Serum levels of cytoskeleton remodeling proteins and their mRNA expression in tumor tissue of metastatic laryngeal and hypopharyngeal cancers

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Abstract
Actin-binding proteins (ABPs) and various signaling systems are involved in the process of squamous cell carcinoma of the larynx and hypopharynx (SCCLH) metastasis. The clinical significance of these proteins has not yet been determined. We analyzed the relationship between the mRNA levels of cofilin 1 (CFL1), profilin 1 (PFN1), adenylyl cyclase-associated protein 1 (CAP1), SNAI1 and RND3 and SCCLH metastasis. The serum levels of the above ABPs were estimated and the relationship between them and their mRNA expressions was analyzed. The expression levels of ABP mRNAs were measured by real-time RT-PCR in paired tissue samples taken from 54 patients with SCCLH (T1-4N0-1M0). Expression analysis was performed using the 2−ΔΔCT method. The levels of ABPs in the blood serum were measured by ELISA. Statistical analysis was carried out using the SPSS Statistica 20.0 software package. No significant difference in the mRNA gene expression in tumor tissue of patients with T1-3N0M0 SCCLH and patients with T2-4N1-2M0 SCCLH was found. High expression of RND3 mRNA was accompanied by an increase in mRNA expression of all studied ABPs. In the blood serum of T2-4N1-2M0 patients, the level of PFN1 was lower by 21% and the level of CAP1 was higher by 75% than those observed in T1-4N0M0 patients. The data obtained showed that RND3 is involved in the regulation of molecular cascades of SCCLH metastasis. PFN1 and CAP1 serum levels can be good classifiers of metastases in patients with SCCLH.

Keywords Actin binding proteins · SNAI1 · RND3 · Squamous cell carcinoma of the larynx and hypopharynx · Metastasis

Introduction
Squamous cell carcinoma of the larynx and hypopharynx (SCCLH) is characterized by a rapid growth, high metastatic and invasive potentials, and aggressive behavior. High mortality rates in patients with SCCLH during the first year after diagnosis are a major challenge. A quarter of patients have clinically undetectable lymph node metastases, which manifest in the late postoperative period [1]. Therefore, further investigation of the mechanisms of metastasis is still needed to improve outcomes for SCCLH patients.

Cancer aggressiveness is determined by various molecular and genetic changes, including the acquisition of the migration ability of tumor cells, accompanied by reorganization of the cytoskeleton [2, 3]. During metastasis, tumor cells can move in the mesenchymal mode with the initiation of epithelial-mesenchymal transition (EMT) and in the amoeboid mode with the activation of the mesenchymal-to-amoeboid transition (MAT) [2, 3, 4, 5]. Tumor transformation of cells is characterized by EMT type 3 [2, 3], accompanied by activation of transcription factors (Snail, Twist, Zeb, etc.), increased production of metalloproteinases, cytoskeleton reorganization by actin-binding proteins (ABPs), and other intracellular events [2, 3]. Switching between EMT and MAT can be quite fast and depends on the properties of the microenvironment. The participation of proteins of the Rho family GTPases is common to all migration routes [6, 7, 8, 9]. The model of the mouse glioblastoma cell line showed that the induced expression of Rnd3 (Rho family—GTPases) inhibited the activity of Snail1, which blocked the migration of tumor cells [10]. Therefore, the study of the relationship
between the level and balance of mRNA of signaling proteins, such as Rnd3 and Snail and the metastatic status in patients with SCCLH is of great importance.

Various ABPs (cofilin, fascin, profilin, adenylyl cyclase-associated protein 1 (CAP-1), ezrin, etc.) are involved in the remodeling of the actin cytoskeleton, which provides the driving force of tumor cells during metastasis [4, 11, 12, 13, 15, 16]. The most important regulator of actin remodeling is the ADF (actin depolymerizing factor) / cofilin family of proteins [17]. Cofilin 1 (non-muscle cofilin) plays an important role in the pathogenesis of various tumors [16, 17], triggers the formation of lamellipodia and ensures the direction of cell transmission [18, 19]. Cofilin 1 works in tandem with ABPs, such as profilin and CAP1 [13, 20; https://www.ebi.ac.uk, https://thebiogrid.org]. Changes in the expression of CAP1, profilin 1 and cofilin 1 were noted in esophageal carcinoma [21, 22]. Profilin-1 is involved in proliferation, motility, endocytosis and other important cellular processes [23, 24]. The activation of the Rho / Rock pathway leads to the phosphorylation of both cofilin and profilin [9, 21, 25]. Moreover, phosphorylated profilin has a high affinity for actin and can significantly affect the migration activity of tumor cells [24, 26]. If the contribution of cofilin to the development of a more aggressive type of tumor is unambiguous, the participation of profilin in tumor progression is still controversial [13, 24].

