Changes of Ki-67 index in squamous cell carcinoma of the cervix during the early course of radiotherapy and prediction of prognosis

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Summary

Purpose: To determine whether changes in the Ki-67 index during the early course of radiotherapy could predict the prognosis in squamous cell carcinoma of the uterine cervix and be of value in clinical practice.

Materials and Methods: Biopsy specimens from 23 cases of histologically confirmed squamous cell carcinoma of the cervix were stained with anti-Ki-67 monoclonal antibody prior to radiotherapy and after 9 Gy. The correlation between the Ki-67 index, local control and distant metastasis was determined by Spearman's correlation test.

Results: Median age of the patients was 49. According to the FIGO staging system four patients had Stage IIA, 16 had Stage IIB, one had Stage IIIA and two had Stage IIIB disease. Among the whole group brachytherapy was applied to 17 patients (17/23) and weekly cisplatin (40 mg/m^2) was applied to 15 patients (15/23). The mean Ki-67 index prior to radiotherapy and after 9 Gy for the entire group were 58.5% and 46.0%, respectively. The Ki-67 index after 9 Gy decreased in most of the patients (74%). During a median follow-up of 23 months four patients developed local recurrence and four patients developed distant metastasis. No significant correlation was detected among the local control and changes in Ki-67 index after 9 Gy, whereas there was a moderate correlation between distant metastasis and changes in Ki-67 index after 9 Gy ($r = 0.51$, $p = 0.01$).

Conclusion: The Ki-67 index can be used safely as a proliferation marker in cervical carcinomas, and changes in the Ki-67 index during the early course of radiotherapy may predict the metastatic potential. However prospective studies including a large number of patients with long-term follow-up are necessary to confirm the clinical utility of this marker in cervical cancer.

Key words: Carcinoma of the cervix; Radiotherapy; Ki-67 index.

Introduction

Radiotherapy (RT) has been the standard, definitive treatment for locally advanced cervical cancer. The overall five-year survival rates range from 15% to 80% depending on the extent of disease [1, 2]. Stage, tumor size, histologic type, grade and lymph-node metastasis are considered to be the most reliable prognostic factors [3, 4]. However the prognosis could be different among patients with tumors of the same size and stage. The lack of accurate criteria to predict prognosis for individual patients remains a major problem. If there is a reliable marker which determines the growth rate, progression and metastatic potential of a tumor more accurately, then the patients at low risk who could be offered a more conservative treatment and the patients at high risk who could be offered more intensive treatment may be identified. This should lead to an improvement in local control and survival.

Ki-67 protein is a nuclear antigen expressed in all phases of the cell cycle except G_0 and early G_1 phases and immunostaining with its monoclonal antibody has been widely used to determine the growth fraction of neoplastic tissues [5]. Many immunohistochemical studies report a relationship between the Ki-67 index and prognosis; however little is known about the effect of radiation on the growth fraction determined by anti-Ki-67 immunostaining.

In this study we investigated the histopathological and morphological characteristics of Ki-67 positive cervical cancer cells, changes in the Ki-67 index during the early course of RT and its correlation with local control and distant metastasis.

Materials and Methods

Forty-six biopsy specimens from 23 patients with invasive squamous cell carcinoma of the cervix who had been treated by definitive RT or radiochemotherapy between August 1999 and September 2000 at Ege University School of Medicine, Department of Radiation Oncology were studied.

Radiation therapy protocol

External RT was delivered by a 6 MV linear accelerator through individually shaped pelvic portals including the tumor and regional lymph nodes using AP/PA or a four-field technique (AP/PA and opposed laterals). Daily fractionation dose was 1.8 Gy, 5 fractions per week. Total external RT dose was 64.8

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Gy (with shrinking fields after 54 Gy) in patients who were not suitable for brachytherapy (6 patients). Midline shielding was performed at 50.4 Gy in patients who were going to receive intracavitary brachytherapy. Two fractions of 8.5 Gy (total dose: 17 Gy) with one-week intervals were applied to point A with the Rotterdamn applicator via micro Selectron high dose rate remote afterloader, Ir-192. External irradiation was stopped at 54 Gy if there was no parametrial involvement. In cases of parametrial involvement 3 fractions of 1.8 Gy of parametrial boost was added after 54 Gy.

