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COLOCALIZATION OF USP1 AND PH DOMAIN OF Bcr-Abl ONCOPROTEIN IN TERMS OF CRONIC MYELOID LEUKEMIA CELL REARRANGEMENTS

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The development of chronic myeloid leukemia (CML) is the result of a reciprocal translocation between chromosomes 9 and 22 due to the emergence of Philadelphia chromosome. The product of this mutation is a hybrid oncoprotein Bcr-Abl. According to the results of mass spectrometric analysis, USP1 protein was identified as a potential candidate for interaction with the PH domain Bcr-Abl oncoprotein. Due to the deubiquitination properties, USP1 protein can prevent proteasomal degradation of Bcr-Abl oncoprotein in a cell and, consequently, contribute to its accumulation, and the progression of the disease. In this work, creating the genetic constructs, we detected the USP1 protein localization in the cell. Also, a nuclear colocalization of USP1 protein with PH domain of Bcr-Abl oncoprotein in HEK293T cells was shown. The results are important for understanding the implications of the Philadelphia chromosome emergence, and the development of new methods for CML treatment, since the recent techniques are not always effective due to the emergence of numerous mutations that cause drug resistance and relapse of the disease.

Key words: *chronic myeloid leukemia, Bcr-Abl protein, PH domain, USP1 protein, deubiquitination, USP1 protein localization, colocalization USP1 protein and PH domain.*

Introduction. Chronic myeloid leukemia (CML) as clonal disease, characterized by proliferation of cells of granulocytic germ hematopoietic system, occupies 10 % of all leukemias. The cause of CML in 95 % of patients is the appearance of the Philadelphia chromosome (Ph+), which is the result of reciprocal translocation between chromosomes 9 and 22 [1, 2]. The product of this mutation is Bcr-Abl oncoprotein. This protein is found to exist in three forms: p190, p210, p230 and associates with the acute lymphoblastic leukemia (ALL), chronic myelogenous (CML) and chronic neutrophilic leukemia (CNL), respectively [3–5]. It is distinguished by the presence of PH and DH domains. From these three forms only p210, p230 contain PH domain. Determination the role of these domains in developing of various forms of leukemia and of novel CML treatments is of great importance [6, 7]. To date, using the existing drugs is unable to completely eliminate the expression of the protein leading to relapse of disease.

According to preliminary results of the mass spectrometric analysis, 23 proteins were identified that are potential candidates for interaction with the PH domain of Bcr-Abl oncoprotein [8]. One such protein is an ubiquitin specific protease 1 (USP1) from the protein cysteine proteases group. *USP*1 gene is localized on chromosome 1. USP1 consists of three domains: two intracellular peptidases and ubiquitin carboxyl-terminal hydrolase. The main function of USP1 protein is deubiquitinating the proteins in the cell [9]. Deubiquitination and ubiquitination, interdependent process, that are involved in numerous cellular functions, including cell cycle regulation, proteasome- and lysosome-dependent protein degradation, gene expression, DNA repair, kinase activation and more [10–13]. USP1 protein possess the regulatory role in response to DNA damage during Fankoni anemia (AF) [14] and translesion synthesis (TLS), plays important role in cancer-related processes [15, 16]. Bearing in the mind that USP1 is often overexpressed in tumors [17], we suggest that this protein may serve as an appropriate target for cancer therapy, which inhibition might help to overcome chemical resistance. Bcr-Abl oncoprotein deubiquitination by USP1 protein can thus prevent its degradation in proteosome, leading to its accumulation in the cell, and disease progression. Furthermore, proteins have strictly determined localization in specific organelles when performing biological functions [18]. Deubiquitination can affect the localization of protein that in turn causes the violations of signaling pathways [19]. Understanding the rules for subcellular localizations of proteins would gain an insight into their function. Due to interaction with each other, proteins necessarily share a common subcellular localization, or an interface between two physically adjacent compartments, at least transiently or conditionally [20–22].

In this work we present data on colocalization of USP1 and PH domain of Bcr-Abl proteins that may help in understanding their functions and participation of different protein complexes in pathological alterations.

