Estrogen receptor, progesterone receptor, and bcl-2 are markers with prognostic significance in CIN III


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There are no known biological markers or technologies to predict the natural history of an individual CIN III. The probability of progression is considered greater with the persistence of high-risk human papillomavirus (HPV) infection and age. p53 polymorphism has been associated with cervical carcinogenesis. Hormone-induced cervical cancer is mediated by estrogen receptor (ER) and progesterone receptor (PR). In cervical cancer, increased bcl-2 and Bax immunoreactivity is generally associated with a better prognosis. The purpose of this study was to evaluate the value of HPV 16 and HPV 18 typing and p53 codon polymorphism genotyping by polymerase chain reaction and ER, PR, bcl-2, and Bax expression by immunohistochemistry in predicting the CIN III clinical behavior of CIN III lesions. We studied the expression of these prognostic factors in the CIN III adjacent to squamous cell microinvasive carcinomas of the cervix (MIC) from 29 patients with FIGO stage IA1 cervical cancer and in 25 patients with CIN III and no documented focus of invasion. In the MIC group, only the CIN III was considered at least 2 mm away from the microinvasive complex. The ER, PR, bcl-2, and Bax immunoreactivity was scored as positive (>10% staining cells) and negative (<10% staining cells). No significant difference was observed between MIC and CIN III group concerning HPV infection and p53 polymorphism. The ER, PR, bcl-2, and Bax immunohistochemical expression was stronger and more frequent in the CIN III group. After multivariable analysis, coexpression of ER, PR, and bcl-2 was the only independent factor in defining low risk of progression for CIN III. Our study suggests that coexpression of ER, PR, and bcl-2 may be a useful tool in identifying the CIN III lesions with low risk of progression to cervical cancer.

KEYWORDS: cervical microinvasive carcinoma, CIN III, immunohistochemistry, prognosis.

Cervical cancer is one of the most frequent cancers in women, with an estimated worldwide incidence of about 371,000 new cases per year and an overall 5-year survival ranging from 44 to 66% for all clinical stages(1).
Invasive cancers are usually accompanied by CIN III, and presumably this lesion precedes most carcinomas\(^2\). The rate of CIN III progression is unknown, but based on follow-up studies of untreated or incompletely treated CIN III, one may assume that risk for cancer progression may be as low as 20% to up to 70%, and regression rate may be as high as 32%\(^3,4\). To prevent invasive cervical cancer, a successful treatment of high-grade CIN, particularly CIN III, is the gold standard management\(^5\). The surveillance schedule for treated patients is not so well defined. Identifying a subset of patients with CIN III with low risk for cancer progression may be helpful in clinical practice for rationalizing follow-up.

HPV infection is present in virtually all cancers and is the strongest epidemiological factor for cervical cancer development\(^6\). Among women with cervical cancer, HPV 16 is the most prevalent HPV type. When determined by polymerase chain reaction (PCR), HPV 16 genoma accounts for more than half of the cases of CIN III and invasive cervical cancer with variations according to race and country\(^6—11\).

Viral integration is important for cell transformation and cervical carcinogenesis. In invasive carcinomas of the cervix, the genoma of HPV 16 and HPV 18 is integrated in up to 70% of the cases\(^12,13\). When viral integration occurs, the HPV genome breaks in the E2 region resulting in loss of its suppressive function on E6 and E7. The E6 gene product of high-risk HPV binds with high affinity to p53, inactivating it by inducing degradation through a selective ubiquitin-dependent proteolytic pathway, promoting uncontrolled cell proliferation and eventual development of cervical neoplasia\(^14,15\). Somatic and germ line mutations of p53 are rarely detected in cervical cancers\(^16,17\); however, at least p53 polymorphisms have been described in both coding and no-coding regions of the genoma\(^18\). Storey et al.\(^19\) were the first to show that the risk for the development of HPV-associated cervical cancer in white British women was significantly higher for arginine homozygosity (Arg/Arg) at codon 72 in the p53 amino acid sequence than for arginine heterozygosity (Arg/Pro) and proline homozygosity. Some studies are in agreement and others have refuted these observations, and these conflicting results have been attributed to ethnic differences in allelic frequencies, sample size, selection bias of controls, DNA source, and the methodology used\(^20,21\).

