The correlation between cytokeratin antigens expression in lymph nodes and morphological features of the tumor front in laryngeal carcionoma – the prognostic significance of the Anneroth, Batsakis and Lunas' classification in the presence of micrometastases

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Introduction. Lymph nodes estimated as pN0 in conventional morphological studies could have focuses of carcinoma cells with a diameter of ≤ 2 mm referred to as micrometastases (pN+) which could be cause of nodal recurrence and poorer survival. New clinico-morphological features are still being assessed in order to find prognostic factors in laryngeal carcinoma which allow to predict the presence of micrometastases as well.

Material and methods. To presented the direct correlation between the morphological features of tumor acc. to the classification of Anneroth, Batsakis and Luna and the probability of micrometastases we have analyzed 22 patients operated for squamous cell carcinoma of the larynx at the ENT Department of the Medical University of Lodz between 1998 and 1999. The total score of classification and particular features were analyzed with the presence of micrometastases as assessed by immunohistochemical methods with a panel of cytokeratin antigens (CKs).

Results. Our study showed that the total morphologic score is very useful in the prediction of micrometastases in patients with laryngeal squamous cell carcinoma of the larynx. The statistical analysis has revealed a significant correlation between the total score, the mode of invasion and the depth of invasion.

Conclusions. The results of our study suggest that it is necessary to extend traditional patomorphologic evaluation by a number of features from the classification of Anneroth, Batsakis and Luna which could aid the diagnosis of micrometastases and thus might influence therapeutic decisions and eventually lead to more appropriate and individualized therapy.

Key words: Laryngeal carcinoma, Anneroth, Batsakis and Lunas' classification, cytokeratin filaments, micrometastases

For over 17 years the Polish population has remained at a high risk of developing laryngeal cancer, while the morbidity and mortality factors for this disease have remained high [16]. Treatment results presented as the number of local and nodal recurrences, and 3- and 5-year survival, remain poor. This explains the ongoing search for potentially prognostic clinico-morphological features based upon detailed immunohistochemical and molecular examination of the tumour and the lymp nodes. In patients with clinically advanced primary tumour and lymphatic node involvement pronounced as cN0 the indications for elective neck dissection remain an unsolved issue. The prognosis of patients, in whom the presence of nodal metastases has not been confirmed in the course of conventional pathological examination (pN0), is also unclear. The problem arises from the possibility of the presence of nodal micrometastases (i.e. lesions less than 2 mm in diameter) (pN+), which may, in turn, be the cause of nodal recurrence and shortened survival. One of the methods of discerning occult foci of malignant cells within lymph nodes is mapping the nodes for the presence of cytokeratin antigens. Cytokeratins are polipeptides belonging to the family of median filamentsa with a diameter of 7-11 nm and high molecular weight (40-60 kDa). They are the markers of epithelial cell differentiation.

Literature reports stress the prognostic value of the morphological features of the front of the primary tumour, i.e. the most invasive and the least differentiated part of the tumour, which determines aggressive malignant invasion and increases the likelihood of micrometastases.

The aim of the study was to analyse the morphological features of the primary tumour, its stroma and the lymph nodes using the modified classification of Anneroth, Batsakis and Luna, and to evaluate the relationship between these parameters and cytokeratin antigen expression within the negative lymph nodes, thus

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attempting to assess the risk of developing micro-metastases.

Material and method

We analyzed tissue samples obtained from 22 patients (17 men, 5 women; age 58-87 yrs; mean age 63±5 yrs) who had undergone surgery for squamous cell carcinoma of the larynx at the Department of Laryngology and Laryngeal Oncology of the Medical University in Lodz between the years 1998-1999. All patients underwent total laryngectomy with uni- or bilateral radical or modified neck dissection. The lesions were assessed according to the TNM criteria.

