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Relationship of intracellular proteolysis with CAP1 and cofilin1 in non-small-cell lung cancer

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The main cause of death in non-small-cell lung cancer (NSCLC) is tumor progression, in which metastasis and invasion play an important role. The metastatic cascade is marked by a change in morphological, biological, biochemical and functional characteristics, including the acquisition of cellular mobility. The migration activity of tumor cells determines the work of actin-binding proteins that cause their functional partners CAP1 and cofilin. Of interest is the study of the regulation of working tandem CAP1/cofilin in NSCLC. The mechanism that regulates the level of proteins in cells is proteolysis, carried out by proteasomes and calpains. Therefore, the aim of this study was to estimate the expression of CAP1/CFL1 mRNA and their protein level in NSCLC tissues, and to analyze the possible mechanisms of their regulation by the proteasome and calpain systems. Samples of NSCLC and histological unchanged lung tissue were used (n = 42). The CAP1 and CFL1 mRNA expressions were determined by real-time PCR, the contents of proteins encoded by them were determined by Western blotting, and the activity of proteasomes and calpains by the fluorimetric method. There was an increase in the expression of mRNA and protein levels of CAP1 and cofilin in the tumor tissue compared with the unchanged lung tissue. The expression of mRNA and the level of CAP1 in tumor tissue increased during growth of the primary tumor. The cofilin level in the tumor tissue decreases against the background of increased expression of its mRNA. At the same time, during tumor growth, the activity of proteasomes and calpains increased. A negative regression relationships between the activity of proteasomes and the levels of CAP1 and cofilin, as well as the activity of calpains and the level of cofilin, were found. It can be assumed that proteasomes and calpains are involved in the degradation of CAP1 and cofilin. The data obtained suggest the importance of CAP1, cofilin and proteolytic systems in the tumor transformation and lymphogenous metastasis.

Keywords. CAP1; Cofilin; Proteasome; Calpain; Proteolysis; Non-small-cell lung cancer (NSCLC)

1. Introduction

The number of malignant neoplasms occupying a leading position in the structure of cancer incidence and mortality includes non-small-cell lung cancer (NSCLC) (Torre *et al*. 2016). The main cause of death in oncological diseases is tumor progression, which is characterized by invasion and metastasis. An important role in these processes is played by the purchase by cells of the ability to mobility, associated with the reorganization of the actin cytoskeleton. More than 100 proteins in the cytoplasm bind to actin, performing various functions: they regulate the volume of the G-actin pool, affect the polymerization rate, stabilize the ends of the threads, cross-link the filaments with each other, and destroy the double helix of F-actin. The activity of these proteins is regulated by protein kinases (Ma *et al*. 2019; Semenyuk and Muronetz 2019).

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Of interest is one of the poorly studied actin-binding proteins, the cyclase-associated protein (CAP1), which is currently being actively studied in various pathologies (Kakurina *et al*. 2018a). CAP1 consists of six domains, the N-terminal domain of which in the human cell is associated with another actin-binding protein cofilin (Zhou *et al*. 2014). Cofilin involved in the actin depolymerization by cutting the actin filament itself or increasing the actin depolymerization rate through the dissociation of actin monomers from the pointed end. With the C-terminus and the WH2 domain, CAP1 can independently with high affinity bind to actin monomers and break its bonds with the tweenfilin protein (Makkonen *et al*. 2013). So, these two mechanisms can allow the CAP1 protein to accelerate the reorganization of F-actin in the cell, which contributes to the formation of actin-rich structures, such as filopodia and lamellopodia, which are necessary for cell migration (Zhou *et al*. 2014).

Over the past 10 years, the involvement of CAP1 in the pathogenesis of cancer of several localizations, including lung cancer, has been shown (Hua *et al*. 2015; Kakurina *et al*. 2018b; Li *et al*. 2013; Liu *et al*. 2014). Using real-time PCR methods, Western-blotting on cell lines of non-invasive (95-C) and invasive (95-D) lung cancer, it was shown that CAP1 protein level increase in lung cancer tissue compared to unchanged tissue, and gene expression CAP1 in the tissue of metastatic tumors significantly exceeds that in the tissue of non-metastatic tumors. In the same work, the relationship of CAP1 gene expression with the stage of lung adenocarcinoma was noted (Tan *et al*. 2013). Other researchers using immunohistochemistry and Western blotting on NSCLC tissues taken from patients at different stages of the disease, built a model using CAP1 to predict the appearance of brain metastases with sensitivity and specificity of 79.5 and 67.1%, respectively (Xie *et al*. 2015).

