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DNA Ploidy, Cyclin D1, bcl-2 and Lymphocytic Infiltration of the Tumor Microenvironment as Prognostic Factors in Laryngeal Cancer Patients

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The aim of this study was to evaluate correlations between DNA ploidy type, the immunostaining of cyclin D1, bcl-2 and the intensity of lymphocytic infiltration of the tumor microenvironment in relation to the histopathological G differentiation and pTNM classification. Thirty two patients were treated surgically for laryngeal cancer with total or partial laryngectomy in the Department of Otolaryngology Zabrze. The percentage of bcl-2 immunostaining was showed in 53% of the cases and was found to correlate with G differentiation and the patients' age. Cyclin D1 antigen stained positively in 24 cases (75%). Expression of cyclin D1 correlated with cancer stage. Cyclin D1 negative stain was found in T4-stage. DNA ploidy was examined in 19 cases. Aneuploidy was found in 5 cases only, while the rest were diploid. DNA ploidy value correlated with cyclin D1 expression. All paraffin sections were found to contain lymphocytic infiltrations of CD43 and CD45RO phenotype in the tumor front. Some cases showed high intensity of lymphocytic infiltrations of CD45RO phenotype. The intensity of CD43 lymphocytic infiltrations in the tumor front was related to the expression of cyclin D1 and bcl-2.

Introduction

Squamous cell carcinoma of the head and neck affects more than 500,000 people worldwide each year. Local-regional recurrence is a common and challenging oncological problem. Identification of local relapse risk factors after appropriate local surgical therapy, radiation or combined treatment continues to be an object of active clinical research. Development of novel molecular markers has encouraged numerous studies aimed at evaluating their prognostic significance and potential clinical utility in identifying patients at risk for local-regional relapse [18].

DNA ploidy analysis in squamous carcinoma of the thorax seems to have much wider prognostic utility. As regards squamous carcinoma of the thorax the opinions voiced by various authors on the role of DNA ploidy analysis are controversial.

Immunohistopathological analysis of the lymphocytic infiltrations that surrounds a tumor is one of the latest pathomorphological methods of defining local immune response to cancerous cells. Defining the number of lymphocytic infiltrations in the area of the tumor edge zone is a component of "tumor front grading", a modern method of evaluating changes at the front of tumor infiltration [20]. And it is here that we face the need to analyze in detail the content of the lymphocytic infiltration, which is one of the natural defensive barriers of the body against cancer.

The aim of the study was to investigate a relation between DNA ploidy, cyclin D1 immunostaining, bcl-2 and the lymphocytic infiltration of the tumor microenvironment in reference to a histopathological grade (G) and pTNM classification in laryngeal cancer patients.

Material and Methods

Our analysis covered laryngeal cancer patients who were treated surgically between 1999 and 2004 at the Department of Otolaryngology, Medical University of Silesia in Zabrze. In the first step of this study, the postoperative material was collected from 32 patients treated with total or partial laryngectomy with simultaneous unilateral or bilateral lymphan-

giectomy. The patients' age ranged from 43 to 72, a mean of 59 years. There were 6 females and 26 males, all residents of the Upper Silesia region.

The neoplasm sites in the analyzed groups was as follows: glottis and epiglottis – 35%; glottis – 26%; glottis and subglottis – 4%; all larynx levels – 35%. According to the local progression stage, one patient was qualified as T1 stage, 4 patients were qualified as T2 stage (no local lymph nodes metastases), 5 patients as T3 (one lymph node metastasis), 22 patients as T4 (11 – N1 stage, 3 – N2 stage). There were 15 patients with lymph node metastases and 17 patients with none. The post operative specimens were prepared at the Pathomorphology Department of the Medical University of Silesia in Zabrze.

The material for study was acquired in one of the following ways:

– paraffin blocks for histopathological and immunohistochemical examinations;
– aspiration and impression cytology for DNA ploidy examination.

