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ЕКСПРЕСИЈАТА НА Ki-67 ВО ИНВАЗИВНИОТ ФРОНТ КАКО ДОПОЛНИТЕЛЕН ЗНАЧАЕН НЕЗАВИСЕН ПРОГНОСТИЧКИ ФАКТОР ЗА ПОЈАВАТА НА РЕЦИДИВИ ВО РАНИТЕ СТАДИУМИ НА КАРЦИНОМОТ НА ГРЛОТО НА МАТКАТА

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Извадок

Целта на оваа студија е да се утврди поврзаноста на промените во клеточната пролиферација, растот, диференцијацијата и протеините кои ја регулираат апоптозата во раните стадиуми на цервикалните карциноми со хуман папилома вирусната (ХПВ) инфекција, хистопатолошките и клиничките параметри и да се процени нивното прогностичко значење.

Експресијата на Ki-67, p53, mdm-2, bcl-2, c-erbB-2/neu, EGFR протеините, како и естрогенските и прогестеронските рецептори е евалуирана имунохистохемиски во оперативните материјали од 83 пациенти со цервикален карцином. Резултатите се оценувани семиквантитативно во површниот, средниот слој и инвазивниот фронт на секоја неоплазма како процент на имунообоени клетки и/или како интензитет на имунообојување за секој протеин. Присуството на ХПВ е одредувано со конвенционална *in situ* хибридизација (ИСХ) и ИСХ со катализирана сигнална амплификација со употреба на мешани биотинизирани проби за идентификација на типовите 6/11, 16/18 и 31/33 или 31/33/51.

Во универијантната анализа како значајни прогностички индикатори за појавата на рецидив меѓу 18 испитувани варијабили, се идентификувани: зафаќањето на пелвичните лимфни јазли (P=0.0008), туморскиот дијаметар (P=0.035), длабочината на стромална инвазија (P=0.029), хистолошкиот тип (P=0.0009), степенот на хистолошка диференцијација (P=0.056), присуството (P=0.056) и типот на ХПВ ДНК (P=0.043), како и експресијата на bcl-2 (P=0.035), mdm-2 (P=0.051), EGFR (P<0.0001) и Ki-67 (P=0.031) во инвазивниот фронт на неоплазмата. Независни значајни прогностички фактори за преживувањето без болест во мултиваријантната анализа се хистолошкиот тип, присуството на ХПВ ДНК и експресијата на Ki-67.

Според тоа, инвазивниот фронт претставува најзначаен дел на неоплазмите за прогноза на болеста, а евалуацијата на Ki-67 може да се употреби заедно со одредувањето на присуството на ХПВ и морфолошките параметри во селекцијата на соодветни терапевтски пристапи кај пациентите во рани стадиуми на цервикалниот карцином.

Клучни зборови: цервикален карцином, хуман папилома вирус, биолошки маркери, имунохистохемија, прогноза.

Ki-67 EXPRESSION IN THE INVASIVE FRONT AS AN ADDITIONAL INDEPENDENT SIGNIFICANT PROGNOSTIC FACTOR INFLUENCING RECURRENCE IN EARLY STAGE CERVICAL CARCINOMAS

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Abstract

The aims of this study were to correlate alterations of cell proliferation, growth, differentiation and apoptosis regulatory proteins in early stage cervical carcinomas with human papillomavirus (HPV) infection, histopathological and clinical parameters, and to estimate their prognostic significance.

Expression of Ki-67, p53, mdm-2, bcl-2, c-erbB-2, EGFR protein, as well as estrogen and progesterone receptors was evaluated by immunohistochemistry in operative specimens of 83 patients with cervical carcinoma. The results were assessed semiquantitatively in the surface area, centre and invasive front of each tumor as a percentage of immunostained cells and/or intensity of immunostaining for each protein. The presence of HPV was assessed by conventional in situ hybridization (ISH) technique and catalyzed reporter deposition signal amplification ISH using mixed biotinylated probes to identify types 6/11, 16/18 and 31/33 or 31/33/51.

Among the 18 variables, pelvic lymph node involvement (P=0.0008), tumor diameter (P=0.035), depth of stromal invasion (P=0.029), histotype (P=0.0009), grade (P=0.056), HPV DNA presence (P=0.056), HPV type (P=0.043), as well as bcl-2 (P=0.035), mdm-2 (P=0.051), EGFR (P<0.0001), and Ki-67 (P=0.031) expression in the tumor's invasive front were identified as important predictive indicators of recurrence in the univariate analysis. Independent significant prognostic factors for disease-free survival in multivariate analysis were the histotype, HPV DNA presence and Ki-67 expression.

The invasive front of carcinomas proved to be the most important area for tumor prognosis. In addition to the detection of HPV presence and morphological parameters, Ki-67 evaluation could also be used in selecting appropriate therapeutical approaches in patients with early stage cervical cancer.

Key words: cervical cancer, human papillomavirus, biological markers, immunohistochemistry, prognosis.

Introduction

The clinical behavior of the carcinoma of the uterine cervix varies and covers a wide spectrum from cases that are relatively indolent to those having a rapidly progressive course. Accurate staging is of utmost importance in determining the prognosis of cervical carcinoma, and primary surgical treatment in patients with early stage carcinoma allows for an accurate determination of the real extent of the disease. Traditionally, clinical and histopathological parameters such as regional lymph node metastases, tumor size, depth of stromal invasion, vessel invasion, and parametrial involvement are used to prognosticate and modify management options both in patients with early stage and locally advanced cervical carcinoma [1-8]. However, many factors are still controversial in their prognostic significance including patient's age, surgical margin involvement, inflammatory stromal reaction, histological type and grade of differentiation. This indicates the need for a more precise quantitative method for determining the prognosis in patients with cervical carcinoma in order to be able to individualize treatment as much as possible.

Recently many subcellular or molecular indicators, such as DNA ploidy status and cellular proliferating index, hormonal status and oncogene amplification or expression, have been investigated as alternatives to histological characteristics in several different tumor types, including cervical carcinoma. Namely, significant progress has resulted from detection and recognition of the human papillomavirus (HPV) role in cervical cancer pathogenesis and recognition of different pathways in cervical carcinogenesis. In recent years it has become clear that carcinogenesis cannot only be explained by the increased stimulation of cell growth, but can also be caused by loss of growth suppression, changes in programmed cell death and alterations in immune surveillance. Therefore, an analysis of markers of cell proliferation and death may provide important intrinsic information regarding the biologic behavior of these tumors. Nevertheless, so far the attempts to determine the prognostic significance of biological markers and their relation to HPV infection in cervical cancer have yielded controversial results.

The purpose of this retrospective study was to correlate alterations of cell proliferation, growth, differentiation and apoptosis regulatory proteins in early stage cervical carcinomas with HPV infection and clinicopathological parameters, and to estimate their prognostic significance. Based on the recent reports [9] that the invasive edge of carcinomas often displays different molecular and morphological characteristics than more superficial parts of the same tumor, we also assessed the aberrant expression and coexpression of these biological markers in the superficial, central parts and invasive front of the tumor in order to determine whether there is a difference in prognostic information.

