

The level of cytoskeleton remodeling proteins in the blood serum and the expression of their mRNA in the tumor tissue in metastasis of the larynx and hypopharynx cancer

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Research Article

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Abstract

Purpose: Actin-binding proteins (ABPs) and various signaling systems are involved in the metastasis of squamous cell carcinoma of the larynx and hypopharynx (SCCLH). The clinical significance of these proteins has not yet been determined. We analyzed the relationship between the mRNA level of cofilin 1 (CFL1), profilin 1 (PFN1), adenylyl cyclase-associated protein 1 (CAP1), SNAIL and RND3 with metastasis in the SCCLH tissue. The serum level of the listed ABPs was estimated and the relationship of them with the expression of the corresponding mRNA was carried out. **Materials and methods:** The expression level of ABPs mRNA was measured by real-time RT-PCR in paired tissue samples taken from 54 patients with SCCLH (T 1-4 N 0-1 M 0). Expression analysis was performed using the $2^{-\Delta\Delta CT}$ method. The level of ABPs in the blood serum was measured by ELISA. Statistical analysis was carried out using the SPSS Statistica 20.0 software package. **Results:** The mRNA expression of the studied genes in tumor tissue of patients with SCCLH T 1-3 N 0 M 0 and T 2-4 N 1-2 M 0 did not differ significantly. High expression of RND3 mRNA was accompanied by an increase in mRNA expression of all studied ABPs. In the blood serum of T 2-4 N 1-2 M 0 patients the level of PFN1 was significantly lower by 21% and the level of CAP1 was higher by 75% compared with the group of patients with T 1-4 N 0 M 0 stage. **Conclusion:** According to our data RND3 is involved in the regulation of molecular cascades SCCLH metastasis. PFN1 and CAP1 serum level can be a good classifier of metastases in patients with SCCLH.

Introduction

Squamous cell carcinoma of the larynx and hypopharynx (SCCLH) is characterized by rapid growth, high metastatic and invasive potential, which determines its high aggressiveness. The high mortality rate in patients with SCCLH (about 40%) in the first year after diagnosis is a significant problem. A quarter of patients have clinically undetectable metastases to the lymph nodes, the manifestation of which occurs in the late postoperative period [1]. Therefore, further investigation on the mechanisms of SCCLH metastasis is necessary to improve patient outcomes.

The aggressiveness of tumors is determined by various molecular and genetic changes, including the acquisition of migration ability by tumor cells, accompanied by reorganization of the cytoskeleton [2, 3]. During metastasis tumor cells can move by the mesenchymal type, with the initiation of epithelial-mesenchymal transition (EMT), and by the amoeboid type, 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-1, 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]. On the cell line of mouse glioblastoma, it was shown that the induced expression of RND3 (Rho family - GTPases) inhibits the activity of Snail1, which blocks the migration of tumor cells [10]. Therefore, the study of the level and balance of mRNA of such signaling proteins as RND3 and SNAIL1 in connection with the metastatic status in patients with SCCLH is of current interest.

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 such ABPs 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]. And if the contribution of cofilin to the development of a more aggressive type of tumor is unambiguous, then the participation of profilin in tumor progression is still being studied [13, 24].

CAP1 accelerates cofilin-mediated disassembly of actin filaments, is involved in mRNA localization and maintenance of cell polarity, motility, and receptor-mediated endocytosis [20]. The increase in CAP1 expression is associated with lymphogenous 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: the EMT marker of the transcription factor SNAI1 and ABPs fascin 1, ezrin, and cyclase-associated protein 1 (CAP1) [11, 12]. The study of the relationship of these ABPs with the level of mRNA expression of regulatory molecules of calpains showed that SCCLH metastasis is associated with a high level of CAPN1 expression, against the background of which the level of CAP1 expression was decreased [14]. Today there is no data on the relationship of EMT-associated markers SNAI1 and RND3 with ABPs cofilin1, profilin 1, and CAP1 in the progression of SCCLH. It is also not known 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) with the processes of lymphogenous metastasis in the tissues of SCCLH. The level of cofilin-1, profilin-1, and CAP1 circulating in the blood stream was assessed in patients with SCCLH. The study will add knowledge about the mechanisms of SCCLH metastasis and will reveal potential markers for predicting the course of the disease.

