Original paper

Prognostic significance of a panel of two biomarkers (E-cadherin and CD105) in laryngeal cancer

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This study aimed to evaluate the clinicopathologic significance of the combined immunohistochemical expression of the epithelial-mesenchymal transition marker E-cadherin and the angiogenesis marker CD105 in laryngeal squamous cell carcinoma and assess correlation of their expression.

Eighty-five patients who underwent complete resection as primary treatment were selected for this study. E-cadherin and CD105 expression levels were determined by immunohistochemistry. The receiver operating curve approach was applied to determine the cut-off value and separate patients with high and low expression of markers. The high-risk group ("CD105 high" and "E-cadherin low" expression) showed statistically significant correlations with age less than 66 years ($p = 0.039$), advanced T-status (T3–4) ($p = 0.046$), aggressive TNM stage (stage III–IV) ($p = 0.003$) and locoregional recurrence of disease ($p = 0.004$). In the Kaplan-Meier analyses, the high-risk group had significantly worse prognoses than other risk groups (log-rank test $\chi^2 = 9.415$, $p = 0.024$).

Spearman's correlation coefficient analysis showed a nonsignificant negative correlation between the expression of E-cadherin and CD105 (rho = –0.073, $p = 0.505$). Simultaneous consideration of E-cadherin and CD105 is a simple panel of markers to determine aggressive tumour phenotype with a higher risk of disease recurrence in patients with laryngeal cancer.

Key words: E-cadherin, CD105, endoglin, larynx, cancer.

Introduction

Laryngeal squamous cell carcinoma (LSCC) represents the second most common malignant tumour in the head and neck, accounting for about 1% of all cancers [1]. The 5-year survival rate for LSCC is 61% and has not significantly improved over the past four decades despite various advances in surgery, radio- and chemotherapy [2]. Established clinical prognostic factors in LSCC are primary tumour site, histopathological grading, and TNM classification, but they do not take into account the biological aggressiveness of the specific tumour [2]. Therefore, identifying biomarkers that may predict the biological behaviour of LSCC could contribute to distinguishing patients with a good prognosis from those with a poor prognosis and improving the clinical treatment.

Biological and clinical data have indicated that epithelial-mesenchymal transition (EMT) and angiogenesis contribute to tumour growth, invasion, and metastasis. Epithelial-mesenchymal transition is the phenomenon in which epithelial cells transform into mesenchymal cells, which is mainly characterised by loss of intercellular adhesion, and an

increase in cellular migration and mobility [3]. Epithelial-mesenchymal transition is classified into three different subtypes that are associated with embryonic development, inflammation, and tumour progression, promoting cancer cell invasion and metastasis [4].

A key molecular reprogramming that occurs during EMT is "cadherin switching", in which the normal expression of E-cadherin, a key component of the adherens junction complexes that maintain cell-cell adhesion and cytoskeletal organization, is down-regulated, while the abnormal expression of N-cadherin, vimentin, and fibronectin is increased [5, 6]. E-cadherin is connected with the cytoskeleton through β-catenin. The down-regulation of E-cadherin is associated with the release of β-catenin, activating Wingless (Wnt) signalling, where the unbound β-catenin enters the nucleus and activates the transcription factors Snail, Slug, Twist, ZEB1, and ZEB2 that directly bind its gene promoter [7, 8]. Several studies have indicated that EMT is involved in the progression, lymphatic invasion, and tumour metastasis of LSCC [8–12].

Tumour angiogenesis, the formation of peritumour and intratumour new blood vessels, is a process fundamental to the growth and metastasis of solid tumours. Epithelial-mesenchymal transition is necessary for angiogenesis, while the same factors drive endothelial cells toward a mesenchymal and proangiogenic phenotype. Tumour angiogenesis, as a complex phenomenon, is controlled by numerous positive and negative factors produced by both malignant and normal cells [13]. The increased growth of the tumour leads to the development of hypoxic areas. In response to hypoxia, tumour cells increase the expression of proangiogenic factors such as vascular endothelial growth factor (VEGF), thus resulting in endothelial cell activation and proliferation [14]. Endoglin (CD105) is a cell-surface coreceptor of transforming growth factor β (TGF-β) which is abundantly expressed in angiogenic endothelial cells and induces the binding of Snail and Smad to promote EMT, thereby promoting carcinogenesis [15]. The assessment of microvessel density (MVD) is a well-established measurement of neoangiogenesis determined with antibodies against endothelial cells [12, 16]. Previous studies have confirmed that quantification of MVD, as determined by immunohistochemical staining for CD105, is a powerful prognostic marker in LSCC [17–19]. However, little is known about the involvement of CD105 in EMT and metastasis [12, 20].

