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THE RELATIONSHIP BETWEEN GENE EXPRESSION OF CYTOSKELETAL PROTEIN GENES AND THE EPITHELIAL-MESENCHYMAL VIMENTIN MARKER IN LARYNGEAL SQUAMOUS CELL CARCINOMA

Aggressive laryngeal squamous cell carcinoma (LSCC) is characterized by a high metastatic potential, which is closely associated with epithelial-mesenchymal transition (EMT). Initiation of EMT is manifested by changes in the expression of some genes, including those associated with cytoskeleton reorganization. Currently, there are no effective methods for predicting metastasis in LSCC patients. In this regard, the study of SCC molecular characteristics remains relevant. In our study we assessed the relationship between the mRNA level of vimentin (VIM) and mRNA of cytoskeleton proteins: fascin-1 (FSCN1), ezrin (EZR), cofilin-1 (CFL1), profilin-1 (PFN1) and adenylyl cyclase-associated protein 1 (CAP1) in LSCC tumor tissue. The analysis was carried out using RT-PCR in paired samples from LSCC patients with and without lymph node metastases. The PFN1 mRNA level was found to be 6.3 times higher in LSCC patients with lymph node metastases than in patients without metastases. The EZR mRNA level was 17 times lower in patients with stage T3-4N0-2M0 LSCC than in patients with stage T1-2N0-1M0 LSCC. High VIM mRNA levels were associated with high FSCN1 and CAP1 mRNA levels and contributed to a stronger association between CFL1 and PFN1 mRNA levels. Thus, no direct relationship between the level of VIM as a marker of EMP and metastasis in a sample of LSCC patients was found. However, the detected relationships between the levels of cytoskeleton protein mRNA and vimentin mRNA may indicate an active reorganization of the cytoskeleton, which ensures high migration and proliferative activity of malignant cells of LSCC.

Keywords: epithelial-mesenchymal transition, actin-binding proteins, laryngeal squamous cell carcinoma, vimentin, intermediate filament proteins, metastasis

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Introduction. The aggressiveness of laryngeal squamous cell carcinoma (LSCC) is associated with its high metastatic potential, which is closely related to the processes of epithelial-mesenchymal transition (EMT) [17]. Initiation of EMT during tumor growth leads to changes in the expression of various genes, including those associated with cytoskeleton reorganization. For tumor cells of epithelial origin, the activation of molecular biological processes associated with EMT is known to promote malignant transformation [11; 13]. The EMT process is accompanied by a cascade of various changes in molecular genetic events, in particular, the activation of transcription factors, increased production of tissue metalloproteinases, loss of intercellular contacts, changes in the content of actin-binding proteins (ABP) and other intracellular events [5;7; 9; 11; 13; 15]. All these processes ultimately lead to the reorganization of the actin cytoskeleton, which is the final step before the onset of tumor cell invasion [7; 8].

Much attention is paid to vimentin, which as an intermediate filament cytoskeleton protein. It is believed that vimentin is mainly expressed in fibroblasts, endothelial cells and lymphocytes. Sufficient evidence has accumulated for the participation of vimentin not only in the regulation of EMT, but also in the regulation of Wnt / β-catenin and GSK-3 / Snail signaling pathways, which allows this protein to be used as a marker of EMT and metastasis [12]. Cytoskeleton remodeling is known to be mediated by multiple ABP, including cofilin-1 (CFL1), profilin-1 (PFN1), ezrin (EZR), fascin-1 (FSCN1) and adenylyl-associated protein-1 (CAP1), which have different functional roles in the cell [6; 9; 19]. In response to extracellular and intracellular signals, ABP and vimentin regulate cytoskeleton reorganization, thereby participating in cancer cell invasion and metastasis [5; 12]. There is virtually no data on the combined effect of vimentin and the cytoskeleton proteins listed above on metastasis in LSCC. In addition, given that laboratory-specific methods for predicting LSCC metastasis have not yet

been introduced into practice, this area of research remains relevant.

