

ORIGINAL PAPER

P53, p63 and Ki-67 assessment in HPV-induced cervical neoplasia

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Abstract

Carcinoma of the uterine cervix is the most frequent malignancy in women, with an incidence of approximately 456.000 cases per year, leading to 200.000 deaths per year. Twenty-six archived formalin-fixed paraffin-embedded samples of squamous cell carcinoma, selected from 30 Papanicolaou-positive smears, have been analyzed using standard HE stain and the IHC indirect tristadial ABC peroxidase method for four antibodies: p53, p63, Ki-67, HPV. Statistical analysis has been done using the Student t-test, one-group two tails, "paired two samples for mean" variant. Two thirds of the cases were medium and poor differentiated carcinomas. The expression pattern of the proliferation and prognostic factors was biologically correlated with the histopathological type and HPV-infection. Two statistically significant correlations were found between p63 and Ki-67 and between p63 and p53 ($p<0.001$). The significant increase of the expression of the analyzed immunomarkers was observed in most of the cases with late stage of cervical neoplasm. P63, followed by Ki-67, showed better correlation with cancer progression than p53. This observation could be used in clinical practice with the purpose of identifying those patients requiring more aggressive treatment.

Keywords: cervical cancer, tumor suppressor genes, prognostic factors.

■ Introduction

Carcinoma of the uterine cervix is the most frequent malignancy in women worldwide, with an incidence of approximately 456.000 cases per year (15% of newly discovered cancers in women), leading to 200.000 deaths per year [1]. In this context, more than 99% of cervical cancers are positive for high-risk human papilloma viruses (HPVs). E6 and E7 genes encode oncoproteins responsible for virus replication, and also for immortalization and transformation of human keratinocytes [2, 3]. The interaction of human papilloma viruses' oncoproteins E6 and E7 with cell cycle proteins leads to disturbances of the cell cycle mechanism and subsequent alteration in the expression of some proteins, such as p53, p63 and Ki-67. The affinity of these viral proteins for the tumor suppressing gene products differs in relation with the oncogenic potential of HPV: the affinity increases for high-risk viruses and decreases for low risk viruses [1]. We have tried to compare the alterations in the expression of these aforementioned proteins in squamous cell carcinoma of the cervix with different patterns.

■ Material and Methods

Tissue samples

We have retrieved from our database, in an interval between 2006–2008, 26-archived formalin-fixed

paraffin-embedded samples of squamous cell carcinoma selected from thirty Papanicolaou-positive smears. The mean age of the women from the studied batch was 35 years ($SE \pm 2.5$). Sections were cut at 5- μ m and stained using the standard H&E stain.

Immunohistochemistry (IHC)

The indirect tristadial ABC peroxidase immunohistochemical method was used for a panel of four antibodies (p53, p63, Ki-67, HPV); details are shown in Table 1.

Table 1 – Antibodies used in the study batch

Antigen	Clone	Specificity	Producer	Dilution
P63	4A4	Nuclear protein of p63 gene	Biogenex	1:20
P53	DO7	Nuclear protein of p53 gene	Neomarkers	1:50
Ki-67	MIB1	Proliferation index	DAKO	1:50
HPV-16	Cam Vir-1	Human papilloma virus capsid	Biogenex	1:50

The immunohistochemistry (IHC) was performed on 3- μ m thick sections from 10% formalin-fixed paraffin-embedded tissues, according to the indirect tristadial avidin-biotin-peroxidase complex method of Hsu SM *et al.* [4], modified by Bussolati G and Gugliotta P [5].

Briefly, the procedure was: deparaffinization in xylene and alcohol series, rehydration, washing in phosphate saline buffer (PBS), incubation with normal

serum, for 20 min., incubation with primary antibody overnight, standard labeled streptavidine-antibody biotin (LSAB) kit (DAKO), washing in carbonate buffer and development in 3,3'-DAB hydrochloride/H₂O₂; microwave antigen retrieval in M-citrate buffer pH 6.0 was performed for certain antibodies.

All specimens were counterstained with Meyer's Hematoxylin, examined and photographed on a Nikon Eclipse E600 microscope. To ensure the reliability of the experimental study, internal quality control of immunohistochemical techniques was performed as a part of an implemented and certified quality assurance system (ISO 15189:2007).

