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E-cadherin, β-catenin and Snail immunoexpression in laryngeal squamous cell carcinoma

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Abstract

E-cadherin, β-catenin and Snail are important molecules involved in cellular adhesion and epithelial–mesenchymal transition. Loss of E-cadherin expression, nuclear relocation of β-catenin and high expression of Snail are connected to tumor progression, rapid cell growth and metastasis. The aim of our study was to analyze the immunohistochemical expression of β-catenin, E-cadherin and Snail, depending on clinico-morphological aspects of the laryngeal squamous cell carcinomas. Our results revealed variable E-cadherin, β-catenin and Snail expression, depending on differentiation degree and tumor stage. These markers can be helpful in identifying the aggressive laryngeal squamous carcinomas.

Keywords: E-cadherin, β-catenin, Snail, laryngeal squamous cell carcinoma.

Introduction

Laryngeal squamous cell carcinoma, like other cancers still has many unknown variables, in terms of pathogenesis, behavior and therapy. The laryngeal squamous cell carcinoma represents approximately 40% of all malignant tumors of the head and neck cancers and 2.4% of all cancers according to the latest statistics [1, 2]. Through the anatomical lesions and functional disturbances liable to cause, this type of cancer is considered to be a major health and social problem. Opposite to other cancers, laryngeal squamous cancer with a rapid diagnostic and an adequate treatment is associated with excellent results in time, with a five-year survival rate of approximately 60% [3].

Local invasion and metastases are the main causes of death and their mechanisms are not entirely understood thereby limiting the development of new treatment methods [4]. In the accomplishment of these aspects are involved numerous proteins like β-catenin and E-cadherin. It has been proved that loss of E-cadherin expression and nuclear relocation of β-catenin are connected to tumor progression, rapid cell growth and metastasis [5]. Snail is another marker involved in tumor progression, high expression being associated to the unfavorable progression of head and neck cancers [6].

We performed a study on the immunohistochemical expression of β-catenin, E-cadherin and Snail in the laryngeal squamous cell carcinomas in relation with the clinicopathological aspects.

Materials and Methods

The study included a number of 38 laryngeal squamous cell carcinomas selected in a period of three years (2010–2012) from the patients admitted and operated in Clinic of Otolaryngology, Emergency County Hospital of Craiova, Romania. The cases were diagnosed in the Department of Pathology of the same Hospital. The selected cases were represented by laryngectomy specimens, which were fixed in 10% buffered formalin, processed by the usual technique with paraffin embedding and Hematoxylin–Eosin (HE) staining. Tumors were histopathological classified and staged according to *World Health Organization* (WHO) recommendation [7]. The analyzed parameters in this study were represented by age, gender, histological grade, depth of invasion (pT), lymph node metastasis (pN) and tumor stage. None of the selected cases presented distant metastases (pM0). The assessment of immunoreactions was performed on serial sections and the antibodies panel used is shown in the table below (Table 1).

Table 1 – ***Panel of antibodies used in the immunohistochemical reactions***

Antibody	Host/Clone/ Manufacturer	Dilution	Pretreatment	External positive control
Beta-catenin	Mouse-antihuman/ β-catenin-1/ DAKO	1:100	Microwaving in citrate buffer, pH 6	Colon adeno-carcinoma
E-cadherin	Mouse-antihuman/ NCH 38/DAKO	1:50	Microwaving in citrate buffer, pH 6	Mammary gland
Snail	Rabbit-antihuman/ Snail-1/ABCAM	1:50	Microwaving in Tris-EDTA buffer, pH 9	Placenta

EDTA: Ethylenediaminetetraacetic acid.