CAP1 accelerates cofilin-mediated disassembly of actin filaments and motility. This protein is involved in mRNA localization, maintenance of cell polarity, and receptor-mediated endocytosis [20]. The increase in CAP1 expression is associated with lymph node metastasis of cancer of various localizations [20, 21]. We have previously shown that the development of SCCLH is associated with a change in the level of mRNA of cell motility proteins: SNAI1 (EMT marker) and ABPs (fascin 1, ezrin, and CAP1) [11, 12]. The study of the relationship between ABPs and the level of mRNA expression of regulatory molecules of calpains showed that SCCLH metastasis is associated with increased level of CAPN1 expression, and decreased level of CAP1 expression [14]. Until now, there is no data on the relationship between EMT-associated markers (SNAI1 and RND3) and ABPs (cofilin1, profilin 1, and CAP1) in the progression of SCCLH. It is also unknown whether the level of these ABPs in the blood serum of SCCLH patients can reflect the progression of the disease. Therefore, the aim of the study was to estimate the relationship between the mRNA levels of EMT-associated signaling molecules (SNAI1, RND3) and functional partners of ABPs (cofilin1, profilin 1, CAP1) and lymph node metastasis in SCCLH tissues. The serum level of cofilin-1, profilin-1, and CAP1 and tissue mRNA level of cofilin 1 (CFL1), profilin 1 (PFN1), adenylyl cyclase-associated protein 1 (CAP1), SNAI1 and RND3 were assessed in patients with SCCLH. The study will clarify the mechanisms of SCCLH metastasis and reveal potential markers for predicting the course of the disease.

## Materials and methods

The study included 54 patients with histologically verified SCCLH. The median age of the patients was 56 years. Patients and tumor characteristics are presented in TABLE 1. Tissue and blood samples were obtained before treatment. Intact epithelial tissue and primary tumor tissue samples were collected during videolaryngoscopy. Tissue samples were placed in an RNAlater solution (Ambion, USA) and stored at − 80 °C.

### Evaluation of mRNA expression level

The total mRNA pool was isolated using the CCR-50 kit (Biosilica, Russia). The quality and integrity of RNA were evaluated using capillary electrophoresis system (Nanodrop-2000, Thermo Scientific, USA and TapeStation, Agilent Technologies, USA) and R6K ScreenTape (Agilent Technologies, USA #5067-5367).

The synthesis of the first strand cDNA from the RNA template was carried out using a set of reagents for reverse transcription OT-1 (Synthol, Russia). The level of gene

| Characteristics | Patients with SCCLH (N=54) |
|-----------------|-----------------------------|
| Gender          |                             |
| Male            | 42                          | 77 |
| Female          | 12                          | 22 |
| Age (years)     |                             |
| ≤ 56            | 29                          | 54 |
| > 56            | 25                          | 46 |
| T classification|                             |
| I               | 8                           | 14.8 |
| II              | 11                          | 20.4 |
| III             | 30                          | 55.6 |
| IV              | 5                           | 9.2 |
| Tumor grade     |                             |
| Low             | 5                           | 9.2 |
| Intermediate    | 30                          | 55.6 |
| High            | 14                          | 25.8 |
| Unstage         | 5                           | 9.2 |
| N classification|                             |
| N−              | 29                          | 54.7 |
| N+              | 25                          | 46.3 |