Methods combining E-cadherin with CD105 might improve patient stratification and be related to clinicopathological features and survival. This study aimed to evaluate the clinicopathologic significance of the combined immunohistochemical expression of the EMT marker E-cadherin and the angiogenesis marker CD105 in laryngeal squamous cell carcinoma and to assess correlation of their expression.

Material and methods

Study samples

Eighty-five patients who underwent complete resection as primary treatment for LSCC were selected for this study. Patients with second primaries or who had received primary radiotherapy and/or chemotherapy were not considered. The clinicopathological data were statistically analysed according to the World Health Organization standard to evaluate the grade of tumour and according to the Eighth Edition of the TNM Head and Neck Cancer Classification to evaluate the TNM stage [21].

The clinical information, including sex, age, histologic grade, primary tumour (T) classification, nodal (N) status, TNM stage, and oncological outcome, was obtained retrospectively from clinical records. Treatment decision-making was based on the clinical stage and the presence or absence of lymph node metastasis at the time of diagnosis. All patients had undergone microlaryngoscopy with laryngeal biopsy, upper aerodigestive tract endoscopy, neck ultrasonography (with or without fine needle aspiration cytology), head and neck contrast-enhanced computerised tomography (CT), and/or magnetic resonance imaging, chest X-ray, and liver ultrasonography. All patients underwent primary partial (61 cases) or total laryngectomy (24 cases) with unilateral or bilateral cervical lymph node dissection. No distant metastases (M) were detected at diagnosis. The clinical follow-up was adjustable to patients' characteristics and it was scheduled as follows: between 4–8 weeks in the first 2 years; every 3 months for year 3; every 6 months for years 4 and 5; and once a year after that. Neck ultrasonography and/or CT, chest X-ray and/or CT, liver ultrasonography, and total-body positron emission tomography were repeated if clinically indicated. Twenty-two (25.9%) of 85 patients developed loco-regional malignancy recurrence (8 local recurrences, 14 recurrences to neck lymph nodes). We defined poor oncological outcome as the recurrence of disease or occurrence of metastasis after treatment. Disease-free survival was calculated from the period of treatment completion until the date of tumour relapse. The median follow-up was 28 months (range 6–60 months).

Ethical considerations

This study was approved by the Institutional Ethical Review Board of the Clinical Center of Montenegro (approval number: 03/01-5068/1, date: 24.03.2022). The research was conducted ethically, with all study procedures being performed in accordance with the

requirements of the World Medical Association's Declaration of Helsinki. All patients preoperatively signed a consent form for disclosure of privacy in managing personal data for scientific purposes. Before undergoing surgery, all patients included in the study signed a detailed informed consent form. We did not need additional informed consent to use the specimens in this study because only archived material was used. Data were examined in compliance with Montenegrin privacy and sensitive data laws.

Tissue processing and immunohistochemistry

Immunohistochemistry was carried out following the manufacturer's recommendations. All included samples originated from complete resection material. We selected the best section from each block showing central and peripheral areas of the tumour, avoiding areas with necrosis. The biopsy specimens were fixed in 10% phosphate-buffered formalin, processed to obtain 4 mm thick paraffin sections, and deparaffinised. The prepared slices were treated with a methanol solution containing 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. Tissue sections were washed with TRIS-buffered saline and then incubated for 30 minutes with mouse monoclonal antibodies against E-cadherin (Clone NCH-38 diluted 1 : 50, DAKO, Denmark) and CD105 (clone SN6h diluted 1 : 20, DAKO, Denmark). All sections were subjected to a heat-induced antigen retrieval process. Immunodetection was performed with the Envision system, DAKO Autostainer, model VL1. Diaminobenzidine (DAB) was used as a chromogen for 10 minutes. The slides were then counterstained with haematoxylin. Laryngeal tissues free of tumour were used as positive controls, and the primary antibody was replaced with TRIS-buffered saline for negative controls.

