in vivo *36*: 1150-1154 (2022)
doi:10.21873/invivo.12814

Loss of E-Cadherin Expression Correlates With Ki-67 in Head and Neck Squamous Cell Carcinoma

CRISTINA STEFANIA DUMITRU, AMALIA RALUCA CEAUSU, SERBAN COMSA and MARIUS RAICA

Department of Microscopic Morphology/Histology, Angiogenesis Research Center, "Victor Babes" University of Medicine and Pharmacy, Timisoara, Romania

Abstract. *Background/Aim: The aim of the study was to evaluate the correlation between the rate of proliferation and immunohistochemical expression of E-cadherin, and their predictive role in patients with head and neck squamous cell carcinoma (HNSCC). Materials and Methods: Samples were collected from 50 patients with HNSCC, and the expression of Ki-67 and E-cadherin was evaluated by immunohistochemistry (IHC). Previously, samples were conventionally stained with haematoxylin and eosin for histological diagnosis and grade. Results: High E-cadherin expression was predominantly associated with less differentiated tumours (p<0.5; p=0.0305). Also, we observed a significant correlation between Ki-67 expression in tumour cells and tumour grade (p=0.0245). A strong correlation was noticed between low E-cadherin expression, increased Ki-67-proliferation rate and advanced T2-T3 tumour stage (p=0.0242). Conclusion: In this study we showed that Ki-67 proliferation rate and E-cadherin expression are important features in patients with HNSCC. Therefore, higher Ki-67 index values correlate with loss of E-cadherin expression, which indicates a poorer prognosis. These aspects support the use of both Ki-67 and E-cadherin as prognostic markers in specimens from patients with HNSCC.*

Head and neck squamous cell carcinoma (HNSCC) represents the sixth most common cancer in the world (3% of all cancers). The incidence of HNSCC continues to rise and it is anticipated to increase by 30% (1.08 million new cases annually) by 2030, according to Global Cancer Observatory (1). Even though significant improvements have been made in the field of prevention, early diagnosis, image diagnosis, and treatment strategies for HNSCC, the survival rates in patients with progressive or metastatic disease remain poor over the last 5 years (2, 3). Prognostic markers remain the real basis for early detection and accurate survival evaluation for HNSCC patients. During the last years, based on the expression of some common markers, many authors tried to perform a molecular classification of HNSCC. Besides cytokeratin, growth factors and other molecules, like E-cadherin and Ki-67, seem to be useful in prognosis of individual patients (4).

As was already demonstrated, intercellular adhesion is primarily maintained by adhesion molecules, specifically E-cadherin (ECAD), a membrane protein encoded by a tumour suppressor gene. Numerous studies suggest that the ECAD gene can predict the prognosis of squamous cell carcinoma. Therefore, low expression of the ECAD gene is encountered in the loss of epithelial cell adhesion, which has been correlated with phenotypic changes in increased invasiveness and motility of cancer cells, associated with cancer invasion and poor prognosis in HNSCC (5).

Many studies have shown that the Ki-67 proliferation index is an important prognostic marker in patients with HNSCC. We know that a higher expression of Ki-67 could indicate a poorer prognosis of patients. Moreover, high Ki67 index value is associated with a higher rate of lymph node metastasis. Ki-67 is expressed strictly in the nucleus, it is not a marker of malignancy, but shows the percentage of cells in division under both normal and pathological conditions (6). Therefore, the prediction of this histopathological parameter could be important and introduced into the clinical routine. Although, several studies have been undertaken on Ki-67 expression in HNSCC, the results differ significantly from one author to another, depending on the type of cases investigated, their anatomical location, the technique used and last but not least the method of interpretation (7).

Correspondence to: Cristina Stefania Dumitru, Piata Eftimie Murgu no. 2, 300041, Timisoara, Timis, Romania. Tel: +40 768009382, Fax: +40 256490626, e-mail: dcristinastefania@gmail.com

Key Words: E-cadherin, Ki-67, immunohistochemistry, head and neck squamous cell carcinoma.

