

**Aim of the study:** Intercellular adhesion molecules present in immunocompetent cells as well as endothelium and tumour cells can regulate cell migration, angiogenesis, apoptosis, proliferation, and metastases in solid tumours. The aim of this study was to analyse the sICAM-1 (soluble intercellular adhesion molecule 1) and sVCAM-1 (soluble vascular cell adhesion molecule 1) expression in peripheral blood mononuclear cell (PBMC) cultures, and to find their relationships with clinicomorphological characteristics in laryngeal cancer.

**Materials and methods:** The analysis included a group of 50 patients with verified squamous cell carcinoma of the larynx. The control group constituted 30 healthy volunteers. The pathological assessment included pTNM, stage, histological grade, and type of invasion according to the tumour front grading. The expression of adhesion molecules was assessed using the enzyme-linked immunosorbent assay (ELISA).

**Results:** Increased expression of sICAM-1 and sVCAM-1 was an indicator of more aggressive laryngeal carcinomas. More advanced local changes evaluated on the pT feature were connected with a higher sVCAM-1 ( $p = 0.017$ ), but not sICAM-1 level. The presence of lymph node metastases correlated with a higher expression of adhesion molecules ( $p = 0.012$  and  $p = 0.003$ , for sICAM-1 and sVCAM-1, respectively). Tumours with more diffuse growth and infiltrating with small cell groups ( $< 15/\text{hpf}$ ) was characterised by the highest level of adhesive proteins ( $p = 0.001$  and  $p = 0.02$  for sICAM and sVCAM, respectively). Moreover, lower levels of sICAM-1 and sVCAM-1 were observed more frequently in patients who lived longer than five years after treatment.

**Conclusions:** The study indicates the importance of the sICAM and sVCAM expression as indicators of advanced changes and prognosis in patients with laryngeal carcinoma.

**Key words:** laryngeal carcinoma, intracellular adhesive molecules, mode of invasion.

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## Expression of cell adhesion molecules in laryngeal carcinoma – preliminary analysis

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### Introduction

Determination proven tumour markers in the blood or other body fluids of patients with various cancer types plays an important role in the diagnosis, treatment planning, assessing prognosis, and monitoring of therapy in modern oncology [1–4]. Unfortunately, despite intensive research, no adequate and sensitive unequivocal immunological markers have been identified so far in the case of various cancers. Laryngeal cancer is the one of the most common malignancies of the head and neck region in the male population, and it remains a major oncological problem in Poland [5]. Neoplastic lesions of the larynx are most often detected at an advanced stage, and proper planning of the extent of surgery frequently presents major difficulties [1, 2, 6]. Identifying biochemical, specific, and sensitive markers of tumour staging would help to optimise the choice of therapy and would improve prognosis in patients with laryngeal cancer.

Intercellular adhesion molecule 1 (ICAM-1) belongs to the immunoglobulin superfamily (IgSF CAMs). This protein is located on the surface of various cells and is involved in binding with other cells or with extracellular matrix elements (ECM). Vascular cell adhesion protein 1 (VCAM-1), also known as cluster of differentiation 106 (CD106), is a protein that mediates the adhesion of lymphocytes, monocytes, eosinophils, and basophils to vascular endothelium. It also functions in leukocyte-endothelial cell signal transduction [7]. ICAM-1 and VCAM-1 are also present on the surface of activated tumour infiltrated leukocytes (TILs) and inflamed vascular endothelial cells within the tumour weave. Many studies indicate a significant share of VCAM-1 and ICAM-1 in the phenomena of carcinogenesis. It has been shown that adhesive proteins are involved in angiogenesis, invasion, and metastasis, as well as in the local inflammatory reaction in response to tumour-associated antigens (TAA) [7, 8]. In many studies the relationships between ICAM-1 polymorphisms and increased incidence of carcinomas with different origin, e.g. brain and oral cavity, have been established [9, 10]. Interestingly, in the case of lung cancer, colon cancer, melanoma, glioblastoma, primary liver cancer, or multiple myeloma an increase of soluble forms of ICAM-1 and VCAM-1 (sICAM-1, sVCAM-1) levels in blood correlated with a higher clinical and morphological tumour stage [1, 3, 6, 8, 11–13]. So far, however, there is no publication describing sICAM-1 and sVCAM-1 levels to assess the severity of squamous cell carcinoma of the larynx. Therefore, it seems to be justified to

analyse the adhesion molecule expression with respect to indication of sICAM-1 and sVCAM-1 as new biomarkers of invasive tumour phenotype laryngeal cancer.

