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CHITINASE 3-LIKE PROTEIN 2 (CHI3L2, YKL-39) ACTIVATES PHOSPHORYLATION OF EXTRACELLULAR SIGNAL-REGULATED KINASES ERK1/ERK2 IN HUMAN EMBRYONIC KIDNEY (HEK293) AND HUMAN GLIOBLASTOMA (U87 MG) CELLS

Human cartilage chitinase 3-like protein 2 (CHI3L2, YKL-39) is secreted by articular chondrocytes, also synoviocytes, lung, and heart. Increased levels of YKL-39 have been demonstrated in synovial fluids of patients with rheumatoid or osteoarthritis as well as in some other pathologies and in malignant tumors, particularly in glioblastomas. It belongs to glycosyl hydrolase family 18 and the most closely related to human cartilage glycoprotein 39 (HC gp-39 or chitinase 3-like protein 1, CHI3L1 or YKL-40), which as it was shown previously, promotes the growth of human synovial cells as well as skin and fetal lung fibroblasts. Dose-dependent growth stimulation was observed when the fibroblastic cell line was exposed to YKL-40 in a concentration range from 0.1 to 2 nM, which is similar to the effective dose of the well characterized mitogen, insulin-like growth factor I. The use of selective inhibitors of the mitogen-activated protein kinase (MAP kinase) signaling pathway indicates that both, YKL-40 and IGF-I are involved in phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2). Thus YKL-40 initiates a signaling cascade which leads to increased cell proliferation, suggesting that this protein could play some role in the inhibition of apoptosis. We report here that YKL-39, which as YKL-40 has significantly increased expression in glioblastomas, also activates signal-regulated kinases ERK1/ERK2 in human embryonic kidney (HEK293) and human glioblastoma (U87 MG) cells.

Introduction. Mammalian genomes, despite lack of chitin (poly-N-glucosamine) synthesis in mammalia, code a set of homologous chitinase-like proteins. On the basis of amino acid sequences and protein structure relationships they have been grouped in glycosyl hydrolase family 18, which also includes bacterial and plant chitinases [1]. In human there are six proteins of this family [2], and two of them, macrophage chitotriosidase-1 (chitinase-1, CHIT1) [3] and acidic mammalian chitinase (AMCase) [4], are catalytically active chitinases. Other chi-lectins are chitinase-3 like 1 (CHI3L1) or human cartilage glycoprotein 39 (HC gp-39) or YKL-40 [5], chitinase-3 like 2 (CHI3L2) or YKL-39 [6], oviductin (oviductal glycoprotein, oviduct-specific glycoprotein precursor, estrogen-dependent oviduct protein, mucin-9, OVGP1) [7] and less homologous chitinase-like protein, which interacts with endocytic/sorting receptor stabilin-1 (stabilin-1 interacting chitinase-like protein, SI-CLP) [8].

YKL-40 is the most investigated protein in chitinase-like signal lectin family. It has molecular weight of about 40 kDa and N-glycosylation at Asn60 (2 β(1, 4)-N-acetyl d-glycosamine, NAG). YKL-40 monomer consists of 383 amino acids, contains signal peptide Met1–Ala21 for secretion and two structure domains [9]. N-terminal amino acids of its mature form are tyrosine (Y), lysine (K), leucine (L) [5]. This lectin acts as a proliferative [10], anti-apoptotic [11, 12], migration and adhesion [13] factor, it enhances bacterial adhesion to colonic epithelial cells through the interaction with bacterial chitin-binding protein [14]. *CHI3L1* gene is expressed in synovial cells, articular cartilage chondrocytes, and liver. It is overexpressed in macrophages during the last stages of differentiation; its expression is increasing significantly under pathological conditions such as inflammation or different tumors notably squamous cell carcinoma of the head and neck, multiple myeloma, acute myeloid leukemia, small cell lung cancer, primary breast cancer, ovarian cancer stage III, colorectal carcinoma, and glioblastoma [15]. *CHI3L1* is overexpressed in glioblastomas in comparison with low-grade gliomas and normal brain [16] and a correlation between high expression of *CHI3L1* and short survival of patients was observed [17]. It was reported that YKL-40 promotes the growth of human synovial cells as well as skin and fetal lung fibroblasts [10]. Dose-dependent growth stimulation was observed when fibrob-