One of the functional partners of CAP1 is cofilin. It is included in the process of metastasis in NSCLC. Numerous studies on NSCLC cell lines show that inhibition of cofilin stops the migration and invasion of tumor cells (Ali *et al*. 2016; Kang *et al*. 2015). In addition, proteomic analysis showed the possible significance of cofilin as a gene for multidrug resistance and radioresistance in lung adenocarcinoma cells (Becker *et al*. 2014). Some researchers suggest to use the cofilin as a diagnostic and prognostic biomarker for NSCLC (Castro *et al*. 2013; Müller *et al*. 2011; Peng *et al*. 2011). In our previous studies, it was also shown that an increase in the level of CAP1 and cofilin in tumor tissue increases the risk of distant metastases (Kolegova *et al*. 2019).

Despite the fact that the pathological mobility of tumor cells provides invasive growth, recurrence, angiogenesis and the formation of distant metastases, the regulation of CAP1 and cofilin in tumor diseases has not been fully defined. The functioning of proteins in the cell is tightly regulated by various post-translational modifications, including phosphorylation, ubiquitination, and acetylation (Ben-Shahar *et al*. 1999; Castro *et al*. 2013). Protein levels depend on synthesis and degradation processes. The last ones are carried out by intracellular proteases including proteasomes and calpains. In some types of cells the possibility of proteasome degradation of actin cytoskeleton proteins, including cofilin has been shown (Yoo *et al*. 2010). There is evidence of the active participation of calpains in the degradation of cytoskeletal proteins, which play an important role in the process of metastasis (Gokhin *et al*. 2014).

Based on the foregoing, the aim of our work was to study the level of mRNA expression and the protein content of CAP1 and cofilin in association with proteasome and calpain activities in NSCLC.

2. Materials and methods

2.1 *Collection of human tissue*

The study was 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 and Tomsk Regional Oncology Center (approvals No. 480/11 and No. 15-07, respectively). All patients enrolled were diagnosed with NSCLC by pathology and did not have other malignancies. The patients were diagnosed with the disease for the first time and did not receive radiotherapy, chemotherapy, or immunotherapy before surgery. The study was carried out in 42 patients with morphologically verified diagnosis of NSCLC: 33 (79%) men and 9 (21%) women, aged 58.5 $\pm$ 1.3 years. Specimens of adjacent (1 cm from tumor) histologically unchanged lung tissue, primary tumor tissue and lymphogenic metastasis tissue were collected from February 2014 to October 2016. All specimens were stored at -80°C until use (samples intended for the isolation of nucleic acids

were additionally stored in RNAlater (Thermo Fisher Scientific, USA)).

2.2 *Preparation of clarified homogenates*

Frozen tissue (100 mg) was homogenized in liquid nitrogen, suspended in 300 µl 50 mM Tris-HCl buffer (pH 7.5) containing (in mM): 2 ATP, 5 magnesium chloride, 1 dithiothreitol, 1 EDTA, and 100 sodium chloride. The homogenate was centrifuged during 60 min at 10,000g and 4°C. The supernatant (clarified homogenate) was used for further studies.

2.3 *Measurement of proteasome and calpain activities*

The proteasome chymotrypsin-like (ChLA), caspase-like (CLA) activities and activity of calpains (AC) were evaluated in clarified homogenates of the studied tissues by hydrolysis of fluorogenic oligopeptides; Suc-LLVY-AMC (Sigma, Japan) served as the substrate for ChLA, Cbz-LLG-AMC (Sigma, Japan) for CLA (Ben-Shahar *et al*. 1999) and Cbz-LLG-AMC (Sigma, Japan) for AC (Sandmann *et al*. 2002) measurements. Proteasome activity inhibitor MG132 (Sigma, Japan) and calpain activity inhibitor MG101 (Sigma, Japan) were added to the samples in order to rule out the contribution of the admixture proteolytic activities. The resultant product was evaluated on a fluorimeter at λex = 380 nm and λem = 440 nm. The quantity of the enzyme at which 1 nmol of the respective substrate was hydrolyzed within 1 min was taken for activity unit. The specific activities of proteasomes and calpains were expressed in activity units/mg protein. Protein was measured by Lowry method.