Materials and methods

Clinical and Histopathological Features

This retrospective study included 83 patients with early stage cervical carcinoma who underwent surgery and postoperative irradiation therapy between November 1988 and June 1997. All patients were treated with radical hysterectomy and bilateral pelvic lymphadenectomy at the University Clinic for Obstetrics and Gynecology or the Special Hospital for Gynecology and Obstetrics in Skopje, and they all received postoperative radiotherapy at the University Clinic for Radiotherapy and Oncology.

The patients were previously included in a larger study (n=484) exploring the prognostic influence of 23 clinicopathological factors and the HPV status on overall and disease-free survival in primarily surgically treated patients with cervical carcinoma [10]. For the purpose of this study only the patients with cervical carcinoma confined to uterine cervix and with defined HPV status were included. Therefore, the selection was made from a group of 111 patients with cervical carcinoma confined to uterine cervix in which the HPV status had been previously determined by conventional colorimetric in situ hybridization (ISH). A total of 87 patients was selected: 77 patients whose HPV status was reevaluated using a novel, more sensitive catalyzed amplification reporter deposition (CARD) ISH method and 10 patients with HPV positive carcinomas. Four of the patients were excluded: one due to different treatment modality implemented (preoperative irradiation), two due to follow-up period less than 18 months and one due to verrucous subtype of squamous carcinoma. The case series was finally made of 83 patients.

The operative specimens were routinely processed and all patients were staged according to postoperative pTNM classification guidelines [11]. Fifty-eight patients were in IB1, 5 in IB2 and 20 in IIIB postoperative stage. In our case series, 73 (88%) patients had a tumor limited to the uterine cervix less than 4 cm in diameter (pT1b1), while 10 (12%) patients had larger neoplasms belonging to pT1b2 category. Pelvic lymph node involvement was found in 20 (24%) patients.

In addition to tumor status and pelvic lymph node status, further prognostic parameters included in the study were the morphometric and morphohistological characteristics including maximum diameter, depth of stromal invasion and thickness of uninvolved cervical stroma, histological type, grade of differentiation, lymph-vascular space invasion and inflammatory infiltrate in the invasive front of the tumor.

Clinical information, including patient age, FIGO stage, date of operation, postoperative treatment and follow-up data were retrieved by reviewing each patient's complete medical records at the University Clinic for Radiotherapy and Oncology. Additional information about the length of disease-free and overall survival and clinical status of some patients were obtained from the Cancer Registry of the Republic of Macedonia and contact with the patients or their families.

During the follow-up period (range, 65-181, mean, 121 months) recurrences were observed in 9 patients. Median follow-up for patients without relapse was 128 (range 18-181) months and for patients still alive (n=72) at the closing date of the study (January 2004) was 130 (range 65-181). The median observation time for the women (6) who died of cervical cancer was 37 (range 4-93) months. Five other patients died of intercurrent disease.

Immunohistochemistry

For immunohistochemistry, paraffin blocks containing a representative part of the superficial, middle layer as well as the invasive front of the tumor were selected, and 5μ thick sections were deparaffinized, rehydrated, and stained using the avidin-biotin peroxidase complex technique (Vectastain universal elite ABC kit, Vector Laboratories, Inc. Burlingame, CA, USA). EnVision™+ System kit (Dako, Glostrup, Denmark) was used for the detection of epidermal growth factor receptor (EGFR). The reaction product was detected with 3,3'-diaminobenzidine chromogen. Details about the primary antibodies used, their sources and working dilutions are listed in Table 1. Antigen retrieval was usually performed by boiling in 10 mM citrate buffer, pH 6.0, for 30 minutes in a microwave oven, while pretreatment with 0.0125% proteinase K was used for EGFR. Positive controls consisted of simultaneously immunostained sections of a carcinoma that was previously shown to be immunoreactive for antibodies used by this technique. Negative controls were performed by omitting the primary antibody.

The results were assessed semiquantitatively in the superficial and middle layers and the invasive front of each tumor as the percentage of the immunostained cells and/or intensity of immunostaining for each protein. All immunostained slides were independently reviewed by two observers in a blinded fashion without knowledge of the clinical data or outcome for an individual patient. In case of discrepancy, the slides were reviewed together and consensus was reached in all cases. For Ki-67, p53, mdm-2, estrogen (ER) and progesterone receptor (PR) analysis nuclear staining, irrespective of intensity of staining, was defined as positive. Brown staining of the cytoplasm and/or nucleus indicated positivity for mdm-2, while perinuclear and cytoplasmic staining indicated positivity for bcl-2 antibody. Brown staining of the plasma membrane indicated positivity for EGFR and c-erbB-2/neu antibodies. The number of cells with nuclear or cytoplasmic staining was determined by counting the number of positive cells and the total number of cells (8-10 high-power fields, x400). An average of 500-1,000 cells in the selected area of the superficial and middle layers and the invasive front of each carcinoma was counted.

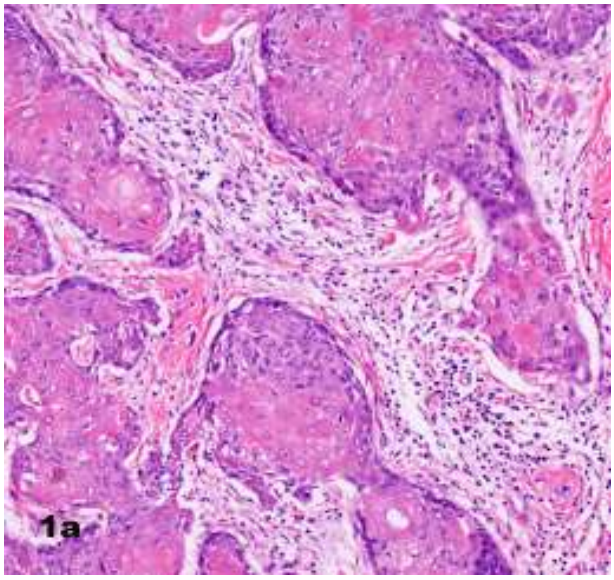
The staining results for Ki-67, p53, bcl-2 and mdm-2 were scored semiquantitatively as the percentage of the immunostained cells. The percent of positivity was determined and the cases were separated in three categories: with less than 10%, 11-20% and more than 20% positive cells for Ki-67, and with less than 5%, 6-10% and more than 10% positive cells for p53, bcl-2 and mdm-2 proteins. Because a threshold of more than 20% of Ki-67 positive cells gave the best separation between subgroups of low and high risk of relapse it was used as a cut-off value between Ki-67 negative and Ki-67 positive carcinomas in the statistical analysis (Figures 1&2). Similarly, a cut-off value of more than 10% was considered to score a case positive for p53 and bcl-2 expression, while mdm-2 expression was considered significant if immunoreactivity was evident in more than 5% of tumor cells.

