Prognostic significance of topoisomerase II alpha and collagen IV immunoexpression in cervical cancer

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Summary

Objective: The immunohistochemical (IHC) expression of topoisomerase II alpha and collagen IV was studied in cervical cancer. The results of IHC expression for both markers were compared to the clinical and histological factors predicting the course of disease. *Methods:* In 114 patients with cervical cancer, treated at the Department of Gynecology and Obstetrics, University Medical Centre Ljubljana between 1995 and 1999, the tumor tissue was analyzed using standard IHC staining (IHS) procedures for topoisomerase II alpha and collagen IV. The obtained results were compared to those obtained by clinical, pathomorphological and morphometric prognostic factors, and the impact of the analyzed factors on the outcome of disease was assessed. *Results:* A high percentage of IHC expression of topoisomerase II alpha was present in 55.3%, and to collagen IV in 28.1% of cervical cancer patients. In the multivariate analysis the IHS intensity to collagen IV was significantly associated with lymphovascular invasion (OR = 5.906; 95% CI 2.18-15.96). Kaplan-Meier analysis showed a statistically significantly better survival in initial cervical cancer stages ($p = 0.001$), in tumors with a higher degree of differentiation ($p = 0.049$), more shallow depth of invasion ($p = 0.004$), smaller horizontal tumor spread ($p = 0.001$), in cases with no lymph node metastases ($p = 0.001$) and no lymphovascular space invasion ($p = 0.001$), in younger age groups ($p = 0.001$) and in women with regular menstrual cycles ($p = 0.001$). *Conclusion:* IHC expression of topoisomerase II alpha and collagen IV was significantly correlated with defense reaction. A negative and weak IHC to collagen IV was a statistically significant independent predictive variable for lymphovascular invasion, related to metastatic spread in the lymphnodes. The two analyzed IHC markers indicate the existence of factors at the molecular level that might complement the assessment of cervical cancer prognosis, resulting in the appropriate choice of type and extent of treatment.

Key words: Topoisomerase II alpha; Collagen IV; Cervical cancer.

Introduction

Cervical cancer continues to remain a major health problem worldwide. According to the latest WHO statistics, cervical cancer is second only to breast cancer among the most frequent female malignancies worldwide [1].

The prognosis of cervical cancer is determined by several predictors, and according to the recent task force on prognostic factors in cervical cancer, there is an urgent need for more specific markers capable of predicting the disease outcome in individual patients.

As the major prognostic factor, the depth of invasion is one of the most widely used parameters of cervical cancer that shows an evident statistically significant relationship with the presence of lymph node metastasis, recurrence and death from cancer in many studies. All other prognostic factors, such as horizontal spread, involvement of lymphovascular space, type of invasion, cell type and additional factors are considered as non-independent prognostic factors. Certain biological factors have been suggested lately that might predict the course of cervical cancer, topoisomerase II alpha and collagen IV being two of them [2, 3].

In the present study, the immunohistochemical expression of topoisomerase II alpha and collagen IV was studied in cervical cancer. The results of immunohistochemical (IHC) expression for both markers were compared to the clinical and histological factors predicting the course of disease.

The major aim of this study was to find which of the analyzed factors has the highest predictive power for predicting cervical cancer outcome in individual patients.

Material and Methods

Patients

Clinical and morphologic data were obtained from 114 patients with cervical cancer, treated at the Department of Gynecology and Obstetrics, University Medical Centre Ljubljana, between 1995 and 1999. The mean patient age was 42.3 ± 11.5 (SD) years (range 25-88 years). After the diagnosis was confirmed, the patients were treated by radical hysterectomy, conization or radiotherapy. After therapy, the patients were closely followed-up; mean follow-up time was 69.64 ± 27.71 (SD) months, range 7-80 months.