The cytological material was collected from the postoperative specimens before they were fixed in formalin. Next the laryngeal cancer specimens were fixed in a 10% solution of buffered formalin for 24–48 hours. Having been fixed, the postoperative preparations were processed with a routine histopathological technique. The sections were collected according to the pTNM classification and processed with the routine histopathological techniques of hematoxylin and eosin staining.

The histopathological preparations were examined with a light microscope (Labophot, Nikkon, Japan) at magnifications ranging between 40× and 400×. The grade of cancers was assessed according to the traditional criteria provided by Broders. The stage was assessed according to the pTNM scale with further regard to the manner of neoplastic expansion and the intensity of lymphocytic infiltrations.

There were two ways of acquiring the cytological material from the specimens of squamous laryngeal carcinoma:

1) Before it was fixed in a formalin solution, the laryngeal cancer preparation had been cut and a preparation was made with an impression method at the site of the tumor occurrence.
2) Cancerous cells were aspired with a needle of 0.7 mm-diameter and cytological smears were made.

Next, the preparations were dried and after 48–72 hours fixed in a 4% formalin solution for 1/5 hour. Examination of DNA ploidy was carried out at the Pathology Unit of the Oncological Centre – M. Curie's Institute in Warsaw. The preparations were stained with Feulgen's method and a set of DNA quantitative analysis agents (Becton-Dickinson Image Cytometry Systems B.V. Leiden, Holland). In the course of Feulgen's reaction, nuclear DNA was hydrolyzed with 5N hydrochloric acid for 60 minutes, washed with distilled water, dehydrated in alcohol, lighted with xylene and "closed" with Permount gel medium (Lipshaw).

The semi-automatic, quantitative analysis of the cell nuclei was made with a static cytophotometer CAS-200 of Becton Dickinson (Reichert MicroStar microscope, a monochromatic video camera, IBM-AT/386 microcomputer, an image monitor, and a text monitor) while the software used was 2.5 Version of QDA (Quantitative DNA Analysis) of Cell Analysis Systems, Inc. Lombard, Illinois, USA. The preparations were made on the basic CAS dish (Becton Dickinson). The apparatus was calibrated *via* assessment of 20–30 reference cells (tetraploid hepatocytes of the rat), provided on the basic dish along with a set for DNA quantitative analysis. The changeability variable (CV), assessed each time in the course of reference cell measurement for the preparations from the same wash, ranged between 0.8 and 2.8%. To avoid some accidental, double measurement of the analyzed cells nuclei the XYSet option of the programme menu was used during the assessment. In each case approximately 100 cell nuclei (fewer in some cases) were measured from the average of 10 fields at the magnification of 400x. The final DNA ploidy was assumed to be the one with the highest DNA index in the main peak. The measurements excluded the nuclei of technically damaged cells which either overlapped or had entered the mitosis phase. The measured cells that lay in groups were separated with a graphic cursor. The changeability variable, assessed in the preparations during the measurement of cancerous cells, ranged from 7% to 15%. Additionally, some 20 non-cancerous cells in each sample were measured as part of internal control.

DNA histograms were classified as follows:

– diploid DNA arrangement, DI (DNA Index) 0.85–1.5 and less than 20% of the cells with DNA content exceeding the diploid DNA population, absence of cells with DI exceeding 2.32;
– aneuploid DNA arrangement, DNA exceeding 1.15;
– hypoploid DNA arrangement, DI less than 0.85.

The immunohistochemical examinations were carried out according to the following methods:

1) DAKOLSAB → 2 KIT Peroxidase [LABELLED STREPTAVIDIN BIOTIN (LSAB) METHOD], DAKO CORPORATION Kit, Catalogue No. K0677.
2) Streptavidin-Biotin-Complex-HRP Duet (Strept.ABC Complex/HRP Duet, Mouse/Rabbit, Dako A/S, Denmark), Catalogue No. K0492.