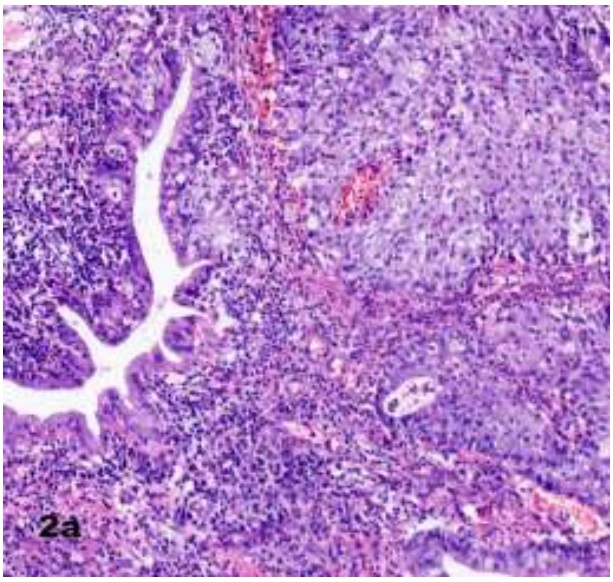
The positivity for ER and PR was determined according to score system proposed by McCarty et al. [12]. The McCarty's score was calculated by multiplication of the intensity of immunostaining (0, no staining; 1, weak; 2, moderate; 3, strong nuclear staining) and the percentage of the immunostained cells (0, no positive cells; 1, less than 10%; 2, 10–50%; 3, 51–80% and 4, more than 80%) for each carcinoma. Therefore, the McCarty's score ranges from 0 to 12, with a cut-off value of more than 5 for steroid receptor positive cervical carcinomas. The intensity of the predominant EGFR and c-erbB-2 oncoprotein immunoreactivity was evaluated semiquantitatively as negative (-), faintly (+), moderately (++), intensively (+++) and very intensively positive (++++). Only the percentage

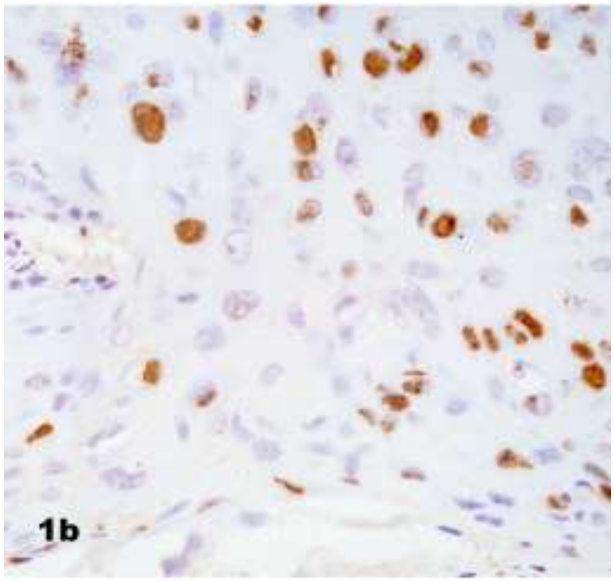
Table 1. Antibodies used in the study

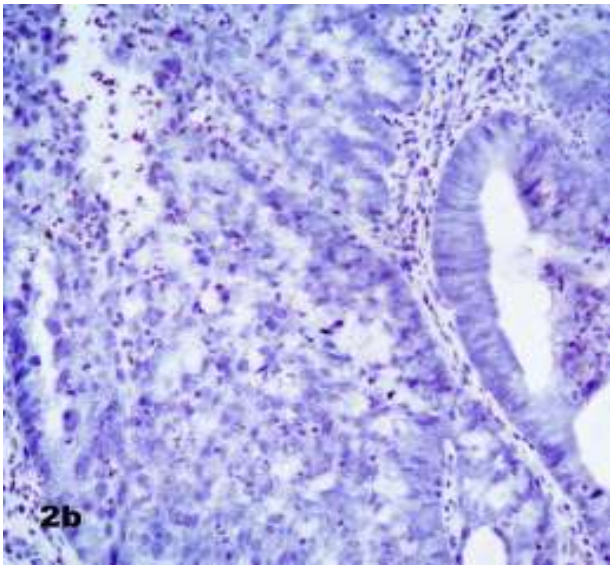
Antibody	Source	Clone	Pretreatment	Dilution
Ki-67	Dako	MIB-1	microwave	1:50
p53	Dako	DO-7	microwave	1:50
bcl-2	Dako	124	microwave	1:80
mdm-2	Dako	SMP14	microwave	1:50
EGFR	Dako	H11	proteinase K	1:150
c-erbB-2/neu	Dako	polyclonal	microwave	1:250
ER	Dako	1D5	microwave	1:50
PR	Dako	PR636	microwave	1:50

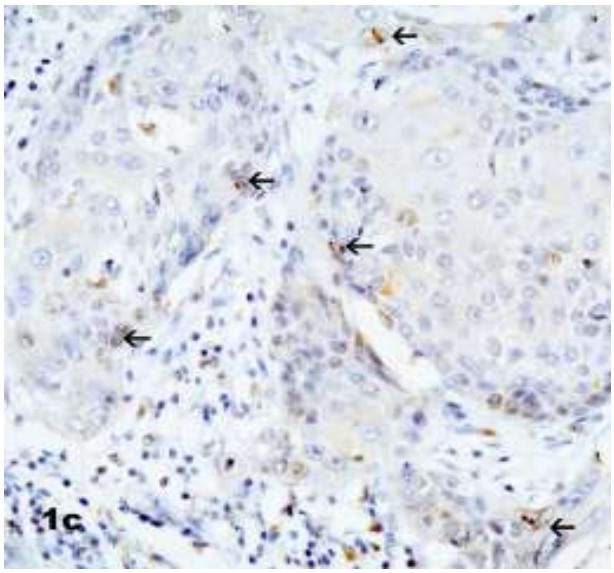
Legend: EGFR, epidermal growth factor receptor; ER, estrogen receptor; PR, progesterone receptor











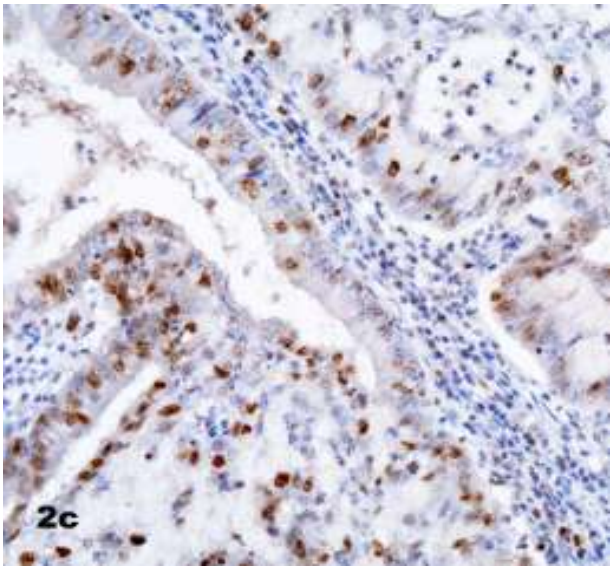


Fig. 1. Squamous cell carcinoma of the uterine cervix (1a, H&E, x100), HPV type 31/33 positive (1b, CARD in situ hybridization, x200), Ki-67 negative (1c, invasive front: 2%, x200)

Fig. 2. Mixed carcinoma of the uterine cervix (2a, H&E, x100), HPV DNA negative (2b, CARD in situ hybridization, type 31/33, x200), and Ki-67 positive (2c, invasive front: 32%, x200)

Table 2. Immunohistochemical expression of the proteins in the tumor's invasive front in relation to clinicopathological characteristics and HPV status in 83 cervical cancer patients (*chi-square or Fisher's exact test*)