The aim of this study was to evaluate the sICAM-1 and sVCAM-1 expression in peripheral blood mononuclear cell (PBMC) cultures, and to find the relationships with clinicomorphological characteristics such as pTNM, stage, grade, and type of invasion in laryngeal cancer.

### Material and methods

The analysis included a group of 50 patients (48 male and 2 female; aged 45–79 years; mean age 62.5 ± 8.3 years) with verified squamous cell carcinoma of the larynx. The control group consisted of 30 healthy volunteers (21 male and 9 female; aged 40–65 years; mean age 54.2 ± 5.9 years). Criteria for patient participation in this study were as follows: a pathologically confirmed diagnosis of *carcinoma planoepitheliale*, primary surgical resection without receiving prior immuno-, radio-, or chemotherapy, and the absence of distant metastasis. Clinicomorphological characteristics of the studied group are shown in Table 1.

### Histological classification and morphological features

The pathological assessment criteria included pTNM, stage S, histological grade G, and type (mode) of invasion according to the tumour front grading (TFG). For histological classification of tumours, archival paraffin-embedded tissue samples were utilised. All specimens were assessed according to the criteria conducted in accordance with the AJCC TNM classification of 2010 for laryngeal cancers

[14]. Morphological estimation of tumour borderline was performed on HE-stained sections in the most invasive, peripheral zones of the tumour, according to tumour front grading (TFG), one of the most reliable pathological methods for the analysis of neoplastic progress and determination of the dynamics of tumour growth [15]. The type of neoplastic infiltration was assessed in at least five different regions of the peripheral part of the tumour (magnification 200×). According to the TFG classification the mode of invasion was graded as follows: 1 point – well-defined borderline; 2 points – cords, less marked borderline; 3 points – groups of cells, no distinct borderline; 4 points – diffuse growth of small cell groups (< 15/hpf). The histological grade of differentiation, G was measured according to the generally accepted three-grade morphological system: G1 (well-differentiated tumour), G2 (moderately-differentiated tumour), G3 (poorly-differentiated tumour).

### Lymphocyte isolation and ELISA tests for sICAM-1 and sVCAM-1

For peripheral blood mononuclear cell (PBMC) isolation, venous blood was obtained from each patient (10 ml) and transferred into test tubes containing heparin (10 U/ml). The control blood samples were obtained from 30 healthy volunteers without a history of malignancies or autoimmune disorders. PBMCs were isolated by Ficoll-Hypaque density gradient and resuspended in RPMI 1649 medium to obtain the concentration of  $1 \times 10^6$  cells/ml. The recovered cells were checked and counted for viability with trypan blue staining method. The isolated PBMC cultures were incubated for 21 hours at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> (Cellstar Incubator) in 96-well plates in a final volume of 0.2 ml (per well). The supernatants of cultures were collected and the secretion pattern of sICAM-1 and sVCAM-1 was measured with specific enzyme-linked immunosorbent assay ELISA Kit (R&D Systems, Inc.; Minneapolis, MN, USA) according to the manufacturer's instructions. Absorbance was measured with an ELISA reader (Multiscan RC 351). The sensitivity of this assay was 0.096 pg/ml for sICAM-1 and 0.6 ng/ml for sVCAM-1. The investigations were performed with the approval of the Bioethical Commission of the Medical University of Lodz and the National Science Council, Poland (No RNN/60/13/KE).