Materials and Methods. *Genetic constructs.* For amplification of full length *USP*1 cDNA the following oligonucleotide primers have been chosen: USP1 fwd AATTGCCTGGTGTCATACCTAGTG) and USP1 rev AGAGACCAATAATATCCAGTAGC). For the choice of primers we used PerlPrimer resource. Genetic construct pCMV-XL5-USP1 carring for the full length *USP*1 coding sequence (obtained from the Department of Molecular Genetics' bank, IMBG) has been used as the matrix plasmid. Components of PCR conditions were set according to the manufacturer's instructions (Thermo Scientific). We cloned full length *USP*1 cDNA into vector pUC18 by *Sma*I site. Then *USP*1 coding sequence was cut from pUC18-USP1 by *Kpn*I and *Eco*RI restriction endonucleases and subcloned into pECFP-C3. To check availability and orientation of insert the following analytical methods were used: restriction, PCR. Also, to avoid genetic mutations derived constructs were sequenced. Genetic construct purification for sequencing was done on silica-based columns (Thermo Scientific). Plasmid DNA purification for transfection was done by non-ionic detergent solution with LiCl.

Cell culture and transfection. The HEK293T cell line was used. The cells were grown until 90 % confluency in DMEM complete at 37 °C and 5 % CO_2 in 6-well plate. To determine the localization of the USP1 protein in cell we transfected 3 μg of pECFP-C3-USP1 into HEK293T cells. Localization of the PH domain was determined in HEK293T cells transfected by 3 μg of pmCitrineC1-PH. pmCitrineC1-PH construct coding for PH domain of Bcr-Abl protein was obtained from the Department of Molecular Genetics' bank, IMBG. To analyze colocalization of USP1 and PH domain of Bcr-Abl protein 3 μg of pECFP-C3-USP1 and 3 μg of pmCitrineC1-PH were used for cotransfection. As a control, pECFP-C3 and pmCitrineC1 vectors in amount of 3 μg each were used. DNA was diluted in 200 μl of DMEM, and appropriate volume of PEI (3:1 μl PEI : μg DNA) was dilluted in 200 μl DMEM. Both solutions were mixed and put at room temperature for 20 min. Transfection mix have been added to cells covered with DMEM complete, and cells were grown for 24 h at 37 °C and 5 % CO_2. Protein expression was analyzed by fluorescent and confocal microscopy.

Fluorescent and confocal microscopy. Before fixation, HEK293T cells were washed twice with PBS warmed to 37 °C (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), fixed for 20 min in fixation solution (4 % paraformaldehyde in PBS) at room temperature and washed thrice in PBS. The samples were incubated for 30 min in 10 mM cupric sulphate in 50 mM ammonium acetate, pH 5.0, to eliminate autofluorescence. After the samples were washed and embedded into Mowiol (Sigma) medium containing 2,5 % DABCO (Sigma). Microscopy studies were performed using Leica DM1000 light microscope (Germany) and Zeiss LSM 510 microscope (Germany). Analysis of the results was performed by means of ImageJ software.

Visual and quantitative analyses. For visual analysis the Zeiss LSM Image Browser software was used. Pearson's correlation coefficient (PCC) and Manders' Colocalization Coefficients (MCC) represent the two major metrics of colocalization used in biomedical research [23–25]. These quantitative indicators were estimated by the Fiji ImageJ software using the ROI function to reduce impact on the outcome of background artifacts.

Results and discussion. *pECFP-C3-USP1 construction. USP*1 gene was amplified by PCR (expected size – 2343 bp). After performing ligation reaction the pUC-USP1 genetic construct was obtained. Then *USP*1 gene was subcloned to create pECFP-C3-USP1 genetic construct. The accuracy of the pECFP-C3-USP1 construction was confirmed by PCR, restriction and sequence analyses. The pECFP-C3-USP1 created was successfully used to determine protein localization in the cell, analysis of protein expression, determining proteins interactions.

Localization of USP1 protein in HEK293T cells. The question of USP1 protein localization in the cell now remains ambiguous. It was shown that USP1 protein has two cNLS fragments that defines nuclear or cytoplasmic localization. Major is cNLS (266-321aa) fragment that defines nuclear localization, another is minor cNLS fragment (1-269aa) that defines USP1 protein localization in cytoplasm [26]. To date, the principle of regulation for protein USP1 location in the cell is unclear. There is

evidence that some mutations in the USP1 protein activate cNLS fragments responsible for cytoplasmic localization, and protein goes into cytoplasm.