"N−"—SCCLH patients without lymph node metastasis (patients with stage T2-4N0M0); "N+"—SCCLH patients with lymph node metastasis (patients with stage T2-4N1-2M0)
mRNA expression was evaluated by real-time PCR (RT-qPCR) using Sybr Green technology on an iCycler amplifier (Bio-Rad, USA). To assess the final product of the PCR reaction for the presence of primer dimers or non-specific products, a melting curve analysis (Melt option) was used. As a reference gene, the “housekeeping” gene of the GAPDH (glyceraldehydes-3-phosphate dehydrogenase) enzyme was used. The PCR amplification parameters were: 94 °C for 10 min (one cycle, pre-denature), 94 °C for 10 s and 60 °C for 20 s (40 cycles). Samples were tested in triplicates and means of obtained Ct values were calculated. Primers were selected using the Vector NTI Advance 11.5 program and the NCBI database: SNAI1 (NM_005985) F5-CCC AAT CGG AAG CCT AAC T-3, R5-AGT AGA GGA GAA GGA CGA AGGA-3; RND3 (NM_001254738) F5-AGA GAG CCA CAA AGC GGA T-3, R5-TAT CCT CTC AAA CGC CTC CTA-3; PFN1 (NM_00125022) F5-TGGAGCAAACCTACCTCTT-3, R5-AGGCGAGACCGAACTTT-3; CFL1 (NM_005507) F5-CGGCTGATGCGCTCTCTA-3, R5-TCTCTTCTGATG CGTCTCTT-3; CAP1 (NM_001105530) F5-CCAAAC GAGCCACAGAGAA-3, R5-ACCCATTACCTGAACCTT GACAT-3. The ΔCt values for each sample were calculated as the difference between the genes of interest (SNAI1, RND3, PFN1, CFL1 and CAP1) and the “housekeeping” (GAPDH). Expression analysis was performed according to the $2^{-\Delta\Delta CT}$ method [27]. A reaction mixture without a matrix and with an RNA matrix without a reverse transcription step was used as a negative control to estimate the contamination of genomic DNA.

**Enzyme-linked immunosorbent assay (ELISA)**

The analysis of circulating ABPs was carried out in blood serum using Multiskan FC 100 microplate ELISA reader (ThermoFisher Scientific), Human Adenylyl cyclase-associated protein 1 ELISA kit (Cusabio), ELISA Kit for Profilin 1 (Cloud-Clone Corp), and ELISA Kit for Cofilin 1 (Cloud-Clone Corp). Blood serum was obtained according to the approved protocol from the above patients.

### Table 2

| Relative mRNA level | Lymph node metastasis (N = 54) | P | Function of the studied genes |
|--------------------|-------------------------------|---|--------------------------------|
|                    | N− (n = 29)                   |   |                                |
| SNAI1              | 0.41 (0.12; 1.44)             | 0.07 | Encoding proteins associated with EMT |
| RND3               | 0.51 (0.06; 3.27)             | 0.18 |                                |
| PFN1               | 2.51 (0.01; 9.08)             | 0.74 | Encoding ABPs                  |
| CFL1               | 0.68 (0.06; 3.60)             | 0.08 |                                |
| CAP1               | 2.09 (0.07; 12.90)            | 0.06 |                                |
|                    | N+ (n = 25)                   |   |                                |

"N−", SCCLH patients without lymph node metastasis (group with stage 1-3N0M0); "N+", SCCLH patients with lymph node metastasis N1-2 (group with stage T2-4N1-2M0); p, significant difference between groups "N−" and "N+" (U-test)
genes. Numerous positive correlations were identified between the studied molecular parameters in tumor tissue. A direct relationship of medium and high strength was found between ABPs: CAP1-CFL1 (r = 0.6; p = 0.03), CFL1-PFN1 (r = 0.7; p = 0.04) and PFN1-CAP1 (r = 0.5; p = 0.03).

There were detected positive correlations between ABPs mRNA expression (PFN1 and CFL1) and EMT-associated proteins mRNA expression (RND3 and SNAI1). The mRNA expression activity of RND3 was associated with the mRNA expression of PFN1 (r = 0.5; p = 0.05) and CFL1 (r = 0.4; p = 0.05). The SNAI1 mRNA level correlated with the PFN1 mRNA level (r = 0.4; p = 0.05).