### Statistical analysis

The statistical analyses were performed using IBM SPSS STATISTICS 21 (Business Machines Corp., USA). Distributions of quantitative variables were described using means and standard deviations. Since the levels of sICAM-1 and sVCAM-1 expression did not show normal distribution (according to the results of the Shapiro-Wilk normality test), non-parametric statistical tests (Mann-Whitney U test, Kruskal-Wallis test) were used to identify the relationship between adhesion molecules expression and clinicopathological parameters. When the results of the Kruskal-Wallis test were significant, post hoc multiple comparisons with Bonferroni correction were done. A *p* value < 0.05 was considered as statistically significant.

**Table 1.** Clinicopathological characteristics of group studied

	Characteristics	n (%)
Age (mean ± SD)	45–79 years (62.5 ± 8.3)	
Overall survival (mean ± SD)	28–79 months (59.5 ± 13.8)	
Gender	Male	48 (96%)
	Female	2 (4%)
Stage (S status)	SI	6 (12%)
	SII	12 (36%)
	SIII	16 (68%)
	SIV	16 (100%)
Tumor size (pT status)	pT1	6 (12%)
	pT2	13 (26%)
	pT3	16 (32%)
	pT4	15 (30%)
Nodal metastases (pN status)	pN0	40 (80%)
	pN1–3	10 (20%)
Degree of differentiation (grade)	G1	9 (18%)
	G2	34 (68%)
	G3	7 (14%)
Mode of invasion (points)	1 – Well-defined borderline	4 (8%)
	2 – Less marked borderline	18 (44%)
	3 – No distinct borderline	16 (76%)
	4 – Diffuse growth	16 (100%)

## Results

### sICAM-1 and sVCAM-1 secretion in cases and control group

The results of this study showed higher average concentrations of both sICAM-1 and sVCAM-1 in the supernatant of incubated PBMCs obtained from patients with laryngeal cancer, as compared to the control group, but the results were of borderline significance ( $p = 0.05$ ). The mean concentration of sVCAM-1 in the cancer group and controls was  $346.5 \pm 391.6$  vs.  $327.2 \pm 404.8$ , respectively. The average values of sICAM-1 concentration in the group studied were  $222.3 \pm 110.5$  vs.  $197.7 \pm 129.3$  for cases and control group, respectively.

### The interconnections of sICAM-1 and sVCAM-1 with clinicomorphological parameters

In the present study the statistical analysis confirmed considerable differences in the level of sICAM-1 among groups of cancers in relation to nodal tumour extension (pN), stage (S), and type of invasion. The presence of the higher content of sICAM-1 in supernatants of PBMCs was more frequent for tumours with positive nodal metastases (pN1-3) ( $p = 0.012$ ). Moreover, significant differences among various subgroups of stage of neoplastic disease were confirmed ( $p = 0.033$ ). The higher concentration of sICAM-1 in PBMC cultures was more characteristic for more clinically advanced tumours (SIII-IV). Similarly, statistical analysis disclosed a positive association between sICAM-1 level and mode of invasion according to TFG classification ( $p < 0.0001$ ). The presence of the higher content

of sICAM-1 in supernatants of PBMCs was more frequent for tumours with more disseminated type of invasion determined by TFG scale and characterised by diffuse growth without distinct borderlines. No statistical dependences were noted for pT and histological tumour differentiation. Statistical evaluation of the quantitative analysis and morphological results showed that the expression of sVCAM-1 in PBMCs isolated from patients was significantly different depending on the local and nodal tumour extension ( $p = 0.017$  and  $p = 0.003$ , respectively), stage ( $p = 0.034$ ), and mode of invasion ( $p = 0.029$ ). The presence of a higher content of sVCAM-1 in supernatants of PBMCs was characteristic for tumours with more advancement determined by pT feature (pT3–pT4), positive nodal status (pN1–3), and higher stage (SIII–IV) and for cancers characterised by diffuse growth without distinct borderlines according to the four-level infiltration-type scale. Unfortunately, no correlation between the level of sVCAM-1 and histological differentiation was noted. The sICAM-1 and sVCAM-1 levels in PBMC cultures in cases and controls are shown in Tables 2 and 3.