The assessment of the relationship between the expression of genes encoding vimentin and the expression activity of genes encoding cytoskeleton proteins in the LSCC tissue, as well as the assessment of their relationship with lymph node metastasis will be useful as new approaches in diagnostics or search for new therapeutic targets. Therefore, **the aim** of the study was to assess the relationship between the levels of vimentin and cytoskeleton proteins: CFL1, PFN1, EZR, fascin-1 FSCN1 and CAP1 in tumor tissue, as well as the association of the expression of these genes with metastasis in patients with LSCC

Material and methods. The study included 43 LSCC patients who were treated at the Cancer Research Institute of Tomsk National Research Medical Center from 2017 to 2021. Tumor (stage T1-4N0-1M0) and normal tissue samples obtained during videolaryngoscopy were the study material. All patients with histologically verified LSCC were divided into the group with regional metastases (n=25) and the group without metastases (n=18). The obtained tissue samples were placed and stored in RNAlater solution (Ambion, USA). The study was conducted in accordance with the Helsinki Declaration of the World Medical Association "Ethical Principles for Medical Research Involving Human Subjects" with the amendments of 2000 and the "Rules of Clinical Practice in the Russian Federation" approved by Order of the Ministry of Health of the RF No. 266 (06/19/2003). Informed consent was obtained from each patient and permission from the Ethics Committee of the Oncology Research Institute of the TNRMC (extract from protocol No. 7 dated 06/24/2019).

Extraction of mRNA and preparation of cDNA. The CCR-50 kit (Biosilica, Novosibirsk) was used to extract the total mRNA pool from paired tissue samples according to the manufacturer's instructions. The concentration and purity of mRNA were assessed using a NanoDrop-2000 spectrophotometer (Thermo Scientific, USA). cDNA synthesis on the RNA matrix was performed using the OT-1 reverse transcription reagent kit (Synthol, Moscow) according to the manufacturer's instructions, and the resulting mixture was then used to perform quantitative real-time polymerase chain reaction (RT-PCR).

Real-time PCR. The level of gene mRNA expression was assessed by RT-PCR using Sybr Green technology and iCycler amplifier (Bio-Rad, USA). Primers were selected using the Vector NTI Advance 11.5 program (Thermo Fisher Scientific, USA) and the NCBI database (http://www.ncbi.nlm.nih.gov/nuccore) [9]. Melting curve analysis (Melt) was used to assess the final PCR product for the presence of primer-dimers or non-specific products. The housekeeping gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzyme was used as a reference gene to normalize the expression of the studied genes. mRNA (cDNA) isolated from morphologically altered laryngeal epithelium was used as a calibrator. Expression analysis was performed using the 2-ΔΔCT method [14].

Statistical analysis of the results was performed using the Statistica 6.0 and IBM SPSS Statistics 22.0 software packages. The normality of the distribution was checked using the Shapiro–Wilk test. The results are presented as Me (Q1; Q3), where Me is the median and Q1; Q3 is the interquartile range, n is the number of patients in the group and P (U-test) is the nonparametric Mann-Whitney U-test. Spearman correlation analysis was used to analyze the relationships. Differences were considered statistically significant at $p<0.05$.

Results and discussion. A 6.3-fold increase in the level of profilin-1 mRNA was observed in tumor tissue of LSCC patients with lymph node metastases compared to that observed in LSCC patients without metastases. The level of cofilin-1 mRNA in tumor tissue of LSCC patients tended to increase in comparison with that observed in patients without metastases (table 1). No significant differences in the level of vimentin mRNA between patients with lymph node metastases and patients without metastases were found. The extent of the tumor involvement influenced the level of mRNA ezrin, which was almost 17 times lower in patients with stage T3-4N0-2M0 compared to that observed in patients with stage T1-2N0-1M0 (Table 1).