The HPV E7 protein binds to the Rb-E2F complexes, releasing E2F protein, and targets Rb protein for ubiquitin-dependent proteolysis\(^22\). E2F-1, the best-characterized member of the E2F family, activates the transcription of genes for S-phase and induces apoptosis in the presence of functional p53 protein\(^23,24\). Thus, during HPV infection, the pro-apoptotic signals generated by E7 may be totally or partially counterbalanced by E6 protein.

The product of the proto-oncogene bcl-2 may block apoptosis. Overexpression of bcl-2 is generally associated with a better prognosis in several malignancies including cervical cancer\(^25—29\), and increased Bax immunoreactivity was described as a favorable prognostic factor in cervical cancer\(^30—33\).

An imbalance between HPV-induced cell proliferation and apoptosis may be not sufficient for the development of invasive cervical cancer, and several possible cofactors have been identified, including the steroid hormones estrogen and progesterone. Most cervical cancers arise in the transformation zone, an area known as the most estrogen- and progesterone-sensitive region of the cervix\(^34\). Expression of estrogen receptors (ERs) and progesterone receptors (PRs) has been reported to be a favorable prognostic factor in breast cancer\(^35\), endometrial carcinoma\(^36\), ovarian cancer\(^37,38\), and cervical adenocarcinoma\(^38\). In squamous cell carcinoma of the cervix, ER and PR expression had minor prognostic value\(^39\), but via their receptors, estrogen and progesterone are involved in cervical carcinogenesis\(^40\) by increasing the genoma expression of HPV 16 and HPV 18\(^41—43\).

The purpose of this study was to evaluate the value of HPV 16 and HPV 18 typing, p53 codon polymorphism genotyping, and ER, PR, bcl-2, and Bax immunohistochemical expression as prognostic factors in CIN III.

### Patients and methods

**Patient characteristics**

For this study, two groups of material were selected

(i) MIC group – the CIN III of 30 patients with the diagnosis of FIGO stage IA1 squamous cell carcinoma of the cervix (MIC) diagnosed between 1996 and 1999;

(ii) CIN III group – the CIN III from the 30 first cases with diagnosis of CIN III, with no documented invasive focus, diagnosed by cone biopsy in 1998 were also retrieved for comparative analysis. All cases were diagnosed, treated, and were followed up at the Gynecology Department of Portuguese Cancer Institute of Coimbra. All patients had documented HIV seronegativity, were not pregnant, and had no history of drug addiction as well as no immunosuppressive therapy in the last 6 months.

Two pathologists (LC and EC) reviewed all slides from punch biopsy, conization, and hysterectomy, and the pathological classification was obtained by consensus between them according to the criteria formulated by the World Health Organization. In the MIC group, only the CIN III at least 2 mm away from the microinvasive complex was studied, and from this group one case was excluded because the material was considered not suitable for immunohistochemistry. From the CIN III group, five cases were excluded: one was lost to follow-up; one due to recurrent CIN III developed 6 months after treatment; one had ASCUS Pap smear at the eighteenth month of follow-up; and two were considered not suitable for immunohistochemistry.

The diagnosis of MIC was obtained by conization in 27 (93.1%) cases and by incidental hysterectomy in two (6.9%). In all cases of MIC, the hysterectomy was the definitive treatment, and in the CIN III group, 15 (60.0%) patients were submitted to subsequent hysterectomy and 10 (40.0%) patients remained in follow-up.

All patients selected for this study had a follow-up of at least 30 months, with no documented CIN or invasive recurrence or abnormal Pap smear.

**Tissue specimens**

The specimens were formalin fixed and paraffin embedded. From each, we selected the two more representative blocks. For immunohistochemistry, 3-μm thick consecutive sections were cut from the respective tumor block.

**Detection and typing of HPV**

HPV DNA was extracted according to standard protocols. Consensus primers for L1 gene were used as described by Kleter et al. Specific primers for HPV 16 E6 gene were used, as described by Shibata et al. and specific primers for HPV 18 genes were used as initially described by Tam and Chow. Agarose gel electrophoresis then confirmed the specificity of the amplified DNA. Controls were always used.

**Detection of p53 codon 72 polymorphic genotypes**

Three to six 10-μm thick sections were cut onto histological slides from each case. p53 codon 72 Pro and Arg genotypes were analyzed by allele-specific PCR according to the method described by Storey et al.

