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Characterization of new cell line stably expressing *CHI3L1* oncogene

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Aim. *To characterize the immortalized 293 cell line after stable transfection with human oncogene (CHI3L1).* ***Methods***. *293 cells, stably transfected with pcDNA3.1_CHI3L1, and 293 cells, stably transfected with pcDNA3.1 as a negative control, were used throughout all experiments. The clones of CHI3L1-expressing 293 cells and 293 cells, transfected with pcDNA3.1, were analyzed by immunofluorescence and confocal microscopy. Cell proliferation was measured using MTT assay; analyses of ERK1/2 and AKT activation and their cellular localization were performed with anti-phospho-ERK and anti-phospho-AKT antibodies. Specific activation of MAP and PI3 kinases was measured by densitometric analysis of Western-blot signals.* ***Results***. *The obtained results show quite modest ability of CHI3L1 to stimulate cell growth and reflect rather an improved cellular plating efficiency of the 293 cells stably transfected with pcDNA3.1_CHI3L1 as compared to the 293 cells transfected with an «empty» vector. ERK1/2 and AKT are activated in the 293_CHI3L1 cells. In these cells phosphorylated ERK1/2 were localized in both cell cytoplasm and nuclei while AKT only in cytoplasm. The 293_CHI3L1 cells differed from the 293 cells, transfected with an «empty» vector, in their size and ability to adhere to the culture plates.* ***Conclusions***. *The overexpression of CHI3L1 is likely to have an important role in tumorigenesis via a mechanism which involves activation of PI3K and ERK1/2 pathways. The tumors which can be induced by orthotopic implantation of the transformed human cells with overexpressed human oncogene CHI3L1 into the rat brain can be used as a target for anticancer drug development.*

Key words: chitinase 3-like 1 protein (CHI3L1), brain tumor, MAP kinase, PI3 kinase.

Introduction. Mouse models of human cancer have been instructional in understanding the basic principles of cancer biology. Recently, we have reported the development of a new method to induce tumors in adult immunocompetent rat brains by the 293 cells stably producing chitinase 3-like 1 oncoprotein (CHI3L1) (Kavsan et al., in press).

CHI3L1 is a member of mammalian chitinase-like proteins [1–3], it is also named YKL-40 based on its three N-terminal amino acids tyrosine (Y), lysine (K) and leucine (L) and molecular mass of 40 kDa [4], or human cartilage glycoprotein-39 (HC gp-39) [1], 38-kDa heparin-binding glycoprotein (Gp38k) [5], breast regression protein 39 kDa (brp-39) [6], and Chondrex [7]. The gene for human CHI3L1 is localized on chromosome 1 and the crystal structure of human CHI3L1 has been described [8, 9].

Increased level of serum CHI3L1 has been suggested as a robust biomarker of various inflammatory/fibrotic diseases [10].

The increased *CHI3L1* expression has been documented in various malignancies [11] and a number of

cancer cell lines [4, 6, 12]. Previously, in an effort to identify genes which could be used as molecular markers of glial tumors, we found *CHI3L1* as one of the most overexpressed in glioblastomas [13]. Furthermore, CHI3L1 plays a role in angiogenesis by stimulating the migration and reorganization of vascular endothelial cells [14, 15]. CHI3L1 also stimulates the migration of endothelial cells and promotes the migration and adhesion of vascular smooth muscle cells [15, 16].

Here we describe further characterization of a new 293 cell line stably producing oncoprotein CHI3L1.

Materials and methods. *Cells*. The 293 cells (Human Embryonic Kidney 293 cells, also often referred to HEK293, or less precisely as HEK cells), were kindly provided by Prof. V. Filonenko; 293 cells, stably transfected with *pcDNA*3.1 («empty» vector) were kindly provided by Dr. V. Grishkova (IMBG, Ukraine); the 293 cells, stably expressing *CHI3L1* (293_*CHI3L1*) we obtained earlier (Kavsan et al., in press).

