ORIGINAL PAPER

Tumor biomarkers in cervical cancer: focus on Ki-67 proliferation factor and E-cadherin expression

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Abstract

Despite recent advances in the immune mechanisms of cervical cancer (CC) and complex management opportunities, relapse remains still an actual issue. While predictive factors are required, current research is directed towards proliferation and tumor aggressiveness biomarkers as potential negative factors in CC. The main objectives were to assess tumor proliferation and invasiveness biomarkers (Ki-67, E-cadherin) and to identify potential correlation between biomarkers and classic prognostic factors in CC. Radical hysterectomy specimens from 61 consecutive CC were immunohistochemically investigated for Ki-67 and E-cadherin. Nuclear immunostaining for Ki-67 proliferation index was assigned scores 1 to 3, "+" meaning low (10–30%), "++" moderate (30–50%), "+++" high-proliferation rate (>50%); cell membrane E-cadherin staining was either negative or positive. Statistical analysis was performed in SPSS–13 software, $p<0.05$. Results: no significant correlation between Ki-67 and classical prognostic factors ($p>0.05$) was reported; however, in relapsed CC, Ki-67 correlates with tumor grading ($r=0.386$, $p<0.05$). Significant correlation between E-cadherin and tumor size ($r=-0.280$, $p=0.029$), relapse ($r=-0.386$, $p=0.002$) and disease free survival ($r=0.374$, $p=0.003$) were demonstrated. Indirect statistically significant moderate correlation between Ki-67 and E-cadherin ($r=-0.461$, $p<0.00001$) was shown, mainly in invasive squamous CC ($r=-0.549$, $p=0.0001$), stage IB ($r=-0.578$, $p=0.009$), IIB ($r=-0.585$, $p=0.003$), relapsed CC ($r=-0.525$, $p<0.01$), HPV-infection ($r=-0.504$, $p=0.033$). Conclusions: CC aggressiveness, particularly in invasive squamous carcinoma, either 16 or 18 HPV-positive cases, FIGO stage IB and IIB, and cases with relapse, depends on two pivotal factors, tumor proliferation rate (Ki-67) and tumor invasiveness (E-cadherin).

***Keywords*:** cervical cancer, biomarkers, Ki-67, E-cadherin.

Introduction

Despite recent advances in the immunopathogenesis of cervical cancer (CC) and complex management opportunities that rely on both modern (anti-*Human Papilloma Virus* vaccine for prevention of the disease) and classic (surgery, adjuvant radio- and/or chemoterapy) aspects, relapse of the disease still remains an actual issue. Several well-known prognostic factors including tumor size (>4 cm), depth of invasion (>1 cm), spread to lymph nodes, capillary lymphatic space tumor invasion, parametrial invasion, positive resection margins, histological type and grading have already been described [1, 2]. However, new predictive biomarkers are needed to identify patients with high risk of relapse and to optimize disease management, especially in early CC. Thus, one major field of research was directed towards tumor proliferation and tumor invasion biomarkers as potential negative prognostic factors [2].

It is well recognized that molecular basis of tumor aggressiveness is related to (i) *uncontrolled tumor proliferation activity,* that can be assess by several cellular proteins including PCNA (proliferating cell nuclear antigen), and Ki-67 antigen; (ii) *adhesion, migration and tumor cell invasiveness*, related especially to E-cadherin adhesion molecule; (iii) tumor (neo) angiogenesis, which can be immunohistochemically evaluated by CD34 expression [2–4]. Thus, (neo) angiogenesis (pro-angiogenic factors released by tumor) – tumor proliferation activity (promoted by newly formed vessels) – invasiveness, result in a complex vicious circle [2].

Ki-67, a proliferation marker known as predictive factor for tumor development, is defined as a nuclear antigen (associated with hetero- and euchromatin) expressed during all active phases of the cell cycle (G1, S, G2, M) except G0; the level of Ki-67 expression is used to determine the cell proliferation status [5–7].

It can be detected through several qualitative and quantitative methods including monoclonal antibody in immunohistochemical assays, electron microscopy, ELISA, flow cytometry, immunocytochemistry [6].

