

A study of Ki-67, c-erbB2 and cyclin D-1 expression in CIN-I, CIN-III and squamous cell carcinoma of the cervix

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Summary. The histological criteria for cervical intraepithelial neoplastic lesions and their follow-ups have been established, but their reproducibility, specificity and sensibility are not certain. Immunohistochemical markers provide more information on each specific case, in order to facilitate its classification and, eventually, its prognosis. Using immunohistochemical techniques, this study analyzes the prognostic value of three markers (Ki-67, c-erbB2 and Cyclin D1) in cases of low grade squamous intraepithelial neoplasia (CIN-I), high grade squamous intraepithelial neoplasia (CIN-III), and infiltrating squamous cell carcinoma (SCC) taken from a group of cervical samples. *In situ* hybridization was performed in order to detect high-risk HPV.

High risk HPV was demonstrated in 82%, 89% and 100% of the LGSIL, HGSIL and SCC cases, respectively.

C-erbB2 expression was detected in 9%, 33% and 50% of the LSIL, HGSIL and SCC cases, respectively. The Ki-67 LI was 25%, 68% and 65.5% in the LGSIL, HGSIL and SCC cases, respectively. Nuclear Cyclin D1 expression was seen in 82%, 11% and 30% of the CIN-I, CIN-III and SCC cases, respectively. We observed that the cytoplasmic cyclin D1 expression increased with the severity of the lesion instead of the nuclear expression decreasing with the progression of the pathology. Nuclear and cytoplasmic Cyclin D1 expression seemed to be related to HPV high risk infection.

We concluded that Cyclin D1, c-erbB2 and The Ki-67 LI expression changed in relation to the severity of the lesion and that they could be helpful in making a differential diagnosis.

Key words: CIN, Squamous cell carcinoma, Cervix, Ki-67 LI, Cyclin D1

Introduction

In recent years, the mortality rate of patients with cervical squamous cell carcinoma (SCC) has decreased, probably due to the use of the Papanicolaou test. The histological criteria for cervical intraepithelial neoplastic lesions and their follow-ups have been established, but their reproducibility, specificity and sensibility are not certain (Robertson et al., 1989). Only around 20% of the cases with low cervical intraepithelial neoplastic lesions (CIN-I) will progress to moderate or severe cervical intraepithelial neoplastic lesions (CIN-III). In fact, we haven't any criteria to predict the follow-up of CIN-I cases.

The oncogenic role of HPV in cervical carcinogenesis, is actually Indisputable. High-risk HPV produces two proteins E6 and E7, called oncoproteins, which link p53 and pRb, respectively. Consequently, E2F is liberated of pRb, independently of its (CD1/CDK4) function. E2F induces p16 synthesis, which deactivates the CD1/CDK4 complex. On the other hand, E2F drives the infected cells to begin cell-cycling, (without the check point induced by p53, deactivated by the action of E6). In this process, cells are submitted to genomic instability and can accumulate a great quantity of mutations. Furthermore, genomic instability increases the possibility that viral DNA can integrate into the cell DNA. In the integration process, the viral DNA breaks into the E2 region, and the E2 protein is not produced. The absence of E2 deregulates E6 and E7 production. The viral DNA integration is produced in different chromosomal loci, and it is possible that some oncogenes or tumor suppressor genes can alter their functions. The CD1 gene maps to 11q13, and it shows the characteristics of a cellular oncogene. CD proteins have CDK-independent properties, which are important for cell growth, metabolism, and cellular differentiation. The over-expression of CD1 is one of the most commonly observed alterations in cervical carcinomas; however, their role in cervical carcinogenesis is still not

clear (Kim and Zhao, 2005).

The immunohistochemical expression of CD1, in cases of SIL and SCC, has been widely studied. It has been detected in cell nuclei. A review of the literature shows variable results ranging from an absence of expression in normal cervical epithelia to a variable degree of expression in SCC (Bae et al., 2001; Kedzia et al., 2002; Skomedal et al., 1999; Cheung et al., 2001). In addition, normal CD1 expression has been observed in normal and CIN-I epithelia, and an absence of expression has been observed in CIN-III cases (Nichols, et al., 1996; Cho et al, 1997; Southern and Herrington, 1998).