Fifteen patients (17/23) with good performance status, normal hemogram and kidney function tests were applied weekly cisplatin (40 mg/m^2) during RT.

Histopathologic study

Twenty-three specimens for pathologic diagnosis were excised before the initiation of RT. Subsequently 23 biopsies were obtained after 9 Gy. Biopsy specimens were fixed in 10% buffered formaldehyde solution for at least 24 hours and embedded in paraffin after tissue processing. The paraffin blocks were cut with a microtome in 5 μm thickness and the slides were stained with hematoxylin-eosin to perform light microscopic examination.

Immunohistochemical study

The paraffin blocks were cut in 5 μm thickness onto poly-L-lysin coated slides and the slides were immunostained with anti-Ki-67 monoclonal antibody (1:100, DAKO Corp, Copenhagen, Denmark) using the avidin-biotin-peroxidase technique. Briefly the sections were first dewaxed in xylene and rehydrated in distilled water. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide solution. After antigen retrieval by heating in 10 mmol/l citrate buffer (pH 6.0) primary antibody was applied for 30 minutes at room temperature, and rinsed in phosphate-buffered saline (PBS) (0.01 mol/l; pH 7.5), the biotinylated secondary antibody and streptavidinperoxidase complex (DAKO Corp, Copenhagen, Denmark) were applied consecutively for ten minutes and rinsed in PBS. The peroxidase activity was visualized with 0.03% 3-3'-diaminobenzidine tetrahydrochloride (DAB) solution (Sigma Chemical, St. Louis, USA) for five minutes. After rinsing in deionised water for five minutes the slides were counterstained with hematoxylin and thereafter dehydrated and mounted.

Immunohistochemical evaluation

The selection of the area for the analysis was based on the availability of sufficient tumor tissue for immunohistochemical evaluation. Counting immunopositive nuclei (brown stained) was performed among 100 tumor cells in at least five representative high power fields (x 400 magnification) across the slide. The Ki-67 index was estimated by the percentage of Ki-67 positive cancer cells among all the counted tumor cells.

Statistical analysis

The SPSS 8.0 computer program was used for statistical analysis, p-values of 0.05 or less were considered statistically significant. All data were evaluated by the χ^2-test, paired samples t-test and Spearman correlation test. Correlations from 0 to 0.25 indicated little or no relationship; those from 0.25 to 0.50 indicated a fair degree of relationship; those from 0.50 to 0.75 a moderate to good relationship and those greater than 0.75 a very good to excellent relationship. A negative value indicated an inverse correlation. If the expected frequencies were small, Fisher's exact test was used instead of the standard χ^2-test.

Results

Patient characteristics

Median age of the patients was 49 (range: 38-77). Four patients (17.4%) had Stage IIA, 16 (69.6%) had Stage IIB, one (4.3%) had Stage IIIA and two (8.7%) had Stage IIIB disease according to the FIGO classification. Thirteen patients (56.5%) had large cell nonkeratinizing type, six (26.1%) had large cell keratinizing type squamous cell carcinoma and subtype could not be specified in four patients. Patient characteristics are indicated in Table 1.

Ki-67 index prior to RT and after 9 Gy

The mean Ki-67 index for the whole group prior to RT and after 9 Gy were 58.5% (range: 21%-88%) and 46.0% (range: 17%-78%), respectively. Ki-67 index decreased after 9 Gy in 74% of the patients and increased in 26% (Table 2).

Morphological assessment

After 9 Gy, giant cells, multinucleate cells, bizarre cells with swollen cytoplasm, karyorrhexis, nuclear enlargement, increased eosinophilia and prominent nuclei were observed in hematoxylin-eosin-stained sections. Hematoxylin-eosin staining patterns prior to RT and after 9 Gy were demonstrated in Figures 1a and 1b, respectively. Various morphologic changes were also observed in the immunostained sections. Staining pattern was localized to the nucleus prior to RT. This uniform pattern disappeared after 9 Gy and a granular, irregular staining pattern was observed. Figures 1c and 1d show the immunostaining patterns prior to RT and after 9 Gy, respectively.