All patients were divided into groups according to the presence of lymph node metastases. In the group of patients with SCCLH T1-3N0M0, the correlation analysis revealed a relationship between the mRNA levels of SNAI1-CFL1 and RND3-PFN1. In the group of patients with SCCLH T2-4N1-2M0, the SNAI1 mRNA level correlated with the CAP1 mRNA level, and RND3 mRNA level correlated with CFL1 and PFN1 mRNA levels. The relationship between the mRNA expressions of CAP1-CFL1-PFN1 was stronger in the group with metastases than in the group without metastases (Table 3). Interestingly, a new correlation relationship between SNAI1 and CAP1 mRNA expressions was observed in tissues of patients with lymph node metastases (r = 0.5; p = 0.04).

To assess the relationship between SNAI1 and RND3 expression levels and metastatic status of SCCLH patients, the total group of patients (N = 54, 100%) was divided into two groups depending on the median expression levels of these genes (Table 4). The first group consisted of patients with a high expression level of SNAI1 and RND3 genes, and the second group consisted of patients with a low expression level of these genes. The low expression level of SNAI1 mRNA (≤ 1.09) was observed in 54% (n = 29) of the total group patients, among whom 20% had lymph node metastases (N+) and 80% did not have metastases (N−). The high level of SNAI1 mRNA (> 1.09) was observed in 46% (n = 25) of the total group patients, 47% of them had lymph node metastases and 53% did not have metastases. The low expression activity of RND3 mRNA (≤ 1.10) was detected in tumor tissues of 44% (n = 24) of the total group patients, among whom, there were 18% with lymph node (N+) metastases and 82% without metastases (N−). The high expression of RND3 mRNA (> 1.10) was observed in 56% (n = 30) of patients, among whom, there were 51% with metastases (N+) and 49% without metastases (N−).

The ABP mRNAs expression were estimated with regard to high or low mRNA expression of the EMT signaling molecules (SNAI1 and RND3) (Fig. 1A, B, respectively). A high level of SNAI1 expression was accompanied by a threefold increase in the level of PFN1 mRNA expression (Fig. 1A). A high expression level of RND3 was accompanied by a four-fold increase in the expression level of CFL1 mRNA (Fig. 1B). No significant differences in the expression activities of PFN1 and CAP1 with regard to the level of SNAI1 were found (Fig. 1A). A high level of SNAI1 mRNA expression was accompanied by a high mRNA expression level of PFN1. A high level of RND3 expression was accompanied by a high level of mRNA expression of PFN1 and CAP1 and CFL1 (Fig. 1B). Moreover, the relationship between the PFN1 and CFL1 mRNA levels and the RND3 expression level was revealed (Table 3).

### Table 3

| mRNA | SNAI1 | RND3 | PFN1 | CFL1 | CAP1 |
|------|-------|------|------|------|------|
| SNAI1 | 0.1   | 0.5* | 0.5* | 0.2  |
| RND3  | 0.3   | 0.4* | 0.2  | 0.5  |
| PFN1  | 0.4*  | 0.7* | 0.6* | 0.4* |
| CFL1  | 0.6*  | 0.7* | 0.5* |
| CAP1  | 0.5*  | 0.4  | 0.7* | 0.8* |

Cells marked in italics contain correlation coefficients for group "N−"; cells marked in bold contain correlation coefficients for group "N+"; *, correlations with p ≤ 0.05

### Table 4

| Levels of expression | SNAI1 | RND3 |
|----------------------|-------|------|
|                      | N−, % | N+, % | N−, % | N+, % |
| Low                  | 80    | 20   | 82    | 18    |
| High                 | 47    | 53   | 49    | 51    |

"N−", SCCLH patients without lymph node metastasis (group with stage T1-3N0M0); "N+", SCCLH patients with lymph node metastasis (group with stage T2-4N1-2M0)

### Quantitative analysis of actin-binding proteins (profiling 1, cofilin1 and CAP1) circulating in the blood serum of patients with squamous cell carcinoma of the larynx and hypopharynx