CD1 expression has been associated with the HPV infection: expression is higher when HPV is considered to be low risk and is lower when HPV is considered to be high risk (Southern et al, 1998). A good correlation has been observed between CD1 and E7 oncoprotein expression (Crish et al, 2000). In cervical lesions and in cell cultures, the expression of CD1 has been detected in the nuclei, but this expression increases in the cytoplasm and decreases in the nuclei, when the cells enter into S phase (Baldin et al., 1993).

C-erbB2 maps to 17q12 and encodes a protein with tyrosine-kinase activity, homologous to the epidermal growth factor receptor (EGF-R). It is expressed in a great number of epithelial tumors, and it is known that c-erbB2 expression is increased in approximately 20-25% of ovarian and/or breast carcinomas, 35-45% of pancreatic carcinomas, and 90% of colorectal carcinomas. In cases of SIL and SCC, it has been observed that there is greater c-erbB2 expression when the severity of the lesion increases. In CIN-I cases, c-erb-B2 expression is low (Brumm et al., 1990). In SCC, the greater expression has been correlated to poor prognosis, an increase in aggression, and lymph node metastases. These data allow us to suspect that c-erb-B2 expression is a late event in cervical carcinogenesis and that it probably acts in a great number of functions and in the activation of the Cyclin D1 function (Chang et al., 1999; Niiibe et al., 2003).

The antibody Ki67 reacts with the nuclear Ki67 antigen, a protein encoded by ki67, on 10q25. Ki-67 is only present in proliferating cells (Gerdes et al., 1984). In normal exocervical epithelia, it is only expressed in the suprabasal layer (Konishi et al., 1991; Resnick et al., 1996), and in CIN cases, it is expressed throughout the different epithelial layers. The Ki-67 labeling index (Ki-67 LI) increases, according to the degree of squamous cervical neoplasia (Payne et al., 1996; McCluggage et al., 1998; Maeda et al., 2001; Alameda et al., 2004).

Our aim in this study was to analyze CD1, c-erbB2 and Ki-67 expression in a group of cervical samples of different pathological stages (CIN-I, CIN-III and SCC).

Materials and methods

Patients

A group of 30 cervical biopsies from the Department

of Pathology at the Hospital del Mar in Barcelona, Spain, were selected. There were 11 cases diagnosed as CIN-I, 9 cases diagnosed as CIN-III and 10 cases diagnosed as SCC. The diagnosis of each sample specimen was established on the basis of a routine histopathological examination (Wright et al., 2002). All biopsy specimens were examined by two experimental pathologists. Cases where there was disagreement on the diagnosis were excluded from the study.

HPV detection

HPV detection was realized using in situ hybridization techniques. It was performed using an automated system (Inform HPV, Ventana), based on a probe for high risk HPV (HPV-HR) and was carried out on paraffin-embedded tissue. After sample deparaffination and rehydration, we incubated the slides in salt sodium citrate buffer with formamide at 75°C. Later, we incubated the slides with the high risk HPV probe, conjugated with FITC. The post-hybridization washes were performed using salt sodium citrate buffer 20x formamide. For amplification and detection, we used an anti-FITC antibody, a second anti-IG biotinylated antibody, an avidin-alkaline phosphatase conjugate that reacted to BICP (5 brome- 4 chlore- 3 indolyl phosphate), and an NBT (Nitro Blue Tetrazolium). The post-hybridization washes were done using salt sodium citrate buffer with formamide. Nuclei were counterstained with ISH red (Ventana). We considered a positive (high-risk HPV) result to be when we observed diffuse or granular, blue nuclei. Positive and negative controls were used.

Immunohistochemical study

The immunohistochemical study was performed using the DakoThechMate Immunostainer (Dako-Cytomation, Glostrup, Denmark). The immunohistochemical staining was developed for the following primary antibodies: CD1 (DCS-6; 1:10; Novocastra, Newcastle, UK), Ki-67 (Mib-1; 1:100; DakoCytomation, Glostrup Denmark), and c-erb-B2 (Polyclonal; 1:350; DakoCytomation; Glostrup, Denmark). The detection was carried out using the EnVision system (Dako Cytomation, Glostrup, Denmark). Positive and negative controls for each antibody were used.

The Ki-67 LI index (percentage of cells with a brown coloration in the nucleus) was obtained by counting 200 cells in a 25 HPF, which included all of the epithelial layers. The positive expression of C-erb-B2 is usually established using a scale of cross-like +1, +2 or +3, and in our study, its expression was considered positive when there was immunoexpression around all of the cytoplasmic membrane. We only considered as positive the cases that showed a score of +3. CD1 expression is usually considered positive in the nucleus, and its positivity is expressed with a percentage of cells. In this study, we observed CD1 expression in the cell cytoplasm, and it was evaluated in both locations.