Since Ki-67 is present only in dividing cells, either normal or tumor, but absent in resting cells, only cells that over-express p53 or p21 may be assessed by using Ki-67 [5, 6]. In normal cervical squamous mucosa, Ki-67 is detected essentially in parabasal epithelial

layers (the main source for cell renewal), but also in certain basal layers [6, 8–10].

E-cadherin adhesion molecule is essentially involved in normal tissue morphogenesis, being responsible not only for cellular interconnection, but also for segregation of cell types, differentiation, signaling, cell motility and proliferation, especially in epithelia [3, 4, 11]. Several recent studies have already focused on changes in intercellular adhesion in different tumors, revealing the pivotal role of E-cadherin during tumor progression and invasion; loss of E-cadherin expression is particularly related to changes in cell phenotype and vital function, increased cell motility and invasiveness [3, 4].

Although a panel of biomarkers (including Ki-67) with predictive value for the progression of cervical dysplasia have been described [5, 6, 8–10], their significance in cervical cancer assessment remains controversial [6, 12].

As new prognostic biomarkers are required and classic factors for tumor aggressiveness may be managed as predictive parameters in CC, we have conceived the current study aiming (i) to assess tumor proliferation and invasiveness biomarkers (Ki-67, E-cadherin), (ii) to identify potential correlation between tumor biomarkers and different classic prognostic factors, and (iii) to demonstrate the importance of such new designed biomarkers as modern prognostic factors in cervical cancer patients.

Material and Methods

Our retrospective observational study was performed on 61 consecutive patients surgically treated for cervical cancer (different histopathological types and FIGO stages), with a mean age of 36.4±5.53 years, who have attended "Cuza-Vodă" Hospital, Iassy, between 2000 and 2003. Data regarding relapse and disease free survival of patients were retrieved from regional oncology files. Paraffin-embedded cervical tissues were processed at the time of diagnosis at the Pathology Department in "Cuza-Vodă" Hospital; the classical and immunohistochemical (IHC) exams were performed at the Immunopathology and Genetics Laboratory of "Sf. Spiridon" Hospital, Iassy.

Ethical Committee approval has been obtained.

The immunohistochemical investigation was performed using Ki-67 and E-cadherin antibodies (DAKO) and streptavidin–biotin method (LSAB Kit, DAKO) [13].

The sections stained for Ki-67 proliferation index (revealed as nuclear staining) were evaluated using scores from 1 to 3: "+" meaning *low-proliferation*, 10–30% positive cells; "++" *moderate proliferation*, 30–50% positive cells; "+++" meaning *high-proliferation* (more than 50% positive cells).

For E-cadherin staining (assessed as brown color of the cell membrane), the following evaluation system was applied: negative reaction defining loss of E-cadherin expression (equivalent with loss of intercellular adhesion and increased tumor invasiveness) and positive non-homogenous reaction defining the presence of E-cadherin expression.

Statistical analysis (descriptive, analytic non-parametric tests) was performed in SPSS–13 software, $p<0.05$.

Results

Baseline assessment of patients with CC enrolled in the current study aimed to establish association with classical negative prognostic factors including: tumor size and grading, histological type, clinical stage FIGO and lymph node invasion, as shown in Table 1.

Table 1 – ***Patient distribution based on classical prognostic factors***

Variable	No. of cases (%)
Tumor size	
<4 cm	31 (50.8)
≥4 cm	30 (49.2)
Tumor grading	
G1 – well-differentiated	15 (24.6)
G2 – moderate differentiated	30 (49.2)
G3 – undifferentiated	6 (9.8)
Gx – cannot assess	10 (16.4)
Histological type	
Invasive squamous cervical carcinoma	42 (68.9)
CC *in situ*	10 (16.4)
Adenocarcinoma	4 (6.6)
Adenosquamous cervical carcinoma	3 (4.9)
Microinvasive carcinoma	2 (3.3)
FIGO classification	
IA	12 (19.7)
IB	19 (31.1)
IIA	3 (4.9)
IIB	24 (39.3)
III	1 (1.6)
IV	2 (3.3)
Lymph node invasion	
present	19 (31.1)
absent	42 (68.9)

Ki-67 assessment and correlation with classical prognostic factors

42.6% (26 cases) displayed mean proliferation values as defined by Ki-67 (Figure 1) and 31.1% (19 cases) high-Ki-67 proliferation (Figure 2), while only 26.2% (16 cases) were defined by low-tumor proliferation (Figure 3). No statistical significant difference between groups defined by Ki-67 expression was demonstrated ($\chi^2=2.590$, $p=0.274$). We have demonstrated a direct statistically significant moderate correlation between Ki-67 expression and age of diagnosis ($r=0.341$, $p=0.007$), suggesting a biological tumor aggressiveness of age-related CC.

