

ORIGINAL ARTICLE

ANALYSIS OF CYTOKERATIN 19 EXPRESSION LEVEL BY IMMUNOCYTOCHEMICAL DIAGNOSTIC METHOD IN LARYNGEAL CANCER PATIENTS WITH REGIONAL METASTASES

DOI: 10.36740/WLek202007109

Oleksander V. Kovtunenکو¹, Bohdan V. Shportko², Serhey M. Tymchuk²¹ STATE ESTABLISHMENT «DNIPROPETROVSK MEDICAL ACADEMY OF HEALTH MINISTRY OF UKRAINE», DNIPRO, UKRAINE² MUNICIPAL INSTITUTION «I.I. MECHNIKOV DNIPROPETROVSK REGIONAL CLINICAL HOSPITAL», DNIPRO, UKRAINE

ABSTRACT

The aim: To investigate the informative content of immunocytochemical studies of lymph node aspirate using a molecular marker of cytokeratin 19 in patients with laryngeal cancer as for regional metastatic spread.

Materials and methods: A comprehensive investigation with immunohistochemical study of squamous cell carcinoma of larynx of 74 patients with laryngeal cancer II – IV stage (T2-4 N0-3 M0) of the second clinical group was conducted.

Results: Statistically significant differences ($p < 0.001$) in the structure of distribution of marker expression between groups were revealed. Thus, a marked expression of cytokeratin 19 was determined in 11 (29.73%) representatives of the group with regional metastases and only in 1 (2.7%) patient without tumor metastases ($p = 0.002$). In the group with metastases the presence of atypical cells was confirmed cytologically in 26 cases (70,27%).

Conclusions: To predict the development of laryngeal metastases in laryngeal cancer patients, it is recommended to carry out an immunocytochemical study using a diagnostic marker of cytokeratin 19. The positive status of cytokeratin 19 increases the chances of regional metastasis of laryngeal cancer by 18.37 times ($p < 0.001$) compared with absence of expression.

KEY WORDS: laryngeal cancer, immunocytochemical study, cytokeratin 19

Wiad Lek. 2020;73(7):1355-1359

INTRODUCTION

Laryngeal cancer is the leader in malignancies of the upper respiratory tract. Despite the progress in world medicine, the survival rate of laryngeal cancer patients has not changed much over the last 20 years. [1,2] Regional metastases into the cervical lymph nodes, the value of which exceeds the size and localization of the primary tumor, play a major role in the prognosis of the disease and are second only to the presence of distant metastases. [3]

The evolution of regional metastasis treatment has gone from a radical operation first described in 1888 to superselective interventions having been performed in recent years. [4,5] Discussion of the volume of cervical dissection is reflected in many publications, which proves the urgency of the problem. [6]

Ultrasound-guided fine needle aspiration biopsy is a standard for the study of the metastatic status of lymph nodes. [7] However, the organ-preserving trend in oncology requires the search for new methods of diagnosing metastatic lymph nodes to accurately select the volume of intervention. Much attention is paid to the study of the expression of molecular markers. In recent years, oncology has paid considerable attention to immunohistochemical, immunocytochemical studies and molecular tumor markers. [8]

The cytoskeleton of all cell types is determined by a set of intermediate filaments, microtubules, and microfilaments. Cytokeratins belong to the group of intermediate filaments, which also include vimentin, desmin, neurofilaments, laminin and glial fibrillar protein. They are characteristic filaments for both epithelial cells and the corresponding epithelial tumors and, due to their high specificity and sensitivity, are used to diagnose the latter. [9]

Nowadays, given the high mortality rate in the first year after diagnosis, treatment and diagnosis of laryngeal cancer, and in particular of its metastases, requires further research, development and implementation of new methods in practice [10,11].

THE AIM

To investigate the informative content of immunocytochemical studies of lymph node aspirate using a molecular marker of cytokeratin 19 in patients with laryngeal cancer as for regional metastatic spread.