lastic cell lines were exposed to YKL-40 in a concentration range from 0.1 to 2 nM, which is similar to the effective dose of the well-characterized mitogen, insulin-like growth factor I. At suboptimal concentrations, the two growth factors work in a synergistic fashion. The use of selective inhibitors of the mitogen-activated protein kinase and the protein kinase B (AKT) signaling pathways indicates that both are involved in mediating the mitogenic response to YKL-40. Phosphorylation of both extracellular signal-regulated kinases 1/2 and AKT occurred in a dose- and time-dependent manner upon addition of YKL-40. Thus, YKL-40 initiates a signaling cascade in connective-tissue cells which leads to increased cell proliferation, suggesting that this protein could play a major role in the pathological conditions leading to tissue fibrosis.

Despite detailed information about structure of many chi-lectins, understanding of their physiological functions is yet limited. YKL-39 is the most closely related to human YKL-40 and has quite high sequence homology (49–53 %) with other mammalian CLPs [6]. The alternative protein name – YKL-39 – refers to the same as in YKL-40 three N-terminal amino acids of the mature protein after cleavage of signal peptide (Met^{1}-Ala^{25}). YKL-39 was identified as a protein in conditioned medium from human articular cartilage chondrocytes primary culture that co-purified with YKL-40. Its amount is approximately 4 % of total proteins in chondrocyte-conditioned medium while content of YKL-40 is 33 %. The highest level of *YKL-39* mRNA was detected in chondrocytes, also in synoviocytes, lung, and heart [6]. No *YKL-39* mRNA was detected in brain, spleen, kidney, pancreas, or liver. *YKL-39* gene, but not *YKL-40*, is upregulated in chondrocytes of patients with osteoarthritis [18]. Previously using Serial Analysis of Gene Expression (SAGE), Northern blotting and PCR-analysis we have shown the increasing *YKL-39* gene expression in glioblastomas. However, Western blot-analysis did not show simultaneous production of YKL-39 and YKL-40 [19] in spite of high degree of their sequence identity.

The work described here is going to demonstrate that YKL-39 also activates extracellular signal-regulated kinase (ERK)-mediated signaling cascade, which is associated with the control of proliferation or differentiation, in both, human embryonic kidney (HEK293) and human glioblastoma (U87 MG) cells.

Materials and Methods. Mouse monoclonal antibody specific for the phosphorylated forms of ERK1/ERK2 as well as rabbit pan-specific polyclonal antibody to this kinases were obtained from «Santa Cruz Biotechnology» (USA), and horseradish peroxidase anti-mouse and anti-rabbit Ig conjugates were obtained from «Promega» (USA). Enhanced chemiluminescence reagents for visualization of bound Igs on Western blots were from «Amersham Pharmacia Biotechnology» (Austria).

After restriction of pGemT-Easy-*CHI3L2* [19], the Nde I/Xho I fragment was subcloned in expression vector pET-24a (+) from «Novagen» (Germany). The nucleotide sequence was checked by the dideoxy method («3130 Genetic Analyzer, Applied Biosystems», USA). *E. coli* (BL21) cells transformed by recombinant plasmid pET-24a (+)-*CHI3L2* were grown, and synthesis of recombinant protein was induced by IPTG. Affinity purification of YKL-39, containing His6 at the C-terminus, was carried out on Ni-NTA agarose (Ni^{2+}-charged nitriloacetic acid-modified agarose) under denaturing conditions in the presence of 8 M urea according to manufacturer's protocol of «Qiagen» (USA). Protein products were analyzed by 12 % SDS-PAGE and Coomassie Brilliant Blue R250 staining. Protein content was determined using the Bradford assay [20]. Purified YKL-39 was stored frozen at minus 20 °C in aliquots to avoid repeated freeze-thawing and protein denaturation.

HEK-293 and U-87 MG cells were obtained from the Bank of cell lines from human and animal tissue (R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, Kyiv, Ukraine). Frozen stocks of cell lines were expanded through two or three passages before use. All cells were grown in Dulbecco's Modification of Eagles Medium (DMEM) supplemented with 10 % FBS and 100 μg/ml penicillin/100 units/ml streptomycin in an environment of 95 % air/5 % CO_2. For investigation of ERK1/ERK2 phosphorylation cells were seeded into 6-well tissue-culture plates in DMEM containing 10 % FBS.