Characteristics	No. of cases (%)	Ki-67 (%) positive	*P*	p53 (%) positive	*P*	bcl-2 (%) positive	*P*	mdm-2 (%) positive	*P*	ER McCarty score positive	*P*	PR McCarty score positive	*P*	EGFR (%) positive	*P*	c-erbB-2 (%) positive	*P*
Age (yrs.)			NS		NS		NS		NS		NS		0.026		NS		NS
≤39	43 (51.8)	27 (63%)		20 (47%)		18 (42%)		33 (77%)		2 (5%)		5 (12%)		2 (5%)		9 (21%)	
>39	40 (48.2)	28 (70%)		25 (63%)		24 (60%)		35 (88%)		2 (5%)		0		3 (8%)		11 (28%)	
Lymph node status (pN)			NS		NS		NS		NS		NS		NS		NS		NS
pN0	63 (75.9)	40 (64%)		34 (54%)		35 (56%)		51 (81%)		4 (6%)		5 (8%)		3 (5%)		16 (25%)	
pN1	20 (24.1)	15 (75%)		11 (55%)		7 (35%)		17 (85%)		0		0		2 (10%)		4 (20%)	
Tumor diameter			NS		NS		NS		NS		NS		NS		NS		NS
≤4 cm	73 (88)	49 (67%)		38 (52%)		37 (51%)		61 (84%)		4 (6%)		5 (7%)		5 (7%)		18 (25%)	
>4 cm	10 (12)	6 (60%)		7 (70%)		5 (50%)		7 (70%)	0	0		0		2 (20%)			
Depth of invasion			NS		0.015				NS		NS		NS	0.048	NS		NS
≤20 mm	73 (88)	48 (66%)		36 (49%)		39 (53%)		61 (84%)		3 (4%)		3 (4%)		4 (6%)		18 (25%)	
>20 mm	10 (12)	7 (70%)		9 (90%)		3 (30%)		7 (70%)		1 (10%)		2 (20%)		1 (10%)		2 (20%)	
Thickness of uninvolved stroma			0.012		NS		0.057		NS		0.073		NS		NS		NS
0-5 mm	37 (44.6)	22 (60%)		22 (60%)		21 (57%)	0.017*	31 (84%)		4 (11%)	0.022^	1 (3%)		3 (8%)		8 (22%)	
6-10 mm	32 (38.5)	27 (84%)		16 (50%)		18 (56%)		25 (78%)		0		3 (9%)		2 (6%)		8 (25%)	
>10 mm	14 (16.9)	6 (43%)		7 (50%)		3 (21%)		12 (86%)		0		1 (7%)		0		4 (29%)	
Histological type			NS		NS		NS		NS		NS		NS		NS		0.001
squamous cell	72 (86.8)	47 (65%)		41 (57%)		38 (53%)		57 (79%)		4 (6%)		5 (7%)		3 (4%)		13 (18%)	
adenocarcinoma	6 (7.2)	4 (67%)		1 (17%)		2 (33%)		6 (100%)		0		0		1 (17%)		5 (83%)	
mixed carcinoma	5 (6)	4 (80%)		3 (60%)		2 (40%)		5 (100%)		0		0		1 (20%)		2 (40%)	
Grade of differentiation			NS		NS		0.037		NS		NS		NS		0.042		NS
well (G1)	3 (3.6)	2 (67%)		1 (33%)		3 (100%)		3 (100%)		0		0		1 (33%)		1 (33%)	
moderate (G2)	31 (37.4)	20 (65%)		15 (48%)		11 (36%)		26 (84%)		1 (3%)		1 (3%)		0		10 (32%)	
poor (G3)	49 (59)	33 (67%)		29 (59%)		28 (57%)		39 (80%)		3 (6%)		4 (8%)		4 (8%)		9 (18%)	
Lymph-vascular invasion			NS		NS		NS		NS		NS		NS		NS		NS
absent	22 (26.5)	17 (77%)		13 (59%)		10 (46%)		20 (91%)		0		1 (5%)		3 (14%)		5 (23%)	
present	61 (73.5)	38 (62%)		32 (53%)		32 (53%)		48 (79%)		4 (7%)		4 (7%)		2 (3%)		15 (25%)	
Lymphocytic infiltration			NS		NS		NS		NS		NS		NS		NS		0.017
scarce	9 (10.8)	5 (56%)		4 (44%)		2 (22%)		7 (78%)		0		0		0		4 (44%)	
moderate	50 (60.2)	36 (72%)		25 (50%)		26 (52%)		44 (88%)		3 (6%)		4 (8%)		4 (8%)		15 (30%)	
abundant	24 (28.9)	14 (58%)		16 (67%)		14 (58%)		17 (71%)		1 (4%)		1 (4%)		1 (4%)		1 (4%)	
HPV presence			NS		NS		NS		NS		NS		NS		NS		NS
negative	31 (37.3)	21 (68%)		13 (42%)		16 (52%)		28 (90%)		2 (7%)		1 (3%)		1 (3%)		9 (29%)	
positive	52 (62.7)	34 (65%)		32 (62%)		26 (50%)		40 (77%)		2 (4%)		4 (8%)		4 (8%)		11 (21%)	
HPV type			NS		NS		NS		NS		NS		NS		NS		NS
16/18	39 (75)	25 (64%)		22 (56%)		18 (46%)		30 (77%)		1 (3%)		2 (5%)		3 (8%)		6 (15%)	0.053#
31/33/51 or 31/33	9 (17.3)	6 (67%)		7 (78%)		5 (56%)		7 (78%)		0		1 (11%)		1 (11%)		4 (44%)	
16/18&31/33/51 or 31/33	4 (7.7)	3 (75%)		3 (75%)		3 (75%)		3 (75%)		1 (25%)		1 (25%)		0		1 (25%)	
Total (%)	83 (100)	55 (66)		45 (54)		42 (51)		68 (82)		4 (5)		5 (6)		5 (6)		20 (24)	

Legend: EGFR, epidermal growth factor receptor; ER, estrogen receptor; G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated; HPV, human papillomavirus; No., number of patients; NS, not significant; P, probability; PR, progesterone receptor; yrs., years; #referring to differences between tumors with thickness of uninvolved stroma ≤10 and >10 mm; ^ referring to differences between tumors with thickness of uninvolved stroma ≤5 and >5 mm; #referring to differences between HPV 16/18 and 31/33/51 or 31/33 positive cervical carcinomas.

of tumor cells with a very intensive positive signal was considered for determining the positivity and carcinomas were grouped in three groups with 0-50%, 51-75% and more than 75% positive cells. The EGFR and c-erbB-2 oncoprotein expression was considered significant if very intensive membrane staining was evident in more than 75% of carcinoma cells.

HPV DNA Detection and Typing

Both conventional and CARD ISH methods were used for the detection and typing of the HPV DNA in the specimens. For the conventional ISH, a detection kit and mixed biotinylated HPV DNA probes for the HPV types 6/11, 16/18 and 31/33/51 were used from Enzo Diagnostics (PathoGene® Human Papillomavirus in situ Typing Assay for Tissue Sections, Enzo Diagnostics, Farmingdale, NY, USA). For the CARD ISH, biotinylated mixed probes for HPV types 6/11, 16/18 and 31/33, as well as the detection kit (GenPoint™ catalyzed signal amplification system for in situ hybridization) were obtained from Dako (Glostrup, Denmark). In addition, in four cases Enzo Diagnostics mixed probes for HPV type 16/18 and 31/33/51 were also used. The technique of tissue preparation and staining procedure for both conventional and CARD ISH, as well as the interpretation of ISH staining has been described previously in detail [13].

