

**ORIGINAL ARTICLE**

**ASSOCIATION OF BIOMARKERS IN PRE-INVASIVE AND INVASIVE  
CERVICAL CARCINOMA**

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**Abstract:**

**Introduction:** Various reports suggest linkage of protein expression to HPV status in cervical carcinomas. The aim of this study was to determine a possible association between HPV type, protein expression and DNA content in both preinvasive (CIN III) and invasive squamous cell carcinoma (SCC) of the cervix, as well as differences between the studied groups in these parameters.

**Materials and methods:** Sections of formalin-fixed paraffin-embedded tumor tissue from 47 cases of CIN III and 60 cases of invasive SCC of the cervix were subjected to HPV genotyping using LiPA (Line immuno-probe assay) and Flow cytometry for DNA content analysis. Also, immunohistochemical staining was performed. Obtained data were analyzed in SPSS using the Chi square test.

**Results:** The major difference ( $p=0.007$ ) between CIN III and invasive SCC of the cervix was found in DNA content - more aneuploid and tetraploid cases in invasive SCC of the cervix compared to CIN III. Although we observed a similar distribution of studied parameters in both groups, no statistically significant association was found between these parameters, except for p53 and pRb ( $p=0.018$ ).

**Conclusion:** The studied groups differ in DNA content. A significant association between analyzed parameters was observed in Invasive SCC of the cervix between p53 and pRb expression.

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**INTRODUCTION**

Pre-invasive, intraepithelial changes of the cervix are often asymptomatic. Cervical intraepithelial neoplasia (CIN) are diagnosed depending on the degree of epithelial thickness that is affected by intraepithelial changes and are described as defined: CIN I (lower grade lesion), CIN II (intermediate grade lesion) and CIN III (higher-grade lesion). CIN III is in fact cervical carcinoma in situ.

Pre-invasive cervical lesions are not rare, but most of the lower graded dysplasia are reversible, although some may progress into higher-level dysplasia. Only 10-15% of CINII progress into carcinoma.<sup>1</sup> Therefore, early intervention is very important.

Cervical carcinoma in situ or CIN III is a malignant tumor, which can persist for years without becoming invasive. In about 30-50% of cases, CIN III progresses into invasive squamous cell carcinoma (SCC) of the cervix.<sup>2,3</sup> The event that makes the cancer invasive is the penetration of the basal membrane.

Patients with invasive cervical SCC have the same epidemiologic characteristics as patients with CIN III, such as lower socio-economic status, early sexual activity, more partners, etc.

It is well known that HPV infection is a key factor in the development of both in situ and invasive cancer.<sup>4</sup> However, years may pass before cervical carcinoma develops following HPV infection. Not every HPV infection will result in carcinoma development. Namely, in some cases it is possible for HPV to spontaneously disappear from the genital tract after 6-12 months of persistence.<sup>5</sup>

Transformation of the host cells, which leads to neoplasia, begins with the disruption of the circular viral genome - most commonly within the E2 gene, followed by viral genome integration into the host genome. Viral integration leads to loss of E2 function, which normally suppresses the transcription of known oncogenes, E6 and E7.<sup>6</sup>

It is also known that p16 expression is associated with activity of the E6 protein in high risk HPV (hrHPV),<sup>6</sup> and that it interferes with the cyclin D/Rb signal pathway by binding to cyclin D.<sup>7</sup>

The roles of proteins p53 and pRb in diseases related to HPV infections have also been previously described.<sup>8,9</sup> Protein p53 is a tumor suppressor whose association with E6 in high risk HPVs leads to loss of p53 activity.<sup>8</sup> Therefore, it is expected that where hrHPV is found, there will be no p53 expression.

Likewise, protein pRb, also a tumor suppressor, binds to another important oncoprotein - E7 in hrHPV. This E7-Rb binding plays an important role in the cell cycle.<sup>9</sup> Therefore, no pRb expression is expected to be found in patients with hrHPV.