Antigen bcl-2 was evaluated with the use of the DAKO M0887 monoclonal antibody, clone 124. Antigen

cyclin D1 was evaluated with DAKO M7155 monoclonal antibody, clone DCS-6. Reaction with the above antibodies was evaluated in two ways: by estimating the percentage of positive tumor cells (0–75% range) in the total volume of the preparation and semi-quantitatively, as the intensity of the reaction using the 0–3 scale in 5 different fields of vision (magnification of 400×).

The lymphocytic infiltration in the tumor microenvironment was analyzed immunohistochemically according to the ABC method (Avidin-Biotin-Complex Kit, Duet DAKO, Copenhagen) with DAKO monoclonal antibodies used for the following antigens: CD20 (Code No. M756), CD43 (Code No. M0786), CD45RO (Code No. M0742). The above reactions were assessed in two ways: estimating the percentage of the cells surrounding the tissue of the positive reaction tumor (0–75% range) in the whole volume of the preparation or semi-quantitatively as the intensity of the reaction using the scale of 0–3 in different high power fields (400x magnification). DAB was used as the chromogen. The intensity of the color reaction was marked as follows: 0 – negative, 1 – slight, 2 – moderate, 3 – strong. Intensive dark brown coloring of the cell cytoplasm was assumed to signify positive reaction. The so-called background was not estimated. A high power field was assumed to be a magnification of 400x. The reaction labeling was carried out by a team of two diagnostic experts. Statistical analysis was carried out with the use of a Pearson correlation test and Spearman correlation test. It was assumed that the significance level was p=0.05.

Results

Squamous cell carcinoma was found histopathologically in all 32 cases but one, the exception being spindle cell carcinoma. The grade was as follows: G1 – 2 cases; G2 – 15 cases; G3 – 15 cases (including the case of spindle cell carcinoma).

Antigen bcl-2 was found in 17 specimens only – 53% of all the specimens examined – in 12 cases the positive color reaction took place in the carcinoma preparations of G2 or G1, while G3 was positively stained in 5 cases only. The percentage of positively stained cells fell within 5–30% range of all cancerous cells. In one case the percentage did not exceed 5% of the cells, while in one it exceeded 50% The infiltration intensity was classified as "1" or "2". Statistical correlation was demonstrated between tumor grade and the intensity of bcl-2 staining (p=0.006). No correlation was found between cancer progress (stage) and bcl-2 expression. Likewise, no correlation emerged between the tumor location and the presence of staining reaction. Analysis of the correlation between the patients' age and the percentage of positively stained reactions (bcl-2) revealed a difference of borderline statistical significance (p=0.059).

Positive reaction with cyclin D1 antigen was present in 24 cases only, which amounted to 75% of the study material. It was absent in 4 carcinoma specimens of G2 and 4 – G3. No staining reaction was found in tumors of pT4 stage (in 4 out of 8 of those specimens metastases developed in local lymph nodes). In 3 tumors the percentage of stained cells was lower than 5%, in one case it exceeded 75% and in the remaining cases ranged between 5 and 30% of the cells. In patients with regional lymph nodes metastases (N1, N2) the positive color reaction with cyclin D1 was present in 8 cases, while in 3 it was negative. The study noted a relation between the degree of cyclin D1 staining and T quality (p=0.008084451). No correlation was found between the tumor grade and the expression of cyclin D1. As regards cyclin D1 antigen, no correlation was noted between the location of the tumor and the presence of positive reaction. The percentage of lymphocytic infiltrations stained for bcl-2 and cyclin D1 is presented in Table 1. The intensity and degree of lymphocytic infiltration of the CD20 phenotype was negligent in the preparations and therefore ignored in the analysis.