### The interconnections of sICAM-1 and sVCAM-1 with 3-year and 5-year patient survival

In our study the relationship between survival period (up to 3 years, between 3–5 years, and over 5 years) and the average level of both, sICAM-1 and sVCAM-1, in the PBMC supernatants in SCLC patients was analysed. Statistical analysis disclosed significant differentiation of both adhesion molecule concentrations in relation to patients' 3- and 5-year survival ( $p = 0.001$  and  $p = 0.032$ , respective-

**Table 2.** Clinicopathological features and expression of sICAM in group studied

	Variable	sICAM [pg/ml]	p
Stage (S)	I–II	117.8 ± 92.8	$p^b = 0.033$
	III	114.0 ± 122.1	$p^a = 0.045$
	IV	220.6 ± 153.7	SI–II vs. SIV
Tumor size (pT status)	pT1	129.6 ± 97.6	ns
	pT2	108.2 ± 91.1	
	pT3	155.4 ± 128.8	
	pT4	213.5 ± 156.4	
Nodal metastases (pN status)	pN0	130.8 ± 103.5	$p^c = 0.012$
	pN1–3	264.2 ± 170.5	
Degree of differentiation (Grade)	G1	122.2 ± 78.4	ns
	G2	170.7 ± 136.8	
	G3	138.5 ± 150.1	
Mode of invasion	1–2 points	93.7 ± 65.5	$p^b < 0.0001$
	3 points	160.1 ± 128.0	$p^a < 0.0001$ 1–2 vs. 4
	4 points	271.0 ± 148.3	$p^a < 0.0001$ 3 vs. 4
Survival	< 3 years	294.8 ± 185.0	$p^b = 0.001$
	3–5 years	211.8 ± 133.7	
	> 5 years	105.5 ± 75.0	

Results are given as mean ± standard deviation

<sup>a</sup>Multiple comparisons were evaluated with Kruskal-Wallis test with Bonferroni correction

<sup>b</sup>Differences between three or four groups were evaluated with Kruskal-Wallis

<sup>c</sup>Differences between two groups were evaluated with Mann-Whitney U test

ns: not significant

**Table 3.** Clinicopathological features and expression of sVCAM in group studied

	Variable	sVCAM [ng/ml]	p
Stage (S)	I–II	383.0 ± 51.3	$p^b = 0.034$
	III	383.9 ± 85.8	$p^a = 0.021$ SI–II vs. SIV
	IV	428.5 ± 50.4	$p^a = 0.034$ SIII vs. SIV
Tumor size (pT status)	pT1	355.0 ± 45.1	$p^b = 0.017$
	pT2	404.2 ± 54.6	$p^a = 0.024$ pT1 vs. pT4
	pT3	374.2 ± 81.6	$p^a = 0.006$ pT3 vs. pT4
	pT4	434.8 ± 45.3	
Nodal metastases (pN status)	pN0	385.6 ± 63.7	$p^c = 0.003$
	pN1–3	446.9 ± 54.9	
Degree of differentiation (Grade)	G1	375.2 ± 65.9	ns
	G2	405.9 ± 62.4	
	G3	388.0 ± 86.2	
Mode of invasion	1–2 points	383.2 ± 54.2	$p^b = 0.029$
	3 points	392.4 ± 83.7	$p^a = 0.019$ 1–2 vs. 4
	4 points	431.9 ± 51.7	
Survival	< 3 years	445.4 ± 32.7	$p^b = 0.032$
	3–5 years	389.3 ± 77.3	
	> 5 years	390.4 ± 64.5	

Results are given as mean ± standard deviation

<sup>a</sup>Multiple comparisons were evaluated with Kruskal-Wallis test with Bonferroni correction

<sup>b</sup>Differences between three or four groups were evaluated with Kruskal-Wallis

<sup>c</sup>Differences between two groups were evaluated with Mann-Whitney U test

ns: not significant

ly). The lower content of both, sICAM-1 and sVCAM-1, in PBMC cultures in patients who lived longer than five years after treatment was observed. The adhesion molecule levels in PBMC cultures in patients with laryngeal carcinoma and statistical analysis results are shown in Fig. 1 and 2.