Based on these data, the aim of the study was to evaluate the interrelation between Ki-67, E-cadherin immunohistochemical expression and their predictive role in patients with HNSCC. We conducted a study on the loss of E-cadherin and the involvement of Ki-67 tumour proliferation rate in HNSCC through immunohistochemical methods (IHC) in order to evaluate their role as predictive markers of tumour proliferation, aggression and lymph node metastasis. Even if these markers are studied separately, we consider that a study on their correlation is required regarding the loss of E-cadherin immunohistochemical expression that correlates with high proliferation rate in HNSCC. The histopathological degree of tumour differentiation should be investigated together with the proliferative index Ki-67 and the ECAD marker in order to consider the overall results, draw conclusions about the clinical evolution and to establish a targeted therapy.

Materials and Methods

Patients and biopsies. The study included 50 cases of HNSCC. Signed consent was obtained from the patient, the principles of the Declaration of Helsinki were respected, and the study was approved by the Institutional Review Board Scientific Research Ethic Committee CECS UMFVBT No.22/September 2019. The specimens were fixed in 10% buffered formalin for 24 h and paraffin embedded. After the morphological evaluation (Broder's system grading), cases were selected for immunochemistry.

Immunohistochemistry. The following steps of the immunohistochemical technique were applied: heat-induced epitope retrieval with Bond Epitope Retrieval Solution 2 (Leica Biosystems, Newcastle Ltd, Newcastle upon Tyne, UK) for 20 min, endogenous peroxidase blocking (5 min), incubation with primary antibodies (20 min) and visualization with The Bond Polymer Refine Detection System (for 15 min). The primary antibodies used were Ki-67 (clone MM1, ready to use, Leica Biosystems) and E-cadherin (clone 26B5, ready to use, Leica Biosystems). The chromogen used was 3.3-diaminobenzidine dihydrochloride. Haematoxylin was used as a counterstain. The chromogen and the counterstain were applied for 10 minutes. The full immunohistochemical procedure was performed with Bond Max Autostainer (Leica Biosystems).

Microscopic evaluation and data analysis. Each case was evaluated regarding its membrane expression (E-cadherin) and nuclear expression (Ki-67). The examination of the sections was performed with the Nikon Eclipse 600 optical photonic microscope and after the general inspection of the sections, ECAD and Ki-67 were evaluated as follows: for the ECAD expression it was used the Intensity Reactivity Score (IRS), where the staining intensity (SI) was assessed as negative (=0), weak (=1), moderate (=2), and strong (=3), and the reactivity was determined by the percentage of positive cells (PP) (8). Ki-67 proliferation index- the density of positive nucleus cells was evaluated qualitatively, as a percentage: value 0 for the absence of reaction in all cells examined by section (regarding the tumour part); +1 (less than 1-3% positive tumour cells); +2 (4-10% positive tumour cells); +3 (11-50% positive tumour cells); +4 (over 50% positive tumour cells) (11,12). The results were statistical analysed using the Chi-squared test and a p-value of <0.05 was considered significant.

Results

The final product of reaction for ECAD expression in tumour cells was predominantly membranic and less cytoplasmic, with an intensely positive reaction in the surface epithelium, a predominantly positive tumour cell percentage >50%, PP=4, and a medium/high IRS (Figure 1C). A highly positive reaction in the tumour cells and in the surface, epithelium was noticed, as well as intensely positive hyperplastic and dysplastic epithelium which respects the decrease towards the surface of the positive reaction. However, certain sample presented a low intensity reaction with a proliferation index over 45-50% (Figure 1A).

We used the average value of cell proliferation calculated according to the available data from the literature. Tumours were classified according to Broder histological criteria, as grade G1 (well differentiated) in 5 cases, grade G2 (moderately differentiated) in 20 cases and grade G3 (weak or undifferentiated based on the degree of keratinization, nuclear pleomorphism and the presence or absence of intercellular bridges) in 25 cases.