Statistical Analysis

The different protein expression patterns were correlated to each other, to the HPV status and to the clinicopathological variables. Correlations were evaluated with the chi-square, Fisher's exact or Student's t-test. Interobserver agreement in the evaluation of the immunohistochemical results was calculated using the kappa statistics. Disease-free and overall survival curves were calculated from the date of operation to relapse or death, respectively or to the date of last follow-up. The 5 patients who died of intercurrent disease were considered as censored observations. Surviving patients were considered at the time of their last clinical control. For the univariate analysis, the percentage of disease-free survival for each group was calculated using the Kaplan-Meier method and comparisons between groups were performed applying log-rank test. Cox's proportional hazard regression model with stepwise selection of variables was used to identify prognostic factors influencing disease-free survival. All prognostic variables that showed statistical significance by univariate analysis (at the 5% or at least 6% level) were thereafter included in the multivariate analysis to define variables of independent significance. Statistical analysis was performed using the BMDP software program. A *P* value of 0.05 or less was considered statistically significant and 95% confidence intervals (CI) were presented as well.

Results

Frequency and Type of HPV

HPV DNA was identified in 62.7% of the 83 carcinomas studied. Types 16/18 were detected in 39 cases; types 31/33/51 or 31/33 were detected in 9 cases and mixed HPV infection was present in 4 cases. In 31 cervical carcinomas, no HPV could be detected by conventional and CARD ISH with the three mixed biotinylated HPV DNA probes used (Table 2, Figures 1&2).

Protein Expression

The distribution of cells positive for cell proliferation, growth, differentiation and apoptosis regulatory proteins varied considerably among the tumors. Ki-67 positive were 60 (72.3%) cases in the superficial layer, 54 (65.1%) cases in the middle layer and 55 (66.3%) cases in the invasive front (Figures 1&2). Positive nuclear p53 staining in more than 10% of the cells was present in 40 (48.2%) cases in the superficial layer, 45 (54.2%) cases in the middle layer and 45 (54.2%) case in the tumor's invasive front. Bcl-2 positive were 47 (56.6%) cases in the superficial layer, 39 (47%) cases in the middle layer and 42 (50.6%) cases in the invasive front, while mdm-2 positive were 69 (83.1%) cases in the superficial layer, 71 (85.5%) cases in the middle layer and 68 (81.9%) cases in the tumor's invasive front. Strong expression of ER and PR (McCarty score over 5) was detected in 3 (36%), 3 (36%) and 4 (4.8%) cases and in 6 (7.2%), 5 (6%) and 5 (6%) cases in the superficial, middle layers or invasive front of the tumor, respectively. Strong positive EGFR staining in

Table 3. Correlation between any two proteins expression in the invasive front of carcinomas (*chi-square and Fisher's exact test*)

Protein	Ki-67	bcl-2	p53	mdm-2	ER	PR	EGFR	c-erbB-2
Ki-67	-	**NS**	**NS**	**0.00034**	**NS**	**NS**	**NS**	**NS**
bcl-2		-	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**
p53			-	**NS**	**NS**	**NS**	**NS**	**NS**
mdm-2				-	**NS**	**NS**	**NS**	**0.016**
ER					-	**NS**	**NS**	**NS**
PR						-	**NS**	**NS**
EGFR							-	**NS**
c-erbB-2								-

Legend: EGFR, epidermal growth factor receptor; ER, estrogen receptor; NS, not significant; PR, progesterone receptor.

Table 4. Five, ten and fifteen year disease-free survival estimates by clinicopathological characteristics, HPV status and expression of biological markers in the invasive front in 83 cervical cancer patients

Variables	*No.*	*Relapses No. (%)*	*Disease-Free Survival Rate (%)*						*Log rank*	*P value*
			5 y	*CI 95%*	*10 y*	*CI 95%*	*15 y*	*CI 95%*		
Age (y)										
≤39	43	3 (7)	93.4	1.4-2.5	93.4	0.1-1.6	93.4	0.9-5.3	1.3010	0.254
>39	40	6 (15)	92.9	3.6-8.6	89.5	1.9-3.5	77.3	2.3-5		
Nodal status (pN)										
pN0	63	3 (4.8)	97.7	0.7-1.3	97.7	1.5-5	90.6	3-3.3	11.205	0.0008
pN1	20	6 (30)	74.3	4.2-10.2	68.7	2.4-4.9	/			
Tumor diameter										
≤4 cm	73	6 (8.2)	94.9	2.4-11.3	93.7	1.6-5.1	88.9	3.3-4.8	4.4624	0.0346
>4 cm	10	3 (30)	79.8	2.2-9.2	79.8	4.3-8.6	68.7	1.3-4.3		
Depth of invasion										
≤20 mm	73	6 (8.2)	94.7	3.6-4.6	94.7	0.5-6.3	88.3	1.4-11.9	4.7679	0.0289
>20 mm	10	3 (30)	79.4	1-4.2	69.3	0.9-3.4	/			
Thickness of US										
0-5 mm	37	6 (16.2)	89.3	4-8	85.6	2.1-2.4	77.3	2.2-3.3	1.9935	0.3691
6-10 mm	32	2 (6.25)	92.3	1.7-11.3	92.3	2.1-3.5	92.3	/		
>10 mm	14	1 (7.1)	97.2	2.3-4.5	97.2	2.4-8.7	97.2	/		
Histological type										
squamous	72	5 (6.9)	94.6	3.5-10.1	94.6	2.4-10	92.3	4.3-12.4	16.268	0.0003
adenocarcinoma	6	1 (16.7)	83.7	1.5-3.9	/		/			
mixed	5	3 (60)	79.7	2.6-3.3	59.4	1.9-2.2	/			
Grade (G)										
well (G1)	3	0	100		100		/		2.9800	0.2253
moderate (G2)	31	6 (19.4)	86.7	1.1-3.1	86.7	0.01-8.1	/			0.056*
poor (G3)	49	3 (6.1)	96.9	3-10.1	94.6	3.3-7.4	76.1	0.7-11.5	3.6471	
LV invasion										
absent	22	3 (13.6)	96.3	1.46-3.6	94.7	1.5-3.9	83.7	2.1-6.5	0.1298	0.7186
present	61	6 (9.8)	86.3	2.8-4.3	86.3	5.2-9.4	/			
LC infiltration										
scarce	9	1 (11.1)	87.6	2.3-5.7	87.6	1.7-4.9	/		0.3478	0.8403
moderate	50	6 (12)	91.2	3.03-7.6	91.2	1.3-3.5	79.8	4.1-9.9		
abundant	24	2 (8.3)	94.3	4.6-9.8	90.7	2.8-9.2	/			
HPV presence										
negative	31	5 (16.1)	86.7	1.9-2.8	82.3	3.9-5.2	82.3	2.71-3.3	3.6479	0.0561
positive	52	4 (7.7)	96.6	3.1-5.1	96.6	2.1-2.9	88.7	0.3-1.7		
HPV type										
16/18	39	3 (7.7)	98.7	1.1-9.5	98.7	4.5-14	/		6.2834	0.0432
31/33/51 - 31/33	9	0	100		100		/			
*mixed types^*	4	1 (25)	73.6	0.7-2.4	73.6	3.3-5.1	/			
Ki-67 (%)										
≤20	28	0	100		100		100		4.6782	0.031
>20	55	9 (16.3)	87.1	0.3-4.3	86.5	6.4-7.9	78.6	3.4-9.9		
p53 (%)										
≤10	38	2 (5.3)	97.5	2.2-3.2	97.2	2.5-9.5	83.1	4.03-1.5	2.5715	0.108
>10	45	7 (15.6)	87.6	4.4-7.7	84.7	4.7-5	/			
bcl-2 (%)										
≤10	41	7 (17.1)	86.7	5.8-6.5	84.7	6-7.4	79.6	4.6-6.5	3.9633	0.047
>10	42	2 (4.8)	97.8	1.4-1.3	97.8	1.4-5.8	91.1	2.6-11.6		
mdm-2 (%)										
≤5	15	0	100		100		/		3.8039	0.0511
>5	68	9 (13.2)	91.2	3.8-5.5	89.5	1.6-5.1	84.2	5.5-6.7		
ER (McCarty score)										
≤5	79	9 (11.4)	92.6	3.7-4.2	91.4	3.9-5.2	84.7	4.9-7.2	2.3457	0.1256
>5	4	0	100		100		/			
PR (McCarty score)										
≤5	78	9 (11.5)	92.6	3.8-4.1	91.4	2.4-4.1	84.7	4.4-7.3	2.4923	0.1144
>5	5	0	100		100		/			
EGFR (%)										
≤75	78	8 (10.3)	91.9	3.8-7.6	91.2	4.4-6.4	85.3	6.7-7.6	1.266	0.2605
>75	5	1 (20)	100		75.2	2.6-8.9	/			
c-erbB-2 (%)										
≤75	63	6 (9.5)	93.6	6.1-9.2	91.8	6.9-10.4	/		1.8086	0.1787
>75	20	3 (15)	89.9	13.3-20.1	89.9	13.3-20.1	77.0	26-31.9		

