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P16INK4a as a progression/regression tumour marker in LSIL cervix lesions: our clinical experience

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Summary

Purpose of investigation: The aim of this prospective study was the evaluation of low-grade intraepithelial lesion (LSIL) lesions evolvement in woman with evidence of high risk HPV infection and p16^{INK4a} negative expression. *Materials and Methods:* 150 women with cytological diagnosis of LSIL were selected to be underwent to three years of follow-up consisting in smear test, colposcopy, and protein p16^{INK4a} investigation every six months and HPV-test every 12 months. *Result:* Final follow-up showed 45 cases of spontaneous lesion regression and 42 cases of persistence with absence of protein p16^{INK4a} in all of them. There were three cases of disease progression to CIN2, two at 18-month follow-up and one at last follow-up. Disease progression was characterized of p16^{INK4a} expression. *Conclusion:* p16^{INK4a} should help to identify which LSIL cases are inclined to the progression of the disease and focalize which patients are eligible for specific treatment.

Key words: Protein p16^{INK4a}; LSIL; Marker of progression; High risk; HPV infection.

Introduction

Infection with oncogenic HPV types is a necessary condition for the development of cervical intraepithelial neoplasia and cervical cancer in, at least, 95% of cases [1]. Over the last years, researchers focused their studies to develop specific diagnostic techniques for the detection and typing of virus isolated in cervical sample, and to identify lesions at high risk for progression [2]. For these reasons, new biomarkers, such as the protein p16^{INK4a} and mRNA-HPV dosage, encoding the high-risk HPV (HR-HPV) oncoproteins E6, E7 have been developed [3,4]. Protein p16^{INK4a} is a cyclin-dependent kinase inhibitor that slows down the cell cycle by inactivating the cyclin-dependent kinases (CDK4/CDK6) involved in the phosphorylation of the retinoblastoma protein (pRb) [5]. In normal cells p16^{INK4a} transcription is epigenetically repressed through a polyrepressive complex-dependent mechanism. HPV16 E7 induces the expression of the cervical cancer biomarker, p16^{INK4a}. The oncogenic p16^{INK4a} activity depends on inhibition of CDK4/CDK6 activity and it needs to be inhibited in order to allow cells to survive [6]. The correlation between the frequency of p16^{INK4a} overexpression and the severity of preneoplastic cervical lesions in cellular and tissue specimens of women with HPV lesions was well described by Tsoumpou *et al.* [7]. P16^{INK4a} dosage shows significantly higher specificity, accuracy, and positive predictive value, but not in sensitivity and negative predictive value than HR-HPV DNA test for predicting high-grade cervical intraepithelial neoplasia (CIN) and cervical cancer evolvement from atypical squamous cells of undetermined significance (ASCUS) and low-grade intraepithelial lesion (LSIL). A correlation between p16 expressions in ASCUS-categorized women with the presence of SILs in follow-up biopsies and positive HR-HPV viral loads is certain. These evidences suggest that p16 expression is an indicator of pathogenic activity of HR-HPV and could be used with more accuracy than HR-HPV viral load for the detection of reactive changes and LSILs from ASCUS-categorized Pap smears [3, 8]. Therefore, p16^{INK4a} protein dosage has been used in routine clinical practice in the last five years. Aim of the present study was to evaluate the evolvement of LSIL lesions characterized to be HR-HPV positive but p16^{INK4a} protein negative. For this purpose, from 2009 to 2013 all patients with LSIL cytological diagnosis referred to the colposcopy clinic of the Division of Obstetrics and Gynaecology of University of Catania had been tested for HPV, virus type research (HR-HPV DNA test), and p16^{INK4a} protein immunostaining dosage. A total of 150 women between 25 and 55 years of age were included in the study and followed-up every six months for three years.

Materials and Methods

Between 2009 to 2013 a total of 177 patients with a cytological diagnosis of LSIL lesion and characterized to be HR-HPV positive and p16^{INK4a} dosage negative were recruited at the Division of Obstetrics and Gynaecology of University of Catania. All women received comprehensive and detailed information about the study and written informed consents were obtained before entering the study in accordance to the Declaration of Helsinki. Patients were enrolled according to their condition and inclusion/exclusion criteria of the prospective study. The study

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Table 1. — *Viral genotypes of HPV assessed with HPV-DNA test.*

Viral genotype	Numbers of cases
HPV16	70 (46.7%)
HPV31	20 (13.3%)
HPV33	17 (11.3%)
HPV18	22 (14.7%)
HPV52	4 (2.7%)
HPV58	5 (3.3%)
HPV53	5 (3.3%)
HPV59	2 (1.3%)
HPV45	2 (1.3%)
HPV35	3 (2%)