Analysis of the expression of CD43 and CD45RO antigens in the tumor microenvironment revealed positive staining in all the specimens. The intensity of the reaction with CD43 antigen was found to be slight and was marked as 1 or 2. The percentage of positive cells ranged between 5 and 50% and in 6 cases the staining reactions of the lymphocytic infiltrations in the tumor front were very weak, falling below 5% (1 case of G2 cancer and 5 cases of G3). No correlation was found between G and T qualities and the expression of CD43 antigen. CD45RO expression was stronger in the lymphocytic infiltration surrounding the tumor. The percentage of positive lymphocytes ranged between 10 and 75%. Only one tumor showed the number of stained cells within the range of 5–10%, while in only one it exceeded 75%. The intensity of positive reaction in most specimens was defined as 2–3, and in 11 cases as mere 3. Only in 3 biopsies was the intensity of reaction marked as 1; the same specimens simultaneously displayed very weak staining reaction with the use of CD43 antibody. No correlation was found between G and pTNM and the expression of CD45RO antigen. A correlation was found between the percentage of CD43- and CD45RO-positive cells (p=0.022) in the preparations. Furthermore, a correlation was encountered between the percent content of the bcl-2- and CD43-positive cells (p=0.0007). As regards the two examined lymphocytic subpopulations no correlation was noted between the intensity of the infiltration on the one hand, and the grade and stage on the other. Likewise,

TABLE 1
Immunohistochemical evaluation of bcl-2 and cyclin D1 antigens in paraffin section of laryngeal cancer

Patient	**Age (years)**	**Sex**	TNM	**Grade**	**Bcl-2** Percentage of positive cells	**Bcl-2** Reaction intensity	**Cyclin D1** Percentage of positive cells	**Cyclin D1** Reaction intensity
1	58	M	T_1N_0	3	0	0	2	2
2	64	M	T_2N_0	2	3	2	2	2
3	60	M	T_2N_0	2	0	0	1	1
4	63	M	T_2N_0	3	0	0	6	3
5	55	M	T_2N_0	2	3	2	2	2
6	49	M	T_3N_0	2	0	0	4	2
7	44	K	T_3N_0	2	5	2	4	2
8	47	M	T_3N_0	3	0	0	2	1
9	62	M	T_3N_0	2	4	2	4	2
10	59	K	T_3N_1	1	2	2	2	2
11	58	M	T_4N_0	3	0	0	0	0
12	48	M	T_4N_0	2	4	2	0	0
13	67	M	T_4N_0	2	0	0	3	2
14	54	M	T_4N_0	3	3	2	3	2
15	54	K	T_4N_0	3	2	1	2	1
16	60	M	T_4N_0	3	0	0	2	1
17	60	M	T_4N_0	3	0	0	0	0
18	62	M	T_4N_0	3	0	0	0	0
19	62	M	T_4N_1	2	1	2	4	2
20	69	M	T_4N_1	3	3	1	0	0
21	72	M	T_4N_1	2	1	1	0	0
22	69	M	T_4N_1	2	2	1	2	1
23	52	M	T_4N_1	1	3	1	2	2
24	71	M	T_4N_1	2	0	0	0	0
25	69	M	T_4N_1	3	2	1	2	1
26	51	K	T_4N_1	2	3	2	3	2
27	53	M	T_4N_1	2	2	2	2	1
28	54	K	T_4N_1	3	0	0	3	3
29	72	M	T_4N_1	2	0	0	1	1
30	56	M	T_4N_2	3	0	0	2	1
31	46	K	T_4N_2	3	0	0	1	1
32	62	M	T_4N_2	3	3	1	0	0

Legend (Tables 1 and 2): Reaction intensity: 0 – negative, 1 – slight, 2 – moderate, 3 – strong; scoring system for bcl-2 and cyclin D1: 0 – negative, 1 – <5% of the cells evaluated, 2 – 5–10% of the cells, 3 – 11–25% of the cells, 4 – 26–50% of the cells, 5 – 51–75% of the cells, 6 – >75% of the cells

no correlation was found between the expression of cyclin D1 antigen and the intensity of lymphocytic infiltration with CD43 and CD45RO phenotypes. The immunohistochemical assessment of the tumor microenvironment antigens – CD43 and CD45RO in the histopathological preparations of laryngeal cancer is presented in Table 2.