Legend: CI, confidence interval; EGFR, epidermal growth factor receptor; ER, estrogen receptor; Grade (G), grade of differentiation; G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated; HPV, human papillomavirus; mixed types^, HPV type 16/18 and 31/33/51 or 31/33 positive; LC, Lymphocytic infiltration; LV, Lymph-vascular invasion; No., number of patients; P, probability; PR, progesterone receptor; US, uninvolved stroma; y, years; *referring to differences between moderately (G2) and poorly (G3) differentiated carcinomas.

more than 75% of the cells was present in 9 (10.8%) cases in the superficial layer, 6 (7.2%) cases in the middle layer and 5 (6%) cases in the invasive front, while positive c-erbB-2 staining was seen in 31 (37.4%) cases in the superficial layer, 22 (26.5%) cases in the middle layer and 20 (24.1%) cases in the tumor's invasive front.

The analysis of the interobserver differences in the evaluation of immunohistochemical expression of the proteins revealed a high level of agreement (92.8-100%, kappa>0.8) in determining the intensity and percentage of immunostained cells for p53, ER, PR, EGFR and c-erbB-2 in all three tumor compartments. In addition, there was a moderate or good agreement (72.3-84.3%, kappa=0.41-0.8) in the assessment of all other proteins except for mdm-2 expression in the invasive front (75.9%, kappa=30.5).

Interestingly, although the frequencies and the median or average values for each protein expression did not differ significantly between the three tumor compartments investigated (data not shown), we often found a heterogeneous staining pattern, with a variable fraction of positive tumor cells located in the superficial and middle layers or the invasive front of a tumor. The additional investigation of the associations between protein expression and HPV status and other clinicopathological characteristics as well as their influence on disease-free survival proved that the invasive front of carcinomas is the most important area for the evaluation of the prognostic significance of the expression of cell proliferation, growth, differentiation and apoptosis regulatory proteins. Correlations between the different staining patterns in all three tumor compartments showed that immunohistochemical expression of the proteins was seldom interrelated (chi-square or Fisher's exact test, Table 3).

Associations of Protein Expression Results with the HPV Status and Clinicopathological Parameters

The various protein expression patterns were correlated with the patients' age, pelvic lymph node status, morphometrical, morphohistological characteristics and HPV status. No significant correlation was observed between protein staining results and HPV status (Table 2) except for the trend of an association between c-erbB-2 oncoprotein and the HPV type present. Preferential expression of c-erbB-2 was detected in HPV type 31/33/51 or 31/33 versus type 16/18 positive carcinomas (44% vs. 15%, P=0.053). Nevertheless, HPV DNA negativity correlated strongly with pelvic lymph node involvement (P=0.0034) and scarce lymphocytic infiltration in the invasive front of the tumor (P=0.0007). No association was found between the HPV type detected and any other clinicopathological variable investigated (data not shown).

Correlations between the expression of the different proteins, HPV status and clinicopathological parameters in the invasive front of the neoplasms are summarized in Table 2.

Survival Analysis

Of 83 patients, 6 (7.2%) women died of cervical cancer. The expected 5-, 10- and 15-year overall survival rate was 94.4%, 92.7% and 92.7%, respectively. Nine of the 83 (10.8%) patients had a recurrence. The actuarial disease-free survival rates for 83 cervical cancer patients at 5, 10, and 15 years were 92.7%, 90.8% and 86.6%, respectively.

The results of the univariate analyses are summarized in Table 4. Lymph node metastases, large tumor diameter, deeper stromal invasion, non-squamous

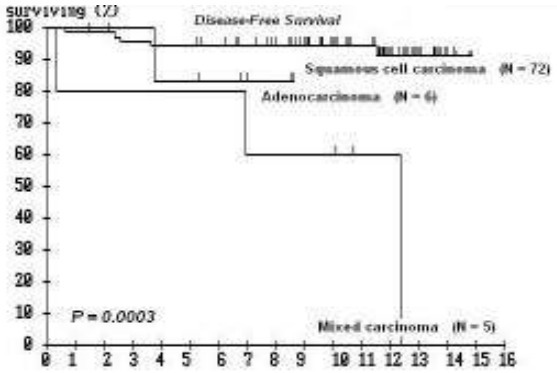


Fig. 3. Disease-free survival of the 83 cervical carcinoma patients distributed by histological type

carcinoma histological type (Figure 3), HPV type, as well as Ki-67 overexpression (Figure 5) and bcl-2 negativity in the tumor's invasive front were highly significant predictors for a shorter duration of disease-free survival in the univariate analysis (P<0.05). In addition, moderate grade of histological differentiation, HPV DNA negativity (Figure 4) and positive mdm-2 staining in the tumor's invasive front were marginally significant predictors for a shorter duration of disease-free survival in the univariate analysis (P<0.06), while all other parameters investigated had no impact on the disease-free survival rate.

Finally, eight variables were selected to be included in the multivariate analysis for disease-free survival: tumor size and depth of stromal invasion as

Table 5. Significant independent prognostic factors for the disease-free survival selected by Cox regression analysis and their relative risk (*chi-square test = 14.399, P = 0.0024*)

Variables	Coefficient (â)	Standard error	P value	RR	CI 95%
Histological type	0.5446987	0.2186494	0.013	2.06	1.07-2.8
HPV presence	0.724837	0.2441279	0.003	1.72	1.4-3.2
Ki-67 expression	0.5127363	0.2536433	0.043	1.67	1.02-2.7

Legend: CI, confidence interval; HPV, human papillomavirus; P, probability; RR, risk ratio or relative risk.