Table 2. — *Follow-up results in number of lesions progression and p16^{INK4a} presentation.*

Follow-up	Lesion progression	Number of cases (%)	p16^{INK4a}
1st follow-up (6 months)	Regression	34 (22.7%)	Negative
	Persistence	116 (77.3%)	Negative
	Progression	0	-
2nd follow-up (12 months)	Regression	64 (42.7%)	Negative
	Persistence	86 (57.3%)	Negative
	Progression	0	-
3rd follow-up (18 months)	Regression	69 (46%)	Negative
	Persistence	79 (52.7%)	Negative
	Progression	2 (1.3%)	Positive
4th follow-up (24 months)	Regression	72 (48%)	Negative
	Persistence	76 (50.7%)	Negative
	Progression	2 (1.3%)	Positive
5th follow-up (30 months)	Regression	75 (50%)	Negative
	Persistence	72 (48%)	Negative
	Progression	3 (2%)	Positive
6th follow-up (36 months)	Regression	75 (50%)	Negative
	Persistence	72 (48%)	Negative
	Progression	3 (2%)	Positive

was not advertised and no remuneration was offered. Colposcopy, HPV test, virus type research, and p16^{INK4a} were performed on all enrolled women. Follow-up was performed every six months for a total duration of three years. Twenty-seven patients dropped out the study, 12 patients asked for immediately surgical treatment pushed by anxiety, and 15 patients were excluded for poor compliance. In the end, the final participants in the study were 150 women between 25 and 55 years, with an average age of 40.2 years ± 8.01 DS. From a careful evaluation of the personal history and risk factors of the enrolled patients, the average numbers of pregnancies was 2.13 ± 1,01 DS; 19 patients (12.6%) had had promiscuous sex during the previous seven years preceding the study, 50 (33.3%) women claimed to have used oral contraceptives in the past, and 26 (17.3%) were under OC treatment during the study. Regarding smoking habits, 54 (36%) patients were regular smokers, 42 (28%) patients were ex-smokers (since at least one year), and 54 (36%) were non-smokers. Virus co-infections cases were present in many patients: 10.7% (n=16) herpes simplex virus-2 (HSV-2), 4.7% (n=7) hepatitis C virus (HCV), and 2.7% (n=4) human immunodeficiency virus (HIV). The authors also found a few cases of super-infection: one (0.6%) case of co-infection with HPV-HCV-HIV and two (0.3%) cases of co-infection with HPV-HSV2-HIV. HPV type test showed that the three most representative viral genotypes found in this study were HPV-16, detected in 70 (46.7%) patients; HPV-18, detected in 22 (14.7%) patients, and HPV-31 detected in 20 (13.3%) patients (Table 1). During the three years of study, all patients underwent an accurate follow-up (cervical smear, colposcopy, p16^{INK4a} protein dosage) every six months and annual HPV-test evaluation. The authors performed a targeted biopsy in 30 cases and p16^{INK4a} protein dosage on the histological sample. Pap-smear test was performed on liquid cytology by THIN prep, a technique that allows to dosage the p16^{INK4a} protein directly. The cytological report followed the Bethesda classification (2001) [9] and the result of p16^{INK4a} protein quantification was performed according to Wentzensen and Bergeron score [10].

Results

The present results showed no lesion progression at first and second follow-ups, performed at six and 12 months. In detail, during the second follow-up, 64 cases in 150 (42.7%) manifested spontaneous lesion regression and 86 (57.3%) cases showed the persistence of the lesion. In all cases with regression and persistent lesions, the p16^{INK4a} protein dosage was negative. During the prospective study, the authors recorded only three (4.5%) cases of lesions progression, respectively, two (3% of all patients) cases at 18-month follow-up, and one (1.5%) at 30-month follow-up evaluation. All three cases were characterized by the positivization of p16^{INK4a} protein dosage. The two cases of lesion progression showed at 18-month follow-up were also recorded during the fourth follow-up. In addition, during the third follow-up at 24 months, the authors registered a slight but significant increment on the numbers of lesion regression cases, passing from 64 (42.7%) cases during the 12-month follow-up to 72 (48%) cases at third follow-up at 18 months. Therefore, they recorded an important reduction of persistence lesion cases that passed from 86 (57.3%) cases at second follow-up to 76 (50.7%) cases during the 24-month follow-up. The p16^{INK4a} protein dosage was still negative in all of regression and persistence lesions cases during the third follow-up. The authors can also confirm that the results obtained during the fourth follow-up at 30 months were absolutely similar to the results recorded during the last follow-up at 36 months. There were 75 (50%) cases of spontaneous lesion regression, especially among young women, and the p16^{INK4a} protein dosage was negative in all of these cases. Furthermore, the 72 (48%) cases of persistence lesion showed an improvement on colposcopy survey associated with a normal cytological report. In these cases the p16^{INK4a} protein dosage was still negative. The greatest data recorded was that the positivization of p16^{INK4a} protein dosage, recorded in the three (2%) cases of lesion progression to CIN-2, steered the pathologists to classify these lesions in high-grade intraepithelial le-

sion (HSIL) instead of LSIL (Table 2).