The clones of 293_*CHI3L1* cells were obtained by limiting dilution cloning of the 293 cells, stably expressing *CHI3L1*. The presence of CHI3L1 in the cloned cells was evaluated by immunofluorescence and confocal microscopy. For this purpose the *CHI3L1* expressing clones of 293 cells and 293 cells, transfected with an «empty» vector were seeded on coverslips and allowed to grow to near-confluence. The cells were washed in cold phosphate-buffered saline (PBS), fixed with 4 % paraformaldehyde and permeabilized with Triton X-100, washed three times for 5 min each with PBS, and blocked with 5 % horse serum («Sigma», USA) in PBS (blocking buffer) for 30 min at room temperature. Incubations were performed at room temperature with antibodies diluted in blocking buffer. Slides were mounted using PVA-DABCO («Sigma») and images were captured with Zeiss LSM 510 Meta confocal microscope (Germany). Presence of CHI3L1 in the conditioned medium from the cloned cells was determined after 24 h of cell starvation by Western blotting, 1/20 part of the cell conditioned medium was used from 3 cm well.

Proteins and antibodies. The goat polyclonal antibodies against human CHI3L1 (S-18) were purchased from «Santa Cruz Biotechnology» (USA), p44/42 mitogen activated protein (MAP) (extracellular signal-regulated kinase (ERK1/2)) (L34F12) Mouse mAb («Cell Signaling Technology», USA); (ERK1) (K-23) rabbit polyclonal IgG («Santa Cruz»); Anti-phospho-Akt1/PKBα (Ser473), clone 11E6, mouse monoclonal IgG («Millipore», USA); Anti-Mouse IgG (H + L), HRP Conjugate, Anti-Rabbit IgG (H + L), HRP Conjugate («Promega», USA), and Rabbit anti-goat IgG (H + + L), HRP Conjugate («Invitrogen», USA) used in Western blot as well as goat anti-rabbit Alexa Fluor 488 and rabbit anti-goat Alexa Fluor 633 antibodies used in immunofluorescence were from «Invitrogen».

Cell proliferation assay. The clone 1 of 293 cells stably expressing *CHI3L1*, and the 293 cells, stably transfected with pcDNA3.1 were seeded in quadruplicates into 96 well plate at density $2 \cdot 10^3$ cells/well and grown in DMEM, supplemented with 10 % FBS, 300 μg/ml geneticin G418 sulphate and 100 μg/ml penicillin/100 units/ml streptomycin (PAA, Austria) for 5 days. Cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) («Sigma») at days 2, 3, 4 and 5, of seeding.

Analysis of ERK1/2 and AKT activation. For investigation of ERK1/2 phosphorylation the 293_*CHI3L1* cells (clone 1) and 293 cells, stably transfected with an «empty» vector, were seeded into 6-well tissue culture plates in DMEM contained 10 % FBS and allowed to grow to near-confluence. Cells were serum-starved for 24 h. Cell layers were washed twice in ice-cold PBS and whole cell lysates were mixed with 2 × Laemmli sample buffer, boiled, proteins were resolved by 10 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to the nitrocellulose membrane.

Membranes were blocked for 1 h at room temperature with 5 % powdered skim milk in Tris buffered saline (TBS) with 0.05 % Tween-100 (TBST), reacted with anti-phospho-ERK or anti-phospho-AKT at 4 °C overnight, and then incubated with HRP-anti-mouse IgG for 1 h. Blots were developed with an ECL detection system. Then membranes were incubated in stripping buffer (0.5 M NaCl, 0.2 M Gly, pH 2.5) for 15 min at 70 °C, washed twice in TBST, blocked, and incubated with anti-ACTB for 1 hr at room temperature. After incubation with HRP-anti-rabbit IgG for 1 h, ACTB was detected with ECL. Specific activation of MAP kinases was measured by densitometric analysis of Western-blot signals using Scion Image 1.62c program (NIH ImageJ; NIH, USA).