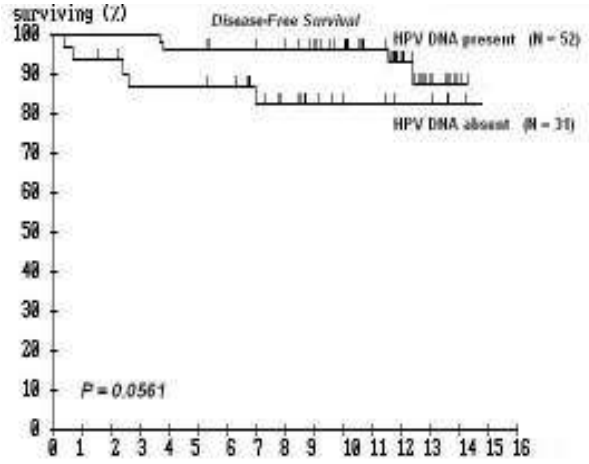


Fig. 4. Disease-free survival of the 83 patients distributed by HPV DNA presence

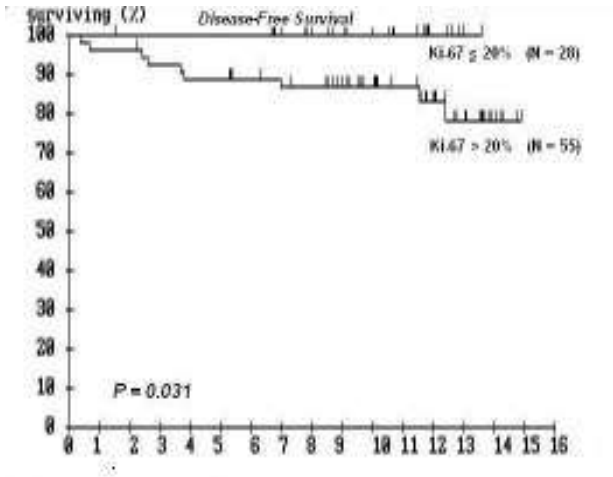


Fig. 5. Disease-free survival of the 83 cervical carcinoma patients distributed by Ki-67 expression in the tumor's invasive front

continuous variables and lymph node status, histological type, grade, HPV DNA presence, Ki-67, bcl-2 and mdm-2 expression in the invasive front as categorized variables. Among these variables, however, the histological type, HPV DNA presence and Ki-67 expression were identified as independent significant prognostic factors for disease-free survival in the multivariate analysis performed by using the Cox's regression model (Table 5).

Discussion

We have studied the prognostic significance of the expression of various cell proliferation, growth, differentiation and apoptosis regulatory proteins in early stage cervical carcinoma in relation to HPV infection and clinicopathological parameters. Bcl-2 negativity, as well as mdm-2 positivity in the tumor's invasive front was correlated with shorter disease-free survival in the univariate, but not in the multivariate analysis.Nevertheless, Ki-67 overexpression in the invasive front was identified as a significant unfavourable prognostic factor influencing recurrence both in univariate and multivariate analysis.P53, EGFR, c-erB-2 protein expression and steroid hormone receptor status had no prognostic significance.

The p53 suppressor gene is located on the short arm of the chromosome at position 17p13.1 and encodes a nuclear protein with a molecular weight of 53 kilo Daltons (kD). The p53 tumor suppressor is a multifunctional protein that plays a central role in the regulation of the normal cell cycle, controlling the switch from late G1 to S phase [14]. This gene is also closely associated with DNA repair and apoptosis. Inactivation of p53 by mutation is a common event in the development of different types of cancer. In addition to very infrequent p53 mutations [15], inactivation of p53 in cervical carcinomas can also be the result of other mechanisms, including increased expression of mdm-2 which binds p53 and blocks transactivation and binding of wild-type p53 by various viral oncoproteins (HPV E6). On the basis of these findings, it is to be expected that tumors containing HPV DNA will contain low amounts of p53 protein.

In our case series, 45 tumors (54%, 32 HPV positive) were p53 positive. Studies of p53 expression by immunohistochemical method yielded widely varying results, reporting rates from 0 to 100% for cervical carcinomas [14-23]. Some variability is undoubtedly the result of the use of different antibodies, fixatives, and immunohistochemical methods, whereas some reflect the use of different threshold for p53 positivity. As with previously reported similar studies, we did not find any correlation between p53 expression and HPV positivity, almost no correlation with the clinicopathological characteristics investigated except for the depth of stromal invasion or with disease-free survival [14-18]. In contrast, Chen et al. [19] claimed that p53 overexpression has prognostic value for patients with stage IB squamous cell carcinoma. Similar findings were reported by three smaller studies for patients with stage I-IV cervical carcinoma [20-22]. Nevertheless, several other larger studies including stage I-IV carcinoma [14-16,23] reported that such results were highly dependent on the stage and independent predictive value of p53 overexpression was not confirmed in multivariate analysis. Thus, at present, no clear relationship exists between p53 expression in cervical carcinomas and patients survival.

The murine double minute 2 (mdm-2) gene, located on chromosome 12q13–14, encodes a 90 kD zinc finger protein, composed of 491 amino acids, which can bind to wild type and mutant p53 [14]. The mdm-2 gene forms an autoregulatory feedback loop with p53. Namely, the mdm-2 oncogene product has been shown to bind to wild type and mutant p53 and to inactivate its physiological role as a transcription factor and cell cycle regulator. Therefore, it might be expected that tumors with HPV infection would not accumulate p53 protein, nor the p53 induced protein mdm-2. Despite the presence of HPV, we have detected mdm-2 in 68 (82%, 40 HPV positive) of cervical carcinomas. In our case series mdm-2 expression correlated to Ki-67 and c-erbB-2 expression and

significantly influenced disease-free survival, although its independent value was not confirmed. This is in contrast to the reported results from three previous studies in which different mdm-2 antibodies were used [14,24,25].

The bcl-2 oncogene is located on chromosome 18 and encodes a 25 kD protein located at the mitochondrial outer membrane, in the nuclear envelope, plasma membrane, endoplasmic reticulum, and in chromosomes. In our case series, 51% (42/83, 26 HPV positive) cervical carcinomas were bcl-2 positive. The rate of bcl-2 expression in early stage cervical carcinomas in the literature ranges from 20% to 63% [18,23,26-29]. In concordance with some prior observations in our case series bcl-2 overexpression was related to tumor grade [29,30], as well as the thickness of uninvolved stroma and longer disease-free survival. Nevertheless, the prognostic value of bcl-2 in cervical carcinomas is controversial, with reports describing bcl-2 as a prognostic factor for improved survival, [18,21,27], decreased survival in patients in more advanced stages treated by radiotherapy [31,32], or having no prognostic relevance [23,28-30,33]. These could be partly explained by differences in cut-off values used, but also by population heterogeneity and various treatment modalities. The putative role of the bcl-2 protein is to extend cell survival by protecting the cell against programmed cell death without affecting cell proliferation in response to a number of stimuli, including radiotherapy and chemotherapy. When overexpressed, bcl-2 produces dramatic extension of cell survival. Therefore, it was proposed that the tumor with overexpression of bcl-2 would resist radiotherapy resulting in worse prognosis [32,34]. In contrast, overexpression of bcl-2 is associated with less aggressive malignant behavior in some carcinomas treated by surgery [20]. These opposing actions of bcl-2 in patients treated by radiation/chemotherapy versus surgery may render the difficulty to explain the association of bcl-2 expression and the patients' outcome in those who received multimodalities of treatment. Thus, most of the studies except two [18,30] that reported on the association of bcl-2 and prognosis in surgically treated cervical cancer patients included patients who also received adjuvant radiotherapy and/or chemotherapy. Some of these studies failed to detect the association between bcl-2 and prognosis, while other studies reported the association between bcl-2 and longer survival in univariate [21] or both univariate and multivariate analysis [18,27]. This is partially in concordance with our results because the independent prognostic influence of bcl-2 expression was not confirmed in the multivariate analysis. Nevertheless, some of these studies did not include some important prognostic factors such as tumor diameter, grade, depth of stromal invasion and lymph-vascular space involvement [18,27].