To perform RT-PCR, total RNA was isolated from HEK-293 and U-87 MG cells using the Tri

Reagent from SIGMA. 5 μg of RNA was reverse-transcribed by oligo (dT) priming (M-MuLV RT, «Fermentas», Lithuania). Relative transcript levels of *CHI3L1* and *CHI3L2* in two cDNA samples and control β-actin (*ACTB*) cDNA were then determined by PCR using the following primers pairs: *CHI3L1* forward 5'-ACACACTCAAGAACAGGAA-3', reverse 5'-TGATTAGGGTGGTAAAATGC-3'; *CHI3L2* forward 5'-TCAAAACCAAGAATCCCAAAC-3', reverse 5'-ATCAGCACAGTGAAATGAGT-3'; *ACTB* forward 5'-AACTACCTTCAACTCCATCA-3', reverse 5'-GTCATACTCCTGCTTGCT-3'. As a positive control for each primer pair were used plasmid vectors containing *CHI3L1* cDNA (pcDNA4 A) and *CHI3L2* cDNA (pET 24a (+). The following thermal cycling parameters were used: 95 °C for 2 min, 35 cycles of 95 °C for 40 s, 59 °C for 30 s, and 72 °C for 30 s. PCR products were resolved on 1,5 % agarose gel containing ethidium bromide. The expected sizes of PCR products were 222, 213 and 261 bp for *CHI3L1*, *YKL-39* and *ACTB*, respectively.

Results and Discussion. Same as *CHI3L1*, SAGE included *CHI3L2* to the list of 44 genes with more than 5-fold increased expression in glioblastoma as compared to normal brain [21]. However, the quantity of YKL-39 protein is much less in glioblastomas than those for YKL-40 [19].

In contrast to chitinase-like glycoprotein CHI3L1, which has been characterized extensively with respect to its expression patterns and possible association with degenerative diseases [15], the features of its closest homologue YKL-39 were poorly described. The fact that one mammalian member of glycohydrolase family 18 has mitogenic activity [10] and the identification of a family of growth factors in the fruit fly *D. melanogaster*, as chitinase-like proteins [22], indicates that this function may be conserved across a wide range of species. A dose-dependent growth stimulation was observed when fibroblast cell lines were exposed to YKL-40 [10, 11], showing that mitogen-activated protein kinase signaling pathway is involved in mediating the mitogenic response and that phosphorylation of ERK1/ ERK2 occurred in a dose-dependent fashion upon addition of YKL-40. High homology of nucleotide and amino acid sequences of YKL-39 and YKL-40 (Fig. 1) suppose some identity of their functions.

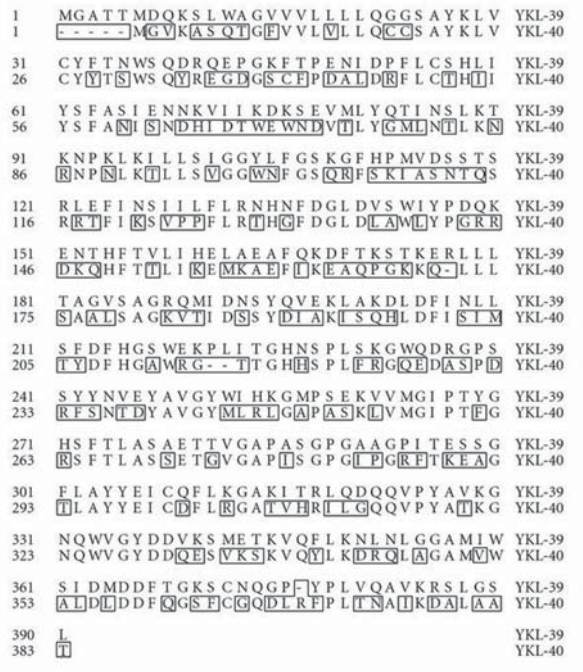

Fig. 1. Structure alignment of CHI3L2 (YKL-39) and CHI3L1 (HC gp-39, YKL-40) proteins. Proteins YKL-39 and HC-gp39 are highly homologous (50.4 %) members of 18 glicosylhydrolases family. Aminoacid residues common for YKL-39 and YKL-40 are boxed