We used for the immunohistochemical reactions LSAB2–HRP (Labeled Streptavidin–Biotin2–Horseradish peroxidase) amplification system (DAKO, Redox, Bucharest,

code K0675) and for the signal visualization 3,3'-diaminobenzidine tetrahydrochloride (DAB, DAKO, code 3467) as chromogen. A semiquantitative quantification of the signals was done using a score, after reviewing the reactions intensity and the percentage of the labeled cells. Therefore, the reactions were considered to be mild (score 1), moderate (score 2) and intense (score 3). Regarding the percentage of the labeled cells, we used the following categories: 1 (<25% marked cells), 2 (>26–50% marked cells), 3 (51–74% marked cells) and 4 (>75% marked cells). The multiplying of reactions intensity and the percentage of the labeled cells provided us a final score, with values between 1 and 12. For the statistical analysis, the final score was considered to be low for the values between 1 and 4 and high for the values between 6 and 12.

Statistical analysis was performed using the Pearson's and χ^2 (*chi*)-square tests within SPSS 17 software and *p*-values <0.05 were considered significant. For the image acquisition were used Nikon Eclipse E600 microscope and Lucia 5 software.

Results

We analyzed a total of 38 laryngeal squamous cell carcinomas and we noticed a predominance in male subjects and an average diagnosis age of 59.8±8.0 years old (Table 2). Most of the tumors were moderate differentiated (22 cases), while the well and poorly differentiated tumors were observed in seven and nine cases. The majority of the carcinomas (26 cases) were without lymph node metastases (pN0). Regarding the tumor stages, the analyses revealed an advanced stage in most of the cases, respectively 19 cases for the stage III and 10 cases for the stage IV (Table 2).

Table 2 – ***Cases distribution according to the investigated clinicopathological parameters***

Parameter	Variable (No. of cases)
Age [years]	<50: 2 >50: 36
Gender	F: 3; M: 35
Differentiation degree	WD: 7; MD: 22; PD: 9
Depth of invasion (pT)	T1: 3; T2: 9; T3: 22; T4: 4
Lymph node metastasis (pN)	N0: 26; N1: 4; N2: 8
Tumor stage	I: 3; II: 6; III: 19; IV: 10

F: Female; M: Male; WD: Well differentiated; MD: Moderate differentiated; PD: Poorly differentiated.

E-cadherin immunoreaction was identified in the tumor cells membrane in 22.8% of the cases. In the well-differentiated cases, the reaction intensity was variable with an average percentage of 53.6±6.6% labeled cells and a mean score of 5.5. In the moderate and poorly differentiated cases, the values were 19.8±5.3% and 17.4±5.8%, the intensity was variable, with mean scores of 1.8 and 1.7, respectively (Table 3; Figure 1, A–C).

Referring to the pathological stages, in the stage I, the average percentage of labeled cells was 32.2±26.8%, with variable intensity and a mean score of 3.6, while in stage II, the values were 29.5±15.7% and 3.3. By comparison, in the stages III and IV, the marked cells values were 22.4±12.8% and 26.8±14.3%, and the scores 2.4 and 1.8, respectively.

In this study, the analysis of *β*-catenin expression revealed membrane, cytoplasmic and/or nuclear positivity in 37.2% of the cases. We have noticed the diminution of membrane expression and the predominance of cytoplasmic and nuclear expression in high-grade carcinomas with deep invasion and in advanced stages. Analyzing the marker expression for well differentiated cases, we found an average marked cells of 47.2±4.1%, variable intensity and a mean score of 5.5, compared to moderate and poorly differentiated cases, where the values were 29.3±3.7% and 48.6±8%, the intensity was variable and the mean scores 4 and 5.6, respectively (Table 3; Figure 1, D–F).

Table 3 – ***Immunostaining scores in relation with clinicopathological parameters***

