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Expression of p16^{INK4a} in relation to histopathology and viral load of 'high-risk' HPV types in cervical neoplastic lesions

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ABSTRACT

A total of 91 cervical archival biopsy series were analysed for the presence and viral load of 'high-risk' types of human papillomavirus (HR-HPV), and p16^{INK4a} expression. The women had various degrees of CIN (cervical intraepithelial neoplasia). HPV 16 was the most prevalent type found, at 47% frequency. The frequency of HPV 16 increased with increasing immunoreactivity to p16^{INK4a}, from 39% to 44% at cases scored low to medium, to 65% at high reactivity. Thirty (33%) of the samples had negative p16^{INK4a} analysis results, but were positive for HR-HPV. There was no significant correlation between viral load and the level of p16^{INK4a} expression, while the grade of CIN correlated to such expressions. Thus, p16^{INK4a} expression analysis yielded information which is consistent with results from the histopathology and might complement the HPV analysis in a clinical prognostic procedure in order to find women at risk for cervical cancer.

1. Introduction

Evidence, confirmed in various studies, reveals certain human papillomavirus (HPV) oncogenic subtypes as being associated with invasive cervical carcinomas[1,2] and cervical intraepithelial neoplasias.[3,4] The presence of high-risk HPV-DNA identifies both women with disease and those who are at a particular risk of progression to disease.[5] Persistent infection with high-risk types of HPV (HR-HPV), particularly HPV16, is regarded as a principal risk factor in the development of squamous cervical lesions and squamous cervical cancer.[6] However, most infections with HPV regress spontaneously, and in cases that do progress to cancer, a long period of latency is normally observed. HPV-infections are prevalent and often transient among younger women, with a peak of 20–25% at 20–24 years of age. With increasing age, there is a decline in the prevalence of HR-HPV to about 7% at 35 years of age.[7] It is likely that HPV-positive women at that age represent a subset of individuals who do not manage to clear their infections spontaneously. Thus, viral persistence appears to be essential for the development of cervical neoplasias. Some observations also suggest an association between high HR-HPV viral loads and disease progression.[8] High HR-HPV viral loads in smears with normal cytology have been associated with

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increased risk of developing dysplasia and carcinoma *in situ* (CIS).[9] In a recent study of varying CIN grades, varying HPV viral load values were found, regardless of CIN grade.[10]

The p16 protein ($p16^{INK4a}$), a CDKN2A gene product, is a tumour suppressor protein that inhibits cyclin-dependent kinases 4 and 6. The $p16^{INK4a}$ protein normally acts as a down-regulator of cell proliferation, but this inhibitory action is ineffective in the context of HR-HPV infected proliferating cells.[11] Therefore, $p16^{INK4a}$ may be a sensitive surrogate marker for such HR-HPV infections.[11] Expression of $p16^{INK4a}$ triggers a negative feedback control of the pRB protein, thereby enhancing $p16^{INK4a}$ levels.[12] Overexpression of $p16^{INK4a}$ is the result of pRb inactivation by the HPV E7 protein.[13] Evidence of elevated $p16^{INK4a}$ levels are observed in HPV transformed cell lines, cervical carcinomas, as well as in high grade cervical intraepithelial neoplasias (HSIL).[14,15] Although studies confirm the overexpression of $p16^{INK4a}$ in practically all HSILs, there are few observations of $p16^{INK4a}$ expression in normal squamous cell epithelium of the cervical mucosa.[16,17] A lower grade of positivity is usually found in cervical (glandular) and metaplastic epithelium.[18] Strong expression of $p16^{INK4a}$ is closely associated with lesions infected by high risk HPV rather than low risk HPV types.[16]

In the present study, our aim was to evaluate the relationship between $p16^{INK4a}$ expression and HR-HPV viral load in a series of paraffin-embedded biopsies with pre-neoplastic and neoplastic lesions.