To test this possibility, we made an attempt to find if YKL-39 also induces phosphorylation of ERK1/ERK2. For the beginning we used HEK-293 cell line which is the same as many other cell types, in monolayer culture can not be maintained in unsupplemented culture medium more than one week without loss of viability. The requirement for addition of supplementation is common for cell lines which do not produce endogenously any growth factor. Cells were grown to near-confluence and serum-starved for 48 hrs followed by exposure to purified YKL-39 at concentrations ranging from 0 to 100 ng/ml or BSA (100 ng/ml) in unsupplemented culture medium. Cells were lysed after 60 min of exposure and cell lysates were analysed by Western blotting with either phosphorylation-specific (P-MAPK) or phosphorylation-independent (Total MAPK) antiserum against the MAP kinases ERK1 and ERK2. The results illustrated in Fig. 2, *a*, suggest that ERK1/ERK2 phosphorylation was stimulated in these cells following addition of YKL-39. The lowest effective concentration of YKL-

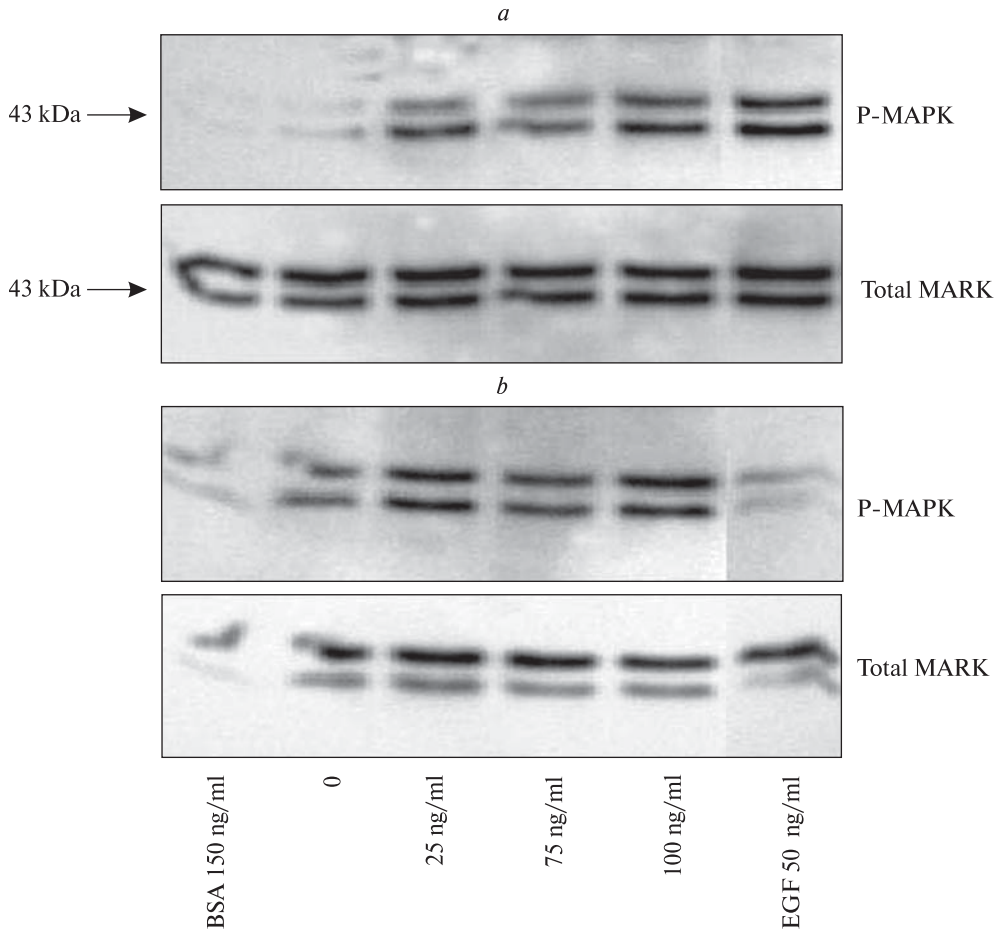

Fig. 2. YKL-39 induces a dose-dependent phosphorylation of ERK1/ERK2 in (*a*) human embryonic kidney 293 (HEK293) and (*b*) human glioblastoma (U87 MG) cells. Cells were grown to near-confluence and serum-starved for 24 hrs followed by exposure to purified YKL-39 at concentrations ranging from 0 to 100 ng/ml or BSA (150 ng/ml) in unsupplemented culture medium. Cells were lysed after 60 min of exposure and cell lysates were analysed by Western blotting with phosphorylation-specific (P-MAPK) antibody following visualization they were stripped and re-probed with a pan-specific (Total MAPK) antibody to determine total ERK1/2 protein

39 was in 5 time larger that eliciting a mitogenic response to YKL-40 [10]. No ERK1/ ERK2 phosphorylation was observed in cells exposed to medium without YKL-39 addition or in cells exposed to medium supplemented with BSA.