With the resulting genetic constructions the expression of protein in the HEK293T cells was revealed. By using fluorescent microscopy, we found that the main place of USP1 protein localization is the nucleus (Fig. 1, *b*, *c*). Also, in some cells the weak signals of USP1 localization in the cytoplasm were noticed (Fig. 1). In the control variant, which envisaged the transfection of pECFP-C3 in HEK293T cells, the ECFP was localized throughout the cell with equally strong intensity of the signal (Fig. 1, *a*).

Colocalization of USP1 protein and PH domain of Bcr-Abl oncoprotein in HEK293T cells. By the co-transfection of pECFP-C3-USP1 and pmCitrineC1-PH of HEK293T cell we have received co-expression of USP1 protein and PH domain – Bcr-Abl oncoprotein. Using confocal microscopy, we conducted the analysis of localization of USP1 and PH proteins and found overlapping localization signal in the nucleus (Fig. 2). Overlapping of USP1 and PH proteins signals indicates their co-localization. For the reliability of the results, we additionally analyzed the data using graphical and quantitative analysis (ImageJ software).

We detected overlapping of localization signals («fusion point») of USP1 and PH proteins in nucleus by means of overlay two images using the Zeiss LSM Image Browser software. Overlap of two signals visually manifested emergence of a joint color indicates the colocalization of USP1 and PH proteins. Results of graphical analysis are shown in scatterplots where the intensity of one color is plotted against the intensity of another color for each pixel, similar to the output provided for flow cytometry data (Fig. 3). Graphical analysis enables to evaluate the results considering the intensity of each of the studied channels. Scattering diagrams, reflecting the intensity of the localization of USP1 and PH proteins clearly shows overlapping of two probes, and therefore, indicates colocalization of proteins in the cell.

Pearson's correlation coefficient (PCC) the linear correlation coefficient measured in the range of −1 to 1, values range from 1 for two images whose fluorescence intensities are perfectly, linearly related, 0 – partial correlation, −1 – absolute difference. In our studies PCC for USP1 and

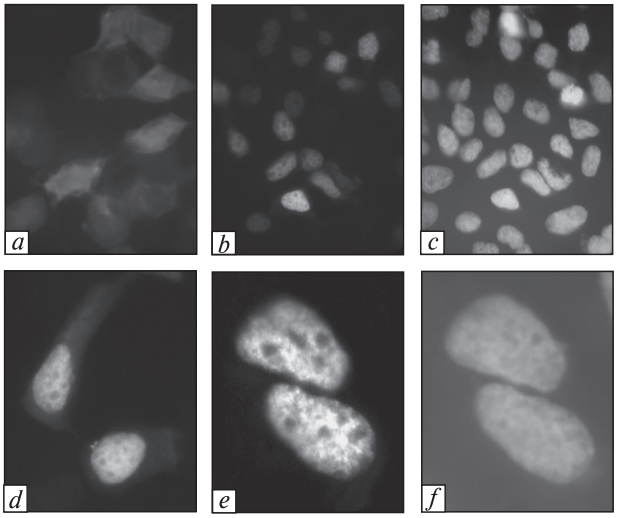

Fig. 1. Expression of pECFP-C3 and pECFP-C3-USP1 in HEK293T cells: *a* – pECFP-C3, ECFP fluorescence (×60); *b* – pECFP-C3-USP1, ECFP fluorescence and *c* – DAPI staining (×40); *d* – pECFP-C3-USP1, ECFP fluorescence (×60) and *e* – (×100), *f* – DAPI staining (×100)

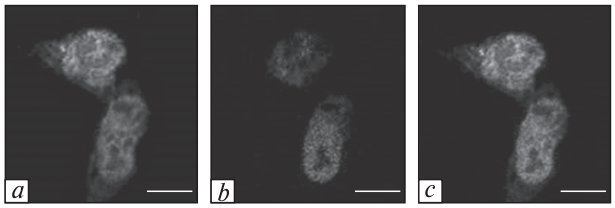

Fig. 2. Co-expression of pECFP-C3-USP1 and pmCitrineC1-PH in HEK293T cells: *a* – ECFP fluorescence (green), *b* – mCitrine fluorescence (red), *c* – merged ECFP and mCitrine signals. Bar – 10 μm