Materials And Methods

The study was carried out in 54 patients with morphologically verified diagnosis SCCLH, aged 56.5 ± 5.4 years. Clinical details were recorded in TABLE 1. Samples were obtained before the special antitumor treatment. Specimens of serum blood and intact epithelial tissue and primary tumor tissue were collected during videolaryngoscopy. Tissue samples were placed in an RNAlater solution (Ambion, USA) and stored at -80°C .

TABLE 1.

Patients and tumor characteristics

Characteristic	SCCLH case (n = 54)	
n	%	
Gender		
Male	42	77%
Female	12	22%
Age		
32-56 years	29	54%
56-76 years	25	46%
Cancer disease stage (T)		
I	8	14,8%
II	11	20%
III	30	55%
IV	4	7.2%
Pathological differentiation		
Low 9	5	9.2%
Middle 31	30	55.6%
High 20	14	25.8%
Unstage	5	9.2%
Nodal stage		
N-	29	54.7%
N+	25	46.3%

Note: "N-" - a group of patients with SCCLH without clinically confirmed lymphogenous metastasis; "N+" - a group of patients with SCCLH with clinically confirmed lymphogenous metastasis N1-2.

Evaluation of mRNA expression level.

The total mRNA pool was isolated using the CCR-50 kit (Biosilica, Russia). The quality and integrity of RNA was evaluated using capillary electrophoresis on a TapeStation device (Agilent Technologies, USA). The synthesis of the first cDNA strand on the RNA template was carried out using a set of reagents for reverse transcription OT-1 (Synthol, Russia). The level of gene 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. Primers were selected using the Vector NTI Advance 11.5 program and the NCBI database: SNAI1(NM_005985) F5-CCCAATCGGAAGCCTAACT-3, R5- AGTAGAGGAGAAGGACGAAGGA-3; RND3 (NM_001254738) F5- AGAGAGCCACAAAGCGGAT-3, R5-TATCCTCTCAAACGCCTCCTA-3; PFN1 (NM_005022) F-5'-TGGAGCAAACCCTACCCTT-3, R5-AGCCCAGACACCGAACTTT-3; CFL1(NM_005507) F5-CTGCCGCTATGCCCTCTA-3, R5-TTCTTCTTGATGGCGTCCTT-3; CAP1 (NM_001105530) F5-CCAAACGAGCCACAAAGAA-3, R5-ACCCATTACCTGAACTTTGACAT-3. Expression analysis was performed according to the $2^{-\Delta\Delta CT}$ method [27]. As negative controls a reaction mixture without a matrix and with an RNA matrix without a reverse transcription step was used to control the contamination of genomic DNA.

Enzyme-linked immunosorbent assay (ELISA)

The analysis of circulating ABPs was carried out in blood serum using a Multiskan FC 100 microplate ELISA reader (ThermoFisher Scientific), using the Human Adenylyl cyclase-associated protein 1 (CAP1) ELISA kit (Cusabio), ELISA Kit for Profilin 1 (PFN1) (Cloud-Clone Corp), ELISA Kit for Cofilin 1 (CFL1) (Cloud-Clone Corp). Blood serum was obtained according to the approved protocol from the above patients.

Statistical analysis.

The data were processed by SPSS Statistica 20.0 software and presented as Me (Q1:Q2) and as the mean \pm standard deviation. The differences were assumed to be significant at $p < 0.05$. Spearman's rank correlation was performed among all molecular targets.

Results

1. *Analysis of mRNA expression of actin-binding proteins PFN1, CFL1, CAP1 and signaling molecules SNAI1 and RND3 in tumor tissue of patients with squamous cell carcinoma of the larynx and hypopharynx*

The study of the expression level of the genes encoding ABPs (PFN1, CFL1, and CAP1) and signaling molecules (SNAI1 and RND3) showed the presence of all parameters in the tissues of SCCLH. The median values of all parameters in the tumor tissue of patients with SCCLH are presented in Table 2. The

expression profile of genes encoding proteins involved in the remodeling of the actin cytoskeleton did not show statistically significant differences between groups of SCCLH patients with lymphogenous metastases (T2-4N1-2M0, n=24) and SCCLH patients without clinically detectable lymphogenous metastases (T2-4N0M0, n=29). However, there was a tendency to an increase of the expression activity of the SNAI1, CFL1 and CAP1 genes in the tumor tissue of patients with SCCLH (T2-4N1-2M0).