To investigate the localization of phosphorylated ERK1/2 and phosphorylated AKT, the 293 cells, stably expressing *CHI3L1* (clone 1), and the 293 cells, stably transfected with an «empty» vector, were prepared as described above. The cells were washed in cold PBS, fixed with 10 % paraformaldehyde and permeabilized in –20 ºC methanol, washed three times for 5 min each with PBS and blocked with blocking buffer (2 % bovine serum albumin, BSA) in PBS for 30 min at room temperature. Incubations were performed at room temperature with antibodies diluted in blocking buffer. Slides were mounted using PVA-DABCO («Fluka, Switzerland») and images were captured with a Zeiss LSM 510 Meta confocal microscope. All images represent a single confocal section, taken from the bottom surface of the cells being analyzed.

Results and discussion. The significant increasing of *CHI3L1* gene expression was shown for a number of tumors [11] including glioblastomas in our and other laboratories [13, 17, 18]. These data were obtained mostly by Serial Analysis of Gene Expression (SAGE) and microarray analysis or polymerase chain reaction (PCR) and were presented mostly as an average gene expression for a given group of patients. The results of *CHI3L1* RNA Northern hybridization which were performed in our lab as described earlier [13, 18] are summarized in Fig. 1 indicating a substantial increase of *CHI3L1* expression in anaplastic astrocytomas – World Health Organization (WHO) III stage of malignancy and glioblastomas – WHO IV stage of malignancy. At the same time *CHI3L1* expression is much lower in diffuse astrocytomas (WHO II stage of malignancy) and in normal adult human brain. However, there appears to be also significant (about 20 %) overlap of the levels in individual samples of tumor and normal brain. Recently it has been shown that the expression of *CHI3L1* does not increase in proneural subtype of glioblastoma comprising up to 30 % of all glioblastomas [19], and the patients with such tumors experience significantly improved outcome. So, the absence of *CHI3L1* expression revealed by Northern hybridization could serve as a prognostic marker.

The clones of 293_*CHI3L1* cells were obtained by limiting dilution cloning of the 293 cells, stably expressing *CHI3L1*. Overproduction of CHI3L1 in the cloned cells was confirmed by Western blot and immunofluorescent analysis (Fig. 2, see inset). Western blot analysis revealed CHI3L1 in the 293_*CHI3L1* cell conditioned medium, showing that CHI3L1 is a secreted protein. Previously it was shown, that CHI3L1 was secreted in large amount by osteosarcoma cell line MG-63 [7]. As it is possible to see, the 293_*CHI3L1* cells also secrete CHI3L1 but at lower level than MG-63 in the same culturing conditions (Fig. 3).

The results presented in the Fig. 4 show the ability of CHI3L1 to promote cellular proliferation. However,

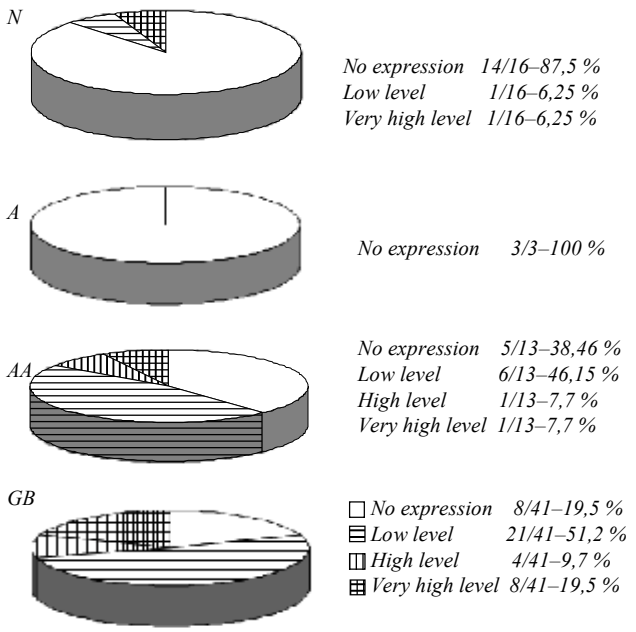

Fig. 1. Results of *CHI3L1* Northern blot analysis in human glial tumors and normal brain. *N* – normal brain; *A* – astrocytoma; *AA* – anaplastic astrocytoma; *GB* – glioblastoma