Statistics

For correlation between IHC parameters used in the study batch, statistical analysis has been done using the Student *t*-test, "paired two samples for mean" variant, one-group two tails, from the Analysis Tool Pak of Microsoft-Excel 2003, running under Windows

XP Professional. A value of *p*<0.05 was considered significant.

Results

The classic histopathology investigation has shown six cases (23.07%) of carcinoma in situ, three cases (11.53%) of well differentiated (G1) squamous cell carcinoma, with keratotic pearls (Figure 1), eight cases (30.76%) of medium differentiated (G2) carcinoma and nine cases (34.61%) of poor differentiated (G3) carcinoma, mostly with non-keratinized large cells (Figure 2).

Some cases with invasive carcinoma had adjacent areas with carcinoma in situ, developed on glandular metaplasia.

Immunohistochemically, HPV16 stain was focally positive in 22% of cases, in the cytoplasm and nuclei of tumor cells. HPV16 was also positive in the areas with dysplasia and carcinoma *in situ* (Figures 3 and 4).

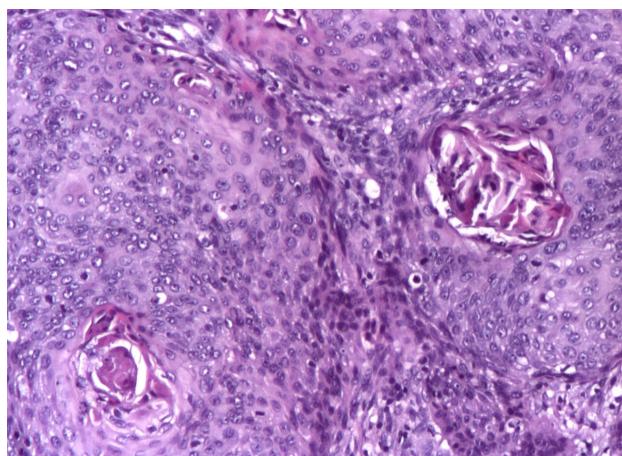


Figure 1 – Keratotic pearls in a keratinizing squamous cell carcinoma of the cervix (HE stain, ob. $\times 10$).

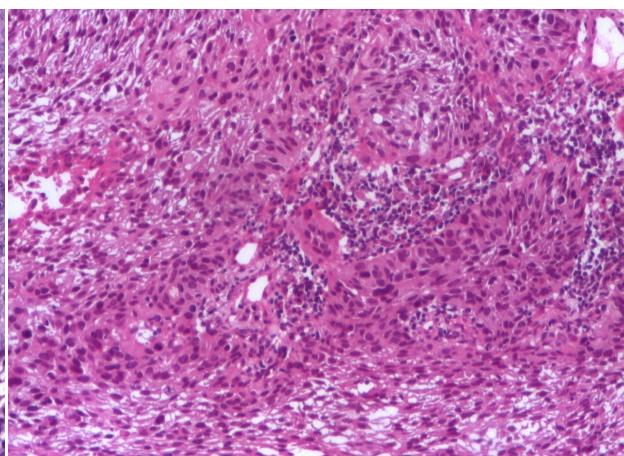


Figure 2 – Large cell non-keratinizing squamous carcinoma of the cervix (HE stain, ob. $\times 10$).

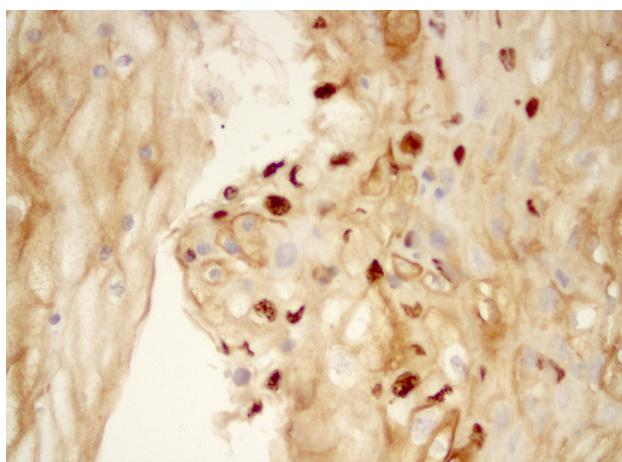


Figure 3 – HPV16 focally positive in dysplastic areas (IHC, ob. $\times 10$).