2.4 *Evaluation of CAP1 and cofilin level*

The expression of CAP1 and cofilin was assessed using Western blotting. Samples containing 20 µg of total protein were separated in a vertical 10% denaturing polyacrylamide gel in Tris-glycine buffer at 200 V and 400 mA, and then transferred to a PVDF membrane (Millipore, USA) in Tris-glycine buffer containing 10% ethanol, at 100V and 350 mA for 1h. Protein production was analyzed using rabbit primary antibodies in 1:1500 dilution to CAP1, cofilin and β-actin (Cell Signaling Technology, USA). Horse anti-rabbit antibodies conjugated with horseradish peroxidase (Cell Signaling Technology, USA) in 1:2000 dilution were used as secondary antibodies. A semi-automated iBind Western System (Thermo Fisher Scientific, USA) was used to bind antibodies and wash the membranes. Then the membrane was treated with the ECL chemiluminescence detection system (GE Healthcare, UK). For immunodetection, the ChemiDoc Touch Imaging System (Bio Rad, USA) was used. The density of the bands was assessed using the Image Lab computer program. Standardization was carried out with respect to β-actin. The results were expressed in relation to the level of the studied protein in unchanged tissue.

2.5 *Isolation of mRNA from tissues and cDNA production*

RNA was isolated using the CCR-50 kit (Biosilica, Russia) according to the manufacturer's recommendations. The concentration and purity of RNA isolation were estimated using a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, USA). RNA concentration ranged from 80 to 250 ng/µl, A260/A280 = 1.95–2.05; A260/A230 = 1.90–2.31. The preparation of cDNA on the RNA template was carried out using a set of reagents for reverse transcription OT-1 (Synthol, Russia). The reaction was carried out for 30 minutes at 42°C, then reverse transcriptase was inactivated at 95°C for 2 min. The resulting reaction mixture containing cDNA in a volume of 3 µl was immediately used as a template for carrying out the polymerase chain reaction (PCR).

2.6 *Real-time PCR*

Relative quantitative polymerase chain reaction in real time was carried out on a Rotor-Gene 6000 amplifier (Corbett Research, Australia). The volume of the PCR mixture had a final volume of 25 µL and contained the following components: BioMaster HS-qPCR (100 mM Tris-HCl, pH 8.5, 100 mM KCl, 0.4 mM dNTPs, 3 mM MgCl2, 0.06 units/µl Taq DNA polymerase, 0.025% Tween-20, HS-Taq DNA polymerase stabilizers, SYBR Green 1 and an inert dye) (Biolabmix, Russia) 12.5 µL, 1 µL forward primer, 1 µL reverse primer, 3 µL cDNA template (≈ 20 ng/µL) and 7.5 µL of deionized water. The GAPDH (glyceraldehydes-3-phosphate dehydrogenase) enzyme gene was used as a control, and the expression level of each target gene was normalized in relation to its expression according to the $2^{-\Delta\Delta CT}$ method. PCR reaction protocol: preheating at 95°C, 6

min, 40 main cycles: denaturation at 95°C, 10 s, annealing and elongation: 62°C, 35 s. As a calibrator, we used mRNA (cDNA) isolated from morphologically unchanged lung tissue. Primers were selected using the Vector NTI Advance 11.5 software and the NCBI database: CFL1 (F 5′-CTGCCGCTATGCC CTCTA-3′; R 5′-TTCTTCTTGATGGCGTCCTT-3′); CAP1 (F 5′-CCAAACGAGCCACAAAGAA-3′;R 5′-ACCCATTACCTGAACTTTGACAT-3′); GAPDH (F 5′- GGAAGTCAGGTGGAGCGA-3′; R 5′-GCA ACAATATCCACTTTACCAGA-3′). The reaction specificity was monitored after each PCR, for each sample and each gene using automatic electrophoresis on a 2200 TapeStation (Agilent Technologies, USA) and R6K ScreenTape kit (Agilent Technologies, USA). A negative control was set: no template + PCR mix + primers, template + PCR mix without primers, RNA + PCR mix + primers. RNA quality was assessed automatically using the RIN (RNA Integrity Number) index, which varied from 4 to 6 for the studied RNA samples. The reaction efficiency for all primers varied from 1.8 to 1.9.

2.7 *Statistical analysis*

The data were processed by SPSS Statistica 20.0 software and presented as median and interquartile range (Me (Q25, Q75)). Verification of the normality of the distribution of the studied samples was performed using the Kolmogorov-Smirnov criterion. The significance of differences between groups was determined using the nonparametric Mann-Whitney test for independent samples. Spearman's rank correlation coefficient, linear and logistic regression methods were used to assess the relationship of studied parameters. The differences were assumed to be significant at $p < 0.05$.