MATERIALS AND METHODS

To perform the research tasks, 74 patients with laryngeal cancer stage III – IV (T3-4N0-3M0) and II clinical group underwent examination, treatment and follow-up in the ENT-Oncology Department of I.I. Mechnikov Dniprop-

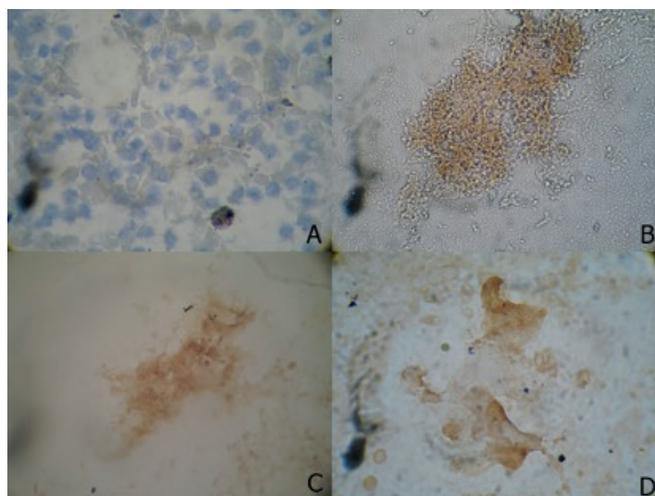


Fig. 1. Defining of cytokeratin 19 expression by immunocytochemical study; microslide stained with monoclonal antibodies cytokeratin 19 (x100). A. - no expression (0%); B. - weak expression level 11-20%; C. - moderate expression level (21-50%); D. - high expression level (>50%).

etrovsk Regional Clinical Hospital from 2011 to 2015. In 37 patients regional metastases of laryngeal cancer were diagnosed and histologically confirmed, the other 37 patients without metastases represented a comparison group.

Transcutaneous aspiration biopsy under ultrasound control was performed in all patients, the obtained cytological preparations were fixed with Cytospray.

The material of the aspirate was examined by cytological and immunocytochemical method. Monoclonal antibodies to cytokeratin 19 (clone RCK108 Dako) were used for the study. For each marker, control studies were performed to eliminate false positive or false negative results. The specimens were examined by light microscopy on a Carl Zeiss microscope. The assessment of marker expression was made with a semi-quantitative method with the following levels: absence of expression – 0-10%, weak – 11-20%, moderate – 21-50% and marked – > 50%.

Modern mathematical and statistical methods of descriptive and analytical statistics were used for the statistical processing of the obtained material, with results in the form of: number of observations (n); arithmetic (M), standard deviation (SD), 95% of confidence interval (95% CI), median (Me), and interquar-

tile range (25-75% percentile). Methods of statistical analysis included: checking the distribution of quantitative attributes for compliance with the normal law by the Shapiro-Wilk test; estimation of probability of differences under conditions of normal distribution by two-sided t-test (Student test), in the other case – by Mann-Whitney test (U test), categorical variables – by Pearson Chi square (χ^2) (Pearson Chi-square), including Yates Chi-square correction for continuity; estimation of the association between categorical variables using Cramer's V (Cramer's V) and Spearman's rank correlation coefficients (ρ); calculation of odds ratio (OR); ROC-analysis.

The data were processed using Statistica v.6.1 Statistical Analysis License Packs. There was studied the level of expression of the molecular marker of cytokeratin 19 by the immunocytochemical method in lymph nodes of 74 patients with stage III-IV cancer. Among the examined, all patients were male, 59.46 – 63.45 years old (95% CI), with no statistically significant differences between groups ($p > 0.05$) in age and degree of tumor differentiation (Table I).

RESULTS

In total, cytokeratin 19 expression was absent in 37 patients (50.0%), low expression was in 12 patients (16.22%), moderate – in 13 (17.57%), and marked – in 12 (16, 22%) (Fig. 1, Table II).