TABLE 2
The immunohistochemical analysis of CD43 and CD45RO antigens in paraffin sections of laryngeal cancer

Patient	Age (years)	Sex	TNM	Grade	CD43 Percentage of positive cells	CD43 Reaction intensity	CD45RO Percentage of positive cells	CD45RO Reaction intensity
1	58	M	T_1N_0	G3	1	1	3	1
2	64	M	T_2N_0	G2	3	2	5	1
3	63	M	T_2N_0	G3	4	2	5	3
4	55	M	T_2N_0	G2	2	1	4	2
5	60	M	T_2N_0	G2	2	1	4	3
6	49	M	T_3N_0	G2	2	1	5	3
7	44	K	T_3N_0	G2	3	1	4	3
8	47	M	T_3N_0	G3	2	1	4	3
9	62	M	T_3N_0	G2	4	1	3	1
10	59	K	T_3N_1	G1	3	1	4	3
11	60	M	T_4N_0	G3	1	1	3	1
12	62	M	T_4N_0	G3	1	1	3	2
13	48	M	T_4N_0	G2	4	2	5	3
14	58	M	T_4N_0	G3	3	1	4	2
15	67	M	T_4N_0	G2	2	1	4	2
16	54	M	T_4N_0	G3	3	2	4	3
17	54	K	T_4N_0	G3	3	1	4	2
18	60	M	T_4N_0	G3	2	1	2	2
19	51	K	T_4N_1	G2	2	1	5	3
20	53	M	T_4N_1	G2	4	3	5	2
21	71	M	T_4N_1	G2	3	1	3	2
22	69	M	T_4N_1	G3	3	1	4	2
23	54	K	T_4N_1	G3	2	1	4	3
24	72	M	T_4N_1	G2	1	1	5	3
25	62	M	T_4N_1	G2	2	1	3	2
26	69	M	T_4N_1	G3	3	2	5	3
27	72	M	T_4N_1	G2	2	1	3	2
28	69	M	T_4N_1	G2	3	2	6	3
29	52	M	T_4N_1	G1	3	2	4	2
30	46	K	T_4N_2	G3	1	1	2	2
31	56	M	T_4N_2	G3	1	1	3	2
32	62	M	T_4N_2	G3	2	1	5	3

Due to technical reasons ploidy examination was carried out on 19 cancer specimens only, the remaining cytological smears were unfit for assessment because of their scarcity. The values of DNA ploidy fell within the range of 0.85–1.80. Five laryngeal tumors of T4 stage were aneuploid. In the remaining 14 cancers the arrangement was diploid. Likewise, diploid turned out to be the arrangement in the specimen of the spindle cell carcinoma. Neither hypoploid nor polyploid arrangement was discovered in the cytological smears (Table 3). What is more, some relation was

TABLE 3
DNA content and ploidy type in relation to the grade and stage in laryngeal cancer patients

Patient (number)	G	TNM	Value of DNA ploidy	Type of ploidy
1	G3	T_1N_0	1.01	Diploidy
2	G2	T_2N_0	0.95	Diploidy
3	G3	T_2N_0	0.88	Diploidy
4	G3	T_3N_0	0.99	Diploidy
5	G1	T_3N_1	0.99	Diploidy
6	G3	T_4N_0	1.03	Diploidy
7	G2	T_4N_0	1.56	Aneuploidy
8	G3	T_4N_0	1.03	Diploidy
9	G3	T_4N_0	1.60	Aneuploidy
10	G3	T_4N_0	1.47	Aneuploidy
11	G3	T_4N_0	1.05	Diploidy
12	G2	T_4N_1	1.59	Aneuploidy
13	G2	T_4N_1	1.03	Diploidy
14	G1	T_4N_1	0. 89	Diploidy
15	G2	T_4N_1	0.91	Diploidy
16	G2	T_4N_1	1.04	Diploidy
17	G3	T_4N_1	0.85	Diploidy
18	G2	T_4N_1	1.80	Aneuploidy
19	G3	T_4N_2	1.01	Diploidy

found between the intensity of cyclin D1 staining and DNA ploidy content (p=0.01). Statistical analysis revealed correlation between DNA ploidy value and the percentage of stained cells with CD43 phenotype (p=0.038) (Table 4).