The results of immunohistochemistry showed positive staining for Ki-67 in all samples. The percentage of positive tumour cells was explained by comparing the nuclei of Ki-67-stained cells with the negative ones in close vicinity. It may be considered that this method of evaluation is also applicable to tumours in other locations and compared to the already known methods on this subject, for practical reasons, we introduced the category +2 (4-10% positive tumour cells) (Figure 1D). The level of invasion can be limited to the epithelium, or to the basal lamina, an aspect called in situ. When it affects only the lamina propria, the surface, it may be considered microinvasive or invasive. Regarding invasive carcinoma, it manifests itself through the destruction of the basal lamina, clearly extending to the underlying tissues, possibly accompanied by the stromal reaction and a strong positive expression over 75% (Figure 1B). In Ki-67 immunoreaction, in the dysplastic epithelium, positive cells are arranged in several rows, in severe dysplasia reaching the immediate vicinity of the free surface. In the metaplastic stratified epithelium, positive cells are located only in the basal area.

We noticed that out of 50 cases, 18% had loss of ECAD, respectively, 82% of primary tumours had a high ECAD response, IRS>10. The high ECAD expression was associated with the predominantly histopathological degree of G3 differentiation, this is also due to the high total number of patients diagnosed with G3 (25 cases), resulting in a significant correlation ($p<0.5$; $p=0.0305$) (Table I). No association was noticed between ECAD expression and patient sex ($p<0.5$; $p=0.6540$), but the increased incidence of men (42 cases) compared to the low number of women who developed HNSCC (8 cases) correspond to epidemiological risk factors, data and studies from the literature. A strong correlation was