**Bax and bcl-2 immunohistochemistry**

The antigens were incubated with monoclonal mouse anti-human bcl-2 (clone 124 (1/40), isotype IgG1; Dako, Glostrup, Denmark) or polyclonal rabbit anti-human Bax (1/500, Dako). To identify antigen–antibody complexes, an immunoperoxidase method employing an avidin–biotin (StreptABC/HRP) peroxidase complex was used for bcl-2 (TechMate 500 Plus, MSIP protocol, Vector Laboratories, Burlingame, CA), and for Bax a catalyzed signal amplification (CSA) system with CSA rabbit link was used. Peroxidase activity was demonstrated by incubating the slides in 3,3′diaminobenzidine (DAB) (Dako) followed by counterstaining with hematoxylin (Richard Allen, Richland, MI) and mounted on a no-aqueous medium (dibutylfталate xylol, DPX). Staining without antibody was performed routinely as a negative control. As positive controls, infiltrating lymphocytes were stained for bcl-2 and Bax.

**ER and PR immunohistochemistry**

The antigens were incubated with monoclonal antibody Novocastra NCL-L-ER-6F11 (1/25) against ERs and monoclonal antibody Novocastra NCL-L-PGR-312 (1/25) against PR.

To identify antigen–antibody complexes, an immunoperoxidase method employing an avidin–biotin (StreptABC/HRP) peroxidase complex (TechMate 500 Plus, MSIP protocol, Vector Laboratories) was used. Peroxidase activity was demonstrated by incubation of the slides in DAB (Dako) followed by counterstaining with hematoxylin (Richard Allen) and mounted using a no-aqueous medium (DPX). Positive control was ER- and PR-positive breast cancer tissue, and specific staining was observed as nuclear brown-colored granules. Negative control was ER- and PR-negative prostate cancer.

**Evaluation of immunohistochemical results**

All hematoxylin and eosin (H&E) and immunostained slides were independently reviewed by two observers (LC and EC). In cases of discrepancy, a consensus was always reached. Cytoplasm immunoreactivity specific for bcl-2 and Bax and nuclear immunoreactivity specific for ER and PR was expressed in terms of the percentage of cells exhibiting specific staining. In all
cases, the intensity of staining was always stronger as more cells were positively stained. A negative staining was considered if less than 10% of the cells showed specific immunoreactivity, independent of the intensity of staining. Similarly, a positive staining was considered if 10% or more of the cells showed specific immunoreactivity. The distribution of the positive cells in the neoplastic epithelium was also recorded. In all cases, 500 cells were randomly counted.

Statistical analysis

Statistical analysis was computed at SSPS 10.0 for Windows (IL). For comparing the mean age, we used independent samples t-test. Chi-square and Fisher’s exact test evaluated the differences in categorical data. The significance was adjusted by computing a binary logistic regression. A P value of <0.05 was considered to reflect a significant difference.

Results

The results of HPV typing, the distribution of the three genotypes of p53 as either Arg and Pro homozygotes or Arg/Pro heterozygote, and the mean age of the patients in both MIC group and CIN III group are outlined in Tables 1, 2, and 3, respectively.

Immunostaining for ER and PR was specially localized to nuclear envelope and occasionally to the cytoplasm (Figs. 1 and 2) and all cases that stained, stained with lower intensity than control. Immunostaining for bcl-2 and Bax was always localized to the cell cytoplasm (Figs.3—5) and occasionally as a finely granular pattern.

In CIN III, the ER, PR, bcl-2, and Bax immunostaining usually involved all epithelial layers, but the expression of bcl-2 (Fig.3) was more intense at the basal two-thirds of the neoplastic epithelium on both CIN III group and MIC group, and the Bax expression (Fig. 4) was stronger at the superficial two-thirds.

The immunohistochemical results for ER, PR, bcl-2, and Bax are outlined in Table 3. After adjustment, by multinomial logistic regression, only coexpression of ER + PR + bcl-2 remained as independent risk factors in defining low risk of progression for CIN III. We computed a sensitivity of 100% and a specificity of 64.4% for ER + PR + bcl-2 as a marker for low risk of progression in CIN III.

For both the MIC group and the CIN III group, there was no statistically significant difference between ER + PR + bcl-2-positive immunostaining and HPV type (P = 0.290) and p53 codon 72 polymorphism (P = 0.755).