Flow cytometry analysis of DNA content in neoplastic processes has both diagnostic and prognostic value. DNA content is actually the number of chromosomes in a cell. The flow cytometer detects changes in numbers of chromosomes, although it cannot detect smaller changes in chromosomes, like deletions or amplifications. The increase or decrease of one or more chromosomes is called aneuploidy, and the enlargement of the whole haploid set is called polyploidy. DNA content in tumor cells often deviates from the normal diploid state. Also, association of aneuploidy with the progression of cervical cancer was found to be significant.<sup>10</sup>

As most cervical lesions are related to HPV infection, the aim of this retrospective study was to determine potential differences in HPV status and HPV-related proteins, p16, pRb and p53, as well as DNA content between CIN III and invasive cervical carcinoma, which could lead to a better understanding of cell proliferation and development of cancer.

Determining an association between p16, pRb and p53 expression, HPV status and DNA content in each of the studied groups could help explain the significance of each parameter in cancer development.

## MATERIAL AND METHODS

### Case selection

Paraffin embedded tumor tissue blocks were randomly selected from the archives of the Pathology Department, Clinical Hospital Center Zagreb and Zagreb University School of Medicine, from patients diagnosed with CIN III and invasive squamous cell carcinoma of the cervix, from 1978 to 2007.

Tumor samples were histologically re-examined on tissue sections of formalin-fixed (10%) and paraffin-embedded tissue stained with hematoxylin-eosin.

There were 47 cases of CIN III in total, mean age 32.3 years, and 60 cases of Invasive SCC of the cervix, mean age 46.82 years.

### HPV detection

DNA extraction from paraffin-embedded tissue sections (6x10 µm) was performed with commercially available QiAmp DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's recommendations. The protocol started with deparaffinization and rehydration, followed by tissue lysis and centrifugation on filter columns.

The detection of HPV DNA was performed using LiPA (Line immuno Probe Assay) by INNO-LiPA HPV genotyping v2 system (Innogenetics, Gent, Belgium), according to their protocol which is based on the amplification of the L1 region of HPV DNA. Results were visually interpreted.

### Protein detection

Immuno-enzymatic staining for detecting protein expression was performed on 4 µm thick paraffin sections immobilized on slides using the DakoCytomation Autostainer Instrument (Dako, Denmark) according to the protocol recommended by the manufacturer of each visualization kit: for p53 EnVision kit (DAKO, Denmark), for pRb LSAB+System-HRP (Dako, Denmark) and for p16 CINTec p16INK4A Histology Kit (Dako, Glostrup, Denmark). Counterstaining was performed with Hematoxylin (Dako). Nuclear and cytoplasmic staining of >30% of cells was considered positive.

### Flow cytometry for DNA analysis

The samples (3x40µm tissue sections) were prepared using the method described by Hedley et al.<sup>11</sup> Briefly, deparaffinization and rehydration of tissue sections preceded tissue lysis and filtration on nylon membranes, after which extracted nuclei were obtained. The nuclei were stained with propidium iodide. Cellular DNA content was analyzed the following day on the FACS Calibur Flow Cytometer (Becton Dickinson, San Jose, CA, SAD), at a wavelength of 488 nm and using red filter. The CellQuest program was used for the collection of 20 000 nuclei per sample, and DNA histograms were analyzed in the ModFit LT V3.0 program (Verity Software House Inc., Topshame, ME and Becton Dickinson, SAD).

Samples with DNA index (DI) equals to one were considered diploid, those with  $1 < DI < 2$  were aneuploid, and those with  $DI = 2$  were tetraploid.

### Statistical analysis

All statistical analyses were performed with the statistical package SPSS for Windows 9.0. The Chi square test was used to compare the studied groups. P values of less than 0.05 ( $p < 0.05$ ) were considered statistically significant.

## RESULTS

### HPV detection

From 47 cases with CIN III, only one (2.1%) was negative for HPV, and one (2.1%) was positive for low risk type of HPV, while the remaining 45 cases (85.7%) were high-risk types of HPV (Figure 1).

From 60 cases of Invasive squamous cell carcinoma of the cervix, only three (5%) were negative for HPV, three (5%) were low-risk types of HPV, and 54 (90%) were high-risk types of HPV (Figure 1).

With a statistical significance at  $p < 0.05$ , we can conclude that there was no difference in HPV prevalence between these two groups ( $p = 0.533$ ).

### Protein detection

Four (8.5%) cases of CIN III could not be analyzed for p53 detection, while 29 from the remaining 43 cases (67.4%) were negative, and 14 of them (32.6%) were positive for p53 protein (Figure 1).