To investigate if YKL-39 may phosphorylate cells derived from glial tumor, we repeated the described above experiment but this time with human glioblastoma U87 MG cell line. ERK1/ ERK2 phosphorylation was also stimulated in these cells (Fig. 2, *b*). However, in contrast to HEK293 cells, ERK1/ERK2 phosphorylation was observed in U87 MG cells exposed to medium without YKL-39 addition or in cells exposed to medium supplemented with BSA suggesting that some YKL-39 or other growth factor is produced in U87 MG cells. Indeed, PCR on total U87 MG cDNA with specific primers to *CHI3L1*, *CHI3L2* and *ACTB* revealed presence of all three RNAs in these cells (Fig. 3, *a*) and only positive control β-actin RNA in HEK293 cells (Fig. 3, *b*).

As it was shown in many publications, ERK1 and ERK2 regulate proliferation and differentiation and form part of a MAPK module that includes Raf MAPKKKs and the MEK1/MEK2 MAPKK. They are activated by mitogenic stimuli such as growth factors, cytokines and phorbol esters, which activate a variety of cell-surface-receptor tyrosine

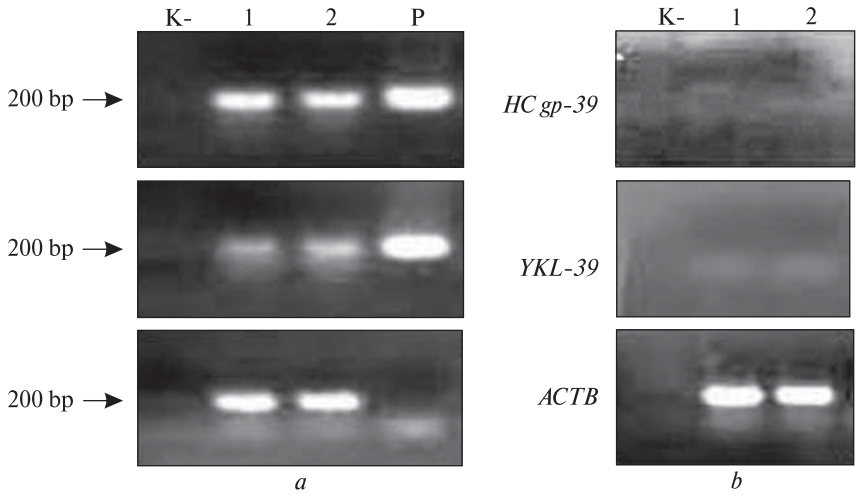

Fig. 3. Result of polymerase chain reaction (PCR) on total U87 MG (*a*) and HEK293 (*b*) cDNA with primers to *CHI3L1*, *CHI3L2* and *ACTB* genes. As a positive control for each primers pair as used plasmid vectors with *CHI3L1* cDNA (pcDNA4 A) or *CHI3L2* cDNA (pET 24a (+). PCR products were resolved on 1,5 % agarose gel containing ethidium bromide. The expected sizes of PCR products were aprox. 250 bp for *CHI3L1*, *CHI3L2* and *ACTB* genes. No PCR products were observed in negative controls (K-)

kinases or G-protein-coupled receptors. This leads to activation of Ras by associated SOS (son of sevenless). Ras-GTP then triggers activation of Raf isoforms (A-Raf, B-Raf, C-Raf/Raf-1) and recruitment of Raf to the plasma membrane, followed by phosphorylation of MEK1/MEK2 and then ERK1, ERK2. This is facilitated by the scaffold protein KSR, which links the three tiers of kinases to Ras. Other scaffold proteins can also tether the ERK module. ERK1/ERK2 have many known targets, including key transcription factors, such as AP-1, NF-B, Myc, kinases, such as Rsk, the cell survival regulator Bcl-2, cPL2 and the cytoskeletal scaffold paxillin. Depending on the strength and duration of stimulation, activation of the ERK1/ERK2 MAPKs can lead to either proliferation or differentiation (for review see [23]).