In the group of patients with regional metastases (n = 37), a positive status of cytokeratin 19 was observed in 30 (81.08%) patients. Of these, a weak reaction was in 8 (21.62% of the total number of patients in the main group), moderate – in 11 (29.73%) and strong – in 11 (29.73%). In the comparison group, expression of cytokeratin 19 was detected in 7 patients (18.92%): weak expression – in 4 patients (10.81% of the total number of patients in the comparison group), moderate – 2 (5.41%), marked expression was observed in 1 patient (2.7%); expression was absent in 30 (81.08%) patients. Statistically significant differences ($p < 0.001$) in the structure of distribution of marker expression between groups were revealed (Table 2). Thus, a marked expression of cytokeratin 19 was determined in 11 (29.73%) representatives of the group with regional metastases and only in 1 (2.7%) patient without tumor metastases ($p = 0.002$).

In the group with metastases the presence of atypical cells was confirmed cytologically in 26 cases (70,27%).

Table I. General characteristics of the examined patients with laryngeal cancer

Characteristics	All examined	Main group with regional metastases	Comparison group without regional metastases	P
Number of patients, n (%)	74 (100 %)	37 (50,0 %)	37 (50,0 %)	-
Age (years)				
M (SD)	61,46 (8,61)	59,84 (7,01)	63,08 (9,79)	0,106*
95 % CI	59,46 - 63,45	57,50 - 62,17	59,82 - 66,35	
Degree of tumor differentiation, n (%)				
High G1	14 (18,92 %)	6 (16,22 %)	8 (21,62 %)	0,553
Moderate G2	51 (68,92 %)	27 (72,97 %)	24 (64,86 %)	0,451
Low G3	9 (12,16 %)	4 (10,81 %)	5 (13,51 %)	0,722

Notes. p - Differences between groups according to Pearson's criterion χ^2 ;

* - by Student's t test

Table II. Expression of cytokeratin 19 in lymph nodes depending on the presence of metastases

Level of expression	All examined (n=70)	Main group (n=37)	Comparison group (n=37)	p
Moderate level, Me (25 %;75 %)	11,22 (5,79; 27,50)	27,50 (12,50; 52,50)	6,58 (3,19; 9,44)	<0.001*
Distribution of level of expression, n (%)				
No expression (0-10 %)	37 (50,0 %)	7 (18,92 %)	30 (81,08 %)	<0.001
Weak expression (11-20 %)	12 (16,22 %)	8 (21,62 %)	4 (10,81 %)	0.207
Moderate expression (21-50 %)	13 (17,57 %)	11 (29,73 %)	2 (5,41 %)	0.006
Marked expression (>50 %)	12 (16,22 %)	11 (29,73 %)	1 (2,7 %)	0.002
Differences and associations between groups**	$\chi^2=30,19$ (p< 0.001); V=0.64; $\rho=0.63$ (p< 0.001)			-
High G1 degree of tumor differentiation				
Moderate level, Me (25 %;75 %)	9,24 (7,47; 17,50)	37,50 (12,50; 57,50)	7,50 (5,92; 9,24)	0.028*
Distribution of level of expression, n (%)				
No expression (0-10 %)	8 (57,14 %)	1 (16,67 %)	7 (87,5 %)	0.035
Weak expression (11-20 %)	3 (21,43 %)	2 (33,33 %)	1 (12,5 %)	0.778
Moderate expression (21-50 %)	0 (0%)	0 (0%)	0 (0%)	-
Marked expression (>50 %)	3 (21,43 %)	3 (50,0 %)	0 (0%)	0.110
Differences and associations between groups**	$\chi^2=7,70$ (p= 0.021); V=0.74; $\rho=0.74$ (p= 0.002)			-
Moderate G2 degree of tumor differentiation				
Moderate, Me (25 %;75 %)	12,50 (5,18; 27,50)	27,50 (12,50; 52,50)	6,19 (2,99; 9,85)	<0.001*
Distribution of level of expression, n (%)				
No expression (0-10 %)	24 (47,06 %)	5 (18,52 %)	19 (79,17 %)	<0.001
Weak expression (11-20 %)	8 (15,69 %)	5 (18,52 %)	3 (12,5 %)	0.838
Moderate expression (21-50 %)	11 (21,57 %)	9 (33,33 %)	2 (8,33 %)	0.068
Marked expression (>50 %)				
Differences and associations between groups**	$\chi^2=21,02$ (p< .001); V=0.64; $\rho=0,64$ (p< 0.001)			-
Low G3 degree of tumor differentiation				
Moderate level, Me (25 %;75 %)	8,85 (4,65; 27,50)	22,50 (13,17; 32,50)	4,65 (0,65; 8,05)	0.178*
Distribution of level of expression, n (%)				
No expression (0-10 %)	5 (55,56 %)	1 (25,0 %)	4 (80,0 %)	0.330
Weak expression (11-20 %)	1 (11,11 %)	1 (25,0 %)	0 (0%)	0.906
Moderate expression (21-50 %)	2 (22,22 %)	2 (50,0 %)	0 (0%)	0.324
Marked expression (>50 %)	1 (11,11 %)	0 (0%)	1 (20,0 %)	0.906
Differences and associations between groups**	$\chi^2=5,76$ (p=0.124); V=0.80; $\rho=0,38$ (p=0.311)			-