Results

The results of this study are summarized in Tables 1-3.

High risk HPV was demonstrated in 82% of the cases of LGSIL, 89% of the cases of HGSIL and in all of the SCC cases.

CD1 expression

In the CIN-I group, nuclear CD1 expression (Figure) was seen in 9/11 cases (82%): In 4 cases, all of them high-risk HPV positive (36%), the expression was only detected in the nuclei with an 11% mean of positive nuclei, and in 5 cases (45%), 4 of them high-risk-HPV positive, we observed expression in the nuclei and the cytoplasm with a mean of 11% positive cells. In the remaining 2 cases, CD1 expression was only cytoplasmic with a mean of 20% positive cells (Table 1).

In the CIN-III group, only one case (high-risk HPV positive) showed nuclear and cytoplasmic positivity with a mean of 40% positive cells. The remaining eight cases only showed the cytoplasmic expression of CD1 with a 76% mean of positive cells (Table 1).

In the SCC group, three cases showed nuclear and cytoplasmic expression of CD1 with a 37% mean of positive cells, and the remaining seven cases showed only cytoplasmic positivity with a 57% mean of positive cells (Table 1).

We observed that nuclear CD1 expression decreased

with the progression of the pathology (9/11 LSIL, 1/9 CIN-III, and 3/10 SCC cases) instead of the cytoplasmic CD1 expression increasing with the severity of the lesion (2/ 11 CIN-I 8/9 CIN-III, and 7/10 SCC cases) (Table 1). Furthermore, the percentage of nuclear and cytoplasmic positive cells increased with the severity of the lesion.

C-erbB2 expression (Fig. 1) was detected in only one CIN-I case that was HPV-HR positive (9%), in 33% of the CIN-III cases, all of them HPV-HR positive, and in 50% of the SCC cases (Table 1). We observed that the expression of c-erbB2 increased with the intensity of the lesion and seemed to be related to the HPV-HR infection, due to the fact that in the HPV-HR negative cases of CIN-I and CIN-III, the expression of c-erbB2 was not detected (Table 1).

The mean Ki-67 LI (Fig. 1) was 25% in the CIN-I cases, 68% in the CIN-III cases and 65.5% in the SCC cases. In the HPV-HR positive cases, the mean Ki-67 LI was 26% in the CIN-I and 70% in the CIN-III HPV-HR cases. In the HPV-HR negative cases, the mean Ki-67 LI was 21% in the cases of CIN-I and 65% in the CIN-III cases (Table 1).

In Table 2 we compared the mean of the Ki-67 LI related to c-erbB2 expression. In those cases, the Ki-67 LI in c-erbB2 positive cases was greater than in the c-erbB2 negative ones.

In Table 3, we summarized the relationship between the Ki-67 LI and CD1 expression. In all of the pathological groups, the Ki-67 LI was higher when CD1 expression was detected in the cytoplasm (Table 3).

Table 1. Summary of results.

LESION	HPV-HR	CD1 N	CD1 N+C	CD1 C	c-erbB2 +	KI-67 LI
CIN-I	POS 9/11 (82%) NEG 2/11 (18%)	4/9 (44%) 0/2 (0%)	4/9 (44%) 1/2 (50%)	1/9 (11%) 1/2 (50%)	1/9 (11%) 0/2 (0%)	26% 21%
TOTAL	11 CASES	4/11 (36%)	5/11 (45%)	2/11 (19%)	1/11 (9%)	25%
CIN-III	POS 8 (89%) NEG 1 (11%)	0/8 (0%) 0/1 (0%)	1/8 (13%) 0/1 (0%)	7/8 (87%) 1/1 (100%)	3/8 (27%) 0/1 (0%)	70% 65%
TOTAL	9 CASES	0/9 (0%)	1/9 (11%)	8/9 (89%)	3/9 (33%)	68%
SCC	POS 10 (100%)	0/10 (0%)	3/10 (30%)	7/10 (70%)	5/10 (50%)	65.5%

CD1, c-erbB2, Ki-67 LI and HPV-High risk positive and negative cases

Table 2. A comparative study between c-erbB2 expression and the Ki-67 LI.

	KI-67 LI
c-erbB2 EXPRESSION	
POS	62.6%
NEG	47.7%

Table 3. Relationship between the Ki-67 LI and CCND1 expression.