Table 1. — *Patient characteristics.*

	No. of patients	%
Age		
Median 49 (range: 38-77)		
Histopathology		
Squamous cell carcinoma*	4	17.4
Large cell keratinized	6	26.1
Large cell nonkeratinized	13	56.5
FIGO stage		
IIA	4	17.4
IIB	16	69.6
IIIA	1	4.3
IIIB	2	8.7
Brachytherapy		
Yes	17	73.9
No	6	26.1
Chemotherapy		
Yes	15	65.2
No	8	34.8
Ki-67 index prior to RT		
Mean 58.5% (range: 21%-88%)		
Ki-67 index after 9 Gy		
Mean 46% (range: 17%-78%)		

*Subtype could not be defined.

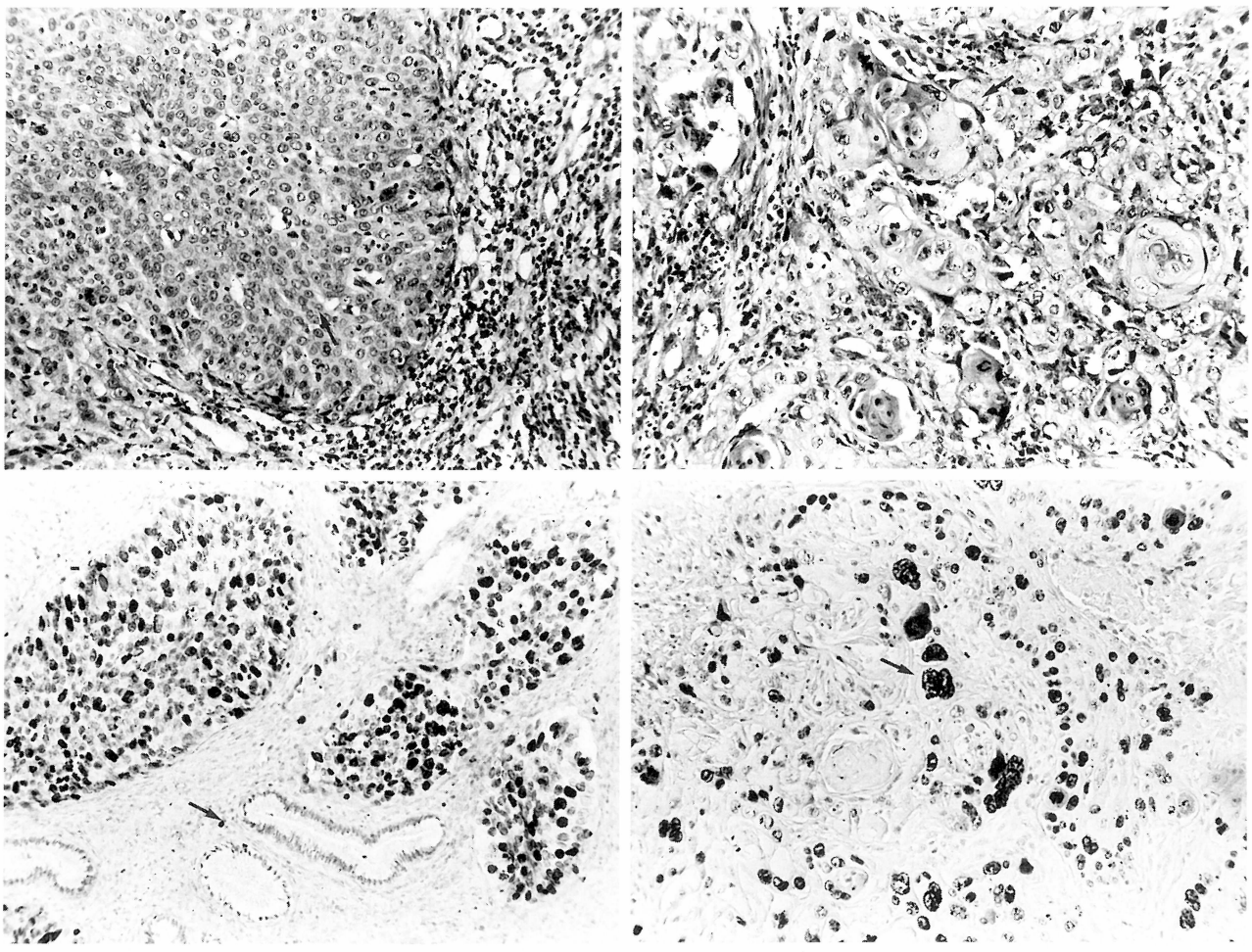

Figure 1. — **A.** Large cell non-keratinizing squamous cell carcinoma of the cervix (arrow) before RT (hematoxylen-eosin staining, x 100). **B.** Changes in the morphologic appearance of cancer cells after 9 Gy (arrow); note the multinucleation, bizarre appearance, prominent nucleoli, dyskeratosis and karyorrhexis (hematoxylen-eosin staining, x 100). **C.** Nuclear Ki-67 immunostaining for squamous cell carcinoma of the cervix before RT; strong Ki-67 immunopositivity of invasive tumor cells forming solid islands neighbouring benign endocervical glands (arrow) (immunoperoxidase staining, x 100). **D** Ki-67 immunostaining after 9 Gy. Note the granular staining in the bizarre cells (arrow) (immunoperoxidase staining, x 100).

Local recurrence and distant metastasis

After a median follow-up duration of 23 months local recurrence was detected in four patients (17.4%). No significant correlation was detected between local recurrence and the accepted prognostic factors such as age, initial hemoglobin level, stage, tumor size, administration of chemotherapy, initial Ki-67 index and changes in Ki-67 index after 9 Gy (Table 3).

Four patients (17.4%) developed distant metastasis and two of them died during the 4th and 12th months of follow-up. No significant correlation was detected between distant metastases and age, initial Hgb level, stage, tumor size, administration of chemotherapy and initial Ki-67 index whereas a moderate correlation was detected between distant metastasis and patients whose Ki-67 index increased after 9 Gy ($r = 0.51$, $p = 0.01$) (Table 4).