We have also investigated Ki-67 distribution among different sub-groups. 63.2% of cases defined as having high-Ki-67 proliferation rate and 61.5% cases with moderate proliferation rate featured relapse; however, relapse was reported in 37.5% of patients with low-tumor proliferation. More over, from total relapsed CC, 17.6 were defined by low Ki-67 tumor proliferation rate. 53.8% of CC that have not survived was distributed in mean Ki-67 proliferation group and 42.1% in high-proliferation group.

There is no significant correlation between Ki-67 and relapse ($p>0.05$), Ki-67 and disease free survival ($p>0.05$); therefore Ki-67 could not be considered as a negative independent prognostic factor.

No correlation between Ki-67 and classical prognostic factors for cervical cancer including tumor size, grading, histological type, lymph node invasion, have been reported.

Only patients with relapse (34 cases, 55.7%) were characterized by a direct statistical significant moderate correlation between *Ki-67 and tumor grading* (r=0.386, p<0.05), suggesting that higher tumor proliferation rate is associated with loss of tumor differentiation.

E-cadherin assessment and correlation with classical prognostic factors

Loss of E-cadherin expression was reported in 47.5% cases; no statistical significant difference between groups regarding E-cadherin expression was defined (χ^2=0.148, p=0.701).

Figures 4–7 show IHC assessment of E-cadherin expression in different histological types of CC.

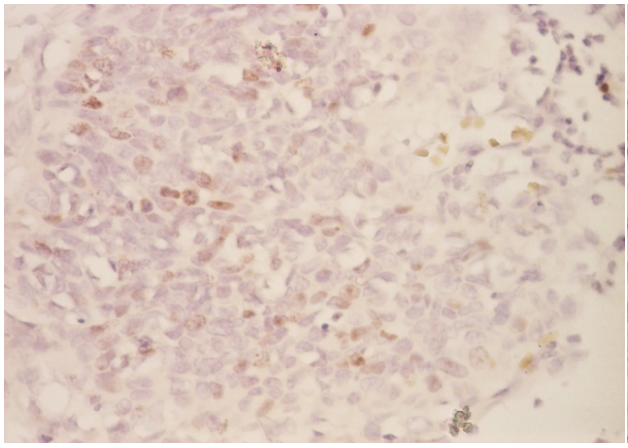

Figure 1 – ***Invasive squamous cervical carcinoma: Ki-67-positive reaction, moderate proliferation rate (40%) (IHC, ob. 20×).***

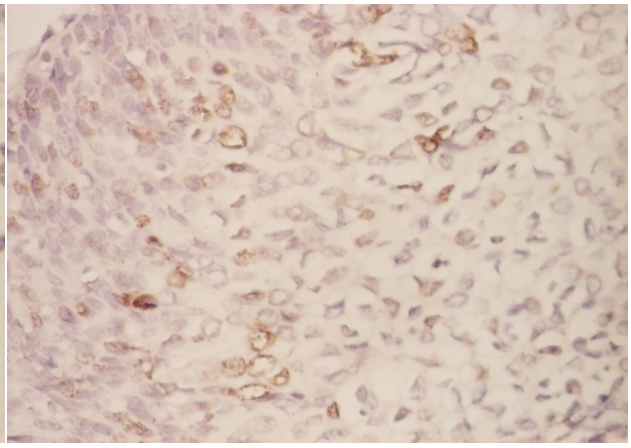

Figure 2 – ***Invasive squamous cervical carcinoma: Ki-67-positive reaction, high-proliferation rate (60%) (IHC, ob. 20×).***