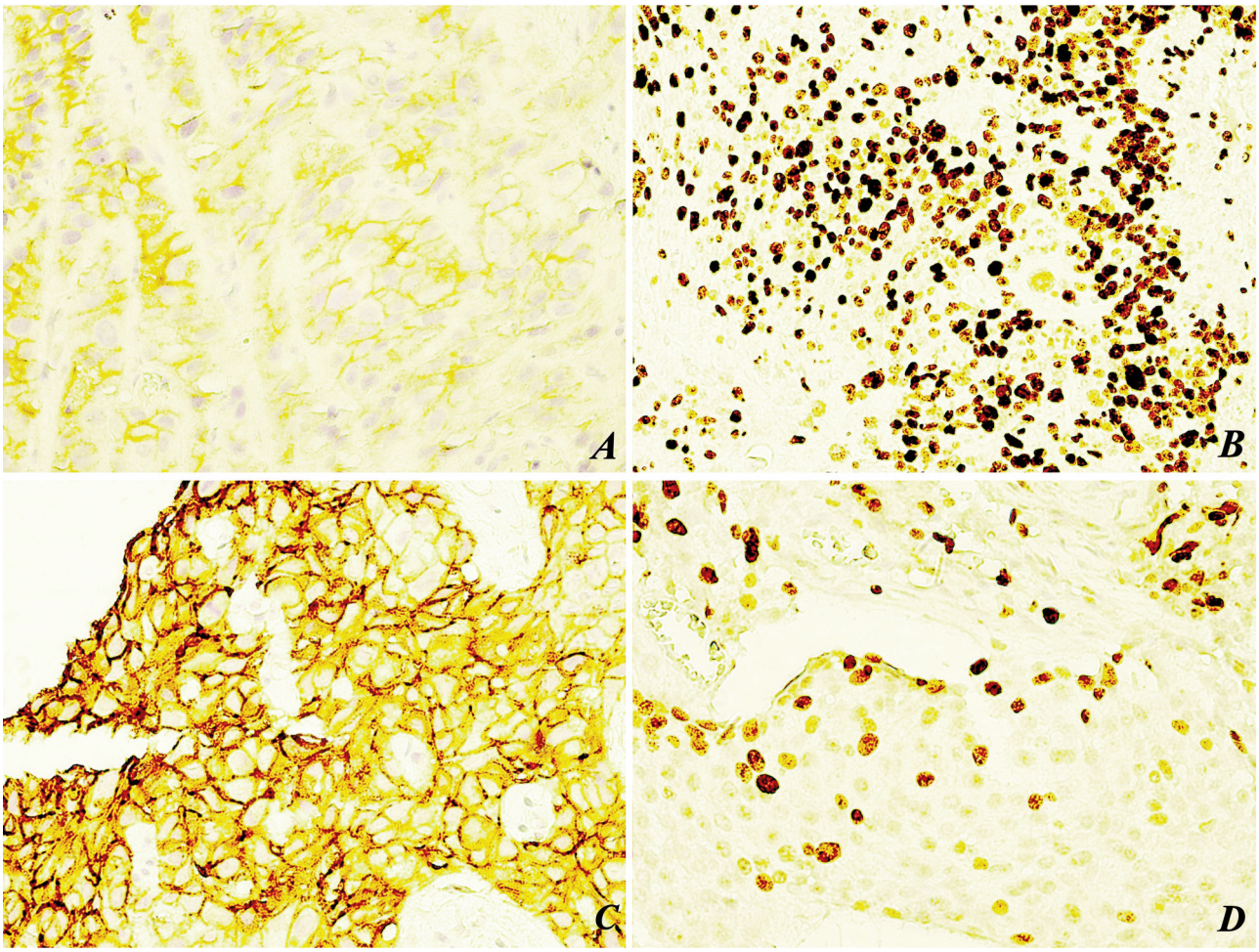

Figure 1. *Immunoexpression of ECAD and Ki-67, original magnification ×400. Weak positive tumour cell cords, low IRS ECAD (A). Proliferation index Ki-67 +4, tumour area with positive cells over 75% (B). Tumour cells with an intensely positive reaction throughout the cell membrane, high IRS ECAD (C). Tumour proliferation index Ki-67 +2 (D). IRS: Intensity Reactivity Score.*

Table I. *ECAD expression in correlation with Broder histological criteria.*

Grading	ECAD weak IRS (0-4)	ECAD moderate IRS (5-9)	ECAD strong IRS (10-15)	*p*-Value
G1	-	1	4	0.0305
G2	1	3	12	
G3	8	2	19	
Total	9	6	35	

ECAD: E-cadherin; IRS: Intensity Reactivity Score.

noticed between low E-cadherin expression, increased proliferation rate and advanced T2-T3 tumour stage. All these aspects suggested the loss of ECAD expression parallel to the increase of tumour proliferation rate (p=0.0242).

Ki-67 nuclear staining was noticed in all of fifty formalin-fixed biopsy samples analysed. The percentage of Ki-67 positive proliferative cells in tumours ranged from 2% to over 75%, with a median of 45-50%. Tumours with less than 10% positive cells were classified as having a low proliferation rate (PR), while tumours with 11-50% and more than 50% of positive cells were defined as having a mean PR medium and, respectively, high. 11 tumours (22%) had a low PR, 21 (42%)

Table II. *Ki-67 index in correlation with Broder histological criteria.*

Grading	Ki-67 tumour proliferation index					*p*-Value
	+1	+2	+3	+4	Total	
G1		2	2	1	5	0.0245
G2		4	6	10	20	
G3	1	4	13	7	25	
Total	1	10	21	18	50	

medium and 18 (36%) a high PR. Most of the tumours with the G1 degree of differentiation had a marking index <50%, while a marking index ≥11% was noticed at the G2, G3 degree of differentiation (Table II). There were significant correlations between Ki-67 tumour expression and tumour histological grade (p=0.0245), clearly showing the IHC importance in assessing HNSCC predictive values and tumour prognosis. However, a significant correlation hasn't been proven regarding Ki-67 expression and tumour stage (p=0.4356), anatomical region (p=0.5382), age (p=0.6456), or lymph node involvement (p=0.5645), in the present study.

Unpredictably, all 9 cases characterized by loss of ECAD expression revealed an increase of Ki-67 tumour proliferation index. Thus, the density of cells with a positive nuclear expression was more than 75%. These cases correlated with the staging of the primary T2-T3 tumour, from which 4 cases showed positive lymph nodes. Consequently, loss of ECAD expression in HNSCC has been associated with a high Ki-67 tumour proliferation index, clinical features of malignancy such as metastases, recurrence, low survival and histopathological-poor tumour differentiation, mentioned by some authors as a marker for high risk of malignancy.