Parameter	Variable (No. of cases)	E-cadherin scores	*β*-Catenin scores	Snail scores
Age [years]	<50: 2	3	5	9
	>50: 36	2.5	4.7	7.7
		p=0.618	*p*=0.754	*p*=0.418
Gender	F: 3	2	4.6	9.6
	M: 35	2.5	4.7	7.6
		p=0.536	*p*=0.821	*p*=0.315
Differentiation degree	WD: 7	5.5	5.5	3.5
	MD: 22	1.8	4	9.1
	PD: 9	1.7	5.6	7.8
		p=0.000	*p*=0.043	*p*=0.004
Depth of invasion (pT)	T1: 3	3.6	4.6	4
	T2: 9	2.6	4.2	6
	T3: 22	2.4	4.9	8.8
	T4: 4	1.7	5	9.2
		p=0.536	*p*=0.942	*p*=0.190
Lymph node metastasis (pN)	N0: 26	2.5	4.5	7.07
	N1: 4	3.7	4.5	8.2
	N2: 8	1.7	5.6	10.1
		p=0.395	*p*=0.304	*p*=0.020
Tumor stage	I: 3	3.6	4.6	4
	II: 6	3.3	4.3	4
	III: 19	2.4	4.4	8.8
	IV: 10	1.8	5.5	9.4
		p=0.380	*p*=0.493	*p*=0.010

F: Female; M: Male; WD: Well differentiated; MD: Moderate differentiated; PD: Poorly differentiated.

In the early stages I and II, the mean values of marked tumor cells were 35.03±7.5% and 40.8%±9.7, the reactions intensity was variable and the mean scores of 4.6 and 4.3, respectively. In the advanced stages III and IV, the average percentage of marked tumor cells were 35±10.8% and 39.8±11.3%, with variable reaction intensity for the both stages and mean scores of 4.4 and 5.5, respectively.

Snail immunostaining was identified at nuclear level in 61.5% of tumor cells. In the well-differentiated cases of laryngeal squamous carcinomas, we detected an average percentage of 23.9±7.6%, mild intensity within the tumor islands and increased in the periphery and the mean score of 3.5. Higher values were present in moderate and poorly differentiated cases, where we obtained an average percentage of marked cells of 69.03±13.2% and 72.7±17.09%, moderate to strong intensities and mean scores of 9.1 and 7.8, respectively (Table 3; Figure 1, G–I).

Referring to the tumor stages, in the stage I we observed a mean value of 38.8±20.8% labeled cells, variable intensity

and an average score of 4, while in stage II, the values were 42.6±2.9% and 3.3. By comparison, in stages III and IV, the values were 66.1±18.4% and 71.05±22.5% for the marked cells, the reaction intensity was variable, and the scores were 8.8 and 9.4, respectively.

The examination of different parameters in our study indicated significant increased E-cadherin values in well-differentiated carcinomas, compared with moderate and poorly differentiated ones (p=0.000, *chi*-square test) (Figure 2A). We also have found significant differences in the expression of β-catenin and Snail, in relation to the tumors degree of differentiation (p=0.043 and p=0.004, *chi*-square test), a high expression of this markers being noticed in the high-grade carcinomas (Figure 2, B and C). Significant differences were obtained between Snail expression and lymph node involvement, the expression being higher in the presence of the lymph node metastasis (p=0.020, *chi*-square test). Our study also showed significant differences for Snail immunoexpression and tumor stage, the expression being higher in the advanced stages (p=0.010, *chi*-square test). We have not found significant differences in the Snail immunoexpression in relation to the depth of invasion, even the reaction was higher in pT3–T4 carcinomas. Also, we did not find any other statistical relation of the markers immunoexpression and clinical parameters.

In addition, the analysis of the E-cadherin and β-catenin labeled cells percentage, indicated a positive linear correlation (p=0.016, Pearson's test) (Figure 2D), while E-cadherin and Snail analysis indicated a negative linear correlation (p=0.000, Pearson's test).

Discussion

The loss of cell adhesion is commonly present in human cancers, including laryngeal squamous cell carcinoma [8]. This process is an important step for the stromal and vascular invasion and the appearance of the metastases. At the basis of the loss of cell–cell adhesion stays the alteration of cadherin/catenin complex [9].

The expression of E-cadherin has been investigated extensively in the literature. Advanced stages, accompanied by metastases and the poorly differentiated tumors have been frequently associated with the reduction or loss of E-cadherin expression [8]. Catenins, such as β- and γ-catenin, bind to the cytoplasmic portion of E-cadherin [8]. The membrane loss of β-catenin is responsible for promoting the invasive character of the malignant tumors, through the reduced cellular adhesion [10].