3. Results

First, mRNA expression and the protein level of CAP1 and cofilin in the tumor, adjacent unchanged and lymphatic metastatic tissue of patients with NSCLC were studied. As a result of the study, a significant increase in the protein level of CAP1 and cofilin in the tissue of the primary tumor and lymphogenic metastases was shown compared with adjacent unchanged lung tissue (figure 1). The expression of CAP1 and cofilin mRNA in 60–61% of patients in tumor tissue was also increased compared to adjacent normal lung tissue (figure 2).

Next, the activity of proteasomes and calpains in the tumor and adjacent unchanged tissue of patients with NSCLC was evaluated (table 1). In the tissue of the primary tumor, ChLA and CLA of proteasomes were higher than in adjacent unchanged tissue by 4.3 and 2.7 times, respectively; in the tissue of lymphogenous metastases – 2.6 and 2.3 times, respectively. AC was also 2.4 times higher in the primary tumor and 1.8 times higher in metastatic lymph nodes compared with adjacent normal tissue.

Illustrative can be considered the result obtained when dividing patients into groups depending on the size of the primary node (T) (table 2). The experiments showed that the growth of the primary tumor in patients with NSCLC is associated with an increase in the mRNA expression of CAP1 and CFL1 in the tumor tissue. The level of CAP1 protein also increased, while the level of cofilin decreased by 3 times in the tumor tissue with tumor progression from stage T2 to T3. At the same time, the activities of the studied proteolytic systems significantly increased 2.5–6 times.

At the final stage of the study, we used correlation and regression analyzes to estimate the relationships between actin-binding proteins CAP1 and cofilin and the activity of proteasomes and calpains and their mutual influence in NSCLC tissue (figure 3). Similar relationships were shown in the tissue of the primary tumor and in the tissue of lymphogenic metastases.

Correlation relationships of average strength were revealed between the parameteres of proteolytic systems: ChLA and CLA of proteasomes are positively interconnected ($R = 0.7$, $p = 0.010$), AC has a positive correlation with CLA of proteasomes ($R = 0.6$, $p = 0.033$). Actin-binding proteins also functioned

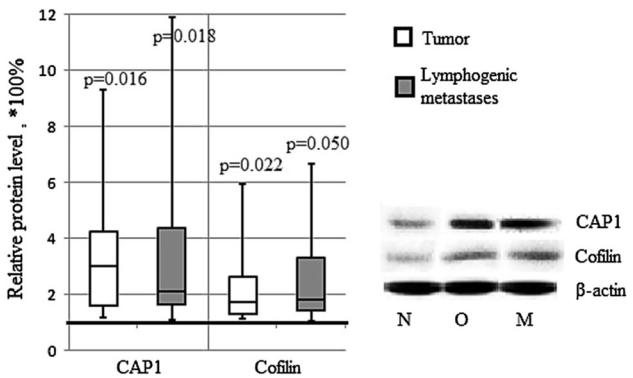

Figure 1. Protein content of CAP1 and cofilin in tumor tissue and lymphogenic metastases of patients with non-small-cell lung cancer. Note:100% is the level of CAP1 and cofilin in intact tissue. N: intact tissue; T: primary tumor tissue; M: metastatic tissue.