	KI-67 LI		
	CIN-I	CIN-III	SCC
CD1 expression			
N	19.3%	-	-
N+C	22%	68.5%	58.3%
C	45%	80%	68.6%

N: nuclear; C: cytoplasmic

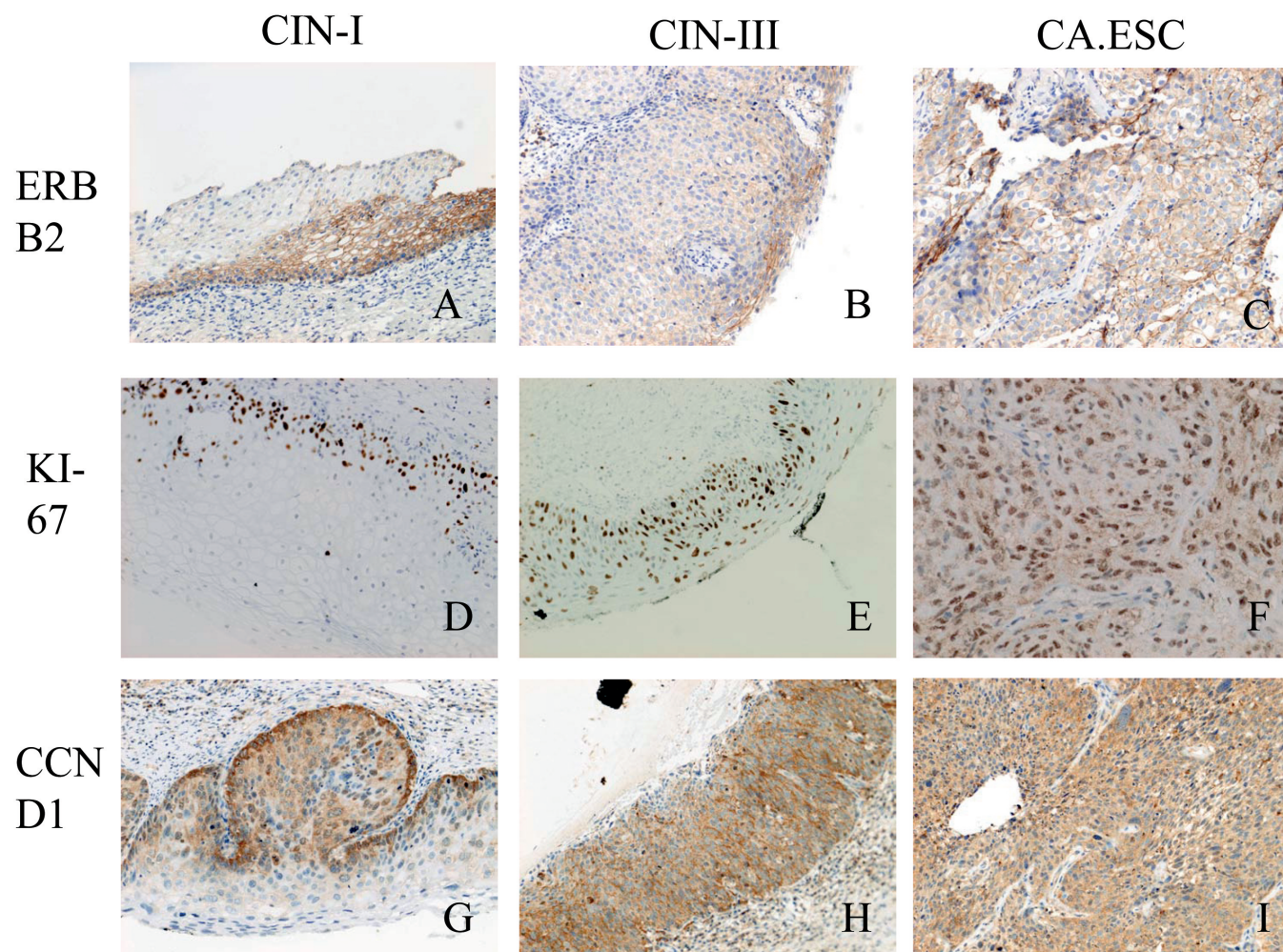


Fig. 1. C-erb-B2. Strong immunoreactivity in the profound half of the epithelium in a case of CIN-I (A), and weak immunoreactivity, but throughout the epithelium, in the cases of CIN-III (B) and a squamous cell carcinoma (C).