Discussion

It has not been possible to predict the natural history of a particular CIN III, even after adequate treatment. Based on the reports of women with CIN III who inadvertently were not treated, we may assume that the progression rate may be high, and this is the reason for treating all women with CIN III(3,4). After treatment, the best management policy in follow-up is not consensual. For the detection of persistent and recurrent high-grade CIN, colposcopy with directed biopsy is a good tool(48), but is very expensive, time consuming, and limited to some medical centers. Cytology is not very sensitive(49,50) and to compensate for its poor sensitivity, clinicians have traditionally used serial repeat cytology. HPV DNA testing for oncogenic HPV types seems comparable to repeated cytology(51). For treatment surveillance, the negative predictive value of HPV testing approaches 100%, but the specificity is low, and this means that oncogenic HPV DNA may persist posttreatment without cytological or histological evidence of disease(52,53).

This study was designed to find out a useful marker to identify a subset of CIN III with low risk of progression to invasive cancer. The immunohistochemical methodology is widely used worldwide. For this reason, we have chosen this technology to evaluate ER, PR, bcl-2, and Bax expression. Formalin-fixed, paraffin-embedded tissue sections with microwave treatment was employed, which is an effective method to expose antigens and advantageous to

Table 1. HPV infection

<table>
<thead>
<tr>
<th></th>
<th>MIC group [n (%)]</th>
<th>CIN III group [n (%)]</th>
</tr>
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<tbody>
<tr>
<td>HPV 16</td>
<td>26 (89.7)</td>
<td>17 (68.0)</td>
</tr>
<tr>
<td>HPV 18</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Other HPV</td>
<td>3 (10.3)</td>
<td>8 (32.0)</td>
</tr>
<tr>
<td>Total</td>
<td>29 (100)</td>
<td>25 (100)</td>
</tr>
</tbody>
</table>

MIC group: CIN III at least 2 mm away from microinvasive complex from patients with the diagnosis of MIC; CIN III group: CIN III from patients with diagnosis of CIN III.

Table 2. p53 codon 72 polymorphism genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>MIC group [n (%)]</th>
<th>CIN III group [n (%)]</th>
</tr>
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<tbody>
<tr>
<td>Arg-Arg</td>
<td>23 (79.3)</td>
<td>18 (72.0)</td>
</tr>
<tr>
<td>Arg-Pro</td>
<td>4 (13.8)</td>
<td>4 (16.0)</td>
</tr>
<tr>
<td>Pro-Pro</td>
<td>2 (6.9)</td>
<td>3 (12.0)</td>
</tr>
<tr>
<td>Total</td>
<td>29 (100)</td>
<td>25 (100)</td>
</tr>
</tbody>
</table>

MIC group: CIN III at least 2 mm away from microinvasive complex from patients with the diagnosis of MIC; CIN III group: CIN III from patients with diagnosis of CIN III.
observe a wider area of tissue sections\textsuperscript{(54)}. The HPV typing and p53 Arg/Pro polymorphism were also studied, as they could be possible confounding variables.

We evaluated the CIN III in 29 patients with MIC, and 25 patients with CIN III were selected as a control group. Probably, this control group belongs to a subset of CIN III with low risk for progression, because all patients had at least 30 months of follow-up without progression or recurrence and no abnormal Pap smear during the follow-up period.

Our results showed that women with MIC were about 5 years older than patients with CIN III group, which is in accordance with a Costa Rica study that suggests that CIN III progresses to subclinical cancer in about 5 years and that symptomatic cancer arises 4 to 5 years later\textsuperscript{(55)}.

We reported a high prevalence of HPV 16 infection in both the studied groups, in agreement with a worldwide study conducted by Bosh \textit{et al}.\textsuperscript{(56)} who found a high prevalence of HPV 16 in cervical cancer in Europe. Identical study on a Portuguese population detected HPV 16 DNA sequences by PCR in 83.3\% of squamous cell cervical cancers\textsuperscript{(57)}.