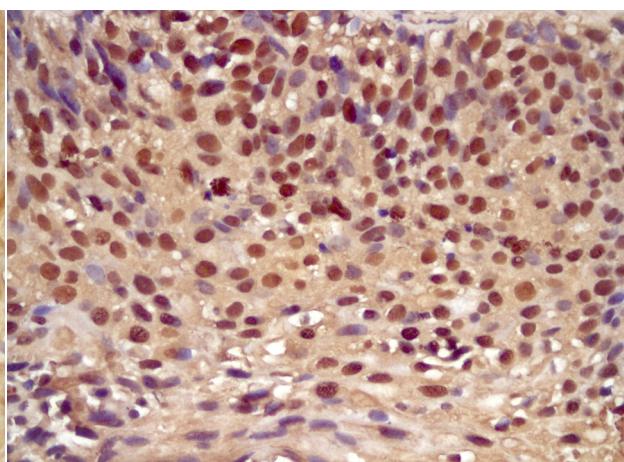


Figure 4 – HPV16-positive in the nuclei of tumor cells from a carcinoma in situ of the cervix (IHC, $\times 10$).

Ki-67 was positive in 20 of 26 cases (76.92%), ranging from 10% to 50% ($m=0.24$, $SE=0.04$) in the tumor cells nuclei (Figure 5).

P53 was expressed in 14 of 26 cases (53.86%), ranging from 5% to 30% ($m=0.05$, $SE=0.01$) – Figure 6, and in the basal cells nuclei of normal epithelium

(positive intern control). P63 was expressed in 25 of 26 cases (96.15%), ranging from 25 to 80% ($m=0.5$, $SE=0.03$), with variable intensity, in different histopathological types (Figures 7 and 8).

The expression pattern of the proliferation and prognostic factors (Ki-67, p53 and p63) was

biologically correlated with the histopathological type and HPV-infection.

Reasonable statistically significant correlations were

found between p63 and Ki-67 ($r=0.33, p<0.001$) – Figure 9, and between p63 and p53 ($r=-0.5, p<0.001$) – Figure 10.

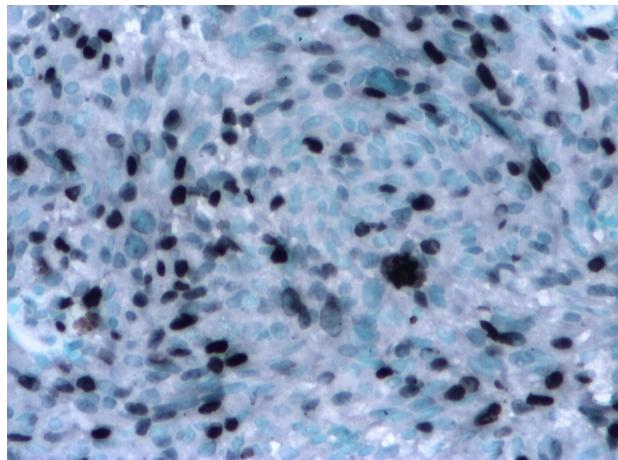


Figure 5 – Ki-67 staining 25–30% of the tumor cells nuclei in a non-keratinizing squamous cell carcinoma (ob. $\times 20$).

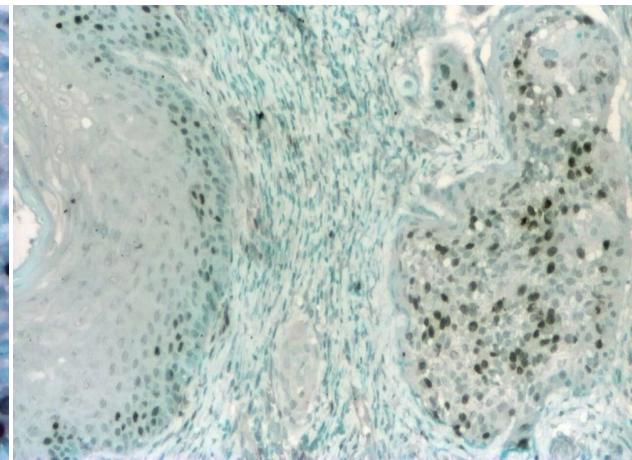


Figure 6 – p53-positive in the basal cells nuclei of normal epithelium and in a tumor nest (25–30%) of a non-keratinizing squamous cell carcinoma of the cervix (ob. $\times 10$).