Notes. p - differences between groups according to Pearson criterion χ^2 , including Yates' correction;

* - by criterion U of Mann-Whitney;

** - associations by Cramer V-criterion (Cramer's V) and Spearman correlation coefficient (ρ)

DISCUSSION

At different degrees of tumor differentiation in laryngeal cancer patients with metastatic neoplasm, the proportion of patients without cytokeratin 19 expression was sig-

nificantly higher than among patients of the comparison group, without statistically significant differences only at a low degree (p=0.035 in G3, p<0.001 in G2 and p=0.124 in G3).

Table III. Assessment of prognostic potential of cytokeratin 19 for regional metastasis of laryngeal cancer depending on the degree of tumor differentiation according to ROC-analysis

Characteristics	Se, %	Sp, %	AUC (95 % CI AUC)	p	Assessment AUC	Optimal cut-off point
Degree of tumor differentiation n (%)						
High G1	83,33	87,50	0,865 (0,581 - 0,985)	0,004	very good	>9,42 %
Moderate G2	81,48	79,17	0,840 (0,710 - 0,927)	<0,001	very good	>9,94 %
Low G3	100,00	80,00	0,800 (0,422 - 0,979)	0,131	very good	>8,05 %
Total	81,08	81,08	0,841 (0,737 - 0,916)	0,134	very good	>9,94 %

Notes. Se - Sensitivity;
 Sp - Specificity;
 AUC - Area Under ROC Curve;
 p - level of significance of AUC;
 AUC - quality assessment of predictability of marker;
 Optimal cut-off point - the optimal cut-off point

Moderate level of marker expression also did not differ in the comparison groups with a low degree of tumor differentiation ($p=0.178$). In cases of moderate and high degree of differentiation, it was significantly higher in the group of patients with metastases compared with patients without metastases ($p < 0.001$ and $p=0.028$, respectively). Overall, a moderate level of expression of cytokeratin 19 in laryngeal cancer patients with metastases was 27.50% (12.50; 52.50) – Me (25%; 75%), whereas in the group without metastases – 6.58% (3,19; 9,44), $p < 0.001$; the difference in median marker values in the comparison groups is 18.14%, 95% CI (10.03 – 25.03).

An evaluation of the association between metastases and the level of cytokeratin 19 expression in lymph nodes according to Cramer criterion ($V=0.64$) and Spearman's rank correlation coefficient ($\rho = 0.63$ $p < 0.001$) showed a significant relationship between them as a whole in all examined and in patients with moderate and high degree of tumor differentiation. This is an evidence of the significant diagnostic value of the expression of this molecular marker in the detection of regional dissemination of tumor. To assess the ability of cytokeratin 19 to predict regional dissemination of tumor in patients with laryngeal cancer, ROC analysis was performed (Fig. 2, Table III).