In the present study, E-cadherin was present in the membrane of tumor cells in 53.6% of the well-differentiated cases, 19.8% of the moderate differentiated cases and 17.4% in the poorly differentiated ones. For laryngeal squamous cell carcinomas, the literature data indicate that the well and moderate differentiated cases maintain the positivity of this marker, while the poorly differentiated cases presents decreased to lost of E-cadherin immunopositivity [8].

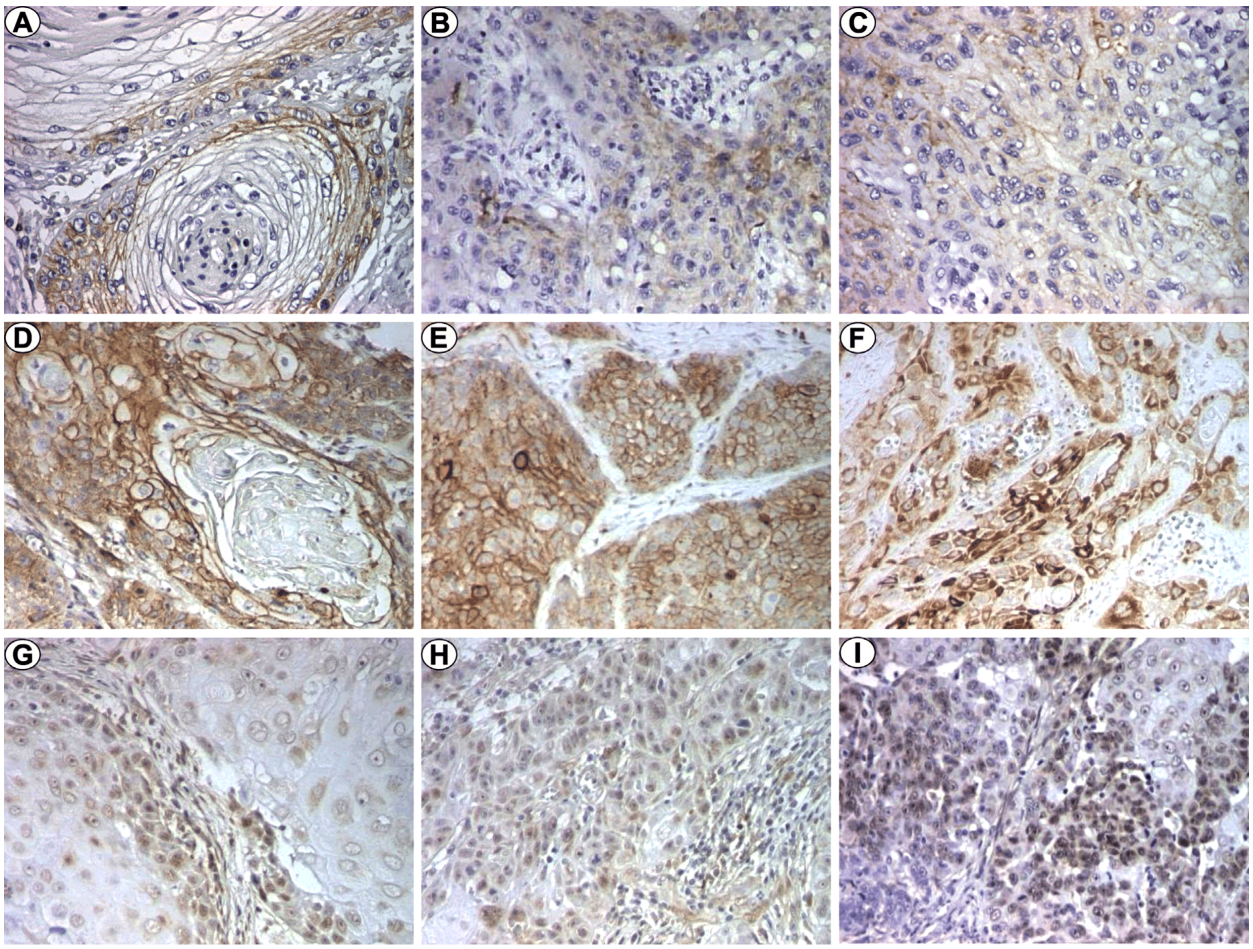

Figure 1 – ***Laryngeal squamous cell carcinoma, E-cadherin (A–C), β-catenin (D–F) and Snail (G–I) immunostaining, ×200: (A, D and G) Well differentiated; (B, E and H) Moderate differentiated; (C, F and I) Poorly differentiated.***