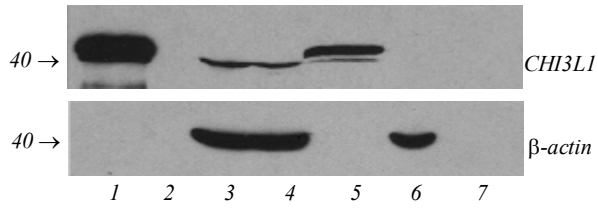

Fig. 3. Western blot analysis of CHI3L1 protein secretion. The conditioned medium in the absence of serum were collected after 24-h culture for analyzing the level of CHI3L1 secreted from 293 cells, stably expressing *CHI3L1* and 293 cells, stably transfected with an «empty» vector. Cell lysates were subjected also to the testing of actin expression; *1* – conditioned medium from MG-63 cells (positive control); *2* – empty track; *3* – 293 cells stably expressing *CHI3L1*; *4* – 293 cells, stably expressing *CHI3L1* in the absence of serum; *5* – conditioned medium from 293 cells, stably expressing *CHI3L1*; *6* – 293 cells, stably transfected with an «empty» vector; *7* – conditioned medium from 293 cells, stably transfected with an «empty» vector

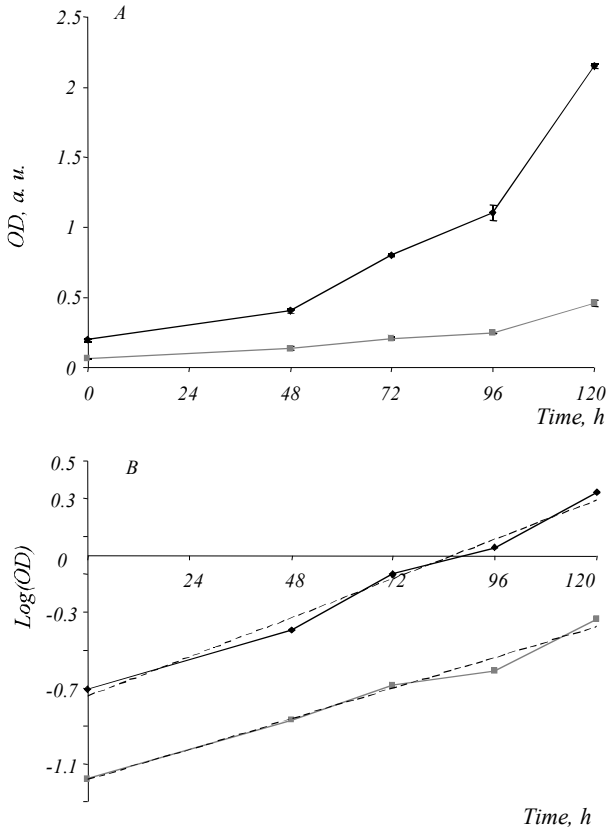

Fig. 4. Proliferation of 293 clones stably expressing *CHI3L1*: *A* – standard coordinates; *B* – semi-logarithmic scale. ■ – 293 cells, transfected with «empty» vector, ♦ – 293 cells, stably expressing *CHI3L1* (clone 1), ··· – trend line. Proliferation was measured by fluorescence after 3 h of cells exposure to MTT reagent. The data shown are the means ± S.D. from four experiments for each cell line

the ability of CHI3L1 to stimulate cell growth when produced endogenously was quite modest, what is consistent with the data of Shao et al. [20]. Five days after the equal numbers of cells were plated, there were approximately 1,6-fold as many the *CHI3L1*-expressing clones as parental cells. The major difference was an improved plating efficiency with the greater number of cells present at the initial time point of 12 h (day 0). After performing linear regression analysis for log scale graph the slopes were 0,0086 for the 293_*CHI3L1* cells and –0,0067 for the 293_3.1 cells, resulting in 1,3-fold increase in cell growth rate. Thereafter, we suppose that the effect of *CHI3L1* transfection was mostly on cellular plating efficiency and perhaps also on clonogenicity, a similar cellular growth phenomenon.