Histology and immunohistochemistry

Both colposcopy biopsies and surgical samples were fixed in 10% buffered formalin, embedded in paraffin, and processed for 5-μm-thick paraffin sections stained with hematoxylin-eosin for routine diagnosis. The morphological and morphometric char-

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Table 1. — *Frequency of histologic types according to WHO classification and frequency of cases grouped into four histologic subtypes, grade, clinical stage according to FIGO, state of lymph nodes, different intensity of defense reaction, and lymphovascular invasion in 114 patients.*

	No. of patients	%
WHO classification into histologic subtypes		
Squamous cell nonkeratinizing	64	56.1
Squamous cell keratinizing	5	4.4
Adenocarcinoma in situ	1	0.9
Adenosquamous carcinoma in situ	1	0.9
Adenocarcinoma, endocervical type	13	11.4
Adenocarcinoma, intestinal type	4	3.5
Adenocarcinoma,endometrioid type	7	6.1
Adenocarcinoma, serose type	1	0.9
Adenosquamous carcinoma	10	8.8
Small cell carcinoma	2	1.8
Mixed cell carcinoma (adeno & squamous)	6	5.3
Four histologic subtypes		
Squamous cell carcinoma	69	60.5
Adenocarcinoma	37	32.5
Others	2	1.8
Mixed cell carcinoma (adeno & squamous)	6	5.3
Grade		
Grade 1	38	33.3
Grade 2	66	57.9
Grade 3	10	8.8
Stage - FIGO classification		
0	3	2.6
IA1	20	17.5
IA2	9	7.9
IB	49	43.0
IIA	1	0.9
IIB	3	2.6
IIIB	15	13.2
IV	14	12.3
State of lymph nodes		
Unknown	42	36.8
Negative state	57	50.0
Positive state	15	13.2
Defense reaction		
No defense reaction	24	21.1
Poor	38	33.3
Moderate	28	24.6
Good	6	5.3
Not assesed	18	15.8
Lymphovascular invasion		
Absent	77	67.5
Present	37	32.5

acteristics of the tumor were evaluated in all cases. Then serial step sections of 114 cervical cancers were analyzed using standard IHC staining procedures for topoisomerase II alpha and collagen IV.

The formalin-fixed, paraffin-embedded tissue sections were stained with monoclonal antibodies to topoisomerase II alpha (Ki-S1) and collagen IV with a standard streptavidin immunohistochemical technique, with antigen retrieval to assess the presence of enzymes. The results were based on nuclear staining and percentage of positivity. Figures 1-2 show tumor cells in the cervix after IHC staining.

Table 2. — *Frequency of immunostaining intensity and the percentage of tumor cells stained for topoisomerase II alpha and to collagen IV.*

	No. of patients	%
Immunostaining intensity for topoisomerase II alpha		
Negative	11	9.6
Poor	11	9.6
Moderate	38	33.3
Intense	45	39.5
Not assessed	9	7.9
Percentage of cells stained for topoisomerase II alpha		
100%	39	34.2
75%	24	21.1
50%	23	20.2
25%	5	4.4
< 25%	3	2.6
Negative	11	9.6
Not assessed	9	7.9
Immunostaining intensity for collagen IV		
Negative	20	17.5
Poor	20	17.5
Moderate	23	20.2
Intense	19	16.7
Not assessed	32	28.1
Percentage of cells stained for collagen IV		
100%	13	11.4
75%	19	16.7
50%	16	14.0
25%	3	2.6
< 25%	11	9.6
Negative	20	17.5
Not assessed	32	17.5

Statistical analyses

The chi-square analysis was used to test the relationship among the variables. Multivariate analysis by Cox's proportional hazards model was used to rank the importance of each prognostic factor in prescribing treatment. Kaplan-Meier survival analysis was used to calculate the survival times. Statistical significance was set at $p < 0.05$.

Results

Clinical characteristics of patients and results of pathomorphological analysis

The frequency of histologic types according to the WHO classification and frequency of cases grouped into four histologic subtypes, grade, clinical stage according to FIGO, state of lymph nodes, different intensity of defense reaction, and lymphovascular invasion in 114 patients are shown in Table 1.

Results of immunohistochemical staining

The frequency of immunostaining intensity and the percentages of tumor cells stained for topoisomerase II alpha and collagen IV are shown in Table 2.

A high percentage of IHC expression of topoisomerase II alpha was present in 55.3% and to collagen IV in 28.1% of cervical cancer patients. IHC reaction to topoi-

Table 3. — *Histologic types compared with percentage of positive immunostaing for topoisomerase II alpha and nuclear grade and defense reaction compared with intensity of immunostaining for topoisomerase II alpha*.