Also, three (5%) cases of Invasive SCC of the cervix could not be analyzed for p53 detection, while 39 of the remaining 57 cases (68.4%) were p53-negative and 18 of them (31.6%) were p53-positive (Figure 1).

With a statistical significance at  $p < 0.05$ , we can conclude that there was no difference in p53 detection expression between these two groups ( $p = 0.917$ ).

From 47 cases of CIN III, four (8.5%) cases could not be analyzed for pRb detection, while from the remaining 43 cases, 30 (69.8%) were negative for pRb protein, and 13 (30.2%) were positive (Figure 1).

There were three (5%) out of 60 cases with Invasive SCC of the cervix that could not be analyzed, while from the remaining 57 cases, 29 (50.9%) were negative for pRb protein and 28 (49.1%) were positive (Figure 1).

With a statistical significance at  $p < 0.05$ , we can conclude that there was no difference in pRb detection expression between these two groups ( $p = 0.057$ ).

There were 11 (23.4%) cases of CIN III that were negative for p16 protein, and 36 (76.6%) were positive (Figure 1).

There was one (1.7%) case of Invasive SCC of the cervix that could not be analyzed for p16 detection, while from the remaining 59 cases, six were (10.2%) negative and 53 (89.8%) were positive for p16 protein (Figure 1).

With a statistical significance at  $p < 0.05$  we can conclude that there was no difference in p16 expression detection between these two groups ( $p = 0.065$ ).

### DNA content

From 47 CIN III cases there were 20 (42.5%) that could not be analyzed for DNA content, while all 27 remaining cases were diploid (Figure 1).

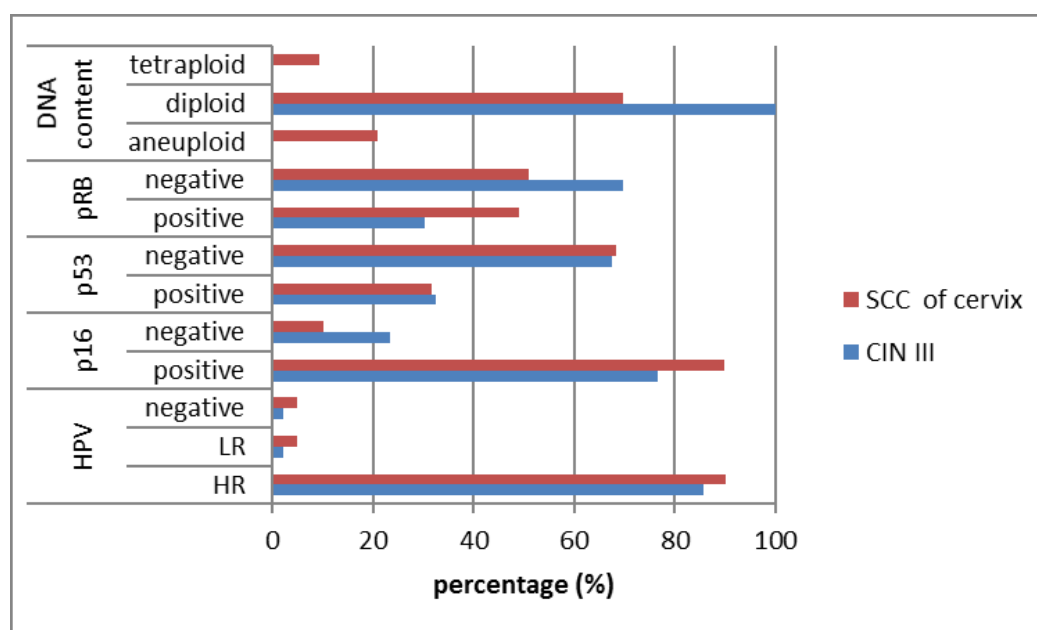


Figure 1. Association of analyzed parameters in study groups (Chi-square test,  $p < 0.05$ )

Also, 17 (28.3%) cases of Invasive SCC of the cervix could not be analyzed, while from the remaining 43 cases, nine (20.9%) were aneuploid, 30 (69.8%) diploid and four (9.3%) tetraploid (Figure 1).

With a statistical significance at  $p < 0.05$  we can conclude that there were fewer aneuploidy and tetraploidy cases and more diploid cases in CIN III compared to Invasive SCC of the cervix ( $p = 0.007$ ).