TABLE 2.

Expression level of mRNA of genes encoding proteins involved in cytoskeleton remodeling depending on the presence of metastases in SCCLH patients

mRNA	T1-4N0-2M0 n=54	T1-3N0M0 n = 29	T2-4N1-2M0 n = 25	P _{T1-3N0M0/ T2-4N1-2M0} (U-test)
snai1	0.78(0.15;4.48)	0.41 (0.12;1.44)	3.01 (0.7;11.4)	<i>0.07</i>
rnd3	0.61(0.0;2.30)	0.51 (0.06;3.27)	1.38 (0.22;4.23)	0.18
pfn1	2.51(0.15;13.24)	2.51 (0.01;9.08)	1.71 (0.5;19.7)	0.74
CFL1	1.16(0.10;18.86)	0.68 (0.06;3.6)	2.76 (0.14;25.5)	<i>0.08</i>
CAP1	1.95(0.03;22.66)	2.09 (0.07;12.9)	9.33 (1.14;19.3)	<i>0.06</i>

Note: italics indicate the significance level at the trend ($0.05 > p < 0.1$); cells marked in gray contain the results of mRNA expression of genes associated with the epithelial-mesenchymal transition; p - significance of differences between groups «T1-3N0M0» and «T2-4N1-2M0»

A correlation analysis was carried out in the general group of SCCLH patients to determine the relationship between the expression activity of the EMT markers and ABPs genes. Numerous positive correlations have been identified between the studied molecular parameters in tumor tissue. The expression activity of RND3 was associated with the expression of PFN1 ($r = 0.5$; $p \leq 0.05$) and CFL1 ($r = 0.4$; $p = 0.047$). The SNAI1 mRNA level correlated with the PFN1 mRNA level ($r = 0.4$; $p = 0.046$). Co-expression of mRNA in group of ABPs was found: CAP1-CFL1 ($r = 0.6$; $p = 0.033$), CFL1-PFN1 ($r = 0.7$; $p = 0.042$) and PFN1-CAP1 ($r = 0.5$; $p = 0.037$).

Further the total sample of SCCLH patients was divided into groups depending on the presence of metastases in the regional lymph nodes. 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 correlated with CFL1 and PFN1, while the strength of the relationship between the CAP1-CFL1-PFN1 ABPs mRNA expression was stronger than in the group without metastases (Table 3).

TABLE 3.

Spearman's correlation coefficients in groups with and without metastases: expression of EMT associated and ABPs genes in tumor tissue of SCCLH patients

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

Note: * - correlations with $p \leq 0.05$; cells marked in white contain correlation coefficients for group of SCCLH patients without metastases (T1-3N0M0); cells marked in gray contain correlation coefficients for group of SCCLH patients with metastases (T2-4N1-2M0)

To assess the relationship between the expression level of SNAI1 and RND3 and the metastatic status of SCCLH patients the total group (N = 54, 100%) was divided depending on the level of expression of these genes by the median. The first group consisted of patients with a high level of expression of the SNAI1 and RND3 genes (fig. 1A), the second - with a low level of expression of these genes (fig. 1B). A low expression level of SNAI1 (0 - 1.09) was observed in 54% SCCLH patients of the total group 20% of which had lymphogenous metastases (N+) and 80% without metastases (N-). A high level of SNAI1 (2 - 76.3) was observed in 46% SCCLH patients of the total group, among whom 47% of patients had lymphogenous metastases and 53% did not.

When analyzing the dependence of the level of ABPs mRNA expression on the level of SNAI1 expression activity significant differences were not found for PFN1 and CAP1 (Fig. 2C). A high level of SNAI1 expression was accompanied by a high level of mRNA expression of PFN1.

Low expression activity of RND3 (0 - 1.1) was detected in tumor tissue in 44% SCCLH patients of the total group among whom the proportion of patients with lymphogenous metastases accounted for 18% with metastases and 82% without them (Fig. 1, A). High expression of RND3 mRNA (1.1-81.29) was observed in 56%, among which 51% of patients were with metastases and 49% without metastases (Fig. 1B). A high level of RND3 expression was accompanied by a high level of mRNA expression of PFN1, CFL1, and CAP1 (Fig. 2D). Moreover the dependence of PFN1 and CFL1 mRNA levels on the level of RND3 expression was confirmed by correlation analysis, the results of which were described in Table 3.