2. Materials and methods

Specimens from 91 biopsies, which represented CIN1-3 lesions, obtained between 2002 and 2003, were collected from the Department of Pathology, Karolinska University Hospital at Huddinge. The original diagnosis were confirmed/revised by one pathologist (BH). The tissues had been fixed in neutral buffered formalin and embedded in paraffin. Serial sections (4 μm thick) were cut from each block. The first section was stained with haematoxylin-eosin and evaluated histopathologically, whereas the following sections were used to prepare DNA for HPV tests. The biopsies were grouped according to morphological diagnosis and the types of HPV present.

2.1. Histological findings

The histological diagnosis has been previously published.[10] The study was comprised of 91 HPV positive cases. According to histopathology, the women had varying degrees of CIN. Among these there were 19 CIN1, 17 CIN2, and 44 CIN3. In eight cases only normal mucosa was found and in three cases the material was insufficient for histological examination.

2.2. Extraction of DNA from tissue sections

The protocols for extraction of DNA from the archival samples have been previously described in detail.[10] Briefly, the analyses were performed on paraffin-embedded (formaldehyde-fixed) tissue sections and extracted using the MagNA Pure LC system (Roche) and a specific Spin Column to bind DNA (Qiagen kit).

2.3. Detection and quantification of HPV "by real-time PCR

The real-time PCR method ('Quantovir') used for typing and quantification of nine high-risk HPV types have been previously published.[10]

2.4. Immunohistochemistry

Immunohistochemical staining was performed with epitope retrieval by heating the sections for 10 min in 10 mM citrate buffer (pH 6.0) in a microwave oven. Endogenous peroxidase activity was abolished with 0.75% hydrogen peroxide (H_2O_2) in methanol and nonspecific binding of primary antibodies to epitomes by a preincubating the sections in 5% normal goat serum for 30 min at room temperature. The primary antibody (DAKO Cytomation, Glostrup, Denmark) was incubated at 4 °C overnight. Reaction products were visualised by a peroxidase labelled secondary antibody (Amersham Biosciences AB, Uppsala, Sweden) and the AEC+substrate-chromogen (DAKO Cytomation, Glostrup, Denmark). Tissue sections containing colon cancer were used as positive controls for $p16^{INK4a}$, while stained negative controls consisted of PBS, pH 7.4, in place of primary antibodies.

The immune staining was evaluated independently by two observers (CF-S and AH) and was considered positive for $p16^{INK4a}$ when the nuclei were clearly positive. In addition, cells with distinct cytoplasmic immunoreaction were scored positive. Image analysis was done as previously described.[19] Scoring of immunohistochemistry results were performed on the basis of both the staining intensity and the percentage of immunoreactive epithelial cells.[14,20] The scoring criteria for $p16^{INK4a}$, as shown in percentages (%): - (negative): (no expression); <20: weak staining ±; 20–30, weak or moderate staining +; 31–50 moderate or strong staining ++; >50 strong staining +++. The scores +, ++ and +++ were considered positive sample for $p16^{INK4a}$.[14,20]

The study was approved by the Local Ethics Committee of the Karolinska Hospital, Stockholm, Sweden.

3. Results

3.1. Frequencies of HR-HPV DNA

The frequencies of the different of HR-HPV types in all the 91 samples analysed with real-time PCR, were: HPV 16 was the most common at 47%, followed by HPV 33 group at 28%, HPV 18/45 at 10%, HPV 31 and HPV 39 at 8% respectively. Multiple infections were found at 10% (9/91) of the samples.