There are no published studies on p53 polymorphism in Portuguese population, but the frequency of homozygous arginine p53 polymorphism has been

Table 3. Age, HPV 16 and ER, PR, bcl-2, and Bax immunohistochemistry positive results

<table>
<thead>
<tr>
<th></th>
<th>MIC group (29 cases)</th>
<th>CIN III group (25 cases)</th>
<th>( P )</th>
<th>Adjusted ( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>41.79 ± 8.89</td>
<td>36.04 ± 10.67</td>
<td>0.035</td>
<td>0.267</td>
</tr>
<tr>
<td>ER positive ([ n %] )</td>
<td>10 (34.5)</td>
<td>11 (44.0)</td>
<td>0.579</td>
<td>–</td>
</tr>
<tr>
<td>PR positive ([ n %] )</td>
<td>8 (27.6)</td>
<td>13 (52.0)</td>
<td>0.094</td>
<td>–</td>
</tr>
<tr>
<td>Bcl-2 positive</td>
<td>16</td>
<td>13</td>
<td>1.000</td>
<td>–</td>
</tr>
<tr>
<td>Bax positive</td>
<td>27</td>
<td>25</td>
<td>0.493</td>
<td>–</td>
</tr>
<tr>
<td>ER + PR positive</td>
<td>3 (10.3)</td>
<td>10 (40.0)</td>
<td>0.023</td>
<td>0.646</td>
</tr>
<tr>
<td>Bcl-2 + Bax positive</td>
<td>14 (48.3)</td>
<td>13 (52.0)</td>
<td>1.000</td>
<td>–</td>
</tr>
<tr>
<td>ER + PR + Bcl-2 positive</td>
<td>0 (0.0)</td>
<td>9 (36.0)</td>
<td>0.0004</td>
<td>0.001</td>
</tr>
<tr>
<td>ER + PR + Bax positive</td>
<td>3 (10.3)</td>
<td>10 (40.0)</td>
<td>0.023</td>
<td>0.646</td>
</tr>
</tbody>
</table>

MIC group: CIN III at least 2 mm away from microinvasive complex from patients with the diagnosis of MIC; CIN III group: CIN III from patients with diagnosis of CIN III. ER, estrogen receptor; PR, progesterone receptor.

Fig. 1. This figure shows a CIN III with nuclear staining positive for ER (\( \times 40 \)).

Fig. 2. This figure shows a CIN III with nuclear staining positive for PR (\( \times 40 \)).

reported as higher as 50% in many Caucasian groups (19, 20, 58, 59) in accordance with the high frequency detected in our samples. We found that p53 polymorphism has no value in predicting progression of CIN III which is in agreement with previous studies reporting that p53 polymorphism has no prognostic significance in cervical cancer (60, 61). In the present study, the p53 polymorphism was not related to HPV type, as reported by others (60-62).

Clinical evidence that estrogens and progesterone may have a carcinogenic effect in HPV-infected cervix was recently reported in two multicentric case-control studies (63, 64). Our ER- and PR-positive results are similar to previous studies relying on ER and PR detection in cervical cancer (39, 65-69). However, wide variation exists between ER and PR reports on cervical cancer probably due to the differences in the methods employed, sample preparation, and different cut-off values for positive results. We found a significantly higher positive coexpression of ER and PR in the CIN III group than in CIN III of the MIC group. As induction of PR is one of the best-recognized responses to estrogen in target tissues (70, 71), we can hypothesize that in the MIC group, most of ER may have no or reduced functional activity. The finding that in all cases in the MIC group with coexpression of ER and PR, the ER staining was stronger and
expressed in more cells than PR staining, which is not true for CIN III group, supports this idea. Moreover, coexpression of ER, PR, and bcl-2 was observed in CIN III group but not in MIC group, which agrees with previous studies suggesting an estrogen-dependent upregulation of bcl-2(72,73).

Several studies have shown that HPV gene expression can be increased in response to both estrogen and progesterone stimulation(41–43). Webster et al.(74) reported that estrogen and progesterone increase the level of apoptosis induced by HPV 16 E2 and E7 proteins, and they suggest that in the presence of E2, these hormones may be protective against cervical cancer via their upregulation of apoptotic cell death.

Apoptosis is regulated by a balanced interaction of genes, among which bcl-2 and Bax are the most important(75–77). The ratio between Bax and bcl-2 genes, among which bcl-2 and Bax are the most cancer via their upregulation of apoptotic cell death.

In conclusion, our study suggests that positive immunohistochemical coexpression of ER, PR, and bcl-2 may be a useful tool in identifying a subset of patients with CIN III that have a low risk for cancer progression, independent of the patient’s age and HPV status. Further studies performed in different populations and in different laboratories must validate these data.

Acknowledgments

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