Immunohistochemical evaluation

The slides were viewed randomly by a pathologist who did not know the clinical data. The immunohistochemical staining scoring system was evaluated according to previously described criteria [22, 23].

E-cadherin

A staining score was given based on the percentage of cells stained (0–100%). All stained cells were considered positive regardless of the intensity of the staining. The staining was predominantly membranous with some cytoplasmatic staining. The receiver operating curve (ROC) approach was applied to determine the cut-off value. The patients were classified as low (Fig. 1) expressers (E-cadherin expression below the cut-off value of the staining scores) or high (Fig. 2) expressers (E-cadherin expression above the cut-off value).

CD105

The intratumoural MVD quantification was performed under light microscopy. The sections were scanned at 40x magnification to select four areas with the highest vascular density ("hot spots"). CD105-positive endothelial cells were counted using a 200x magnification. The rounded mean value of the vessel count in four fields for each case was used as the final MVD value. Any endoglin-stained single cell or cell cluster that was separated from the adjacent microvessels, tumour cells, or other elements of connective tissue was considered to be a countable vessel. A visible vascular lumen was not required to count as a microvessel. A cut-off point identified by the ROC was chosen to separate patients with high and low MVD. Figure 3 shows a representative field of high CD105 expression in laryngeal SCCs. For

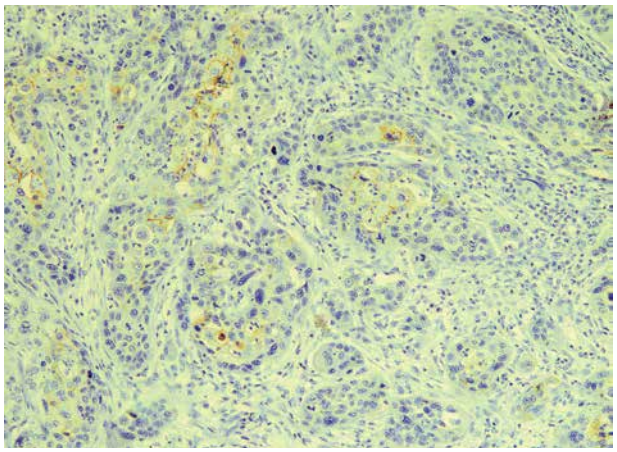

Fig. 1. Low expression of E-cadherin in laryngeal squamous cell carcinoma, 100×

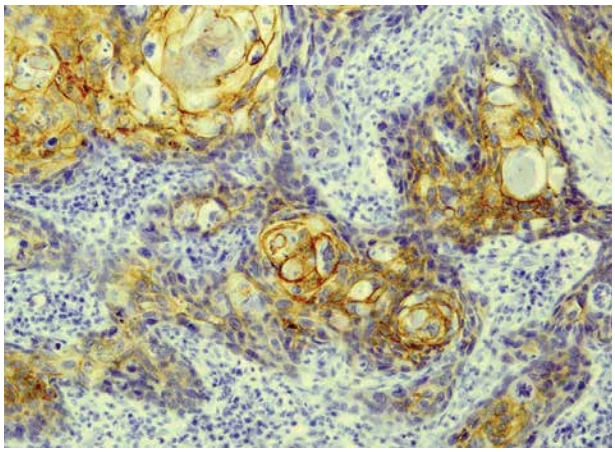

Fig. 2. High expression of E-cadherin in laryngeal squamous cell carcinoma, 100×

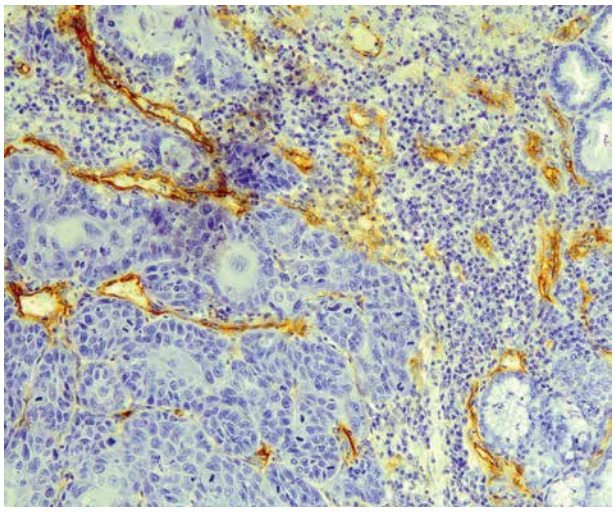

Fig. 3. High expression of CD105 in laryngeal squamous cell carcinoma, 100×

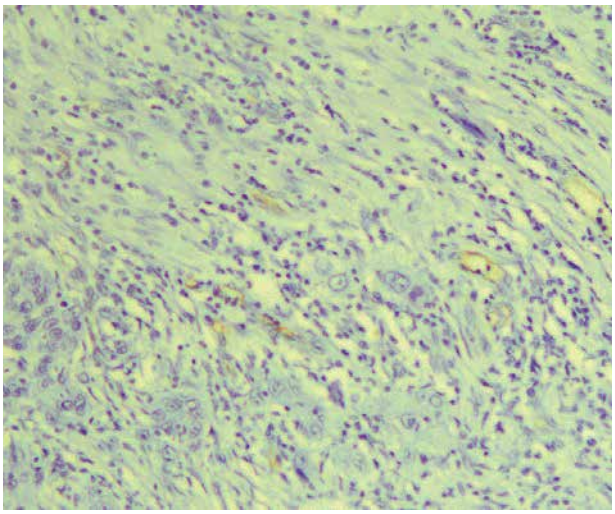

Fig. 4. Low expression of CD105 in laryngeal squamous cell carcinoma, 100×