1. Quantitative analysis of actin-binding proteins PFN1, CFL1 and CAP1 circulating in the blood serum of patients with squamous cell carcinoma of the larynx and hypopharynx

The next stage of the study was to assess level of circulating ABPs in the blood serum of SCCLH patients by ELISA. Table 4 shows the median values of ABPs level in the blood serum of SCCLH patients in the

general group (T1-4N0-2M0) and in groups depending on the presence of lymphogenous metastasis (T1-3N0M0, T2-4N1-2M0).

TABLE 4.

Level of ABPs in the blood serum of SCCLH patients depending on the metastatic lesion of regional lymph nodes.

ABPs	T1-4N0-2M0 n=54	T1-3N0M0 n = 29	T2-4N1-2M0 n = 25	P (U-test)
PFN1, ng\ml	0.30(0.24;0.37)	0.33(0.27;0.41)	0.26(0.23;0.36)	P =0.05
CFL1, ng\ml	0.84(0.63;1.20)	0.86(0.60;1.01)	0.78(0.65;1.28)	P=0.17
CAP1, ng\ml	0.11(0,08;0.15)	0.08(0.06;0.15)	0.14(0.08;0.19)	P= 0.02

Note: p - significance of differences between groups «T1-3N0M0» and «T2-4N1-2M0»

There was a significant decrease in the level of profilin-1 by 21% ($p = 0.05$) and an increase in the level of CAP1 by 75% ($p = 0.02$) in the blood serum of patients with clinically confirmed metastases to the regional lymphatic nodes compared with the group of patients without lymphogenous metastases. It should be noted that in spite of the fact that no significant links were revealed by correlation analysis the direction of changes in the levels of mRNA expression of PFN 1 and CAP1 in tissues was similar to the serum levels of these proteins. At the same time the expression level of CFL1 in the tumor tissue of SCCLH patients with lymphogenous metastases significantly increased ($p = 0.08$) while the protein level in the blood serum of these patients remained practically unchanged.

ROC analysis was carried out to estimate the possibility to use ABPs level 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 PFN1 and CAP1 were the most significant classifiers (area under the curve AUC = 0.75 (95% CI: 0.06 ~ 0.45) and area under the curve AUC = 0.81 (95% CI: 0.65 ~ 0.97 respectively).

Discussion

Analysis of the expression activity of genes encoding proteins involved in the remodeling of the actin cytoskeleton in the tumor tissue of SCCLH patients depending on the presence of lymphogenous metastases did not reveal statistically significant differences. However, it should be noted that there was a tendency to an increase in the expression activity of the SNAI1, CFL1 and CAP1 genes in the tumor tissue of SCCLH patients T2-4N1-2M0. Tumor tissue obtained by biopsy contains 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. So podosomes, short-lived membrane structures, are formed in cells of the monocytic line such as macrophages, monocytes, dendritic cells, etc. and control matrix remodeling. Podosome superstructures are organized in endothelial cells to control vascular remodeling [29]. Thus the processes of cytoskeleton remodeling are taking place in the tumor microenvironment actively which may be reflected in changes in the total expression and level of ABPs.

Co-expression of the ABPs 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 expression of EMT associated genes (SNAI1, RND3) and functional partners of ABPs (cofilin-1, profilin-1 and CAP1) in the 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 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 CAP1 and cofilin expression is associated with the invasiveness of tumor cells in cancer of some localizations [32]. Later, using structural biology methods it was shown that CAP works in tandem with cofilin accelerating the depolymerization of actin filaments by almost 100 times [30]. However data indicating a more complex role of CAP1 in the implementation of migration activity and invasiveness of tumor cells are accumulating. The knockdown of the CAP1 gene can lead to FAK/Rap1-mediated stimulation of cell adhesion and the formation of lamellipodia with a subsequent increase in cell motility [33]. The role of cofilin and its functional partner profilin 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. The close relationship between the expression of cofilin 1 and profilin 1 in SCCLH patients is revealed and shown by us for the first time. This data requires further more detailed study in respect that changes in the expression activity of the studied proteins mRNA are multidirectional. The functional partnership between PFN1 and CFL1 in the tumor process is described in the literature [13]. The study of mRNA co-expression of profilin 1 and CAP1 was carried out for the first time. However given the rather contradictory data on the role of the PFN1 and CFL1 tandem in the development of aggressiveness of various tumors [13, 34, 35, 36] further studies of the relationship of these genes and their proteins with SCCLH progression are required.