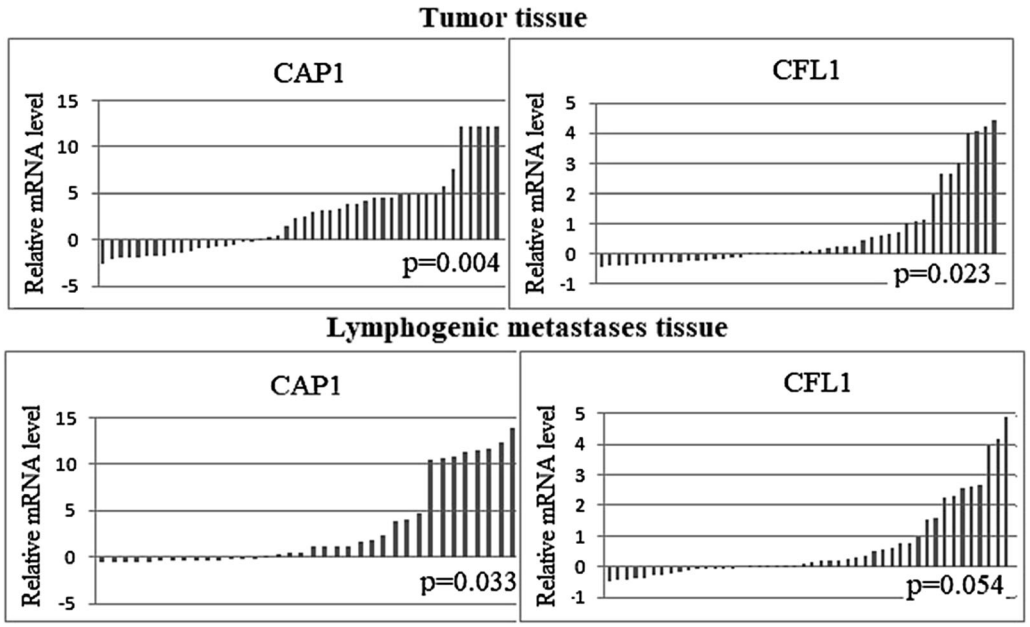

Figure 2. Level of mRNA expression of CAP1 and cofilin in tumor tissue and lymphogenic metastases of patients with non-small-cell lung cancer. Note: 0 is the level of CAP1 and cofilin in intact tissue.

Table 1. Proteasome chymotrypsin-like (ChLA), caspase-like (CLA) activities and activity of calpains (AC) in intact tissue, primary tumors, and lymphogenic metastases of patients with non-small-cell lung cancer

Parameter	Intact tissue n = 42	Tumor tissue n = 42	Lymphogenic metastases n = 31	p
ChLA (10^3 U/mg protein)	12.43 (6.77; 19.52)	53.17 (26.12; 102.17)	33.33 (21.76; 53.97)	$p_{12} < 0.001$; $p_{13} < 0.001$; $p_{23} = 0.114$
CLA (10^3 U/mg protein)	14.19 (10.16; 19.78)	37.45 (20.76; 74.57)	33.33 (13.09; 63.98)	$p_{12} = 0.009$; $p_{13} = 0.038$; $p_{23} = 0.225$
AC (10^3 U/mg protein)	36.38 (17.00; 57.19)	87.69 (46.97; 178.42)	67.01 (32.81; 161.11)	$p_{12} = 0.047$; $p_{13} = 0.010$; $p_{23} = 0.445$

Table 2. Proteasome chymotrypsin-like (ChLA), caspase-like (CLA) activities and activity of calpains (AC), protein and mRNA levels of CAP1 and cofilin 1 in the tissue of primary tumor depending on the size of the primary node

Parameter	$T_2N_{0-2}M_0$ n = 24	$T_3N_{0-2}M_0$ n = 18	p
CAP1 (relative mRNA level)	1.05 (0.30; 2.30)	4.60 (2.55; 9.90)	0.005
CFL1 (relative mRNA level)	1.60 (0.30; 3.40)	5.20 (4.40; 5.80)	< 0.001
CAP1 (relative protein level)	2.13 (2.06; 3.34)	4.42 (3.67; 6.60)	0.031
Cofilin (relative protein level)	1.91 (1.48; 2.01)	0.65 (0.23; 1.05)	0.033
ChLA (10^3 U/mg protein)	17.68 (5.91; 26.49)	41.42 (29.26; 100.45)	0.015
CLA (10^3 U/mg protein)	10.74 (3.59; 20.08)	63.15 (23.20; 95.88)	0.007
AC (10^3 U/mg protein)	46.97 (36.06; 87.70)	113.25 (69.66; 194.57)	0.022

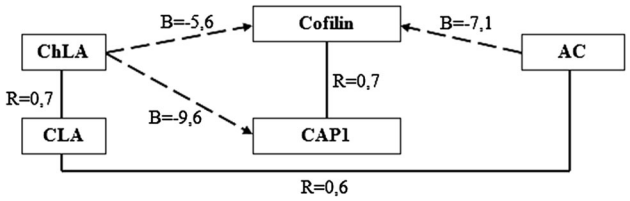

Figure 3. Correlation and regression relationships of CAP1, cofilin and activities of proteasomes and calpains in tumor tissue of patients with non-small-cell lung cancer. Note: The straight line indicates the correlation relationships (R is the Spearman correlation coefficient), the dashed line indicates the regression relationships (B is the coefficient in the linear regression equation), ChLA is the chimotrypsin-like activity of the proteasomes, CLA is the caspase-like activity of the proteasomes, AC is the activity of calpains; all presented values have statistical significance $p < 0.05$.

interconnectedly: positive correlation between the level of CAP1 and cofilin was noted ($R = 0.7$, $p = 0.004$), and quite strong co-expression of their genes was observed ($R = 0.8$, $p < 0.001$).