In the first step of our study, the relationship between ABP and EMT-associated proteins mRNAs expression and SCCLH metastasis was found; therefore, we compared the serum level of ABPs with tissue level of ABP mRNAs in SCCLH patients. We assessed whether the studied parameters can be used as markers of lymph node metastasis in this category of patients. In the second step of our study, the level of circulating ABPs in the blood serum of SCCLH patients using ELISA was assessed. Table 5 shows the median values of ABP levels in the blood serum of all SCCLH patients.
The serum level of profilin-1 was significantly lower (by 21%) and the serum level of CAP1 was significantly higher (by 75%) in patients with lymph node metastases than in patients without lymph node metastases. It should be noted that, despite the fact that the correlation analysis did not reveal significant associations, the changes in the mRNA expression levels of PFN1 and CAP1 in tissues were similar to those observed in blood serum. However, the expression level of CFL1 mRNA in the tumor tissue of SCCLH patients with lymph node metastases was significantly increased, while the protein level in the blood serum of these patients remained unchanged.

ROC analysis was carried out to estimate the possibility to use ABP serum levels for predicting SCCLH metastasis. ROC analysis is proposed as a simple and practical tool for assessing the ability of candidate predictive factors in a binary classification [28]. Serum levels of profiling 1 and CAP1 were the most significant classifiers (AUC = 0.75, 95% CI (Confidence Interval) = 0.63 – 0.84 and AUC = 0.81, 95% CI (Confidence Interval) = 0.65 – 0.97, respectively).

### Discussion

No significant differences in the expression activity of genes encoding ABPs between tumor tissues of SCCLH patients having lymph node metastases and tumor tissues of patients having no metastases were found. However, the expression activity of SNAI1, CFL1, and CAP1 genes in tumor tissues of patients with T2-4N1-2M0 SCCLH tended to increase. Tumor biopsies contain not only tumor cells but also cells of the tumor microenvironment, which consists of a complex network of structural components and non-malignant stromal cells, such as endothelial cells, pericytes, fibroblasts and immune cells. Both tumor cells and stromal cells undergo different types of cytoskeleton remodeling during their vital activity. Podosomes, short-lived membrane structures, are usually found in monocyctic cells, such as macrophages, monocytes, dendritic cells, etc. and control matrix remodeling. Podosome superstructures are organized in endothelial cells to control vascular remodeling [29]. Thus, cytoskeleton remodeling is actively taking place in the tumor microenvironment which may be reflected in changes in the total mRNA expression and serum level of ABPs.

Co-expression of ABP genes (CAP1, PFN1, and CFL1) was revealed as a result of the assessment of the presence and nature of the relationship between the levels of EMT-associated genes expression (SNAI1, RND3) and functional
partners of ABP genes expression (\textit{CAP1}, \textit{PFN1}, and \textit{CFL1}) in tumor tissue of SCCLH patients. Changes in these mRNAs during tumor progression do not contradict the literature data [13, 17, 18, 30]. The involvement of cofilin 1 in the formation of the malignant phenotype of tumor cells is still under study. It has been shown that in vitro stimulation of metastatic cells MTLn3 by epidermal growth factor (EGF) leads to an increase in the level and activity of cofilin 1 at the anterior membrane edge, which leads to the generation of free barbed ends necessary for activation of lamellipodia formation and chemotaxis to EGF, which promotes invasion and metastasis [17, 31]. It is known that CAP1 promotes disassembly of cofilin-linked actin filaments and acceleration of actin dynamics [17]. An increased level of \textit{CAP1} and cofilin expression is associated with cancer cell invasiveness [32]. The structural biology methods have shown that \textit{CAP1} works in tandem with cofilin 1 accelerating the depolymerization of actin filaments by almost 100 times [30]. However, data indicating a more complex role of \textit{CAP1} in the implementation of migration activity and invasiveness of tumor cells are accumulating. The knockdown of the \textit{CAP1} gene can lead to FAKRap1-mediated stimulation of cell adhesion and the formation of lamellipodia with a subsequent increase in cell motility [33]. The role of cofilin 1 and its functional partner profiling 1 in the aggressiveness of cancer is well covered in the literature [13]. However, the dynamics and interconnection between these two proteins and their joint contribution to the formation of the metastatic phenotype of tumor cells have not been studied. In our study we revealed the close relationship between the expressions of cofilin 1 and profiling 1 in SCCLH patients. These data require further more detailed study due to the fact that changes in the mRNA expression of the studied proteins are multidirectional. The functional partnership between cofilin 1 and profiling 1 in the tumors is described in the literature [13]. The study of mRNA co-expression of profiling 1 and \textit{CAP1} was carried out for the first time. However, considering contradictory data on the role of the profiling 1 and cofilin 1 tandem in the development of aggressiveness of various tumors [13, 34–36], further studies of the relationship between these genes/their proteins and SCCLH progression are required.