Discussion

Morphological features have been widely used in the diagnosis and prediction of biological behavior and prognosis of cancer. These parameters also assist in treatment selection. Besides morphological data, proliferative activity of neoplastic cells is of great importance in predicting prognosis. There are many methods which determine the cell kinetic characteristics of the tumor. A monoclonal antibody against Ki-67 protein, first defined by Gerdes *et al.*, demonstrates proliferating cells in G_1, S, G_2, and M phases of the cell cycle [6]. The antibody stains Ki-67 protein expressed by all proliferating cells during the G_1, S, M and G_2 phases of the cell cycle, while cells in G_0 and early G_1 phase lack this antigen expression. Therefore it provides a convenient guide to tumor growth fraction and has been applied to study the growth fraction and cytokinetic activities in various cancers.

Table 2. — *Ki-67 index before radiotherapy and after 9 Gy, histopathology and tumor response.*

				Ki-67 index (%)			
Patient no.	Age	Stage	Treatment	0 Gy	9 Gy	Histopathology	Response
1	45	IIB	CRT	88	36	NOS	CR
2	46	IIB	RT	64	30	K	PR
3	66	IIA	CRT	67	53	K	CR
4	59	IIA	CRT	46	20	K	PR
5	43	IIA	CRT	68	73	NOS	PR
6	40	IIB	RT	21	71	NK	PR
7	43	IIB	CRT	83	45	NK	CR
8	55	IIA	CRT	65	25	NOS	CR
9	66	IIIB	CRT	50	30	NK	PR
10	38	IIB	RT	87	65	NK	PR
11	63	IIB	RT	76	57	NK	CR
12	46	IIB	CRT	73	37	NK	CR
13	77	IIB	RT	65	50	NK	CR
14	49	IIB	CRT	76	40	NK	CR
15	44	IIB	CRT	43	78	NK	PR
16	46	IIB	CRT	50	26	NK	CR
17	62	IIIB	CRT	38	73	NK	PR
18	63	IIB	RT	48	37	K	CR
19	60	IIB	CRT	40	17	NK	CR
20	40	IIB	RT	21	60	K	PR
21	51	IIIA	RT	53	68	K	PR
22	39	IIB	CRT	40	30	K	CR
23	50	IIB	CRT	85	38	NOS	CR

NOS: Not otherwise specified. K: Large cell keratinizing. NK: Large cell nonkeratinizing. CR: Complete response. PR: Partial response. CRT: Chemoradiotherapy. RT: Radiotherapy alone.