comparison, Figure 4 depicts a representative field of laryngeal SCCs with low staining for CD105.

Statistical analysis

Descriptive statistics were presented as mean ± standard deviation or median (minimum–maximum) for continuous variables, and frequency and percentage for categorical variables. The correlation of E-cadherin and CD105 expression levels with clinicopathological features was tested using the χ^2 test or Fisher exact test. The receiver operating curve approach was applied to determine the analytically best-fitting cut-off points of the variables selected for the subsequent survival analysis. The associations between the expression levels of E-cadherin and CD105 and LSCC prognosis were analysed using Kaplan-Meier survival analysis, and the log-rank test was used in the univariate analysis. The relationship between E-cadherin and CD105 was performed using Spearman's rank correlation analysis. *P*-values lower than 0.05 were considered statistically significant. All statistical analyses were conducted with the package SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA).

Table I. Number of patients according to E-cadherin and CD105 expression

CD 105 expressionb	E-cadherin expressiona		Total
	Low	High	
High	16	30	46
Low	12	27	39
Total	28	57	85
$\chi^2 = 0.026, p = 0.872$c			

Results

The present study involved 85 cases, 43 of them with glottic and 42 with supraglottic LSCC treated with primary surgery. There were 43 patients with early (TNM stage I and II) and 42 patients with advanced (TNM stage III and IV) cancer. The mean age of the patients was 59.3 ±9.1 (range 37–81) years. The median age was 58 years. There were 66 (77.6%) male patients and 19 (22.4%) female patients.

E-cadherin expression was associated with the cell membrane and varied greatly among tissue samples 2–75 (median 25, mean 28.9 ±19.9). The cut-off value for E-cadherin expression identified by ROC was 16.5 (sensitivity 64%, specificity 68%). Using the calculated cut-off value, 28 patients (32.9%) were classified as low expressers and 57 (67.1%) as high expressers.