PH proteins in different cells ranged from 0.48 to 0.58, which indicates the average level of correlation. Considering that the joint localization does not necessarily mean proportional distribution, we used Manders' Colocalization Coefficients (MCC) because it is more useful for data that are poorly suited to the simple, linear model that underlies PCC. Because MCC provides not only effective statistical processing, it also reflects the number of protein localization signals with overlapping. Also MCC provides two components: the fraction of A with B and the fraction of B with A, this is important when the probes distribute to different kinds of compartments. The MCC is determined in a range from 0 to 1, where 0 is the lack of colocalization

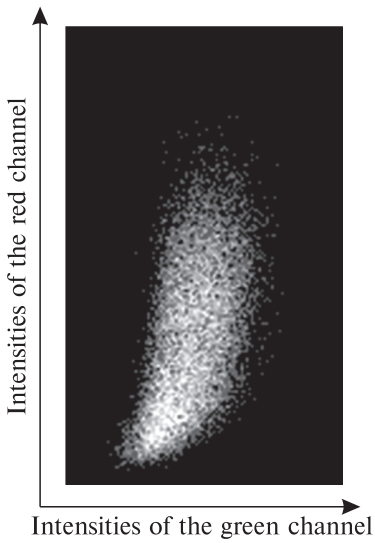

Fig. 3. Scattering diagram of intensity shown by graphical quantitative analysis of USP1 protein and PH domain expression in HEK293T cells (Fiji ImageJ software). Area of signals localization, which do not overlap is shown in blue, area of signals colocalization is shown in yellow

and 1 – full overlapping localization signal proteins. In our experiment MCC ranges from 0.8 to 1 units indicating a high level and even at full overlap USP1 protein and PH domain in different cells as was estimated according to Fiji ImageJ software. Also interesting is how coefficient data fluctuate in different cells that may indicate the relationship of colocalization of USP1 and PH proteins due to the period of the cell cycle.

Conclusion. We created pUC18-USP1, pECFP-C3-USP1 genetic structures that allow the protein expression in eukaryotic systems. Using the fluorescent target in pECFP-C3-USP1, we found that the main place of USP1 protein localization is the nucleus. We also detected weak signals of the USP1 protein sites in the cytoplasm. Using confocal microscopy, we found overlapping localization signals of USP1 protein and PH domain Bcr-Abl oncoprotein in the nucleus in HEK293T cells. Pearson's correlation coefficient for USP1 and PH proteins ranged from 0.48 to 0.58 indicates the positive level of correlation. Manders' Colocalization Coefficients ranged from 0.8 to 1 units, indicating a high level and even at full overlap USP1 protein and PH domain. By this we confirmed the colocalization of USP1 protein and PH domain Bcr-Abl oncoprotein in the nucleus of HEK293T cells. Correlation between USP1 and PH domain indicates their colocalization in cell, as well as their joint distribution and/or interaction with similar molecular complexes. USP1 protein and PH domain are characterized to have nuclear localization, but for Bcr-Abl oncoprotein only the cytoplasmic localization has been shown.

КОЛОКАЛИЗАЦИЯ БЕЛКА USP1 И ДОМЕНА PH ОНКОБЕЛКА Bcr-Abl КАК ПОТЕНЦИАЛЬНЫЙ МАРКЕР КЛЕТОЧНЫХ ПЕРЕСТРОЕК ПРИ ХРОНИЧЕСКОЙ МИЕЛОИДНОЙ ЛЕЙКЕМИИ

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Развитие хронической миелоидной лейкемии (ХМЛ) вызывает филадельфийская хромосома, результат реципрокной транслокации между хромосомами 9 и 22. Продуктом мутации является гибридный онкобелок Bcr-Abl. По результатам масс-спектрометрического анализа как потенциальный кандидат на взаимодействие с PH доменом онкобелка Bcr-Abl определен белок USP1. За счет деубиквитинирующих свойств белок USP1 может предотвратить протеосомную деградацию онкобелка Bcr-Abl в клетке и, как следствие, способствовать его накоплению и прогрессированию заболевания. С помощью созданных нами генетических конструкций определена локализация белка USP1 в клетке, а также установлена ядерная колокализация белка USP1 с PH доменом онкобелка Bcr-Abl в клетках HEK293T. Полученные результаты важны для понимания последствий появления филадельфийской хромосомы и при разработке новых методов терапии ХМЛ, поскольку современные методы борьбы не всегда эффективны вследствие появления многочисленных мутаций, которые вызывают устойчивость к препаратам и рецидив заболевания.

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