Histologic type	Percentage of nuclei immunostained for topoisomerase II alpha							
	100%	75%	50%	25%	< 25%	Negative	Not estimated	Total
Squamous cell carcinoma	25 (36.2)	18 (26.1)	14 (20.3)	3 (4.3)	2 (2.9)	3 (4.3)	4 (5.8)	69 (100.0)
Adenocarcinoma	11 (29.7)	4 (10.8)	8 (21.6)	0 (0.0)	1 (2.7)	8 (21.6)	5 (13.5)	37 (100.0)
Others	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)
Adeno&squamous cell carcinoma	1 (16.7)	2 (33.3)	1 (16.7)	2 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	6 (100.0)
Total	3 (34.2)	24 (21.1)	23 (202)	5 (4.4)	3 (2.6)	11 (9.6)	9 (7.9)	114 (100.0)

Nuclear grade	Immunostaining intensity for topoisomerase II alpha					
	Negative	Poor	Moderate	Intensive	Not estimated	Total
Grade 1	9 (23.7)	5 (13.2)	12 (31.6)	9 (23.7)	3 (7.9)	38 (100.0)
Grade 2	2 (3.0)	5 (7.6)	23 (34.8)	31 (47.0)	5 (7.6)	66 (100.0)
Grade 3	0 (0.0)	1 (10.0)	3 (30.0)	5 (50.0)	1 (10.0)	10 (100.0)
Total	11 (9.6)	11 (9.6)	38 (33.3)	45 (39.5)	9 (7.9)	114 (100.0)

Defense reaction	Immunostaining intensity for topoisomerase II alpha					
	Negative	Poor	Moderate	Intensive	Not estimated	Total
None	6 (25.0)	5 (20.8)	6 (25.0)	7 (29.2)	0 (0.0)	24 (100.0)
Poor	2 (5.3)	4 (10.5)	17 (44.7)	15 (39.5)	0 (0.0)	38 (100.0)
Moderate	2 (7.1)	2 (7.1)	11 (39.3)	13 (46.4)	0 (0.0)	28 (100.0)
Strong	0 (0.0)	0 (0.0)	2 (33.3)	4 (66.7)	0 (0.0)	6 (100.0)
Not estimated	1 (5.6)	0 (0.0)	2 (11.1)	6 (33.3)	9 (50.0)	18 (100.0)
Total	11 (9.6)	11 (9.6)	38 (33.3)	45 (39.5)	9 (7.9)	114 (100.0)

somerase II lpha was significantly decreased in adenocarcinoma compared to intense IHC in squamous cell carcinoma and was significantly associated with nuclear grade ($p = 0.038$) and defense reaction ($p = 0.001$).

Using the chi-square test we found statistically significant correlations between various degrees of defense reaction in the presence of carcinoma as well as between the intensity of IHC expression of collagen IV and the percentage of cells stained for collagen IV. In less intense IHS to collagen IV, which reflects the loss of components of the basal membrane and the thinning of collagen fibres in invasive carcinomas, a well expressed defense reaction was found present.

Table 3 shows histologic types compared with percentage of positive immunostaing for topoisomerase II alpha and nuclear grade and defense reaction compared with intensity of immunostaining for topoisomerase II alpha.

Survival

The mean patient age was 42.3 years (± SD 11.5 years, range 25-88 years). According to data from the Cancer Registry of Slovenia, 83 patients (72.8%) were alive, 23 (20.2%) had died, and the information for eight (7%) patients was lost by 30 April 2004, the end of the observation period. The mean survival time by then was 69.64 months (± 27.71 months).

The intensity and share of immunostaining for topoisomerase II alpha had no significant influence on mean patient survival time. However the mean survival time was significantly shorter in cases with negative and weak immunostaining for collagen IV ($p = 0.011$).

Kaplan-Meier analysis showed statistically significantly better survival in women with initial stages of cervical cancer ($p = 0.001$), higher degree of tumor differentiation ($p = 0.049$), more shallow depth of tumor invasion ($p = 0.004$), smaller horizontal tumor spread ($p = 0.001$), in cases without lymph node metastases ($p = 0.001$) and lymphovascular space invasion ($p = 0.001$), in younger age groups ($p = 0.001$) and in women with regular menstrual cycle ($p = 0.001$).