Discussion

HNSCC is the most common neoplasm in the ENT field and its prognosis depends on the size of the lesion, the level of local invasion and the presence of lymph node metastases. Tumour suppressor genes, especially ECAD and regulatory apoptosis genes are variable in tumour progression. Cell proliferation is a fundamental biological mechanism in oncogenesis and in the detection of cell growth markers, such as the Ki-67 index, used as a prognostic factor. In HNSCC, Ki-67 is the one most frequently used cell proliferation index, and ECAD is an important tumour suppressor (9, 10).

The level of ECAD expression is frequently low or may be absent in some cancers, while loss of intercellular junctions is thought to precede tumour invasion and metastasis. Loss or reduction of E-cadherin-mediated cell adhesion defines the declaration of invasion and metastases in many carcinomas, including HNSCC. Few studies have approached the loss of ECAD expression in HNSCC associated with high tumour proliferation (11).

We studied the importance of loss of E-cadherin immunohistochemical expression correlated with high proliferation rate in HNSCC. Thus, the prediction of these immunohistochemical parameters could be important and introduced into clinical routine. In 9 cases of loss of ECAD expression we observed increased presence of the Ki-67 tumour proliferation index, with a positive nucleus cell density of over 75%. The studies of von Zeidler SV *et al.* and Gabriella Szentkúti *et al.* were consistent with the above findings (12, 13).

The Ki-67 proliferation index has been shown to be an important feature of HNSCC. Thus, a higher expression of Ki-67 could indicate a poorer prognosis of patients. Moreover, it is associated with a higher rate of lymph node metastasis, as we saw in our study. Thus, the prediction of this histopathological parameter could be important and introduced in the clinical routine (14). Therefore, the histopathological degree of tumour differentiation should be investigated together with the proliferative index Ki-67 and other markers in order to take into account the overall results, draw conclusions about the clinical evolution and outline a targeted therapy (15).

High proliferative activity (>50%) is associated with an increased risk of recurrence after surgery in patients with stage I tumours, making Ki-67 a potentially useful marker for patients in need of extensive surgical treatment. The high rate of metastases in stage T1-T2 tumours are in line with previous studies showing a failure rate of 20-40%. Previous studies of Ki-67 expression in recurrent locoregional oral cancers have shown conflicting results (16). In our study, we observed a relationship between histological grade and Ki-67 proliferation factor expression. Most studies to date support a positive correlation between the expression of the Ki-67 proliferative index and the histological grade, as well as Mondal *et al.* which found a significant correlation between them (17).

Numerous studies have reported that the ECAD gene is a good or bad predictor in HNSCC, depending on the increase or loss of IHC expression. In our study we showed that loss of expression was associated with a proliferation index Ki-67 over 75%, with a poor prognosis of malignancy. This could have a particular therapeutic significance in the future biological therapy. Therefore, the histopathological degree of tumour differentiation should be investigated together with the proliferative index Ki-67 and the expression of ECAD in order to consider the overall results, draw conclusions about the clinical evolution and outline a targeted therapy.

Conclusion

E-cadherin and Ki-67 immunohistochemical expression are potential predictive markers of HNSCC. In our study, the Ki-67 proliferation index and ECAD expression were shown to be

important features of HNSCC. Thus, higher Ki-67 expression correlates with loss of IHC ECAD expression, which indicates a poorer prognosis of patients. Moreover, this correlation is associated with a higher rate of lymph node metastasis.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

CSD, design of the study and written of the manuscript; ARC and SC, immunohistochemistry and independent evaluation; MR, revising the text and supervisor.

Acknowledgements

The Authors are grateful to 'Victor Babes' University of Medicine and Pharmacy Timisoara, Romania. Also, we would like to thank to our histotechnologist Ciprian Onica for the excellent technical support.

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Received February 12, 2022
Revised March 14, 2022
Accepted March 30, 2022