The 293_*CHI3L1* cloned cells were increased in size as compared to the «empty» vector transfected human 293 cells, both cell lines were oblong but the 293_*CHI3L1* cloned cells were more prone to grow in well-defined monolayer while the «empty» vector transfected human 293 cells tended to grow in foci (Fig. 5, see inset).

The MAPK and phosphoinositide 3-kinase (PI3K) pathways are strongly associated with cell survival. ERK1 and ERK2 were identified as growth factor-stimulated protein kinases phosphorylating microtubule-associated protein-2 (MAP-2) and myelin basic protein (MBP). Activation of ERK1/2 is involved in many cellular responses such as cell motility, proliferation, differentiation and survival [21]. We found that overproduction of CHI3L1 in the cells stably transfected with *CHI3L1* induced activation of ERK1/2 with equivalent cytoplasmic and nuclear localization. Moreover, AKT the main component of PI3K signaling pathway, was also phosphorylated in these cells and confocal laser scanning microscopy showed phosphorylated AKT to be located in the cytoplasm (Fig. 6, see inset). Although AKT is best known for promoting cell survival and growth through pathways parallel to ERK's control over cell proliferation, AKT activation can also stimulate proliferation through multiple downstream targets impinging on the cell-cycle regulation [22]. It has been reported that AKT migrated into the nucleus in response to a variety of stimuli, where it can block FOXO-mediated transcription of target genes that promote apoptosis, cell-cycle arrest, and metabolic processes [23]. On the other hand, a function of AKT is to phosphorylate and inhibit proapoptotic components of the intrinsic cell death machinery within the cytoplasm [24]. It is possible to hypothesize, that in the case of the 293_*CHI3L1* cells AKT can play this role. So, CHI3L1 has a different effect on cellular localization of ERK1/2 and AKT. The results suggest that biological effects of CHI3L1 are mediated by association with unknown cell surface receptor promoting proliferation of the 293 cells through Ras/MAPK/AKT pathways and support a role of CHI3L1 in the malignant phenotype as a cellular survival factor.

It is interesting that the 293 cells (HEK293) were obtained by transformation of the human embryonic kidney cell culture with sheared adenovirus 5 DNA [25]. The observation that the 293 cells stain strongly and specifically with antibodies to several NF proteins, which are generally thought of as excellent markers for

neuronal lineage cells, stimulated detailed DNA microarray analysis of the 293 cells. More than 60 mRNA normally expressed specifically in neuronal cells were detected in the 293 cells by this approach. Microarray analysis of the 293 cells using Affymetrix and Clontech arrays revealed also mRNAs encoding many other proteins normally expressed in neuronal lineage cells. The pattern of expression was quite similar to that seen in the PC12 and Ntera-2 cells, which also express a mix of neurofilament subunits and two basic keratins. So, the 293 cells were similar in several respects to the two well defined neuronal lineage cell lines [26].

Tumor formation by the 293 cells stably expressing *CHI3L1* in rat brains strongly suggests that this gene is likely to be critical in the tumor development, and tumors which can be induced after orthotopic implantation of transformed human cells with overexpressed human oncogene *CHI3L1* in the rat brain can be used as a target for anticancer drug development.