Results of multivariate analysis

To determine the importance of each individual prognostic factor in deciding on the treatment of cervical cancer, multivariate analysis with the Cox proportional hazards model was performed.

In the logistic regression model we put the following variables: FIGO stage of disease, patient age, tumor differentiation degree, defense reaction, lymphovascular space invasion, and intensity of IHC staining for topoisomerase II alpha and collagen IV. The patient survival time was the dependent variable. Using the step-by-step method we found that the most important variable in the survival time prediction for women with cervical cancer was lymphovascular space invasion followed by intensity of IHC staining to collagen IV (Table 4).

Table 4. — *Results of logistic regression step by step method - regression model*.

Variable	Regression coefficient	Standard deviation	*p* value	Odds ratio with 95% confidence interval
Lymphovascular space invasion	3.302	1.141	0.004	27.1 (2.9-254.3)
Negative and weak expression of IHC staining to collagen IV	2.145	0.954	0.025	8.5 (1.3-55.4)

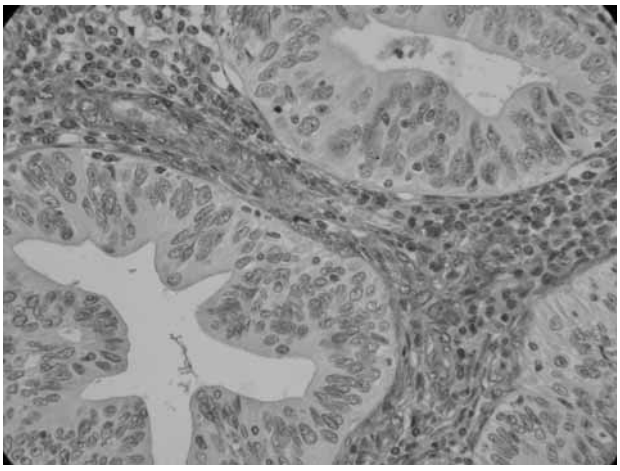

Figure 1. — Collagen fibres encircling adenocarcinoma. Intense immunohistochemical staining of collagen IV.

In our study a specific prognostic factor for lymphovascular space invasion, which is a poor prognostic factor as it permits lymphogenic carcinoma metastasis, proved to be the intensity of the IHC staining to collagen IV.

In the multivariate analysis the intensity of IHS to collagen IV was significantly associated with lymphovascular invasion (OR = 5.906; 95% CI 2.18-15.96).

Discussion

Cervical cancer is the only cancer that can be efficiently prevented by detection of precancerous lesions [4]. Efficient early detection and treatment of cervical cancer involves knowledge of molecular mechanisms [5, 6]. Currently, the main focus is given to the interaction between the tumor and the matrix, interaction between growth factors and components of intercellular, processes of angiogenesis and biochemical markers [5, 6]. Determination of these biochemical markers may be helpful in the diagnosis and prediction of the disease course, and in the decision on the treatment modality, and in early detection of recurrent and metastatic disease.

The stage of cervical cancer is the most important prognostic factor for the course of disease, even if it is detected early (stage IB, IIA) and treated surgically. A greater tumor size represents a higher risk of lymph node metastases and predicts a poorer course of disease [7]. Thus, in our study the women with early stages of disease at diagnosis (IA and IB) also had a significantly longer survival time than those with the disease diagnosed at Stage II or higher ($p = 0.001$).

Depth of invasion ≥ 5 mm combined with horizontal spread ≥ 7 mm meant a significantly shorther mean survival time. Depth of invasion is the major criterion used by the histopathologist when precisely assessing growth of invasion under microscope examination, and it also affects the decision on a greater or lesser radicality of surgery. A number of recent studies have shown that increasing depth of invasion in cervical cancer increases the risk of lymph node metastases, recurrence of disease and death by cervical cancer [8]. The depth of invasion is one of the major prognostic factors for lymph node metastases [9].

The mean survival time of the women enrolled in our study was significantly longer in women with endometrioid type of adenocarcinoma or with squamous cell carcinoma. The survival time was the shortest in women with small cell carcinoma. This is in line with other authors claiming that small cell non-differentiated carcinomas have a high malignant potential and an extremely quick spread of metastatic lymph nodes via lymphovascular spaces [10, 11].