The correlation analysis revealed that metastasis affected the strength and number of correlations between expressed genes encoding cytoskeletal proteins. Thus, in LSCC patients without lymph node metastasis (N0), one strong correlation between the mRNA level of profilin 1 and cofilin 1 was found ($r=0.8$; $p<0.05$) (Fig. 1A).

In the group of LSCC patients with metastases (Fig. 1B), moderate-strength relationships were found between the expression levels of almost all the genes studied ($r=0.5$-0.6; $p<0.05$), with the VIM mRNA level positively associated with the expression activity of the FSCN1, EZR, PFN1 and CAP1 genes ($r=0.6$; $p<0.05$). A positive relationship was also found between the expression activity of the genes encoding PFN1, CAP1 and CFL1 ($r=0.6$; $p=0.04$), with the mRNA levels of PFN1 and CFL1 being less strongly associated ($r=0.5$; $p<0.5$). Thus, the presence of lymph node metastasis increased the number of co-expressed genes, with a positive correlation observed.

The total sample of patients with LSCC was divided into the groups with respect to the median level of vimentin mRNA: patients with a low VIM expression level (below 0.65 U) in the tumor tissue, and patients with a high VIM expression level (above 0.65 U) (Table 2).

Table 1

The relative mRNA level of actin-binding proteins and vimentin in tumor tissue of LSCC patients with and without lymph node metastasis

мРНК	T1-2N0-1M0	T3-4N0-2M0	N0	N1-2	P1	P2
VIM	0.31 (0.01;41.93)	1.27 (0.22;7.31)	0.39(0.01;41.93)	1.44(0.22;18.00)	0.58	0.28
FSCN1	1.31 (0.03;7.15)	0.94 (0.12;8.00)	0.32(0.04;6.60)	1.73(0.21;14.40)	0.70	0.11
EZR	12.50 (0.57;34.32)	0.72 (0.05;4.67)	3.46(0.06;21.99)	1.53(0.07;8.17)	**0.03**	0.50
PFN1	1.19 (0.01;7.30)	2.09 (0.18;18.82)	0.40(0.00;4.69)	2.51(0.76;25.99)	0.22	**0.01**
CFL1	1.53 (0.08;13.77)	1.15 (0.19;6.60)	0.38(0.04;7.75)	3.10(0.19;27.00)	0.94	*0.06*
CAP1	5.46 (1.07;26.93)	3.30 (0.10;29.25)	3.52(0.22;29.33)	4.55(1.07;28.62)	0.87	0.38

Note: a p-value is a level of statistically significant difference between the groups (Mann–Whitney U test)

The group of LSCC patients with a high level of VIM mRNA consisted of 9 patients with lymph node metastasis and 13 patients without metastasis. A similar distribution was observed for the group of patients with a low level of VIM mRNA (Table 2). Although lymph node metastases in the presented patient sample were not associated with VIM mRNA levels, their increase resulted in a significant increase in CAP1 and FSCN-1 expression (Fig. 2).

In addition, the VIM mRNA level affected the relationships between gene expression activities in cancer tissue. Thus, at a low VIM mRNA level, one correlation was observed between PFN1 and CFL1 mRNA (r=0.4; p<0.05) (Fig. 1C), while at a high VIM gene expression, the number of relationships between the ABP mRNA levels increased, and the strength of the relationship between CFL1 and PFN1 mRNA also increased (r=0.8; p=0.04).

The study found that neither lymph node metastasis nor the extent of laryngeal cancer involvement correlated with the expression of the gene encoding type III intermediate filament protein VIM. This fact is partly supported by information obtained from the GEPIA2 database (http://gepia2.cancer-pku.cn/), a resource for gene expression analysis based on tumor and normal samples from TCGA and GTEx data. Expression of the vimentin gene is not associated with the extent of squamous cell carcinoma of the head and neck (Fig. 3A), is not expressed at a sufficiently high level (Fig. 3B) in comparison with conditionally normal tissue and other genes studied (Fig. 3C). Although the high level of vimentin mRNA, did not demonstrate a significant difference in the groups of patients with and without metastases, its increase was combined with a significant increase in the mRNA level of the actin-associated protein PFN1 (Table 2).