The linear regression method showed the possible regulatory effect of proteasome and calpain activities on the CAP1 and cofilin levels in NSCLC tissues. It is likely that the ChLA of proteasomes degrades the actin-binding proteins CAP1 ($B = -9.6$, $p < 0.001$) and cofilin ($B = -5.6$, $p = 0.005$), and AC is involved in the degradation of cofilin ($B = -7.1$, $p = 0.002$).

4. Discussion

CAP1 and cofilin play an important role in the reorganization of the cytoskeleton, which can affect cell motility, cell division and other processes. Destroying actin filaments from one end, they contribute to active actin threadmilling in the cell. Probably, the noted increase in actin-binding proteins and mRNA levels in the tumor tissue compared with adjacent unchanged tissue may indicate active invasion of tumor cells, as well as their preparation for metastatic processes.

The revealed increase in the expression activity of CAP1 in the primary tumor in comparison with the practically unchanged level of expression of cofilin 1 mRNA, possibly indicates the tissue-specificity of the participation of these actin binding proteins in tumor growth in NSCLC. Considering the data on the protein level of CAP1 and cofilin 1 obtained by the Western blotting method, which are consistent with the results of RT-PCR, it is possible that CAP1 plays a significant role in the functioning of cofilin 1, and it is this ratio of CAP1 and cofilin that is important for the metastatic process in NSCLC.

In the tissue of the primary tumor and lymphogenic metastases, an increase in the proteasome and calpain-dependent proteolysis was revealed in comparison with the corresponding adjacent unchanged tissue. The results obtained on the increase in the activity of proteasomes and calpains in malignant tissue compared to normal tissue are consistent with the literature data in other localizations (Ivanova *et al*. 2014; Lakshmikuttyamma *et al*. 2004; Shashova *et al*. 2017). It is likely that the proteolytic activation in NSCLC occurs due to the increasing intensity of intracellular processes. Tumor growth is accompanied by a violation of the cell cycle, increased proliferation and inhibition of apoptosis. In order to carry out effective regulation of the level of proteins and preservation of the cellular proteome, the activity of proteases is enhanced in tumors, substrates for which are cell cycle regulating proteins, estrogen and progesterone receptors, components of the system of insulin-like growth factors, many transcription factors, pRb and p53 proteins, an NF-inhibitor κB IκB, signaling pathway components (Rothberg *et al*. 2012).

With a change in the size of the primary tumor (T), a persistent increase in the mRNA expression and the protein level of CAP1 was found, while the expression of cofilin mRNA rose and its protein level decreased. This inconsistence in the changes in the level of protein and its mRNA in the tumor is interesting and requires further study. It can be hypothesized that the decrease in cofilin level in the tumor results from activation of some regulatory mechanisms at the posttranslational level. For instance, it was shown that the level of cofilin in the cell can be regulated by different miRNAs due to RNA-interference (Li *et al*. 2017; Tian *et al*. 2015), as well as by proteolytic systems, e.g. proteasomes and calpains. Our results showed the activation of proteasome and calpain proteolytic systems with increasing tumor size T, which increases the degradation of waste proteins, including actin binding proteins (Larsen *et al*. 2008).

When conducting correlation analysis, it was confirmed that intracellular proteases functioned as a single network, including actin-binding proteins. Regression analysis showed a rather strong negative relationship between the chymotrypsin-like activity of the proteasomes and the level of CAP1 and cofilin, and the activity of calpains with cofilin level. Some of the data we have obtained have similar results in world literature. On 293T and HeLa cell cultures, degradation of

cofilin proteasomes after phosphorylation was confirmed (Yoo *et al*. 2010).

As a result, it was shown that in NSCLC there is an increase in mRNA and protein levels of CAP1 and cofilin, as well as the activity of proteasomes and calpains, with compared to unchanged tissue. Based on the correlation and regression relationships, it can be assumed that the proteasome and calpain systems are responsible for the degradation of the CAP1 and cofilin proteins in the tumor tissue of NSCLC. The data obtained suggest the importance of the work of the CAP1/cofilin tandem and intracellular proteolytic systems in the processes of tumor transformation and lymphogenous metastasis of NSCLC. In general, the results provide new theoretical data on the mechanisms of tumor progression both in general and in NSCLC.

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