Metastases of cervical cancer in neighbouring or remote lymph nodes are among the major prognostic factors for the outcome of cervical squamous cell and adenocarcinomas [12]. Numerous studies over the past years aimed at finding the complicated mechanisms of morphologic reactions in lymph nodes, occurring as the host's immune response to carcinoma [12]. According to many authors vascular invasion is a poor prognostic factor for the course of cervical cancer [11]. In our series the women without lymph node metastases had a significantly longer mean survival time than the women with detected metastases.

Stromal reaction, the so-called defense reaction around carcinoma, as an interaction between the tumor and the host is still insufficiently investigated. Roughly we know two types of stromal reactions: reaction of the connective tissue and infiltration with immunocompetent cells. Sano et al. have found that an increased number of plasma cells, reticular fibres and a high collagen/collagenase ratio act as a protective mechanism against the invasion of carcinoma [13].

Proliferation tumor activity was in our study manifested as increased intensity of IHC-stained topoisomerase II alpha and good defense reaction around the tumor. When the defense reaction is good, collagen is thinned due to increased number of plasma cells and reticular fibres, which is reflected in less intense IHC-stained collagen IV, and decreases the invasiveness of carcinoma. Similar conclusions have been reached by some other authors [14-19].

In 114 women the presence of topoisomerase II alpha and collagen IV in tumor tissue was determined using IHC. When the percentage of fibres stained for collagen IV using IHC was 100%, the intensity of topoisomerase II alpha expression was negative, and vice versa when the fibres stained for collagen IV remained uncolored, the intensity of topoisomerase II alpha expression was the highest. Topoisomerase is a marker of cell proliferation, thus the higher the intensity of topoisomerase II alpha expression, the quicker the carcinoma growth. The same conclusion was obtained by Gibbons et al. after they investigated topoisomerase II alpha and MIB-1 expression in changed squamous epithelium of the uterine cervix and assessed their power as new proliferation markers. The number of positive nuclei in the epithelium increased according to progressive changes in the epithelium from the basal layer to its entire depth [15].

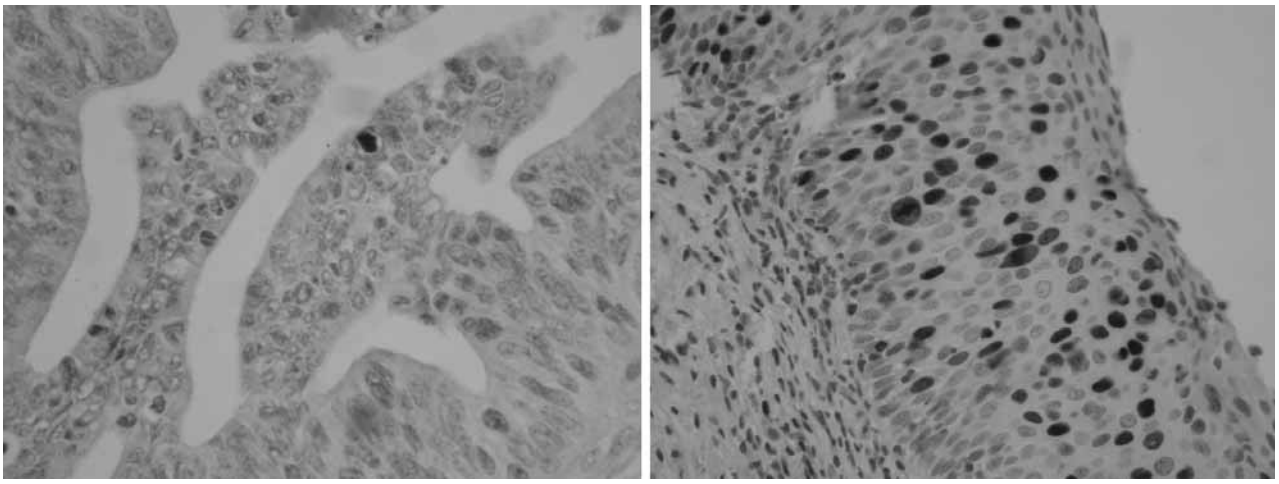

Figure 2. — Immunohistochemical staining of topoisomerase II alpha: poor staining intensity and a small percentage of stained nuclei of adenocarcinoma (left) and extremely strong staining intensity and a high percentage of stained nuclei in CIN 3 (right).