CD105-assessed MVD in considered LSCC varied among tissue samples from 5 to 24 (median 13, average 12.8 ±4.1). The cut-off value identified by ROC for survival analysis was 12.5 for CD105 expression (sensitivity 91%, specificity 41%). Using the calculated cut-off value, 46 (54.1%) tumours were classified in the "high MVD" group, and the rest, 39 (45.9%) tumours, constituted the "low MVD" group.

To evaluate the prognostic value of the combined E-cadherin and CD105 expression, the patients were divided into four groups: Group I: "MVD high" and "E-cadherin low", Group II: "MVD high" and "E-cadherin high", Group III: "MVD low" and "E-cadherin low", and Group IV: "MVD low" and "E-cadherin high". Group I (16 cases) was considered as high-risk, group IV (27 cases) as low-risk and Group II (30 cases) and Group III (12 cases) as intermediate risk groups ($\chi^2 = 0.026, p = 0.872$) (Table I).

Table II. Correlation of combined expression of CD105 and E-cadherin and clinico-pathologic characteristics of 85 patients with laryngeal squamous cell carcinoma

Variables	Number of patients	E-cadherin expression[a] and CD 105 expression[b]				*p*-value
		E-cadherin low, CD105 high	E-cadherin high, CD105 high	E-cadherin low, CD105 low	E-cadherin high, CD105 low	
Sex						0.058
Male	66	9	25	12	20	
Female	19	7	5	0	7	
Age (years)						0.039
≤ 65 years	64	14	25	10	15	
> 65 years	21	2	5	2	12	
Primary tumour site						0.485
Glottic	43	8	12	7	16	
Supraglottic	42	8	18	5	11	
T classification						0.046
T1 and T2	57	7	19	11	20	
T3 and T4	28	9	11	1	7	
N status						0.197
N0	66	10	22	11	23	
N+	19	6	8	1	4	
TNM stage						0.003
I and II	43	3	13	10	17	
III and IV	42	13	17	2	10	
Histologic grade						0.273
G1	36	7	15	4	10	
G2 and G3	49	9	15	8	17	
Loco-regional recurrence						0.004
L–R rec. no	63	8	19	11	25	
L–R rec. yes	22	8	11	1	2	

[a]*Low E-cadherin expression below 16.5, high E-cadherin expression above 16.5*
[b]*Low CD105 expression below 12.5, high CD105 expression above 12.5*
[c]χ^2 *test*

The correlation of the combined MVD and E-cadherin expression with clinico-pathologic parameters is summarised in Table II. The high-risk group (Group I) showed statistically significant correlations with age less than 66 years (≤ 65 years vs. > 65 years) ($p = 0.039$), advanced T-status (T3–4 vs. T1–2) ($p = 0.046$), aggressive TNM stage (stage III–IV vs. stage I–II) ($p = 0.003$) and poor outcome (locoregional recurrence of disease) ($p = 0.004$) (Table II).

In the Kaplan-Meier analyses, the high-risk group (Group I) had significantly worse prognoses compared with other risk groups (log-rank test $\chi^2 = 9.415$, $p = 0.024$) (Fig. 5). Spearman's correlation coefficient analysis showed a nonsignificant negative correlation between expression of E-cadherin and CD105 (rho = –0.073, $p = 0.505$).

Discussion

Advances in diagnostic and therapeutic strategies has not considerably improved the survival rate of LSCC in the last few decades. Local tumour invasion and lymph node metastasis are still the most important unfavourable prognostic factors for LSCC. Identifying novel biomarkers for the progression and metastasis of LSCC is necessary in head and neck oncology.

This study was undertaken to elucidate the correlation between the expression of E-cadherin as an EMT marker and CD105-assessed MVD in LSCC. Loss and reduction of intercellular adhesion proteins such as E-cadherin promote EMT and migratory cell phenotype and are related to the local invasion and