Ki-67. Immunoreactivity in parabasal and some intermediate cells in a CIN-I case (D) and throughout the epithelium of a CIN-III case (E) and in a case of infiltrating squamous cell carcinoma (F).

Cyclin D1. Nuclear and cytoplasmic immunoreactivity in a CIN-I case (G), and mainly cytoplasmic immunoreactivity in the cases of a CIN-III (H) and an infiltrating squamous cell carcinoma (I). x 10

Discussion

Our results revealed different expression patterns for the studied markers related to the lesions, mainly when we considered the CIN-I cases as compared to the CIN-III and SCC cases.

Ki-67 is expressed only in proliferating cells (Kim and Zhao, 2005). Our cases showed an increase in the Ki-67 LI in relation to increases in the severity of the lesions. Our results showed a Ki-67 LI of 25% in the CIN-I cases, a Ki-67 LI of 70% in the CIN-III cases, and a Ki67 LI of 65.5% in the SCCs. These data are in

accordance with the literature. The Ki-67 LI can be useful in distinguishing the different grades of dysplasia, though not in predicting their behavior (Alameda et al., 2004). There were no differences in the Ki67 LI in the high-risk HPV positive cases and the rest of the studied cases.

Keeping in mind that only the cases with a +3 score were considered positive, in our cases c.erb-B2 expression, increased according to the severity of the lesions as shown in the literature (Brumm et al., 1990).

We observed a relationship between the Ki-67 LI and c-erbB2 immunoexpression. C-erbB2 is a protein

with tyrosine-kinase activity, homologous to the epidermal growth factor receptor (EGF-R). C-erb-B2 acts by stimulating cell proliferation. In CIN-I and CIN-III cases there is a relationship between high-risk HPV-HR positivity and c-erb-b2 immunoexpression. All of the cases without c-erb-B2 immunoexpression were negative for HPV-HR. We do not have an explanation for this fact, as it appears that c-erb-B2 amplification is a late event in cervical carcinogenesis (Chang et al., 1999; Niibe et al., 2003).

The nuclear expression of CD1 decreases with an increase in the severity of the lesion. In the literature, a loss of nuclear CD1 expression has been reported in SCC (Nichols et al., 1996; Bae et al., 2001). Our cases showed nuclear CD1 expression in 9/11 cases of CIN-I, in only 1/9 cases of CIN-III and in 3/10 cases of SCC. The percentage of positive CD1 increased with the severity of the lesion. Nuclear CD1 expression has been inversely associated with HR-HPV infection (Southern and Herrington, 1998). Our cases showed CD1 nuclear expression in all of the CIN-I cases that were HPV-HR negative and only in 77% of the LGSIL HR-HPV positive cases. There is no data in the literature related to cytoplasmic CD1 expression. A possible explanation for this fact may be the following: CD1 binds CDK4 and phosphorylates pRB in the G1 cell phase (Diehl et al., 1997a,b). In HPV-HR related lesions the E7 oncoprotein binds pRb and deactivates it directly by ubiquitination. Under these conditions CD1 is not necessary for the cell to enter into S phase (Cho et al., 2002). Under normal conditions CD1 is located in the nucleus in the G1 phase, and it is phosphorylated, ubiquitinated, and carried to the cytoplasm in the S phase (Diehl et al., 1997a,b). In our cases, the cytoplasmic expression of CD1 increased according to the severity of the lesion (7 of 11 CIN-I cases and all of the CIN-III and SCC cases), and the CD1 expression changed to be predominantly nuclear in the CIN-I cases and predominantly cytoplasmic in the CIN-III and SCC cases. An increase in the severity of the lesion is in correlation with an increase in the number of cells in the S phase, when CD1 is found in the cytoplasm of the cell (Baldin et al., 1993). Consequently, if we accept that neoplastic tissue contains a greater number of cells than normal tissue, then the neoplastic cells would be able to show cytoplasmic CD1 expression, and the cytoplasmic immunoexpression of CD1 would be seen with more intensity and more extensively in cases of SCC than in cases of CIN-I.

We concluded that CD1, cerbB2 and the Ki-67 LI markers increased in relation to the severity of the lesions and that they could be helpful in making a differential diagnosis. The cytoplasmic expression of CD1 and the absence of nuclear CD1 expression in SCC could also be of help in diagnosis. More studies should be conducted to elucidate the importance of cytoplasmic CD1 expression.

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