Discussion

Assessing DNA ploidy type, examining apoptosis marker (e.g. bcl-2) and proliferation antigens, assessment of Tumor Front Grading with the definition of the inflammatory infiltration phenotype composition belong to modern research methods that complement the traditional criteria of histopathological cancer evaluation [6, 9].

Bcl-2 antigen is the apoptotic molecule and it plays a role in the prolongation of cell survival, which may subsequently contribute to the development of human laryngeal cancer [4, 6].

DNA ploidy is a recognized prognostic factor and it is assumed that the diploid arrangement of the carcinoma may indicate better prognostication, although in the cases of head and neck carcinoma opinions vary [1, 10, 17]. In laryngeal cancer a high percentage of diploid tumors is encountered in tumors of well or moderate differentiation, while those of poor differentiation tend to be aneuploid [8]. Carcinomas of the T3–T4 stages present the aneuploid type [19]. Metastases in regional lymph nodes appear more frequently in poorly differentiated tumors of the aneuploid type [16, 22], though some researchers do not confirm this statement [14]. Our study encountered aneuploid arrangement in 5 cases of advanced carcinoma. Furthermore, the relation was found between the ploidy value and cyclin D1 antigen expression on the one hand and the intensity of CD43 lymphocytic infiltration on the other.

Cyclin D1 has a utility as a prognostic factor in the laryngeal cancer research. Cyclin D1 presence in the cancerous tissue was found to correlate with tobacco smoking [15]. More frequent cyclin D1 expression was encountered in the carcinomas situated in the epiglottis area. This expression might be related to the presence of lymph node metastases and the stage [3]. Other authors failed to find significant relation between cyclin D1 staining and the clinical course of the disease [12, 13]. This study noted a relation between bcl-2 staining and the tumor stage. It is controver-

TABLE 4
Statistical correlation between the factors analyzed

	G	Age	Bcl-2	Cyclin D1	T quality	CD43	CD45RO	DNA ploidy
G								
Age								
Bcl-2	**p=0.006**	**p=0.059**				**p=0.0007**		
Cyclin D1					**p=0.008084451**	**p=0.038**		**p=0.01**
T quality								
CD43							**p=0. 022**	

sial whether bcl-2 antibody labeling in the specimens of laryngeal cancer is a prognostic factor as no significant relation was found between the analyzed factors [2, 11, 15]. Our study demonstrated a relation between bcl-2 expression, tumor grade, patients' age and lymphatic infiltration intensity (CD43).

Examination of the neoplasm microenvironment plays a major role in the analysis of prognostic factors. Lymphocytic infiltrations in laryngeal cancer tend to be present in two locations – tumor mass and tumor stroma [5]. The lymphocytes of the CD43 order as a prognostic factor play a positive role, which has been demonstrated in previous research [4, 7]. The immune response in laryngeal cancer appears mostly as the T-cell response, what accounts for distinct CD45RO-immunopositivity visible in lymphocytes [7, 21].

Conclusions

1) Examinations of the microenvironment of cancer confirm the major role played by T-cell response *via* the presence of lymphocytes of the CD45RO and CD43 phenotypes.
2) DNA ploidy is an important prognostic factor in laryngeal cancer. The aneuploid arrangement seems to be associated with the advanced stage of laryngeal cancer.
3) Cyclin D1 antigen expression correlates with the tumor stage and DNA ploidy value.
4) bcl-2 antigen labeling in laryngeal cancer has some diagnostic utility and may correlate with cancer grade and patients' age.
5) The intensity of CD43 lymphocytic infiltrations in the tumor front is related with the expression of cyclin D1 and bcl-2 antigens.

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