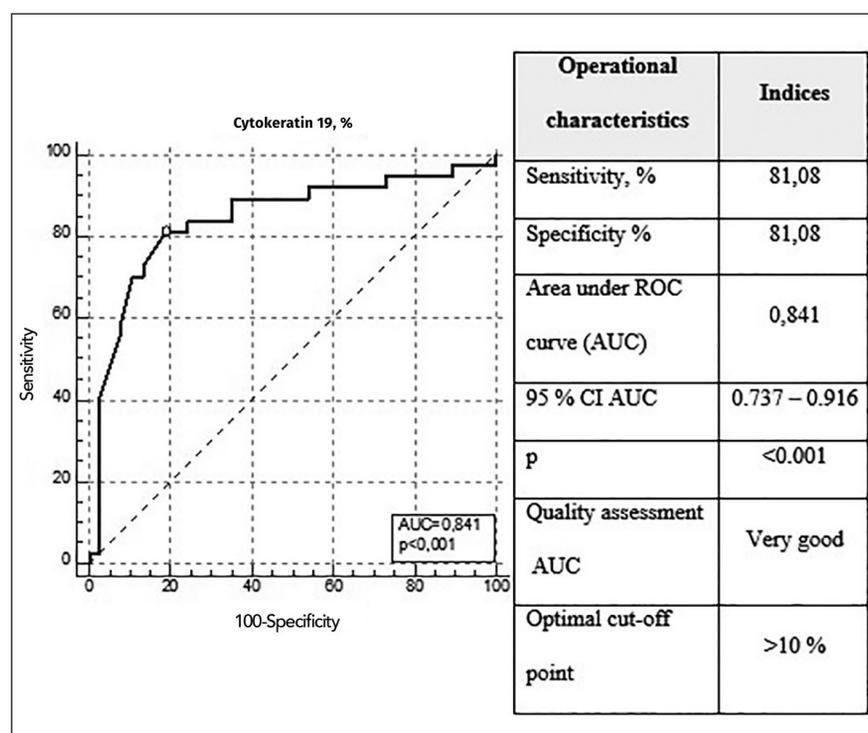


Fig. 2. Assessment of the possibility of predicting regional metastases of laryngeal cancer based on the level of cytokeratin 19 expression in lymph nodes according to ROC-analysis.

Statistically significant ($p < 0.001$), very good operational characteristics in cytokeratin 19 for the prediction of regional metastases of laryngeal cancer were found: sensitivity – 81.08%; specificity – 81.08%; area under the ROC curve AUC=0.841, 95% CI (0.737 – 0.916).

An assessment of the prognostic potential of cytokeratin 19 for regional laryngeal cancer metastases showed that this marker had very good operational characteristics regardless of the degree of tumor differentiation (no statistically significant AUC was obtained for low tumor differentiation).

These results of ROC-analysis suggest that prediction of regional laryngeal cancer metastases is possible on the basis of cytokeratin 19 expression. The critical value of the marker expression level by the optimal cut-off point is $> 9.94\%$, i.e. a weak expression level.

In weak and higher levels of cytokeratin 19 expression patients have a higher chance of regional metastasis of laryngeal cancer – the odds ratio OR is=18.37 (95% CI 5.74 – 58.79; $p < 0.001$).

CONCLUSIONS

1. In immunocytochemical study of the expression level of cytokeratin 19 in patients with squamous cell laryngeal cancer, positive expression was determined in 37 (50.0%) patients with a low level – 11.22% (5.79; 27.50); in more than a third of patients with moderate (13 patients – 17.57%) and marked (12 patients – 16.22%) expression of the marker.
2. In laryngeal cancer patients with regional metastases, the values of expression of the molecular marker were 18.14% higher (95% CI 10.03 – 25.03) by median values.
3. The diagnostic value of cytokeratin 19 expression in relation to regional metastasis was determined by Cramer criterion ($V = 0.64$), Spearman correlation coefficient ($\rho = 0.63$, $p < 0.001$), ROC- analysis (very good operational characteristics: sensitivity – 81,08%; specificity – 81,08%; AUC = 0.841, $p < 0.001$).
4. To predict the development of laryngeal metastases in laryngeal cancer patients, it is recommended to carry out an immunocytochemical study using a diagnostic marker of cytokeratin 19. The positive status of cytokeratin 19 increases the chances of regional metastasis of laryngeal cancer by 18.37 times ($p < 0.001$) compared with absence of expression.
5. The prospects for further research are aimed at investigating additional molecular markers for determining optimal therapeutic and diagnostic tactics in regional laryngeal cancer metastases.