Directly after surgery the material was preserved in a 10% solution of buffered formaldehyde. Samples from the primary tumour designated for microscopic analysis were obtained according to standardised procedure. The depth of invasion was assessed at the point of the deepest invasion of the surrounding tissues. One or two samples were obtained from every lymph node after it had been dissected along its greatest diameter. The tissue samples were embedded in paraffin, routinely cut into 4-5 μm sections (at least 3-4 from the primary tumour and each lymph node), attached to glass slides and H&E stained. Pathological analysis was performed according to an own modification of the classification of Anneroth, Batsakis and Luna (Table I). We assessed 10 pathological features of the tumour (degree of keratosis, nuclear differen-tiation, mitotic figures) and the type of interaction between the tumour and the surrounding tissues (type and stage of invasion, the presence of eosinophil and lymphoid invasion, the presence of spindle-like cells, vascular invasion and micrometastses). The analysis was performed under a light microscope (magn. 200x, number of mitoses magn. 400x), going by the areas of deepest invasion of the surrounding tissues. Tumour assessment has been presented as the number of scored points. Immunohistochemical reactions with policlonal antibodies (NCL-C11, Multi-Cytokeratin 4/5/6/8/10/13/18, RTU-D

Table I. Modified classification of Anneroth, Batsakis and Luna

Feature	Characterization	Scale
Cytoplasmic differentiation	High (>50% keratinized)	1
	Moderate (20-50% keratinized)	2
	Poor (5-20% keratinized)	3
	None (<5% keratinized)	4
Nuclear differentiation (polymorphism)	High (>75% mature cells)	1
	Moderate (50-75% mature cells)	2
	Poor (25-50% mature cells)	3
	None (<25% mature cells)	4
Number of mitoses	Single (0-1)	1
	Moderate number (2-3)	2
	Large number (4-5)	3
	Very numerous (>5)	4
Mode of invasion	Well-defined bordeline	1
	Cords; less marked bordeline	2
	Groups of cells; no distinct bordeline	3
	Diffuse growth	4
Stage of invasion (depth)	Misroinvasion (few cords) – to *lamina propria*	1
	Nodular into submucosa – to *lamina muscularis*	2
	Invasion deeper than *submucosa* with *periosteum*	3
Plasmalymphocytic invasion	Marked (continuous rim)	1
	Moderate (many large patches)	2
	Slight (few small patches)	3
	None	4
Eosinophil infiltration	21-30 cells/ high-power field	1
	11-20 cells/ high-power field	2
	3-10 cells/ high-power field	3
	0-2 cells/ high-power field	4
Spindle cells	None	1
	Present	2
Capillary embolus	None	1
	Present	2
Micrometastases	None	1
	Present	2

Novostatin Universal Detection Kit, NCL-L-DAB Liquid DAB Substrate Kit; Novocastra UK) were performed on 2-3 sections obtained from the same lymph node (3-4 μm sections attached to polysin-covered glass slides) in accordance with the manufacturer's directions. For the sake of pathological analysis of the lymph nodes it was assumed that a micrometastasis is a focus ≤3 mm in diameter. The results of nodal cytokeratin microfilament expression were compared with the morphological features of the tumour. In the course of the statistical analysis we applied a model of unifactorial logistic regression to evaluate the risk of developing micrometastases; the significance level was set at p=0.05.

Results

A majority of the patients (18/22; 81.8%) suffered from advanced cancer involving the entire larynx and originating from the subglottic area. The most numerous group of patients (20/22; 90%) had stage III and stage IV tumours.

16 patients (72.7%) underwent unilateral lymphadenectomy, in 2 cases in the form of radical neck dissection. The remaining 6 patients (27.3%) underwent bilateral lymphadenectomy. Pathological assessment of H&E stained lymph nodes did not confirm the presence of metastases, however immunohistochemical evaluation with policlonal anti-CK antibodies confirmed the presence of cancer cells (micrometastases) within the lymph nodes of 11 patients (50%).