Table 3. — *The correlation between local recurrence and accepted prognostic factors.*

Prognostic factors	r value	p value
Age		
> 50 vs ≤ 50	0.17	0.43
Hemoglobin level		
> 12.5 g/dl vs ≤ 12.5 g/dl	0.09	0.66
Stage		
II vs III	0.04	0.84
Tumor size		
> 5 cm vs ≤ 5 cm	0.09	0.66
Chemotherapy		
(+) vs (–)	0.21	0.33
Initial Ki-67 index	0.10	0.64
Changes in Ki-67 index after 9 Gy		
Increase vs decrease	0.27	0.20

However, little is known about the correlation between the growth fraction and radiation response or prognosis after RT. A wide variation exists in response of tumors to RT. Nine or 10 Gy is recommended as a critical dose in squamous cell carcinomas to evaluate the effect of radiotherapy because this dose achieves initial abatement of the neoplastic mass without impairing cell morphology and antigenic characteristics. RT appeared to cause liquefaction necrosis in cancer at doses of 18 Gy or more [7, 8]. The same findings were also described using the labeling index technique [9].

Table 4. — *The correlation between distant metastasis and accepted prognostic factors.*

Prognostic factors	r value	p value
Age		
> 50 vs ≤ 50	0.17	0.43
Hemoglobin level		
> 12.5 g/dl vs ≤ 12.5 g/dl	0.38	0.06
Stage		
II vs III	0.22	0.30
Tumor size		
> 5 cm vs ≤ 5 cm	0.33	0.18
Chemotherapy		
(+) vs (–)	0.14	0.50
Initial Ki-67 index	0.13	0.54
Changes in Ki-67 index after 9 Gy		
Increase vs decrease	0.51	0.01

Cervical tumors show areas of heterogenity of Ki-67 labelling. This finding reflects the population heterogeneity and intraclonal heterogeneity of a tumor [10]. In the present study diffuse nuclear-stainings and dot-stainings were observed prior to RT, whereas after 9 Gy Ki-67 positive cancer cells mainly showed dot-staining, ring-staining, large, irregular spot-staining; diffuse nuclear-staining disappeared. Histopathologic evaluation with hematoxylin-eosin staining after 9 Gy showed multinucleated cancer cells, bizzare cells with swollen or clear cytoplasms mixed with intact cancer cells. These findings are consistent with the literature [7, 8, 11, 12]. All these morphometric changes may be explained by the physical effects of radiotherapy on the cells.

In this study, the mean Ki-67 index prior to RT was 58.5% (range: 21%-88%). This index was 41% (range: 20%-86%) in Nakano *et al's* study [7]; 67% (range: 21%-99%) in Avall-Lundqvist *et al's* study [13]; 42% (range: 26%-66%) in Oka *et al's* study [12] and 57.1% (range: 23.2%-90.1%) in Ming *et al's* study [3] about cervical cancer.

The percentage of the Ki-67 index is highly variable during RT. Some of the studies reported a decrease in Ki-67 index while some reported an increase. Nakano studied the changes in the Ki-67 index during the radiotherapy of 29 cases of cervical carcinoma and found that the mean Ki-67 index which was 41% prior to radiotherapy significantly increased reaching a peak value of 68% at doses of 9 Gy and then decreased to 39% and 20% at doses of 18 Gy and 27 Gy, respectively [7]. Oka *et al.* [12] also reported that the Ki-67 index increased reaching a peak value at 9 Gy and then decreased at doses of 18 Gy and 27 Gy thus assuming that this transient increase could have been attributable to the combined effects of the recruitment phenomenon and G_1 or G_2 block [12]. In a study of oral squamous cell carcinoma, after 10 Gy radiotherapy the Ki-67 index decreased in 29 of 31 cases, whereas an increase was observed in two cases [8]. Abatement of the growth fraction after 10 Gy of radiotherapy was significantly correlated with complete response ($p < 0.01$) providing a predictive factor of