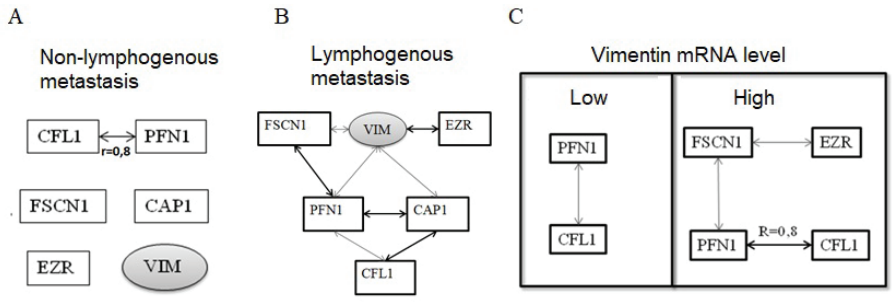

Fig. 1. Expression of genes encoding cytoskeleton proteins in tumor tissue of patients with laryngeal cancer: Spearman correlation coefficients.
Note: bold arrows show coefficient r=0.8, thin arrows show r=0.5-0.6

Table 2

Expression of the gene encoding vimentin in tumor tissue of patients with laryngeal cancer

Groups of patients with LSCC		Level of VIM mRNA		Total number of patients
		low	high	
Lymph node metastasis	no	12	13	25
	yes	9	9	18
Total number of patients		21	22	43

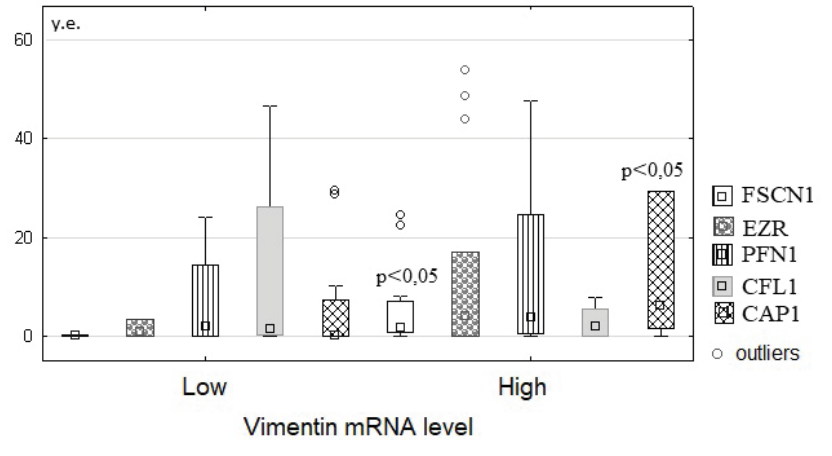

Fig. 2. The expression levels of actin-binding protein genes in tumor tissue of patients with laryngeal cancer depending on the different expression activity of the gene encoding vimentin. (U-test)