The revealed relationship between the expression activity of RND3 and PFN1, CFL1, and CAP1 mRNA in the presented study probably indicates that a special place in molecular events during tumor metastasis belongs to RND3. It has been shown that RND3 is involved in various signaling cascades and regulation of cytoskeleton organization [8, 9, 10, 25]. The multidirectional changes in Rnd3 expression in tumors are mentioned and associated with the mutational status [37, 38]. An increased level of RND3 expression in combination with changes in the mRNA levels of CAP1, PFN1, and CFL1 and the absence of a clear

association with the presence of lymphogenous metastases possibly indicates the molecular features of SCCLH progression. Further studies are required for final conclusions about the role of Rnd3 in lymphogenous metastasis in SCCLH patients including in vitro studies which will expand the knowledge about the regulation of ABPs.

The study of profilin 1, cofilin 1 and CAP1 level in the blood serum of SCCLH patients also showed dependence on the metastatic status. The detected decrease in the level of profilin-1 and an increase in the level of CAP1 in the blood serum in patients with clinically detectable metastases in the regional lymphatic nodes do not contradict the available literature data [13, 17, 18, 32]. But data on the role of these ABPs in the formation of the metastatic profile of tumor cells in SCCLH have not been described and the mechanisms of their possible functional partnership have not been disclosed. Probably 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 the direction of changes in the expression levels of CAP1 and PFN1 mRNA in tumor tissues was similar to changes in the serum protein level. The revealed dependence of the serum levels of profilin 1 and CAP1 on the presence of regional metastases indicates their participation in the pathogenesis of SCCLH. The multidirectional change in the level of these ABPs in the blood serum of SCCLH patients has been shown for the first time.

Conclusion

Thus serum levels of profilin 1 and CAP1 were identified as the most significant candidate markers of SCCLH metastasis on the presented group of SCCLH patients. The revealed interrelationships in 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 mRNA of ABPs possibly provides a high migration, proliferative activity and the development of a more malignant phenotype of SCCLH. The results obtained also indicate that ABPs cofilin 1, profilin 1, and CAP1 function as partners. In general the data obtained can be used as a theoretical platform for the development of additional criteria for the prognosis of lymphogenous metastasis in SCCLH.