the outcome of the treatment [8]. In another study, the changes in the Ki-67 index during radiotherapy of various tumors was investigated [14]. In four patients with primary breast carcinoma Ki-67 indices decreased after 2 Gy but increased after 26 Gy; in one patient there was an increase after 2 Gy followed by a decrease after 26 Gy and in another patient there was a decrease in 6 Gy. In one patient with poorly differentiated bronchial squamous cell carcinoma and one patient with well differentiated adenocarcinoma the Ki-67 index increased after 2 Gy. In our study the Ki-67 index decreased after 9 Gy in 17 patients whereas it increased in six patients. Controversial results were achieved from studies investigating the prognostic significance of Ki-67 in cervical carcinoma [3, 7, 10-13, 15-18]. In most of them there was no significant correlation between the Ki-67 index and survival and the accepted prognostic parameters such as FIGO stage, histologic type and grade, lymphovascular space invasion and pelvic lymph-node metastases [3, 13, 16, 17, 19]. In a study by Cole *et al.* no correlation between growth fraction and age, clinical stage, lymph-node involvement or short-term survival was determined among 28 cases of cervical carcinoma [17]. Oka *et al.* [18] reported that tumors with a high growth fraction had a good prognosis in 45 patients with locally advanced cervical cancer treated by RT, and the 3-year survival rate was significantly better in patients with a high Ki-67 index and this was attributed to the fact that the higher the tumor proliferative rate the greater the sensitivity to radiotherapy [18]. In their subsequent study, Nakano *et al.* [20] reported longer survival in patients with a high Ki-67 index. However Garzetti *et al.* [19] suggested that the Ki-67 index was particularly high in the presence of lymphatic spread and in patients who relapsed and tumors with a more rapid growth rate would behave more aggressively than those growing less rapidly.

In the present study no significant correlation was detected between local recurrence and the initial Ki-67 index and changes in the Ki-67 index after 9 Gy. There was no significant correlation between the initial Ki-67 index and distant metastasis, whereas a moderate correlation was detected between distant metastasis and patients whose Ki-67 index increased after 9 Gy ($r = 0.51$, $p = 0.01$). If we consider that there are many factors affecting response to radiotherapy, it seems advantageous to evaluate the early changes in cell biology to predict tumor response and to manage the treatment. Early changes observed in the proliferation index may predict the sensitivity of the tumor to that treatment.

Response to radiotherapy may not be limited to neoplastic tissues only. In cell cultures it has been shown that radiotherapy is more effective in some phases of the cell cycle such as before G_2-M while its effect is lower during the late S phase. Thus, in a tumor if most of the cells are in the S phase, the tumor will proliferate more rapidly but also be more radioresistant. Therefore it would be difficult to predict the responsiveness to RT using the S-phase determination. Resistant clones with higher proliferative activity may dominate or the quiescent cells may re-enter the cell cycle. This can explain the increase in the Ki-67 index during the treatment and the treatment failure.

Although, in the present study, there were patients who received radiochemotherapy (15 patients), no difference was observed between two groups regarding local control or distant metastases; however the number of patients must be increased in order to conclude that the results were independent from the type of treatment. A correlation between the Ki-67 index and survival has not been attempted because of insufficient follow-up time.

The ultimate goal of studying the Ki-67 index is to identify different prognostic groups thus to alter the treatment plan in order to determine the treatment outcome. Prediction of the outcome of RT would eventually facilitate the early choice of an adequate treatment. The Ki-67 index may serve as an effective parameter for potential proliferative activity of a tumor and it may contribute to the individualization of a treatment. Although the Ki-67 protein is extensively used as a proliferation marker, its functional significance still remains unclear and other markers are needed to determine the proliferation rate of a tumor. Prospective studies including a large number of patients, possibly multicentric, with long-term follow-up are necessary to confirm the clinical utility of Ki-67 in cervical cancer.

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