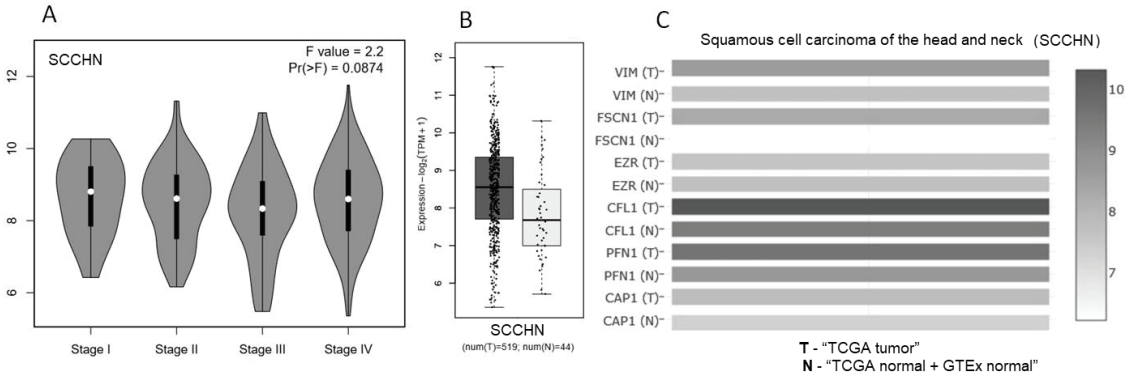

Fig. 3. Vimentin gene expression depending on the extent of head and neck squamous cell carcinoma (A), in normal and tumor tissue of head and neck squamous cell carcinoma (B), and in comparison with the expression of the genes fascin-1, ezrin, cofilin 1, profilin-1 and adenylyl-associated protein 1 in tumor and normal tissue of head and neck squamous cell carcinoma (C)

Correlation analysis revealed that the high expression of EMT marker vimentin enhanced the positive relationships between the mRNA levels of CFL1 and PFN1 and contributed to the development of multiple relationships between the mRNA levels of other proteins. Considering the fact that during cancer metastasis, the establishment of multiple positive relationships between the mRNA expression levels of the studied proteins was also observed, it can be concluded that vimentin has an indirect effect on metastasis. There is evidence that ABP (cytoskeletal proteins) not only regulate the locomotor activity of cells, but also participate in the transcription, since actin and ABPs are present not only in the cytoplasm, but also in the cell nucleus. The cell nucleus contains the monomeric actin, the amount of which depends on the key ABPs – PFN1 and CFL1 [1; 2]. It is possible that the coactivation of the studied genes is related not only to the mobility of tumor cells during metastasis, but also to the processes of transcription, and further to the proliferative activity of the tumor itself.

In our study we also found that the mRNA expression levels of CAP1 and FSCN1 significantly increased with the evidence of high vimentin expression, however, a positive correlation was shown only between the expression of FSCN1, EZR, PFN1 genes, and a strong positive correlation was established between PFN1 and CFL1. Our results are consistent with other recent studies, in which co-expression of actin-binding proteins CAP1, PFN1 and CFL1 and the participation of these proteins in the metastatic cascade were revealed [6; 9; 18]. In our study, the ezrin mRNA level decreased by 16 times with increasing stage from T1-2N0-1M0 to T3-4N0-2M0, but the level of significance was low (p=0.5). Ezrin, a protein which links the cell cytoskeleton, membrane, and extracellular matrix, is involved not only in cell locomotion, but also in adhesion, differentiation, proliferation, signaling, blebbing, and entosis [3; 4; 16], including metastasis [4; 10]. To analyze the relationship between EZR and tumor progression, it is likely necessary to take into account the presence of mutations in its gene, as well as the expression activity of genes of the ERM (ezrin-radixin-moesin) family [19]. In addition, the decrease in ezrin expression may be associated with a disruption of the adhesive properties of tumor cells, which is important for invasion and metastasis. The role of ezrin in tumor invasion, metastasis, progression, and drug resistance is still under investigation [4; 10].

Conclusion. Our data supplement the knowledge about the molecular genetic mechanisms of metastasis in LSCC and their relationship with the EMT processes Considering our data and the fact that the reorganization of the cytoskeleton during the EMT process is controlled by various molecules, we can conclude that almost all the proteins studied, as well as other proteins, participate in molecular biological changes in lymph node metastasis from LSCC. For a more complete analysis of possible mechanisms and confirmation of the results obtained, further in vitro studies, which will expand the knowledge about the regulation of ABP, are required. Nevertheless, the discovered relationships can supplement the knowledge about cancer development and progression, which will be useful in the development of new diagnostic techniques or therapeutic targets.

The authors declare no conflict of interest in the submitted article.

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