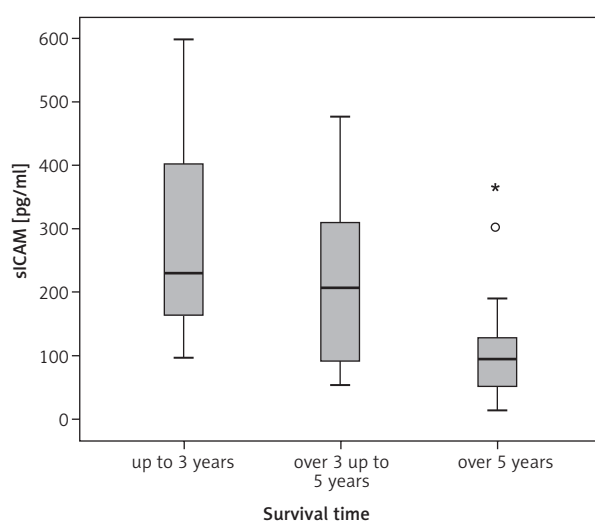
## Discussion

This study was aimed to investigate the relationship between the levels of adhesion molecules sICAM-1 and sVCAM-1 in the supernatant of cultured PBMCs of patients and the morphological and clinical level of severity of laryngeal squamous cell carcinoma. Changes of concentration of the soluble fraction of protein studied were assessed in relation to tumour progression, depending on clinicomorphological parameters such as pTNM and stage, the degree of histological differentiation, and the type of tumour invasion, classified according to the invasive tumour front grading scale (TFG). Our results indicate a strong positive correlation between the level of both, sICAM-1 and sVCAM-1, and the tumour aggressiveness. Unfortunately, a literature review revealed no reports about the possibility of using measurements of sICAM-1 and sVCAM-1 activity in terms of finding an unequivocal and sensitive indicator of the degree of advancement of laryngeal cancer. Identification of such a biomarker can help to optimise the therapy and assess prognosis in patients with this type of carcinoma.

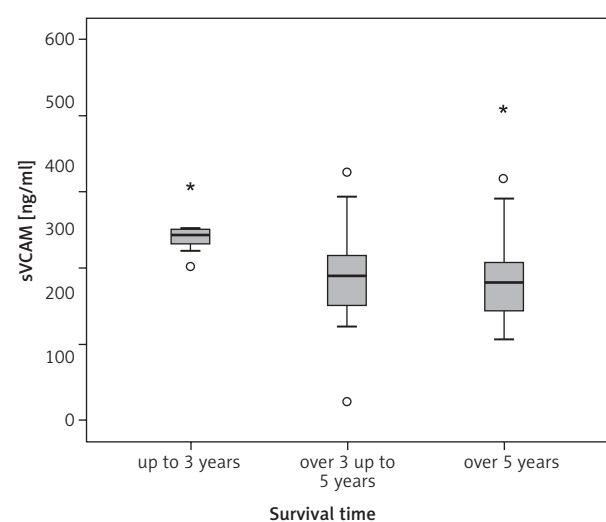
The literature survey indicated the usefulness of measuring blood levels of proteins from the family of CAMs as potential markers of severity of cancer invasion [6, 8, 11, 13, 16]. Chuang *et al.* [11] observed that ICAM-1 mediates IL-6-dependent increase of migration of oral squamous cell carcinoma cells. Similarly, recently Usami *et al.* [1] showed that ICAM-1 plays an important role in tongue squamous cell carcinoma progression, which may result from the cancer cell activity, tumour angiogenic activity, lymphangiogenic activity, and increased macrophage/