REFERENCES

1. Johnson N. W., Amarasinghe H. K. Epidemiology and Aetiology of Head and Neck Cancers. In: Jacques Bernier eds. Head and Neck Cancer Multimodality Management. New York: Springer; 2011, p.1-57
2. Economopoulou P., de Bree R., Kotsantis I., Psyrri A. Diagnostic Tumor Markers in Head and Neck Squamous Cell Carcinoma (HNSCC) in the Clinical Setting. *Front. Oncol.* 2019; 9:827. doi: 10.3389/fonc.2019.00827
3. Woolgar J.A., Triantafyllou A., Lewis J.S. Jr. et al. Prognostic biological features in neck dissection specimens. *Eur Arch Otorhinolaryngology.* 2013;270(5):1581-1592
4. Robbins K.T., Ferlito A., Shah J.P. et al. The evolving role of selective neck dissection for head and neck squamous cell carcinoma. *Eur Arch Otorhinolaryngology.* 2013;270(4):1195-1202
5. de Kort W. B., Maas S. L. N., Van Es R. J. J. et al. Prognostic value of the nodal yield in head and neck squamous cell carcinoma: a systematic review. *Head and Neck.* 2019;41(8):1209-1219
6. Rodrigo J.P., Grilli G., Shah J.P. et al. Selective neck dissection in surgically treated head and neck squamous cell carcinoma patients with a clinically positive neck: Systematic review. *European Journal of Surgical Oncology.* 2018;44:395-403
7. Flach G.B., Tenhagen M., de Bree R. et al. Outcome of patients with early stage oral cancer managed by an observation strategy towards the N0 neck using ultrasound guided fine needle aspiration cytology: no survival difference as compared to elective neck dissection. *Oral Oncology.* 2013;49:157-164
8. Shores C.G., Yin X., Funkhouser W. et al. Clinical evaluation of a new molecular method for detection of micrometastases in head and neck squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg.* 2004;130(8):937-942
9. Werner S., Keller L., Pantel K. Epithelial keratins: biology and implications as diagnostic markers for liquid biopsies. *Mol. Aspects Med.* 2019. <https://doi.org/10.1016/j.mam.2019.09.001>
10. Ali A.N., Switchenko J.M., Kim S. et al. A model and nomogram to predict tumor site origin for squamous cell cancer confined to cervical lymph nodes. *Cancer.* 2014;1:1-8
11. Coskun H.H., Medina J.E., Robbins K.T. et al. Current philosophy in the surgical management of neck metastases for head and neck squamous cell carcinoma. *Head Neck.* 2015;37:915-926.

The work is performed within the framework of the department's research: "Optimization of diagnosis and treatment of diseases of the upper respiratory tract and ears", № state registration 0118U006634, deadline 2022 year.

ORCID and contributionship:

Oleksander V. Kovtunencko: 0000-0002-0562-7684 ^A

Bohdan V. Shportko: 0000-0002-4242-2784 ^{B,C,D,F}

Serhey M. Tymchuk: 0000-0002-8706-2313 ^E

Conflict of interest:

The Authors declare no conflict of interest.

CORRESPONDING AUTHOR

Bohdan V. Shportko

Municipal Institution

«I. Mechnikov Dnipropetrovsk Regional Clinical Hospital»

7/428 Monitorna St., 49018 Dnipro, Ukraine

tel: +380505920776

e-mail: bogdan.shportko@gmail.com

Received: 18.01.2020

Accepted: 10.06.2020

A – Work concept and design, B – Data collection and analysis, C – Responsibility for statistical analysis, D – Writing the article, E – Critical review, F – Final approval of the article