3.2. $P16^{INK4a}$ expression in relation to CIN grade

In 85 cases the material sufficed for immunological analysis of the $p16^{INK4a}$ antigen (Fig. 1). Reactivity to this antigen could only be demonstrated in 47% (9/19) of CIN 1 lesions, in 65% (11/17) of CIN 2 and in 77% (34/44) of CIN 3 cases. The level of reactivity correlated with CIN grade (chi-square exact test for trend, $p = 0.022$), Spearman rho = 0.476, $p < 0.001$. Presence of the $p16^{INK4a}$ antigen could be seen in one of the eight cases with only normal mucosa present in the biopsy while reactive

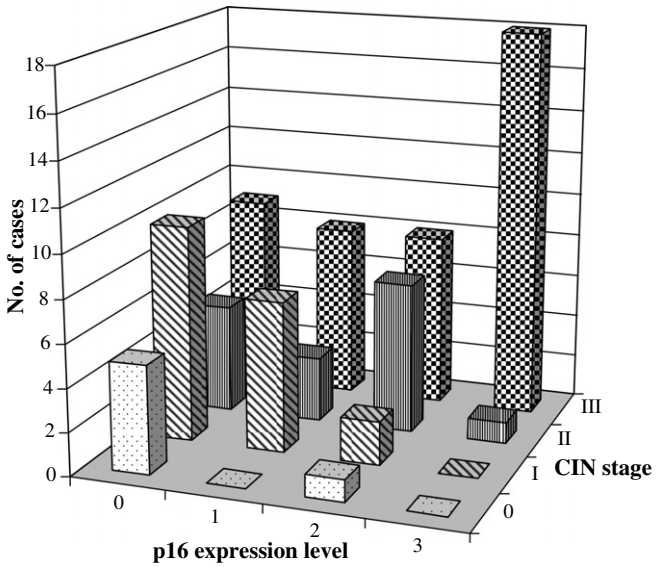

Fig. 1 – Frequencies of the HR-HPV types in samples from different scores of p16 expression. Frequencies of the different scores of p16 antigene expression in total 85 samples, distributed over the three CIN stages, are shown. CIN1 (19 cases) – crosshatched bars, CIN2 (16 cases) – vertical hatched bars, CIN3 (43 cases) – chess hatched bars, and six cases with normal histology punctuated bars. One case is not shown in the figure because this case belongs to cases where the material was insufficient for the histological analysis and the expression of p16 was scored as 2++.

cells could be seen in one of the cases insufficient for CIN grading.

3.3. *p16^{INK4a} expression in relation to different types of HR-HPV*

In Table 1, results are summarised regarding the relationship between the different types of HR-HPV and the expression of p16^{INK4a} antigen. The percentage of samples, which were positive, was dependent on the HPV type. The frequency of HPV 16 positive cases at different expression levels of p16 was 44% in + scored, 39% in ++ scored and 65% in +++ scored samples. Of the samples negative for p16^{INK4a}, we found 41% HPV 16 positive, 7% 18/45 HPV positive, 14% HPV 31 positive, 34% HPV 33 positive and 7% HPV 39 positive results.

Table 1 – p16 expression level in relation to presence of different HR-HPV types

P16 expression level	0	1 (+)	2 (++)	3 (+++)
HPV 16	12/29 (41%)	8/18 (44%)	7/18 (39%)	13/20 (65%)
HPV 18/45	2/29 (7%)	3/18 (17%)	3/18 (17%)	2/20 (10%)
HPV 31	4/29 (14%)	0/18 (0%)	1/18 (6%)	2/20 (10%)
HPV 33/52/58/67	10/29 (34%)	4/18 (22%)	8/18 (44%)	5/20 (25%)
HPV 39	2/29 (7%)	4/18 (22%)	1/18 (6%)	1/20 (5%)[a]

The number of multiple infected cases was 9/85 (11%). Presence of each type was scored also in co-infected cases.
a This case was co-infected with HPV 16 and 33/52/58/67 group.