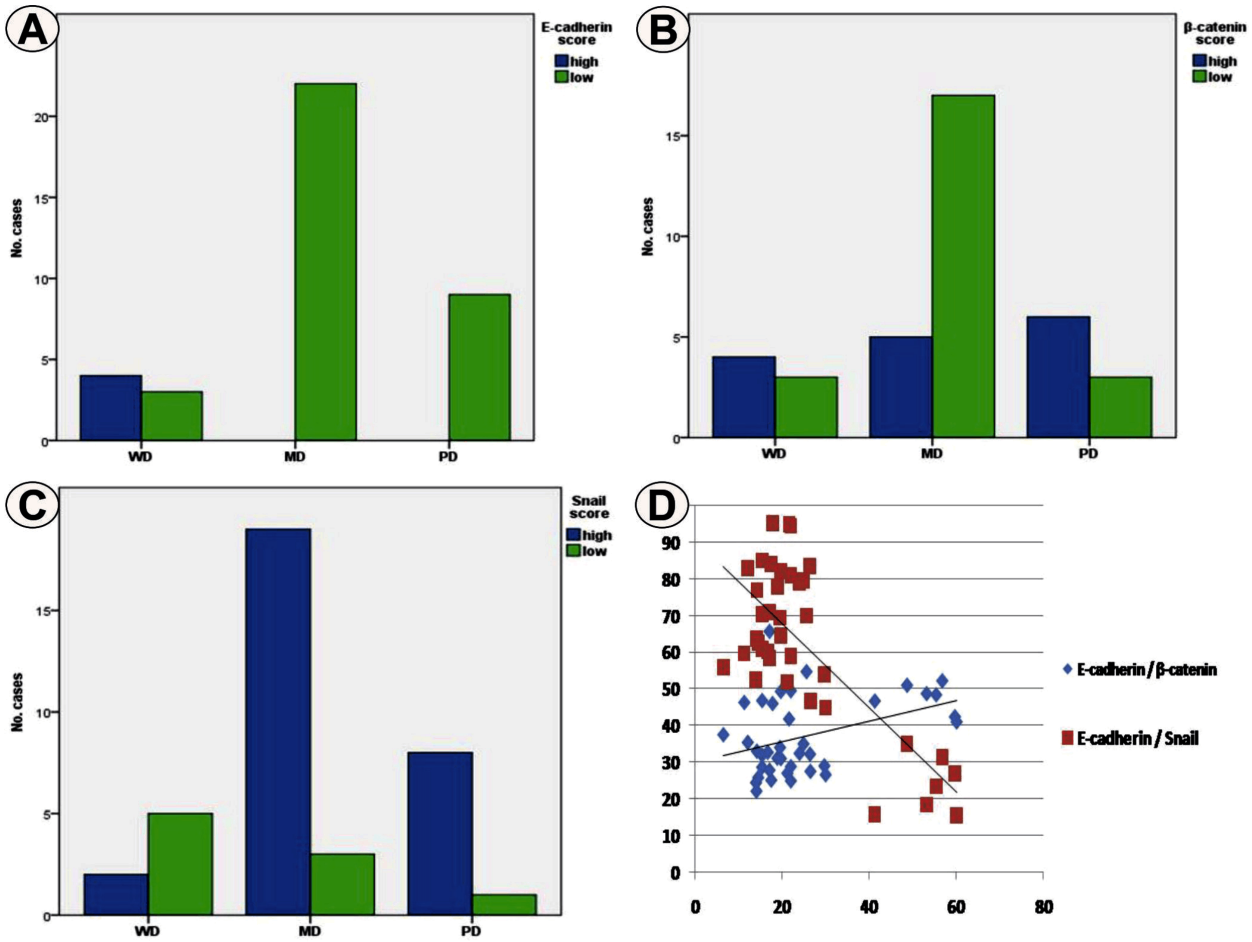

Figure 2 – ***Cases distribution according to the scores of E-cadherin (A), β-catenin (B) and Snail (C) and the markers values distribution (D). WD: Well differentiated; MD: Moderate differentiated; PD: Poorly differentiated.***

E-cadherin expression decrease is linked to a higher tumor aggressiveness, justifying the high invasion capacity in the surrounding tissues and the lymph nodes [8, 11]. The expression of this marker has been studied in other types of cancer where similar aspects were found. Therefore, in breast cancer, Kowalski *et al.* noticed that the E-cadherin losses associated with poor prognosis and tumor invasion [12]. Also, in prostate cancer, it has been demonstrated that the decrease of E-cadherin expression is associated to a higher invasion capacity and the absence of its expression is related with an increased rate of bone metastases [13].

β-Catenin can be seen in different cellular locations, like membrane, cytoplasm or nucleus, and depending on this having different roles. Membrane localization is associated to negative tumor growth, while the other locations are related to Wnt pathway of carcinogenesis [14, 15]. This marker is described as having a dual role; first, it is involved in the cellular adhesion and on the other way is involved in the Wnt pathway of carcinogenesis [16]. In the present study, *β*-catenin was identified in all locations, in 37.2% of cases, the immunostaining being more frequently cytoplasmic and nuclear in high grade and advanced stage carcinomas. Normally, *β*-catenin is observed in the membrane location the loss of this expression is considered an important moment in the appearance of dedifferentiation and metastases [9, 17]. By contrary, the nuclear expression is associated to poor prognosis in head and neck squamous cell carcinomas [18]. The literature indicates an intense reaction of this marker in other types of cancers, like colon, esophagus or prostate [19–21]. It has been also observed a biomolecular functional connection between *β*-catenin and other proteins involved in cell adhesion and migration [22].