The gene for the human EGFR is located on chromosome 7 and encodes a transmembrane cell surface 170 kD glycoprotein that binds epidermal growth factor, transforming growth factor-alfa, amphiregulin, and heparin-binding epidermal growth factor. Binding of the ligand to the extracellular domain initiates biological responses ultimately resulting in DNA replication and cell division. Due to the different techniques or different antibodies and diverse cut-off levels used, EGFR overexpression ranging widely as 6-100% has been reported in cervical carcinomas [25,35-40]. In our case series, only 6% (5/83) of the carcinomas stained positively for EGFR. Studies investigating the role of EGFR expression present conflicting results; some studies reported that overexpression of the EGFR in cervical carcinomas correlates with a poor prognosis [25,35-37], while others, in accordance with our results, found no relation of EGFR overexpression with prognosis [38-40].

The c-*erbB-2/neu* gene on chromosome 17q21 encodes a 185-190 kD transmembrane class I tyrosine kinase receptor protein structurally related to the EGFR. This gene can be activated by point mutation or amplification. Although it has been suggested that higher levels of c-*erbB*-2 expression are seen at later stages of cervical carcinoma [41], a wide range (19-77%) of positive rate of c-erbB-2 immunostaining has been reported [25,36,39-43]. These series, however, utilized different monoclonal antibodies and were composed of different tumor types, with lower rates reported squamous cell carcinomas, which is in concordance with our results. Certain studies report a relatively higher rate of c-*erbB*-2 positivity indicating a significantly poorer survival [41-43], whilst series similar to ours (24%), with lower incidence of positivity, reported no survival significance [25,36,39,40], which indicates clinical limitations of the test depending on laboratory methodologies as well as differences in case selection. Thus, at present, high expression of c-erbB-2/neu has been associated with a poor prognosis, especially in more advanced stages of cervical cancer and in adenocarcinomas [41,42].

Since the uterine cervix is one of the target tissues of sex steroid hormones, the growth and differentiation of the cervical epithelia are assumed to be subtly regulated by sex steroid hormone via specific binding proteins known as estrogen receptors (ER) and progesterone receptors (PR). The studies investigating their association with HPV infection, as well as their role in cervical progression and prognosis have yielded controversial results. Nonogaki et al. [44] suggest that ER expression in cervical carcinomas may be related to HPV types present. Their observations were not supported in our case series, because ER was demonstrable in a variety of HPV types. Contrary to earlier biochemical studies [45], the results of immunohistochemical studies showed, similar to our findings (5%), a decrease or loss of ER in neoplastic cells [12,44] and lower percentage of positive invasive cervical carcinomas. In addition, in concordance with our results, majority of the immunohistochemical studies have found that steroid receptor status has no impact on either disease-free or overall survival in cervical carcinoma patients [12,46].

The proliferation marker Ki-67 antigen is a nuclear non-histone protein of 395 and 345 kD, expressed in the nuclei of proliferating cells during the cell cycle, except during the resting G0 phase. The Ki-67 coding gene is localized on the long arm of chromosome 10. So far, most

studies which applied Ki-67 or MIB-1 antibody for the evaluation of cervical carcinoma have not found a correlation between Ki-67 and the conventional prognostic parameters, [14,47-49] or survival [48,50-52]. Nevertheless, cell proliferation is described as an additional parameter useful in the prognostic evaluation of locally advanced cervical carcinoma [53]. The explanation for the latter observation is that cancers with high proliferative activity tend to possess higher rate sensitivity to radiotherapy, although these findings were not confirmed in all subsequent studies [54]. In early stages, where a large proportion of patients is not treated by radiation therapy, two studies found an increased Ki-67 expression to relate to a worse outcome [49,55]. Thus, studies on its prognostic significance have reported conflicting results showing better, worse, or no different outcome with increasing Ki-67 staining. Using a small group of patients for the study [48,49], including tumors at different stages [20,47,48], including patients treated by different therapeutical modalities and short follow-up period [48, 49], probably affected the results of these series or made the results difficult to interpret. In order to avoid the above situations, we used a reasonably large group of patients who had early stage carcinomas confined to uterine cervix, which had been uniformly treated and followed for a long period of time. Our study of the relation between Ki-67 and other clinicopathological parameters showed that Ki-67 was only related to the thickness of uninvolved cervical stroma and its overexpression could predict disease-free survival.

The established clinicopathological variables such as lymph node metastases, tumor diameter, depth of stromal invasion [1-6,10,14,28,33], histological type as well as grade [14,28] were of prognostic significance in univariate analysis. HPV type, as well as HPV DNA presence, had a significant influence on disease-free survival in the univariate analysis. In multivariate analysis, only histological type and HPV DNA presence were statistically significant. With regard to histological type, it has been reported earlier that survival rate for patients with adenocarcinoma or mixed carcinoma was significantly poorer than that for patients with squamous cell carcinoma [3,6-8,47]. In addition, our results are in concordance with reported data that HPV-negative tumors are more aggressive and show greater metastatic potential and worse prognosis than HPV-positive cancers [22,56,57].

Our data also suggest that the invasive front of carcinomas is the most important area for the evaluation of the prognostic significance of the expression of cell proliferation, growth, differentiation and apoptosis regulatory proteins. Namely, we have tried to investigate the concept of Bryne et al. [9] about the prognostic value of invasive front for head and neck squamous carcinomas, later adopted by some investigators for early stage squamous cell carcinoma of the uterine cervix [2,28]. According to their results, the invasive front of carcinomas proved to be the most important area for tumor prognosis. Thus, similarly to determining the morphohistological characteristics such as grade or inflammatory infiltrate, the evaluation of the expression of proteins in the invasive front of the cervical carcinomas may be more biologically relevant because this area is possibly more important for the determination of invasive and metastatic capacity than the rest of the tumor. In addition, such an approach is less time-consuming and possibly more reproducible since the major part of the tumor can be disregarded.

In conclusion, the current study confirmed previous data on prognostic factors in early stage cervical carcinoma, insofar as the univariate analysis demonstrated that regional lymph node status, tumor diameter, depth of invasion, and histological type were of important prognostic significance in postoperative pT1b1/1b2 disease. In addition, according to the results of the multivariate analysis, non-squamous cell histological type, HPV DNA absence and Ki-67 expression in the tumor's invasive front in more than 20% of carcinoma cells have now been identified as independent predictors of recurrence. These prognosticators may assist in choosing between surgery and surgery followed by radiotherapy in patients with early stage cervical carcinoma.

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