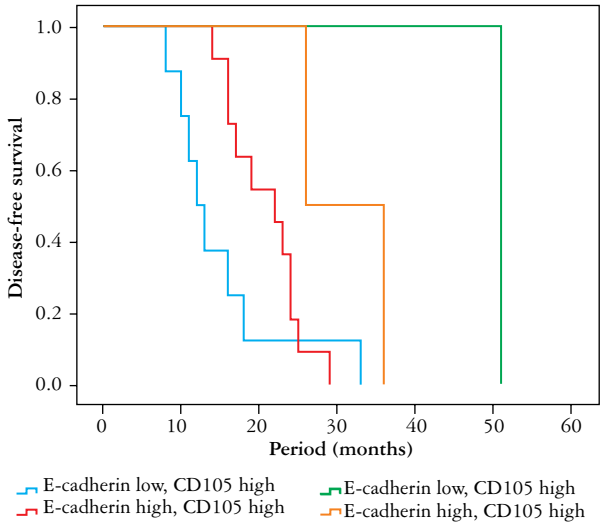

Fig. 5. Disease-free survival according to combined expression of CD105 and E-cadherin

distant metastasis of malignant tumours [4, 24]. The available literature proved the importance of EMT in the progression of LSCC. Greco *et al*., in a cohort of 82 patients with LSCC, found that E-cadherin overexpression was an independent risk factor for worse 3-year overall survival [9]. In the study by Zhu *et al*. negative E-cadherin expression was associated with poor overall survival [10]. Angiogenesis is crucial for growth of the tumour, its invasion, and metastasis. CD105 is upregulated in endothelial cells in new blood vessels in or around the tumour.

Very few studies have implicated the involvement of CD105 in EMT and metastasis. Mitselou *et al*., in their cohort of 69 patients with colorectal cancer, found a significant correlation between CD105 tumour epithelial cell expression and E-cadherin expression ($p = 0.001$) [7]. Hu *et al*., analysing tissue specimens of patients with clear cell renal cell carcinoma, found that CD105 was involved in the EMT with low expression of E-cadherin and stem cell traits but not metastasis [20]. Zhang *et al*. conducted a study in ovarian cancer cells and concluded that the effect of CD105 on cancer metastasis may be related to E-cadherin expressions, leading to the promotion of EMT and invasion [25]. Franz *et al*., in a cohort of 37 LSCC patients, conducted the first study in this cancer type to investigate the relationship between EMT markers (E-cadherin, N-cadherin, Snail, Slug, Zeb1, and Zeb2) and CD105-assessed MVD [12]. They found a non-significant correlation between CD105-assessed MVD and two EMT markers' (Snail and Zeb2) expression (Spearman's rank correlation, rho = 0.30, $p = 0.07$, rho = 0.27, $p = 0.11$, respectively).

According to the data available to us, this is one of the very rare studies in which the combined influence of EMT and angiogenesis in LSCC has been analysed. Based on our previous research, we chose to investigate two biomarkers using immunohistochemistry on the surgical specimens of LSCC. We divided patients into a high-risk group (MVD high and E-cadherin low), a low-risk group (MVD low and E-cadherin high), and an intermediate-risk group (other combinations of CD105 and E-cadherin expression) [10]. The high-risk group showed statistically significant correlations with advanced T-status, aggressive TNM stage (stage III–IV), and lower disease-free survival. These results indicate that EMT and angiogenesis influence tumour aggressiveness in LSCC. On the other hand, we only found a negative correlation between CD105-assessed MVD and expression of the EMT marker E-cadherin without statistical significance.

The main limitation of our investigation lies in the retrospective single-centre setting with a limited number of cases. The cut-off value for separating tumours with high vs. low expression of different markers is not standardized. The median and the quartiles of measurements are commonly selected cut-off values for immunopositivity [26]. Receiver operating curve have been increasingly used to determine cut-off scores in many cancers [27], and we used them in our study. The main strengths of our study concern the homogeneity of the patient population since only squamous cell carcinomas were considered, all patients underwent surgery performed by the same team, and only surgical specimens (not biopsies) of LSCC were assessed.

Conclusions

Simultaneous consideration of both E-cadherin and CD105 is a potentially simple panel of markers to determine an aggressive tumour phenotype with a higher risk of disease recurrence in patients with LSCC, who might benefit from a more aggressive primary treatment. To better evaluate the predictive potential of a prognostic strategy based on EMT and angiogenesis, additional randomised prospective studies with larger patient groups are needed.

The authors declare no conflict of interest.

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