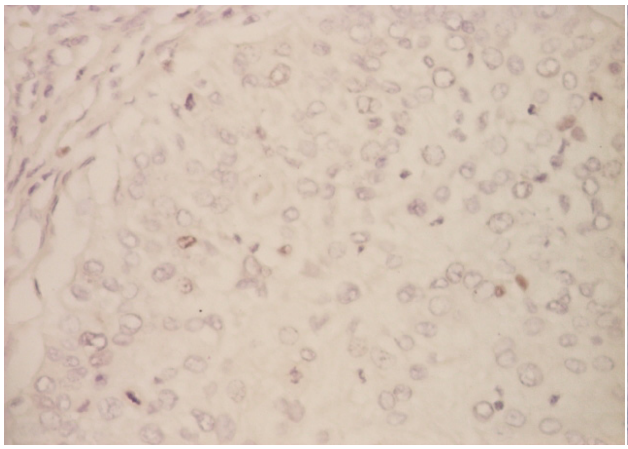

Figure 3 – ***Invasive squamous cervical carcinoma: Ki-67-positive reaction, low-proliferation rate (20%) (IHC, ob. 20×).***

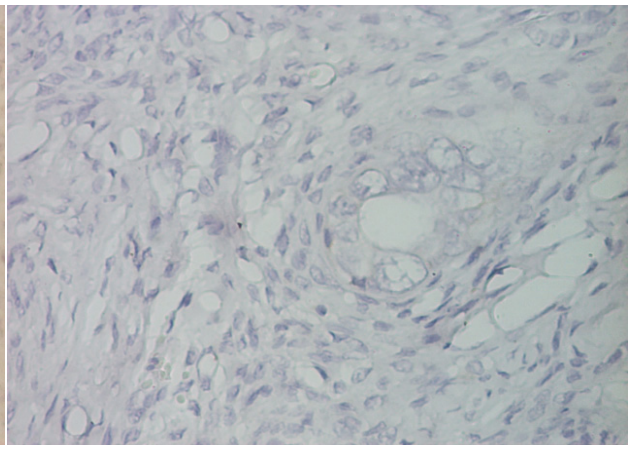

Figure 4 – ***Loss of E-cadherin expression (IHC, ob. 20×).***

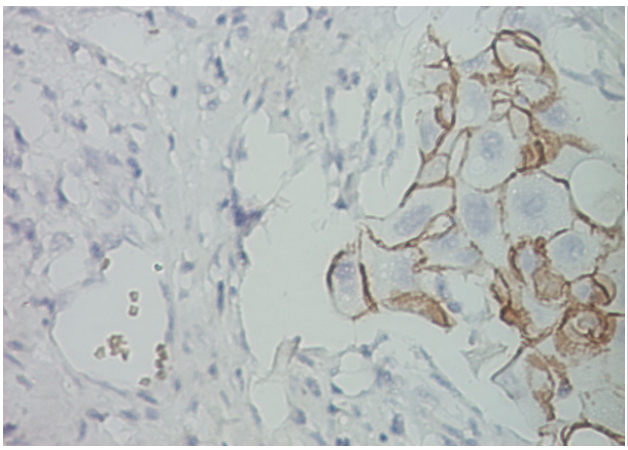

Figure 5 – ***Invasive squamous cervical carcinoma: E-cadherin-positive reaction (IHC, ob. 20×).***