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Figures

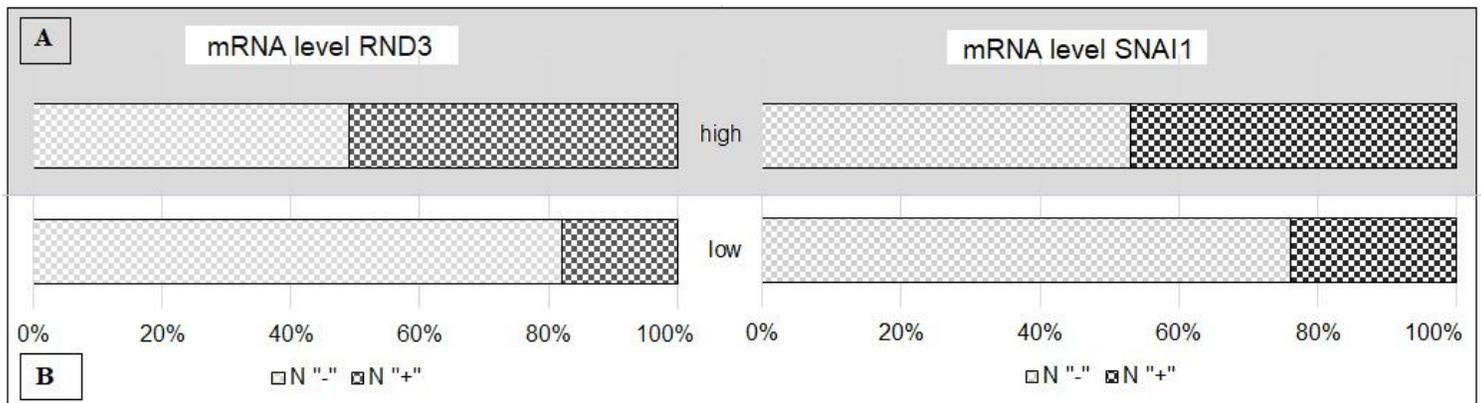


Figure 1

Distribution of SCCLH patients relative to the level of mRNA SNAI1 and Rho –GTPase RND3 in tumor tissue and depending on the metastatic process. (A) - low level of mRNA expression (low), (B) - high level of mRNA expression (high). Note: "N-" - SCCLH patients without clinically confirmed lymphogenous metastasis; "N+" - SCCLH patients with clinically confirmed lymphogenous metastasis N1-2. The expression level data in Figures 1A and 1C are standardized.

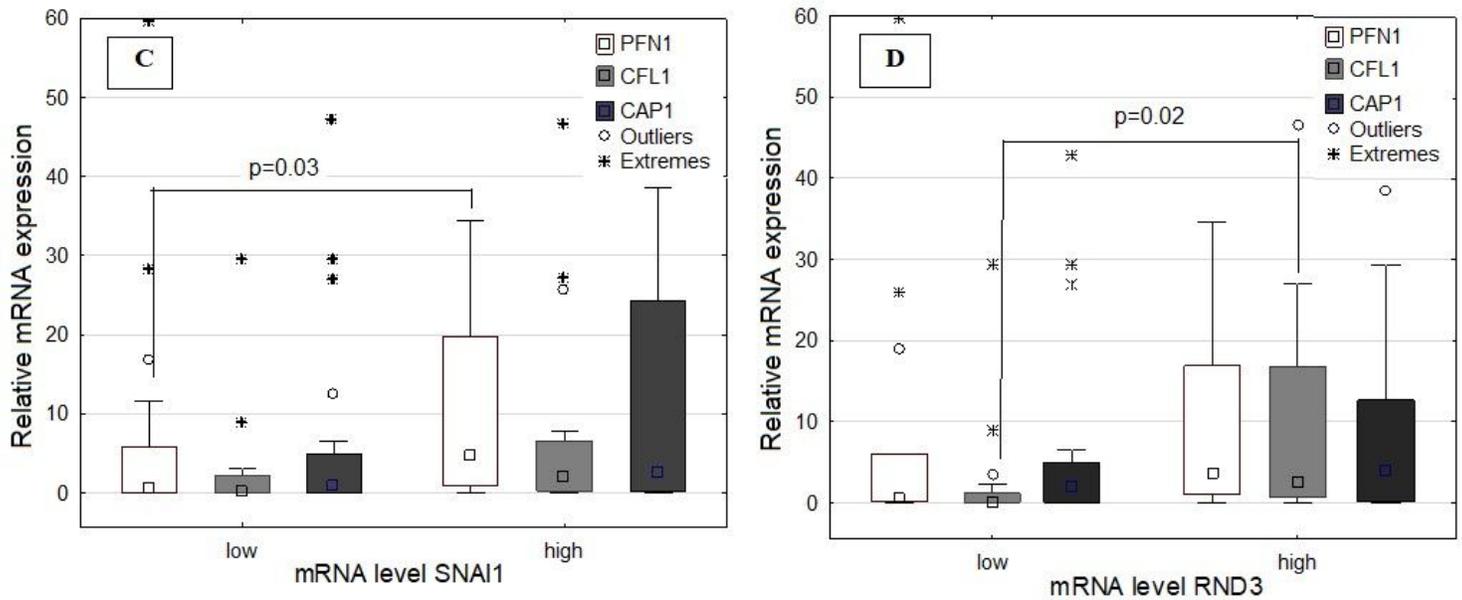


Figure 2

Expression level of genes encoding cytoskeleton proteins PFN1, CFL1, and CAP1 against the background of different expression activities of SNAI1 (C) and RND3 (D) mRNAs. Relative expression was calculated by Δ Ct Method and histograms represent Mean (\pm SD) expressions of mRNA for PFN1, CFL1 and CAP1. Note: Box plots showing the significance of the Mann-Whitney U test (p) between groups of different expression activities of SNAI1 (C) and RND3 (D) mRNAs. The lines in the boxes represent the medians; boundaries and whiskers indicate the 25th/75th percentiles, respectively

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