3.4. *Viral loads*

3.4.1. *HR-HPV*

The viral loads obtained for HR-HPV in relation to expression of p16 antigene is shown in Fig. 2. There was a distribution of viral load values of several orders of magnitude at all scoring grades. The span of values of HR-HPV copies per cell ranged from 0.02 to 36,000. The number of copies per cell had a mean value of: + scored 2032 (SD = 8478); ++ scored 677 (SD = 2004), and +++ scored samples 83 (SD = 267). However, by using one-way ANOVA analysis, there was no statistically significant difference between the average values. In the samples, which were negative for the expression of the p16^{INK4a} antigene, the mean values of the copies per cell was 888 (SD = 3934).

3.4.2. *HPV 16*

Since HPV 16 is the most predominant type of HPV in this material, as well as in cervical squamous carcinomas, viral

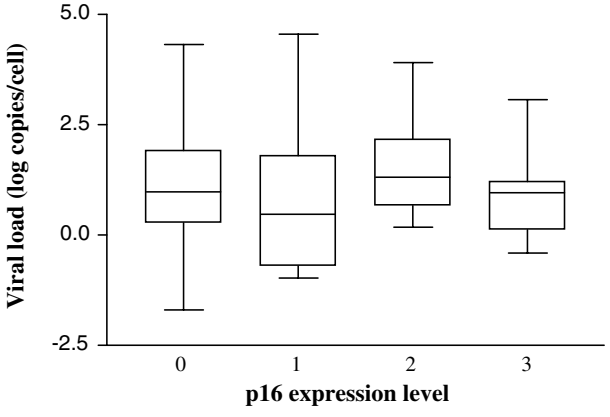

Fig. 2 – HR-HPV viral load and p16 antigene expression. Distribution of the log-values for viral load at different scores of p16 expression is shown. The horizontal line denotes average log-values.

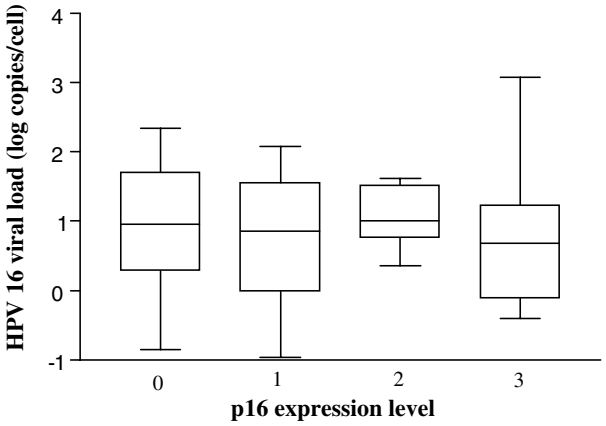

Fig. 3 – HPV 16 viral load and p16 antigene expression. Distribution of the log-values is shown for viral load at different scores of p16 expression. The horizontal line denotes average log-values.

load values were examined separately for this HPV type in correlation to $p16^{INK4a}$ expression (Fig. 3). The mean values of the copies per cell were: + scored 27 (SD = 42); in ++ scored 16 (SD = 14), and in +++ scored samples 107 (SD = 330). However, by using the one-way ANOVA analysis, there were no significant difference between the values. In the samples which were negative for expression of $p16^{INK4a}$ antigene expression had a mean value of 41 (SD = 66) as the number of copies per cell.