Snail is a transcription factor involved in embryogenesis and cancer development. It is known the fact that this marker is needed for neural crest development and in cancer acts like a suppressor for E-cadherin and an initiator factor for the epithelial–mesenchymal transition [23]. Epithelial–mesenchymal transition is a process involved in invasion and metastases of cancer [24]. Loss of cellular adhesion, invasion capacity and mobility are the main cellular characteristics involved in the epithelial–mesenchymal transition [25]. All these characteristics are the result of a synergistic action of Snail and other transcription factors, which are implicated in the disruption of cellular plasticity, cell death resistance, and the increasing capacity of dissemination and metastasis [26, 27]. The overexpression of Snail is related to poor prognosis and shorter survival in carcinomas, including head and neck squamous cell carcinoma [28–30].

Significant differences were noticed in Snail relation to the degree of differentiation, the reaction being significantly superior in the moderate and poorly differentiated

cases compared to the well-differentiated ones. Other studies indicated that a positive, intense Snail immunoreaction is correlated to the degree of differentiation and the depth of the invasion. These studies described a Snail higher expression in the poorly differentiated invasive carcinomas [31, 32]. Different studies revealed an inverse relation between Snail and E-cadherin, indicating a decreased E-cadherin expression in highly expressed Snail tumors [33, 34].

Conclusions

In this study, the laryngeal squamous carcinomas with high grade and advanced stages indicated diminished E-cadherin expression, increased Snail expression and cytoplasmic/nuclear β-catenin immunostaining. E-cadherin reactions revealed linear negative correlation with Snail and a linear positive one with β-catenin. The immunoexpression assessment of E-cadherin, Snail and β-catenin can be helpful in identifying the aggressive laryngeal squamous carcinomas.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper. All authors read and approved the final manuscript.

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