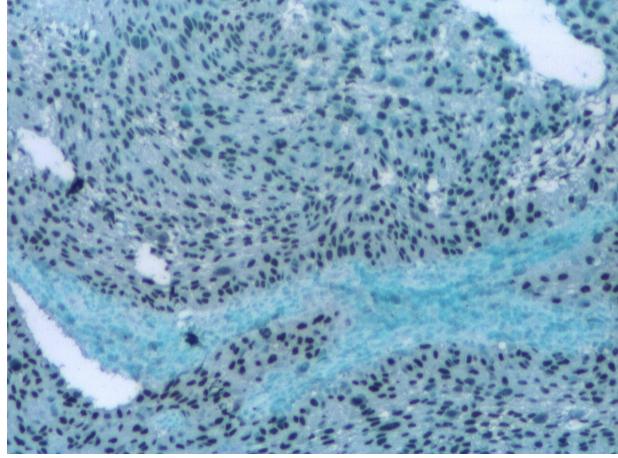


Figure 7 – p63-positive in the tumor cells nuclei (75–80%) in a non-keratinizing squamous cell carcinoma (ob. $\times 4$).

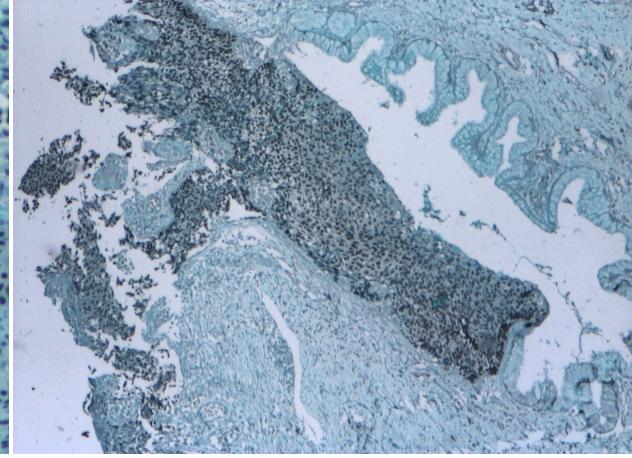


Figure 8 – p63-positive in the tumor cells nuclei (75–80%) in an in situ squamous cell carcinoma developed on a glandular metaplasia (ob. $\times 4$).

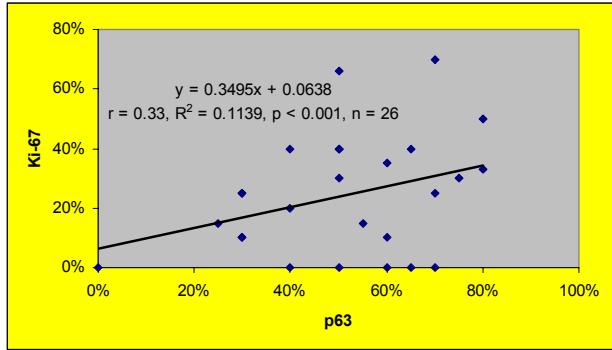


Figure 9 – Correlation between p63 and Ki-67 in squamous cell carcinoma of the cervix.

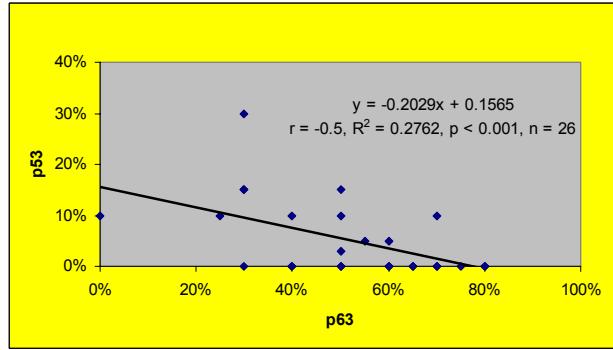


Figure 10 – Correlation between p63 and p53 in squamous cell carcinoma of the cervix.

