos

HPV: Human papilloma virus; Pos: positive result; Neg: negative result; NM: not monitored; AIS: adenocarcinoma *in situ*; LSIL: low-grade squamous intraepithelial lesion; ASC-US: atypical squamous cells of undetermined significance; ?*: non-measurable sample due to lack of cellularity.

these data, however, is the low number of women with persistently positive dual staining in the absence of abnormal colposcopic findings, since those with suspicious findings confirmed on biopsy were obviously treated. However, although women persistently positive throughout the one-year follow-up were few, it must be stressed again that among those who remained positive after an otherwise normal colposcopy, one in five developed CIN2+ during that one-year period. Conversely, women with a normal colposcopy and negativity for p16/Ki-67 in the initial check-up had a low (3%) risk of developing a CIN2+ lesion during one year of follow-up.

Our results suggest that dual staining might be useful for predicting an uneventful follow-up after LEEP treatment, since all our patients thus treated presented negative dual staining after one year of follow-up and had normal control colposcopies, regardless of their high-risk HPV tests. However, it should be considered that our data are limited and more observational studies are needed.

According to a recent publication, our findings, if confirmed in larger series, could eventually help in reducing the number of repeat colposcopies (14) in the case of persistent negative dual immunohistochemical staining for p16/Ki67 after a normal colposcopy.

In conclusion, we believe that the results of our study are encouraging and that the use of this additional test could eventually lead to a reduction in the number of repeat colposcopy referrals, which would have both economical and emotional implications, reducing costs in procedures and reducing stress, anxiety and potential harm in the women monitored.

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Received July 27, 2015
Revised September 7, 2015
Accepted September 16, 2015