Discussion

HPV prevalence infection has its highest peek among young women between 20 and 24 years in Europe [1]. In the last decade, the introduction in clinical practice of HPV DNA test increased the low sensibility of the Pap smear test alone, especially in cases of ASCUS or LSIL on cytological report. HPV DNA testing is particularly indicated for women with cervical abnormal cytological evaluation, because of its significantly higher sensitivity than repeated cytology. However, it has a significantly lower specificity [11]. According to literature, the specificity of the HPV DNA testing with cytology triage for CIN 2+ was 99.2%, whereas the specificities of the HPV DNA test alone and cytology were respectively, 93.0% and 99.1%; moreover, the association reached better results among women aged at least 35 years [12]. HPV test performed alone has a good rate of false positive characterized by high sensibility and low specificity and, in according to this evidence, it would create false alarmism among young women where the probability of spontaneous lesion regression is very high [13]. On the other hand, the association HPV test + cytology markedly decreases the number of false-positive screening test results. During this decade, other markers were evaluated to predict cervical cancer progression, such as $p16^{INK4a}$ protein, with the aim to increase the specificity and the predictive positive value (PPV) of HPV test. $P16^{INK4a}$ protein is the product of a tumour suppressor gene and inhibits formation of enzymatically active complexes of cyclin-dependent kinases 4 and 6 (CDK4/6) with D-type cyclins that, preventing the phosphorylation of pRb (retinoblastoma protein), stops the progression of cellular cycle locking the cells on G1 phase. Oncogenic stress induces $p16^{INK4a}$ expression, which in turn triggers cellular senescence through activation of the retinoblastoma tumour suppressor. HPV-associated tumours express high levels of $p16^{INK4a}$ in response to E7 oncoprotein expression. HPV E7 expression causes an acute dependence on KDM6B expression for cell survival [14]. In normal conditions, the $p16^{INK4a}$ protein is not detectable in the cell, but in cells with dysplasia or carcinoma of the cervix, the overexpression of the protein $p16^{INK4a}$ is peculiar. This is a reflex mechanism of defence against viral activity operated by tumour oncoprotein E7 [10]. The main oncogenic activity of E7 of HR-HPV is to prevent the pRB function that, cooperating with the transcription of E2F factor, leads to the transcription of genes that promote cell proliferation [15]. Thus, an increased expression of $p16^{INK4a}$, detectable by immunocytochemistry reaction may represent a useful marker to highlight cells transformed by HPV, which have failed to control the cell cycle. $P16^{INK4a}$ is not the only method to predict the molecular lesion suspicious behaviour, but it is used in several studies, showing the usefulness of different biomarkers association, such as $p16^{INK4a}$ with K – 67, in the management of cervical neoplastic lesions. These evidences underline how physicians' decisions could be refined by the new specific molecular processes knowledge associating with cytological results [16]. HPV testing is not an indication of the presence of the disease; in fact only a small percentage of women who are positive of HPV test will develop cervical cancer in the future. The positivity of $p16^{INK4a}$, however, indicates the presence of disease rather than the risk of developing the disease over time and would therefore be a sensitive marker such as HPV testing, but more specific than existing disease [17, 18]. Nieh *et al.*, comparing the performance of $p16^{INK4a}$ associating with the use of HPV testing in the triage of ASCUS, showed a sensitivity of $p16^{INK4a}$ of 95%, slightly higher but not statistically significant different than sensitivity of the HPV test used alone (86%). Conversely, the specificity was 96%, statistically higher ($p < 0.001$) than HPV test sensitivity (31%) [8].

Conclusion

The quantification of $p16^{INK4a}$ protein helps us to identify, among LSIL lesions, which cases were eligible of specific treatment. The evaluation of $p16^{INK4a}$ protein should assist to expose not well-defined cases or borderline cases helping to better differentiate the mild lesions, and consequently, to evaluate the possible adverse effects of overtreatment in those patients overvalued morphologically. Furthermore, a LSIL lesion negative for $p16^{INK4a}$ progresses very rarely to HSIL, consequently women who have LSIL HR-HPV lesions but $p16^{INK4a}$ negative may benefit from routine screenings.

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