Pathological analysis of the primary tumour revealed that a majority of the patients presented with squamous cell carcinoma of intermediate differentiation G2 (12 pts, 54.5%). The total score obtained in the course of primary tumour assessment acc. to the classification of Anneroth, Batsakis and Luna oscillated between 11 and 20

(mean: 15.6 points) for CK poli+ tumours and between 10 and 16 (mean: 12.1 points) for Ck poli- tumours. A majority of CK poli + tumours scored 16-20 points (7 pts, 63.6%); in the case of CK poli- tumours a majority of cases scored 11-15 points (6 pts, 54.5%). An analysis of selected features of the primary tumour in relation to nodal policytokeratin expression has shown, that the most numerous subgroup consisted of tumours with intermediate cell differentiation – 9 cases of CK poli+ (81.8%) and 5 cases for CK poli- (45.4%). The number of mitotic figures within the CK poli+ cells was, in a majority of cases, 2-3 per microscopic field (5 pts, 45.5%) and 3-4 per microscopic field (5 pts, 45.4%). In the CDK poli- group we found an intermediate number of mitotic figures to be the most common (6 pts, 54.5%). In case of CK poli+ tumours there was a predominance of deep cancerous invasion of the surrounding tissues with cartilage destruction (7 pts, 63.3%). In case of CK poli- tumours invasion was predominantly submucosal (6 pts, 54.5%). An analysis of the course of invasion in patients with confirmed micrometastases revealed the a majority of cases the tumour invaded the surrounding tissues with small, diffused cell clusters (5 pts, 45.5%).

12 patients (54.4%) achieved 3-year survival, and 7 (31.8%) – 5-year survival. An analysis of the total score acc. to the classification of Anneroth, Batsakis and Luna for CK poli+ tumours revealed that 3-year survival was achieved by 4 pts. (36.4%) with a mean score of 16.8 points, while 5-year survival was achieved by only 1 patient with a total score of 18 points. In the CK poli- group 3- and 5-year survival was achieved by 8 pts (72.7%) with a mean score of 12.7 points and 6 pts (54.5%) with a mean score of 11.4 points, respectively.

Statistical analysis aimed at the assessment of the risk of developing nodal micrometastases in relation to

Table II. Analysis of the micrometastatic risk dependant on the morphologic features of the primary tumor

Feature	Regresion coefficient	SE	Relative risk	95% range for relative risk	
Number of mitoses					
1 – 2 mitoses			1.000		
2 – 3 mitoses	0.576	0.321	1.957	0.931	4.154
3 – 4 mitoses	**0.724**	**0.361**	**2.539**	**1.218**	**5.210**
>5 mitoses	0.306	0.536	1.458	0.556	4.041
Depth of invasion					
in situ and *lamina propria*			1.000		
lamina propria + lamina muscularis	**0.969**	**0.415**	**2.656**	**1.168**	**5.950**
deep invasion with *periosteum*	**1.428**	**0.343**	**4.349**	**2.090**	**7.999**
Mode of invasion					
well-defined bordeline			1.000		
cords; less marked bordeline	-0.110	0.651	0.896	0.250	3.205
groups of cells; no distinct bordeline	0.356	0.639	1.433	0.399	5.078
diffuse growth	**0.511**	**0.861**	**1.657**	**1.308**	**9.014**
Total score ABL classification					
6 – 10			1.000		
11 – 15	0.223	0.783	3.125	0.713	14.39
16 – 20	**2.184**	**0.729**	**7.470**	**1.561**	**34.60**
21 – 25	**2.605**	**0.851**	**11.540**	**2.349**	**65.75**

the results of pathological analysis revealed the strongest statistical significance (p<0.0005) for cases with a total score exceeding 16 points. The morphological features showing the strongest statistical correlation (p<0.05) were the mode and the depth of invasion (Table II).