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Характеристика нової клітинної лінії, що стабільно експресує онкоген *CHI3L1*

Резюме

Мета. *Охарактеризувати імморталізовану клітинну лінію 293 після стабільної трансфекції онкогена CHI3L1.* ***Методи***. *Клітини 293, стабільно трансфековані pcDNA3.1_CHI3L1, та клітини 293, стабільно трансфековані pcDNA3.1 як негативний контроль, використано в усіх експериментах. Клони 293 експресуючих CHI3L1 клітин та клітини 293, трансфековані «порожнім» вектором, проаналізовано методами імунофлуоресценції та конфокальної мікроскопії. Клітинну проліферацію визначено за допомогою MTT, активацію ERK1/2 і AKT та їхню локалізацію в клітинах – із застосуванням анти-фосфо-ERK- та анти-фосфо-AKT-антитіл. Специфічну активацію кіназ MAP і PI3 визначено денситометричним аналізом Вестерн-блот сигналів.* ***Результати***. *Отримані результати демонструють помірну здатність CHI3L1 стимулювати клітинний ріст, але відображають скоріше підвищену здатність до прикріплення 293 клітин, стабільно трансфекованих pcDNA3.1_CHI3L1 порівняно з клітинами 293, трансфекованими «порожнім» вектором. У 293_CHI3L1 клітинах ERK1/2 та AKT перебувають в активованому стані. У цих клітинах фосфорильовані ERK1/2 локалізовані як у цитоплазмі, так і в ядрі, тоді як AKT – лише в цитоплазмі. 293_CHI3L1-клітини відрізняються від клітин 293_pcDNA3.1 за морфологією та їхньою здатністю до прикріплення до культуральних чашок.* ***Висновки***. *Надекспресія CHI3L1, очевидно, має важливе значення в пухлиноутворенні і опосередковується активацією PI3K- і MAPK- сигнальних шляхів. Пухлини, спричинені ортотопічною імплантацією трансформованих клітин людини з надекспресованим CHI3L1 у мозок щурів, стають вірогідною мішенню для антиракової терапії.*

Ключові слова: хітиназа 3-подібний білок 1 (CHI3L1), пухлини головного мозку, MAP-кіназа, PI3-кіназа.

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Характеристика новой клеточной линии, стабильно экспрессирующей онкоген *CHI3L1*

Резюме

Цель. *Охарактеризовать иммортализованную клеточную линию 293 после стабильной трансфекции онкогена CHI3L1.* ***Методы***. *Клетки 293, стабильно трансфецированные pcDNA3.1_CHI3L1, и клетки 293, стабильно трансфецированные pcDNA3.1 в качестве отрицательного контроля, использованы во всех экспериментах. Клоны 293_CHI3L1 и клетки 293_ pcDNA3.1 анализировали методами иммунофлюоресценции и конфокальной микроскопии. Клеточную пролиферацию определяли с помощью MTT, активацию и локализацию ERK1/2 и AKT анализировали с применением анти-фосфо-ERK- и анти-фосфо-AKT-антител. Специфическую активацию MAP- и PI3-киназ определяли денситометрическим анализом Вестерн-блот сигналов.* ***Результаты***. *Полученные результаты демонстрируют умеренную способность CHI3L1 стимулировать клеточный рост и отражают скорее повышенную способность к прикреплению клеток, стабильно трансфецированных pcDNA3.1_CHI3L1 по сравнению с 293-клетками, трансфецированными «пустым» вектором. ERK1/2 и AKT в клетках 293_CHI3L1 находятся в активированном состоянии. В этих клетках фосфорилированные ERK1/2 локализованы как в цитоплазме, так и в ядре, в то время как AKT – только в цитоплазме. Клетки 293_CHI3L1 отличаются от клеток 293_pcDNA3.1 по морфологии и способности прикрепления к культуральным чашкам.* ***Выводы***. *Сверхэкспрессия CHI3L1, очевидно, имеет важное значение в опухолеобразовании и опосредуется активацией PI3K- и MAPK-сигнальных путей. Опухоли, которые индуцируются ортотопической имплантацией трансформированных человеческих клеток со сверхэкспрессированным CHI3L1 в мозг крыс, могут служить мишенью для антираковой терапии.*

Ключевые слова: хитиназа 3-подобный белок 1 (CHI3L1), опухоли головного мозга, MAP-киназа, PI3-киназа.