The intensity of topoisomerase II alpha expression and the percentage of stained tumor cells were significantly higher in women after conization than in those after hysterectomy or radiotherapy. This indicates a high degree of proliferation activity of early-stage cervical carcinomas with a small volume, which should not be underestimated for this very activity. The fact that we are dealing with malignant neoplasmas is confirmed by the development of carcinoma in lymphovascular spaces in cases of initial invasion of carcinoma Stage IA when the depth of invasion does not exceed or is even less than 3 mm. It can be presumed that increased carcinoma volume leads to relatively poorer blood supply, hence the decreased carcinoma growth, and consequently the poorer expression of proliferation markers.

The percentage of cell nuclei stained for topoisomerase II alpha was significantly lower in adenocarcinomas than in squamous cell carcinomas. Also, the course of disease was more favorable to the women with the former compared to those with the latter carcinoma. The results of studies on the predictive value of histologic carcinoma subtype for the course of cervical cancer are contradicting [20]. Villoglandular adenocarcinoma, adenoid and basal cell carcinomas all predict a good course of disease. Poorer is the prognosis for the course of serous papillary carcinoma. Waldenstrom *et al.* have recently published survival results for women with adenocarcinoma in Sweden [20]. The 5-year survival rate of women with adenocarcinoma was 64%, and of those with squamous cell carcinoma 66%. The authors concluded that the survival rates are similar under the condition that their treatments are similar.

In our study we have found statistically significant correlations between the intensity of topoisomerase II alpha expression and degrees of tumor differentiation. With grade 1, the intensity of expression was negative in 23.7%, whereas with grade 3, it was highly positive in 50% of cases. Today, the degree of tumor differentiation is generally not considered to have an important predictive value in squamous cell carcinoma [21], whereas in adenocarcinoma it is considered a reliable prognostic factor for the course of cervical cancer [14].

Collagen IV plays an important role in tumor invasion and metastasis; immunohistochemically it is displayed in the basal membrane on the borderline between the epithelium and the stroma. Collagen IV shows whether the basal mambrane in the cancerous tissue is discontinued or completely erased. Therefore, a negative and weak IHC expression to collagen IV reflects a complete absence of collagen fibres or decreased collagen in the basal membrane and in the connective stroma around the cancerous tissue of invasive carcinoma [19, 22-26].

Favret *et al.* analyzed the distribution of laminin, collagen IV and fibronectin in dysplasias and neoplastic changes on the uterine cervix using the IHC method [22]. The analysis was made on normal cervical tissue (16 cases), in cervical dysplasia (14 cases) and in invasive carcinomas (45 cases). It was found that in normal and dysplastic epithelium the distribution of laminin and collagen IV in the basal membrane was linear and continuous. In in situ carcinoma small discontinuations and changes in linearity were seen; whereas in microinvasive carcinomas erasement of the basal membrane was observed. In well differentiated invasive carcinoma dotted discontinuations of the basal membrane around neoplastic islands were noted. In contrast to moderate and poorly differentiated carcinomas, the decrease and loss of positive stain reaction to laminin and collagen IV was even more progressive. The results of this study show that the distribution pattern of intrinsic components of the basal membrane is proportional to the histologic grade of cervical neoplasia and the invasion ability [23]. Toki *et al.* investigated laminin and collagen IV spread in the basal mambrane of 45 cervical adenocarcinoma cases using the IHC method [23]. They found the staining pattern to be in agreement with the grade of differentiation.

Conclusion

In conclusion IHC expression of topoisomerase II alpha and collagen IV was significantly correlated with defense reaction. A negative and weak IHS to collagen IV was a statistically significant independent predictive variable for lymphovascular invasion, related to metastatic spread in the lymph nodes. The two analyzed IHC markers indicate the existence of factors at the molecular level that might complement the assessment of prognosis of cervical cancer, resulting in appropriate adjustment of the type and extension of cervical cancer treatment.

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