### **Association of HPV genotype, protein expression and DNA content**

No statistically significant association was found between the analyzed parameters in CIN III. In Invasive SCC of the cervix, a statistically significant association was only found between p53 expression and pRb expression ( $p = 0.018$ ) (Table 1).

**Table 1. Association of analyzed parameters in study groups (Chi-square test,  $p < 0.05$ )**

Studied Parameters	CIN III (p value)	Invasive SCC of the cervix (p value)
HPV-p53	0.622	0.264
HPV-pRb	0.74	0.307
HPV-p16	0.755	0.582
HPV-ploidy	0.804	0.507
p53-pRb	0.182	<b>0.018</b>
P53-p16	0.844	0.541
p53-ploidy	0.387	0.707
pRb-p16	0.599	0.058
pRb-ploidy	0.555	0.193
p16-ploidy	0.064	0.319

Legend: SCC - squamous cell carcinoma

### **DISCUSSION**

The results for HPV prevalence in CIN III as well as in Invasive SCC of the cervix are as expected and as previously reported<sup>5, 12, 13</sup> - they show a high incidence of hrHPV types. However, in our study there are more negative cases than in the previously reported studies mentioned earlier.

It is possible that some negative cases are in fact false negative results because the DNA isolation was performed on paraffin-embedded tissue, which disrupts DNA, and therefore negatively affects HPV detection.<sup>5</sup> In addition, some parts of the viral genome may be lost during the integration process,<sup>6</sup> for instance, the L1 region. This can possibly lead to false negative results of HPV detection, because most commercially available detection methods are based on amplifying the above-mentioned region.

Our results show that p53 expression has not been detected in most cases of CIN III and Invasive SCC of the cervix, which is contrary to some reported data,<sup>14, 15</sup> but consistent with earlier findings reporting that p53 expression is in negative correlation with HPV infection.<sup>15</sup> However, this could not be confirmed in our study.

In most of CIN III cases, pRb expression was not detected, while in cases of Invasive SCC of the cervix, pRb expression was detected in less than 50% of the cases. Our results are not consistent with some previous reports<sup>14</sup> where pRb was detected in nearly all cases in both studied groups. We expected to find little or no pRb expression as was previously reported in another study.<sup>16</sup> However, our results also showed an increase of pRb expression as the disease progresses, which is contrary to our expectations and previously reported results where the expression of pRb in cases with CIN III was higher compared to cases with Invasive SCC of the cervix.<sup>16</sup>

In concordance with previously reported data, p16 expression was detected in CIN III and in Invasive SCC of the cervix. Higher levels of p16 are expected to be found in higher levels of HPV-related malignancies<sup>17, 18</sup> Only one study has shown that no significant correlation between p16 expression and HPV types was found in cervical neoplasia.<sup>15</sup> Our study also showed no statistically significant association between p16 expression and hrHPV detection, although we observed that hrHPV, as well as p16 expression, was detected in both study groups. Although previously reported data show a high percentage (>80%) of aneuploidy in CIN III, our cases are all diploid. It was also reported that aneuploidy is positively correlated with hrHPV,<sup>19, 20</sup> which could not be confirmed in our study.

A high percentage of aneuploidy was also reported in Invasive SCC of the cervix. Though our results show a high incidence of diploid cases, our results also show aneuploidy in some cases. Interestingly, we found tetraploidy (9%) in Invasive SCC of the cervix, which was not previously reported.<sup>9, 21</sup>

Also, as previously reported<sup>9</sup> we confirmed with statistical significance ( $p = 0.007$ ) that DNA content changes with progression of cervical cancer.

A possible explanation for all these differences is the uneven distribution of successfully analyzed cases in studied groups. Unfortunately, not all cases could be analyzed due to low material quality, which is common in older paraffin-embedded samples.

Although we expected to find p16 expression and no p53 and pRb expression in cases with hrHPV, as well as changes in DNA content, we could not find any statistically significant association between studied parameters, except between p53 and pRb expression.

In conclusion, we found no statistically significant differences in HPV prevalence and protein expression between CIN III and Invasive SCC of the cervix. The only statistically significant difference between studied groups was in DNA content.

Also, the only statistically significant association of studied parameters was found in Invasive SCC of the cervix between p53 expression and pRb expression ( $p=0.018$ ).

Although we found some differences between the studied groups, further investigation is needed for a better understanding of cervical carcinoma development.

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