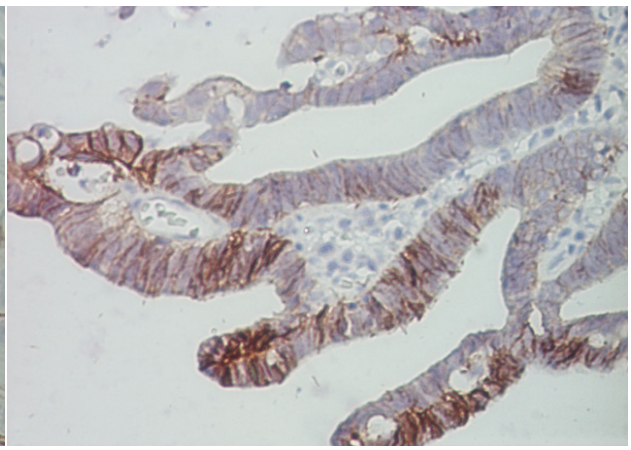

Figure 6 – ***Adenocarcinoma: E-cadherin-positive reaction (IHC, ob. 20×).***

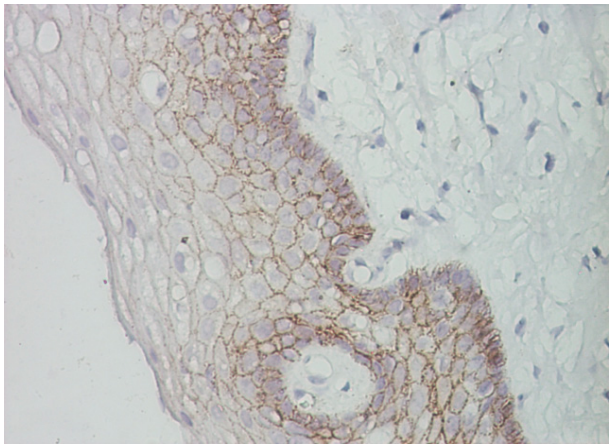

Figure 7 – *In situ cervical carcinoma: E-cadherin-positive reaction (IHC, ob. 20×).*

An indirect statistically significant mean correlation has been reported between E-cadherin and patient's age (r=-0.302, p=0.018), supporting the relationship between advanced biological age and tumor aggressiveness.

Statistical analysis has demonstrated that 75% CC characterized by loss of E-cadherin expression have relapsed in maximum five years from the diagnosis and only 37.5% of those with different patterns of positive E-cadherin staining. Besides, from all relapsed patients the majority (67.7%) presented loss of E-cadherin expression. 62.1% of patients with loss of E-cadherin died, as compared with 25% of those that maintained E-cadherin expression.

Consequently, we have identified two correlation, (i) *E-cadherin and relapse* – an indirect statistically significant moderate correlation (r=-0.386, p=0.002), and (ii) *E-cadherin and disease free survival* – a direct statistically significant moderate correlation (r=0.374, p=0.003). No significant correlation between *E-cadherin* expression and other classical prognostic factors have been reported, except with *tumor size*: indirect, statistically significant weak correlation (r=-0.280, p=0.029). As with Ki-67 in subgroup analysis, loss of *E-cadherin* expression is directly related to low-*tumor differentiation* (r=-0.457, p=0,003).

Ki-67–E-cadherin correlation

We have demonstrated an indirect statistically significant moderate correlation between the two tumor biomarkers as assessed by IHC (r=-0.461, p<0.00001).

Detailed sub-group analysis has demonstrated that statistical significant indirect correlation between *Ki-67 proliferation index and E-cadherin* is maintained among: (i) women with invasive squamous cervical carcinoma (42 cases) (r=-0.549, p=0.0001), (ii) clinical stage IB (19 cases) (r=-0.578, p=0.009) and IIB (24 cases) (r=-0.585, p=0.003), and (iii) CC with relapse (34 cases) (r=-0.525, p<0.01).

In 16 and/or 18 type HPV-positive women (18 cases) (as demonstrated by *in situ* hybridization technique), we have shown an indirect statistically significant moderate correlation between *Ki-67 expression and E-cadherin* (r=-0.504, p=0.033); tumor invasiveness correlates with proliferation rate among patients with demonstrated HPV-infection.

Discussion

Carcinogenesis is generally based on uncontrolled cell proliferation, assessed by Ki-67 biomarker, and abnormal invasiveness and cell motility, evaluated by E-cadherin expression [3–5].

Recent advances in the immunopathogenesis of cancer, especially in cervical cancer, have been related to IHC evaluation of two important tumor biomarkers, (i) Ki-67 antigen, a human nuclear protein expressed through the entire cell cycle except G0 phase, which is widely used to assess proliferation status, and (ii) E-cadherin, an intercellular adhesion molecule, usually affected during tumor progression and invasion [5, 8–10, 14–20].

Until now, the majority of studies were mainly directed towards tumor biomarker involvement in cervical dysplasia; few researchers were interested in demonstrating prognostic implications of such biomarkers in CC.