cancer cell adhesion. Similar results were also reported for colon carcinoma cases and in brain tumours [6, 9, 13]. The increased expression of adhesion molecules on tumour cells correlated with higher cancer progression and worse prognosis. Moreover, the highest concentration of ICAM-1 and E selectins on endothelial cells in colorectal cancer were associated with the presence of liver metastasis. Higher levels of ICAM-1 and sVCAM-1 family proteins were also detected in the serum of patients with other cancers [11, 16]. In a study by Chuang *et al.* [11] the incidence of metastases was also determined by a higher level of ICAM-1. Dymicka-Piekarska *et al.* [16] showed that increased levels of soluble adhesion molecules sVCAM-1 and sICAM-1 correlated with tumour size and metastasis formation in patients with colorectal cancer [14]. These results are consistent with our observations. In the present study the higher levels of sICAM-1 and sVCAM-1 in the supernatant of mononuclear cells in patients with laryngeal cancer associated with metastases to the lymph nodes. Similar correlation was observed between the level of adhesion molecules examined and the progression of tumour in the four-grade scale of invasion type.

The various mechanisms explaining the role of adhesion proteins in the processes of carcinogenesis have been discussed [18–20]. Proper adhesion process is essential for intercellular communication, cell movement, and intracellular signalling. The family of CAM molecules is involved at different levels in the regulation of inflammation development, the cellular immune response, and programmed cell death [20, 21]. In the case of tumour cells, a disrupted apoptosis mechanism and uncontrolled proliferation are typically observed. Moreover, the most important characteristics of the neoplasm, from the clinical point of view, are its ability to migrate tumour cells within the surrounding environment, their ability to penetrate the blood and lymph vessels, transport to distant tissues and organs, and metastasis tumour formation. All these processes are preceded by the loss of proper adhesion between the cells



**Fig. 1.** The concentration of sICAM-1 in the group studied depending on the 3-year and 5-year survival time



**Fig. 2.** The concentration of sVCAM-1 in the group studied depending on the 3-year and 5-year survival time

and intercellular matrix components. Interestingly, it was also shown that CAM family proteins may inhibit T-cells in the receptor-blocking mechanism, which allows tumour cells to evade the immune system [20]. In other studies, authors have tried to identify the mechanism binding the increased expression of ICAM-1 and the invasion and metastatic status. For instance, Chuang *et al.* [11] showed that the increased level of ICAM-1 in tumour cell lines of oral cancer may be associated with increased IL-6 levels, which in an autocrine manner, through the Syk kinase/JNK/AP-1 pathway, promotes tumour cell migration ability. Most of the research has been devoted to the molecular mechanisms that may be responsible for the expression of adhesion molecules in neoplastic disease, and not to the assessment of the relationship between sICAM-1 and sVCAM-1 levels and clinicomorphological markers of invasiveness of individual tumours. Such studies have recently been performed for colorectal, liver, lung, and brain cancer [6, 16–18].

Some works also suggest the possibility of anticancer therapy based on compounds capable of inhibiting adhesion molecules [8, 21, 22]. Interestingly, a few studies suggest that increased expression of ICAM-1 on the surface of tumour cells can support their destruction by immunocompetent host cells [23]. It seems that a clear understanding of the biological roles of ICAM-1 and VCAM-1 in cancer patients in relation to practical use requires further detailed research.

It should be emphasised that our results must be regarded as preliminary. Assessing the suitability of a potential marker requires verification of its sensitivity and specificity for an adequately numerous group of patients. Considering the fact that adhesion proteins are involved in most inflammatory conditions, the criterion of specificity for the measurement of sICAM-1 and sVCAM-1 concentration may be particularly difficult to achieve. Of note, there are also limitations in the comparison of our results with the data from literature, coming not only from the variability in type/location of tumours, but also material used for analysis (plasma, tissues, cell lines, animal models) and analytical methods (ELISA, RT-PCR, Western blotting). Hence, we would like to indicate the necessity of performing further studies confirming our findings in a larger group and with regard to relations with other immunological parameters that affect the function of immunocompetent cells and tumour phenotype [24, 25]. Clearly, the question of the clinical usefulness of sICAM-1 and/or sVCAM-1 as biomarkers of progression of laryngeal carcinoma requires further research.

Despite these limitations, the results of this study indicate the importance of the sICAM-1 and sVCAM-1 expression as indicators of advanced changes and progression in patients with laryngeal carcinoma; however, further research into the use of measurements of adhesion protein concentrations are needed.

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