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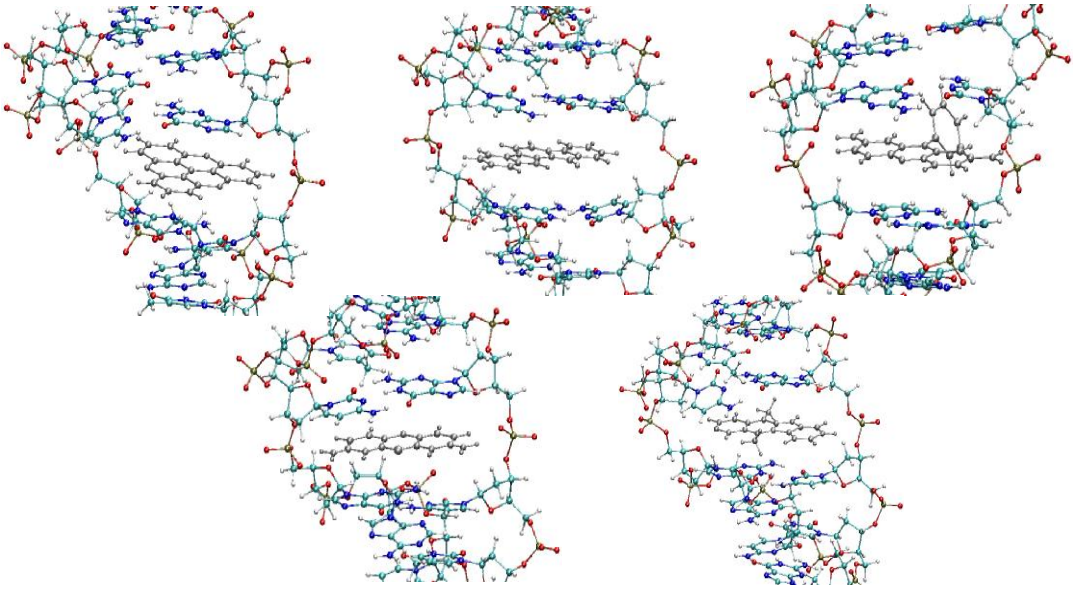

Рис. 2. Центральные участки расчетных структур комплексов гетероциклических лигандов с двуспиральной ДНК

Figures to article by O. V. Balynska et al.