Ki-67 proliferation marker is already recognized and validated as specific and sensitive biomarker in cervical intraepithelial neoplasia [9, 10, 14, 15, 17]. It is well demonstrated that Ki-67 expression is increased in upper layers of cervical epithelia, being of major significance for the differentiation of non-neoplastic lesions that can mimic cancer [7, 15, 16]. Moreover, Ki-67 protein could be a biomarker of the proliferative activity and progressive potential of normal, dysplastic and neoplastic cervical changes, with certain therapeutic implications [4, 7]. Several studies have also suggested that Ki-67 may be a sensitive biological indicator of progression independent of age and menopausal status [10, 12]; Ki-67 can be used as an independent prognostic factor for the progression and biological behavior of cervical dysplasia, especially when HPV-infection assessment is missing [10].

A relationship between increased proliferative activity and cervical dysplasia relapse has already been proposed [20].

Despite advances in understanding role of Ki-67 in assessing cervical dysplastic lesion, the prognostic value in CC is still controversial. Although several authors have not demonstrated any relation concerning Ki-67 and prognosis in CC, others have suggested the importance of Ki-67 for the evaluation of cell kinetics in response to radiation therapy [6–8]. Thus, the tumor growth fraction as estimated by Ki-67 is considered of value for radiation response and prognosis after radiation therapy [6, 8]; also, early changes in Ki-67 expression during radiotherapy may characterize subsequent metastasis invasion [8].

As supported by Garzetti GG *et al.*, in 1995, Ki-67 proliferation index is significantly related to several parameters including tumor size, lymph node invasion and disease free survival in women with stage IB of CC. Furthermore, a statistical significant difference regarding Ki-67 level among young and elder population, signifying a biologic aggressiveness of age-related cervical carcinoma [10, 12].

Ki-67 is associated with invasive cervix carcinoma, mainly with squamous keratinizing histological type

[24], while increased biomarker expression is related to lymphatic invasion [2].

Recent studies related on tumor invasiveness aimed to demonstrate changes in both expression and sub-cellular localization, not only in E-cadherin but also in α- and β-catenins, during progression of cervical dysplasia; moreover, P-cadherin expression becomes predominant and can be used to discriminate between malignant and benign cervical lesions [3, 4, 11, 21–23]. Quantitative and qualitative changes of the components of the intercellular junction have been extensively investigated in normal, metaplastic and pre-malignant cervical epithelial lesions [3, 11, 21–23].

The current study was designed in order to investigate tumor proliferative activity (Ki-67) and invasiveness (E-cadherin) as main determinants of cancer aggressiveness, biomarker distribution among histological CC type, FIGO classification, disease free survival and relapse; we were interested in demonstrating potential correlations concerning tumor biomarkers and classic prognostic factors.

Statistical analysis underwent has revealed certain statistical significant correlations, supporting either already known data from literature, either new information. Thus, there have been demonstrated a significant relation between two biomarkers known to define tumor proliferation (Ki-67) and invasiveness (E-cadherin), critical elements for cervical cancer aggressiveness. Higher is the tumor-cell proliferation rate, more suppressed is E-cadherin expression, meaning increased cell invasiveness and motility. Furthermore, this negative statistically significant correlation regarding Ki-67 and E-cadherin is maintained in sub-groups analysis: (i) invasive squamous carcinoma, (ii) FIGO IB and IIB stage, (iii) relapse, and (iv) HPV-positive group. Also, the complex relationship between Ki-67 and age support information obtained from Garzetti GG *et al.*, related to increased tumor aggressiveness with biological age in CC, while connection between E-cadherin expression and age enhance the well-known tumor link tumor aggressiveness–age at diagnosis in CC [10, 12].

No correlation between tumor markers and classic negative prognostic factors including lymph node metastasis, cancer histological type, clinical stage, has been shown in this study, apart sub-group analysis.

Conclusions

Cervical cancer aggressiveness, particularly in invasive squamous carcinoma, either 16 or 18 HPV-positive cases, FIGO stage IB and IIB, and cases with relapse, depends on two pivotal factors, tumor proliferation rate (Ki-67 biomarker), and tumor invasiveness (E-cadherin biomarker).

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