Discussion

Ki-67 is a proliferation marker, which correlates with the histological grade of cervical neoplasia. Recently, it was found that in patients with high-risk HPV the viral load (detected by hybrid capture II

method) is positively correlated with the expression of Ki-67 and CIN grade [6].

There were also some correlations of high-risk HPV load to cervical intraepithelial neoplasia grades, expression of Ki-67 and P16ink4a: the high-risk HPV load had positive correlation to the progression of

cervical lesion and increased malignancy of cells [6].

Biological behavior of preneoplastic lesions could be predictable by multiple parameter logistic regression models with Ki-67 labeling index, chromosome index for chromosome 1 and aneusomy for chromosome 1 in cervical smears [7].

Higher MIB-1 levels were seen in tumors with a lower grade and higher stage at diagnosis, being associated with poorer outcome. Moreover, MIB-1 levels seem to be higher in tumors due to infection with HPV16 and 18 compared with HPV-45 [8].

A tissue microarray study using a panel of cervical biomarkers, such as p16, involucrin, Ki-67, and HPV L1 proteins may improve the final reporting of various HPV-induced (pre)neoplastic epithelial lesions [9].

The p53 family proteins, p63 and p73 are similar to p53 in some aspects such as structural homology, transactivation of p53-downstream genes, and induction of apoptosis, but they also differ from p53, because in particular, they are not inhibited by viral oncproteins such as HPV-E6. Therefore, it was suggested that the p53 family proteins could be therapeutic agents for HPV-associated uterine cervical cancers and ESM6 mediated expression of the p53 family proteins would be a safe and strong tumor targeting strategy [10].

In tumor cells, HPV-16 (E6) was predominantly located in nuclei of wild-type p53 cells, and it was able to induce phosphorylation of p53 at multiple sites [11].

Regarding p53, its expression did not correlate with tumor recurrence in a study done on 30 patients. According to this study, immunohistochemistry for p53 protein appears to provide no prognostic information in the patients with early stage cervical cancer treated by surgery [12]. However, it still remains a prognostic factor for the aggressive behavior of the tumor, when it exceeds more than 30% positivity in tumor cells nuclei.

Supporting this data, another study done on 53 patients with follow-up in Serbia, has shown a low incidence of p53 mutations and prevalence of Arg/Arg genotype polymorphic variant of codon 72 of p53 gene in early stages of cervical carcinoma [13].

Using proteomic analysis, TACC3 (transforming acidic coiled coil) was thought to be the critical molecule in mediating the anticancer mechanisms in p53-inactivated cells of HPV-18-positive cervical carcinomas, by inducing G2/M arrest and apoptosis, preliminary data suggesting that the overexpression of TACC3 may be associated with the mechanisms of chemoresistance, tumor progression, cell proliferation and metastasis [14].

P63 is the precursor of p53 and stains the basal cells, being a useful marker of squamous neoplasms within the cervix.

Based on RT-PCR and western blot analysis in cervical cancer cell lines, beta isoform of p63 (possibly DeltaN) may be considered as an important marker in uterine cervical squamous cell carcinogenesis [15].

Also, it was shown that DEK proto-oncogene over-expression disrupts the normal differentiation program of cervical cells independent of p53 or cell death, these phenotypes being accompanied by elevated p63-expression in the absence of p53 destabilization [16].

P63 is of value in distinguishing small cell neuroendocrine carcinoma (p63-negative) from small cell squamous carcinoma (p63-positive) and in confirming that a poorly differentiated carcinoma is squamous in type [17].

Also, it is useful for distinction between glandular and squamous lesions in cervico-vaginal specimens [18] and it seems to be a suitable marker, together with CK17, for identification of cervical stem cell, which is thought to be the HPV target cell [19].

Related to this marker, it was recently discovered, using high-density micro arrays for human cDNA sequences, that a new E6/P63 pathway, together with a strong E7/E2F mitotic pathway, modulates the transcriptome in cervical cancer cells [20].

Conclusions

A significant increase of the expression of the analyzed immunomarkers was observed in most of the cases with late stage of cervical neoplasm. P63, followed by Ki-67, showed better correlation with cancer progression than p53. This observation could be useful in clinical practice in order to identify those patients requiring more aggressive treatment.

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