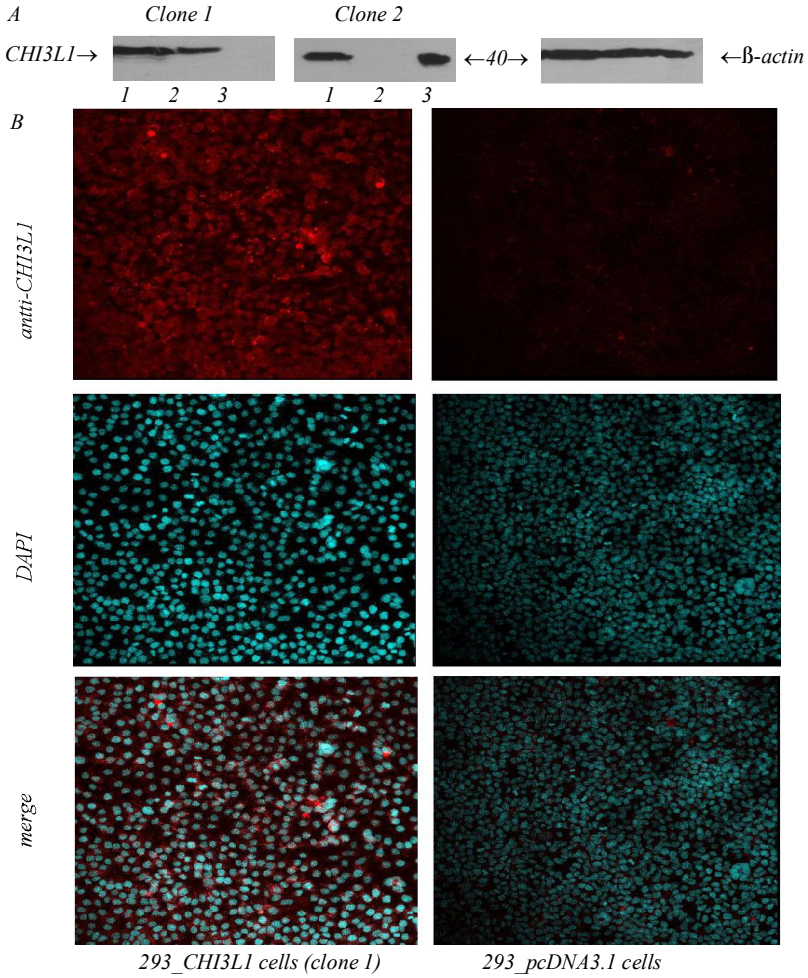

Fig. 2. *A* – Western blot analysis of CHI3L1 protein in 293_*CHI3L1* cells clone 1: *1* – 293 cells stably expressing *CHI3L1*; *2* – glioblastoma total lysate, prepared earlier; *3* – 293 cells, stably transfected with an «empty» vector clone 2: *1* – 293 cells stably expressing *CHI3L1*; *2* – 293 cells, stably transfected with an «empty» vector; *3* – glioblastoma total lysate, prepared earlier [27]; *B* – Immunofluorescent analysis of CHI3L1 in paraformaldehyde fixed 293 cells, stably expressing *CHI3L1* (clone 1) and in 293_pcDNA3.1 cells

Figures to article by O. V. Balynska et al.

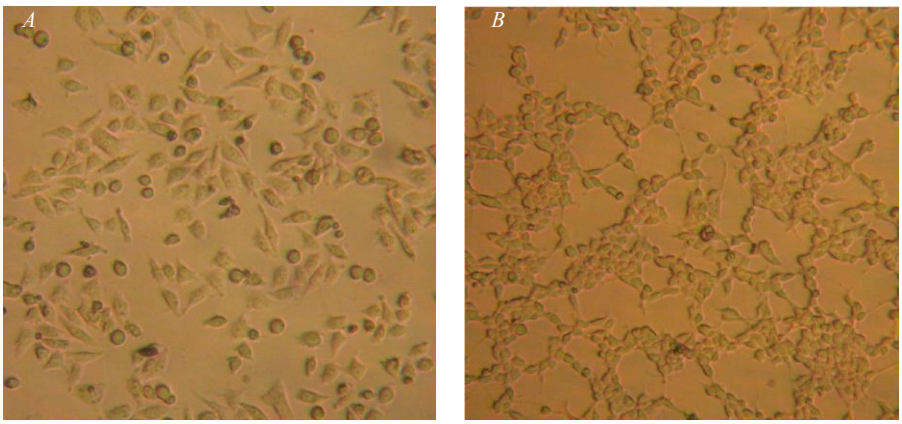

Fig. 5. Phase-contrast features of human 293_*CHI3L1* cells (*A*) and 293 cells, stably transfected with an «empty» vector (*B*)

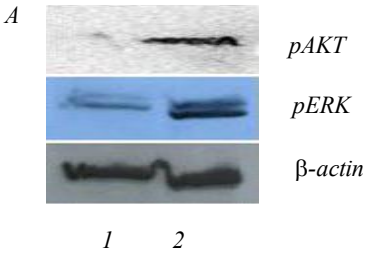

Fig. 6. ERK1/2 and AKT activation: *A* – Western blot analysis of AKT and ERK1/2 activation in starved 293 cells, stably transfected with an «empty» vector (*1*), 293 cells, stably expressing *CHI3L1* (*2*); *B* – immunofluorescent analysis of ERK1/2 and *C* – AKT activation in starved 293 cells, stably expressing CHI3L1 and 293 cells, stably transfected with an «empty» vector