The relationship between the expression activity of RND3 and \textit{PFN1} mRNAs; CFL1 and \textit{CAP1} mRNAs revealed in our study most likely indicates that RND3 plays an important role in molecular events during metastatic progression. It has been shown that Rnd3 is involved in various signaling cascades and regulation of cytoskeleton organization [8–10, 25]. The multidirectional changes in Rnd3 expression in tumors are associated with the mutational status [37, 38]. The increased level of RND3 mRNA expression in combination with changes in the mRNA expression levels of \textit{CAP1}, \textit{PFN1}, and \textit{CFL1} as well as the absence of a clear association with the presence of lymph node metastases possibly indicates the molecular features of SCCLH progression. Further studies are required for final conclusions about the role of Rnd3 in lymph node metastasis in SCCLH patients including in vitro studies which will expand the knowledge about the regulation of ABPs.

The study of profiling 1, cofilin 1 and \textit{CAP1} levels in the blood serum of SCCLH patients showed association with the metastatic status. Our study results were consistent with other studies which demonstrated the decreased level of profiling-1 and increased level of \textit{CAP1} in the blood serum of patients with clinically detectable metastases in regional lymphatic nodes [13, 17, 18, 32]. However, there is little data on the role of these ABPs in the formation of the metastatic profile of SCCLH tumor cells and the mechanisms of their possible functional partnership until now. It is likely that not only tumor cells but also immunocompetent cells can be a source of circulating ABPs in blood serum and the results obtained may reflect the body’s immune response to tumor progression [39, 40].

It should be noted that changes in the mRNA expression levels of \textit{CAP1} and \textit{PFN1} in tumor tissues were similar to those in the serum. The revealed relationship between the serum levels of profiling 1 and \textit{CAP1} and the presence of regional metastases indicated that these proteins participated in the pathogenesis of SCCLH. The multidirectional changes in the serum levels of these ABPs in SCCLH patients were shown for the first time.

**Conclusion**

Thus, serum levels of profiling 1 and \textit{CAP1} were identified as the most significant candidate markers of SCCLH metastasis on the presented group of SCCLH patients. The revealed relationship between the expression activity of genes associated with EMTs and ABPs genes are likely to make a certain contribution to tumor progression providing active rearrangement of the cytoskeleton. Co-expression of RND3 and ABP mRNA is likely to provide a high migration, proliferative activity and the development of a more malignant phenotype of SCCLH. The results obtained also indicate that ABPs, such as cofilin 1, profiling 1, and \textit{CAP1} function as partners. The data obtained can be used as a theoretical platform for the development of additional criteria for predicting lymph node metastasis in SCCLH.

**Author contributions** All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [GVK], [OVČ], [EES] and [ESK]. The first draft of the
manuscript was written by [GVK] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. Example: CRediT taxonomy: Conceptualization: [GVK], [ELC], [IVK]; Methodology: [GVK], [ESK]; Formal analysis and investigation: [GVK], [ESK], [EES]; Writing—original draft preparation: [GVK]; Writing—review and editing: [ELC], [IVK]; Funding acquisition: [IVK]; Resources: [OVC]; Supervision: [ELC], [IVK].

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Data Availability Database will be made available if required.

Declarations

Conflicts of interest The authors declare that there is no conflict of interest.

Consent for publication Patients signed informed consent regarding publishing their data.

Ethical approval The manipulations were carried out under conditions of voluntary participation and confidentiality in accordance with the Helsinki Declaration of the World Medical Association “Ethical Principles for Conducting Scientific Medical Research with Human Participation” as amended in 2000. The study was allowed by Ethic Committees of Tomsk National Research Medical Center.

Informed consent Informed consent was obtained from all individual participants included in the study.

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