4. Discussion

The gene for the tumour suppressor protein $p16^{INK4a}$ has been reported to be inactivated in many forms of human cancer, whereas in cervical cancer, a strong nuclear and cytoplasmic overexpression of $p16^{INK4a}$ has been observed.[21] This overexpression of $p16^{INK4a}$ in cervical cancer, and its precursor lesions, is supposed to result from the functional inactivation of the pRb protein by the HR-HPV E7 oncoprotein.[15] It has been suggested that $p16^{INK4a}$ transcription may be directly induced by the transcription factor E2F, released from pRb after binding to the HR-HPV E7 protein.[12] Klaes et al. demonstrated that the use of $p16^{INK4a}$ immunostaining allows precise identification of CIN and cervical cancer lesions in cervical biopsy specimens and can significantly reduce false-negative and false-positive interpretation in cervical cancer screening.[22] The specificity of the staining has been discussed by Murphy et al., who found that the method has pitfalls in cervical glandular lesions and showed a limited applicability for $p16^{INK4a}$ in relation to such lesions. Nielsen et al. demonstrated $p16^{INK4a}$ staining in some areas of squamous metaplasia with only relatively few Ki-67 positive cells in the same area.[23] Ki-67 (MIB1) has been suggested as alternative specific marker of progression both in cytology[24] and in histopathology.[25,26] Cameron et al. suggested a combined use of these two markers in histopathology.[27] In our study there was a correlation between CIN grade and $p16^{INK4a}$ expression levels with more advanced lesions showing stronger reactivity. However, when weaker reactivities were encountered, only 47–77% of different CIN groups were reactive, indicating a lower sensitivity for detecting such a lesion. Comparing our data with those of Benevolo et al. we find a less stringent correlation between CIN grade and $p16^{INK4a}$ expression.[28]

The lack of $p16^{INK4a}$ expression in some HR-HPV positive lesions might be explained by the regressive nature of these particular cases.

Preinvasive lesions of the cervix are frequent, especially in young women, with a peak in incidence between the age of 25 and 40 years. There is of yet no reliable way to predict if a CIN lesion will progress to invasive cancer or if it will remain stable or regress to normal. One third of the samples in our study that were negative in the $p16^{INK4a}$ analysis, were positive for HPV. Since HR-HPV infections result in progression to cervical cancer in only a small percentage of infected women, after a long period of latency,[29] detection of $p16^{INK4a}$ in addition to HR-HPV may be more useful than only HPV DNA tests for the detection of active and potentially persistent infection.

A combination of organised and opportunistic screening has reduced the incidence of squamous carcinoma substantially during the last few decades in Sweden. Studies have shown that 15–28% of HPV-DNA-positive women with normal cytology developed CIN within 2 years, compared with only 1–3% of HPV-DNA negative women.[30] Against this background a screening strategy for cervical cancer has been presented in which HPV testing is combined with cytological examinations.[2] On the other hand, the abundance of transient infections and lack of treatment for HPV infections make it impractical to follow up all infected women in such a screening design. HPV testing was identified to have higher sensitivity and equal specificity compared to repeated Pap smear collections as a triage for CIN2-3.[31] Viral load and integration have been proposed as parameters for increasing the specificity of HPV tests. Thus, it has been found that women infected with a high viral load of HPV 16 are at an increased risk of developing cervical carcinoma *in situ* several years before diagnosis, compared to women infected with a low viral dose.[8,9] By using quantitative methods to measure HPV viral load, we may have a sensitive tool for identifying women with an overrisk for cervical carcinoma. This would have important applications for cervical screening.

In the present study we found a positive correlation between grade of dysplasia and $p16^{INK4a}$ staining, Spearman rho = 0.476, $p < 0.001$.

No such relationship was seen for viral load and we did not find any relationship between $p16^{INK4a}$ and viral load either. These findings indicate that the viral load *per se* may not be the decisive factor for progression, not even for HPV 16. The finding by others in retrospective investigations[9] may be interpreted more in terms of viral load affecting the host defence and the persistence of the virus, rather than any factor directly related to oncogenesis. Our interpretation is that the measurement of $p16^{INK4a}$ expression has a potential for use in a secondary screening context, while viral load is a weaker candidate. The use of $p16^{INK4a}$ in histopathology appears to be standardising the evaluation of dysplasia, possibly together with Ki-67.

In conclusion the demonstration of $p16^{INK4a}$ accumulation in the cell nucleus is one simple way of emphasising the presence of dysplastic cells, and this staining can be applied to

cytological samples, particularly when used on liquid based samples. Although in the future, vaccination might prevent up to 80% of cervical cancers worldwide, the need for screening in some form is likely to remain.

Conflict of interest statement

None declared.

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