Discussion

One of the methods of estimating prognosis in patients treated for malignancy is the morphological evaluation of the tumour, and especially its peripheral parts. The discrepancies observed between the conclusions affecting pathology-dependant prognosis are usually explained by the differences in the character of growth of the central and peripheral areas of malignant invasion [1-8]. The classification presented by Anneroth, Batsakis and Luna [9] was primarily concerned with *carcinoma planoepitheliale* of the oral cavity. It included assorted pathological features of the tumour and the types of interactions between the cancerous tissue and the stroma of the tumour. We are also convinced that this classification provides the basis for prognosis among these patients. The application of this classification system has allowed us to identify patients with significantly better prognosis (total score <10 points). Our own studies performed on the postoperative material obtained from a large number of patients which were aimed at assessing the correlation between the total score acc. to the modified classification of Anneroth, Batsakis and Luna and 5-year survival of laryngeal cancer patients have shown that the achieved score relates to prognosis. The results presented here, obtained from 22 laryngeal cancer patients, also confirm the value of this classification in prognosing the development of nodal micrometastases. A high total score, i.e. exceeding 16 points, relates to an increased risk of the presence of foci of single cancer cells within lymph nodes which were originally, in the course of routine assessment, pronounced non-metastatic (pN0). Such a high score also correlates with a decrease in 3- and 5-year survival of laryngeal cancer patients. A number of literature reports on laryngeal cancer present attempts at multifactorial morphological assessment of the tumour aimed at the identification of prognostic factors and provide similar results [4, 9-20]. Jacobsson [13] has reported that the total score acc. to his scale and some selected features (i.e. nuclear differentiation and mode of invasion) are significant prognostic factors. The classification presented by Crissman [4, 10] is also an independent prognostic factor. Other authors, who had performed assessments basing upon this classification, have also found a correlation between a low histologic grade of the tumour and the presence of both cancer cell emboli within the vessel lumen and clinically enlarged lymph nodes [15]. Another scoring system which allows to analyse the survival time and the presence of nodal metastases is the method of assessing tumour malignancy suggested by Lund [16]. Reports stressing the prognostic value of morphological assessment of the peripheral parts of the tumour and presenting it as more reliable are

becoming more common [5, 8]. Tumours assessed at below 14 points according to the TFG score (Tumour Front Grading) had significantly poorer prognosis [5-7]. Welkoborsky et al. [8] have noted a statistically significant correlation of the TFG score with disease-free survival and with the presence of malignancy. Similar results have been reported by Gabriel et al. [5], thus confirming the prognostic value of the TFG scale and pronouncing it as adequate both for conveying the dynamics of tumour growth and for predicting the likely length of survival.

The grading of laryngeal cancer, one of the most commonly conveyed features of malignancy, remains a matter for discussion. Our studies have not confirmed the value of the G feature for assessing the risk of micrometastases. Many other authors have also failed to show the correlation between the grading of laryngeal cancer and the course of disease [8, 15, 21, 22]. However, some authors point out the prognostic value of tumour grade, stressing that this, predominantly, concerns tumours of low differentiation [18, 23]. The prognostic value of the mitotic index in laryngeal cancer patients also remains unclear [14, 18]. Our study results have shown that the number of mitoses within the tumour cells has no impact on patient survival, while Stefanowa-Urbaniak et al. [18] have confirmed such a correlation. One has to stress that in papers dealing with laryngeal cancer this feature often appears as an element of numerous classifications and morphological assessment scores, thus the mitotic index does not usually appear as a separate issue and is hardly ever reported in the form of a number [4, 10, 13, 15, 18].

Important factors influencing the probability of nodal micrometastases, and affecting patient prognosis, include the depth and mode of laryngeal wall invasion [18, 24, 25]. Tumours characterized by diffuse invasion with small foci of malignancy were found to be more commonly associated with treatment failure and local/nodal recurrence. Our studies have shown the value of the depth and mode of invasion for assessing the risk of the presence of occult foci of cancer cells within the lymph nodes. While studying the prognostic factors of laryngeal cancer, pathologists also assess the interactions between the tumour and the surrounding tissues, however there exist no reports as to the independent prognostic value of these morphological features. Nevertheless, they begin to appear as elements of numerous classifications and scores for the assessment of the primary tumour [4, 9-20].

Conclusions

A more detailed morphological assessment of laryngeal cancer specimens based on the modified classification of Anneroth, Batsakis and Luna might improve the diagnosis of nodal micrometastases.

The risk of nodal micrometastases in laryngeal cancer increases with a high total Anneroth, Batsakis and Luna score (>16 points), diffuse cancerous invasion with

single cells and deep invasion of the laryngeal wall involving the cartilages.

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