Thus, the results presented here demonstrate that YKL-39 may initiate the phosphorylation of ERK1/ERK2 leading to the initiation of MAP kinase signaling cascade in human embryonic kidney (HEK293) and human glioblastoma (U87 MG) cells. The finding is consistent with the well established role of this signaling pathway in the propagation of mitogenic signals [24]. The activation of cytoplasmic signal-transduction pathway suggests that YKL-40 and YKL-39 interact with one or several signaling components on the plasma membrane.

The inhibition of the mitogenic response of the cells to YKL-40 by either the MAP kinase or PI-3K inhibitors suggested that both pathways are required for the cells to complete progression through the mitotic cycle and only one of these pathways is insufficient for the cells to complete mitosis [10]. Recent data suggest that PI-3K activation is required for the progression of mitosis, promoting the entry of quiescent cells into the S phase [25], and the downstream phosphorylation of AKT is in part responsible for the propagation of this signal. The possible participation YKL-39 also in activation of PI-3K signaling passway is currently under investigation.

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ХИТИНАЗА 3-ПОДОБНЫЙ БЕЛОК 2 (CHI3L2, YKL-39) АКТИВИРУЕТ ФОСФОРИЛИРОВАНИЕ ВНЕКЛЕТОЧНЫХ СИГНАЛ-РЕГУЛИРУЕМЫХ КИНАЗ ERK1/ERK2 В КЛЕТКАХ ЭМБРИОНАЛЬНОЙ ПОЧКИ ЧЕЛОВЕКА (HEK293) И ГЛИОБЛАСТОМЫ ЧЕЛОВЕКА (U87 MG)

Хитиназа 3-подобный белок 2 (CHI3L2, YKL-39) человека секретируется хондроцитами суставного хряща, синовиоцитами, в легких и сердце. Продемонстрированы повышенные уровни YKL-39 в синовиальной жидкости пациентов с ревматоидным артритом или остеоартритом, а также при некоторых других заболеваниях и злокачественных опухолях, в частности глиобластомах. Этот белок принадлежит к семейству гликогидролаз 18, более всего структурно сходен с хитиназа 3-подобным белком 1 (CHI3L1, HC gp-39 или YKL-40) и, как ранее показано, способствует росту синовиальных клеток человека, а также фибробластов кожи и эмбриональных фибробластов легких. Зависимую от дозы стимуляцию роста зафиксировали при действии YKL-40 на несколько линий фибробластов в диапазоне концентраций от 0.1 до 2 нМ, эта концентрация близка к эффективной дозе хорошо охарактеризованного митогена – инсулиноподобного фактора роста I. Использование селективных ингибиторов сигнального пути митоген-активированной протеинкиназы (MAP киназы) указывает на вовлеченность обоих белков, YKL-40 и IGF-I, в фосфорилирование внеклеточных сигнал-регулируемых киназ 1 и 2 (ERK1/ERK2). Таким образом, YKL-40 инициирует сигнальный каскад, который усиливает клеточную пролиферацию, и это может свидетельствовать об определенной роли YKL-40 в ингибировании апоптоза. В настоящей работе мы сообщаем о том, что YKL-39, как и YKL-40, имеет существенно повышенную экспрессию в глиобластомах, а также активирует сигнал-регулируемые киназы ERK1/ERK2 в клетках эмбриональной почки человека (HEK-293) и глиобластомы человека (U87 MG).

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ХІТИНАЗА 3-ПОДІБНИЙ БІЛОК 2 (CHI3L2, YKL-39) АКТИВУЄ ФОСФОРИЛЮВАННЯ ПОЗАКЛІТИННИХ СИГНАЛ-РЕГУЛЬОВАНИХ КІНАЗ ERK1/ERK2 В КЛІТИНАХ ЕМБРІОНАЛЬНОЇ НИРКИ ЛЮДИНИ (HEK293) ТА ГЛІОБЛАСТОМИ ЛЮДИНИ (U87 MG)

Хітиназа 3-подібний білок 2 (CHI3L2, YKL-39) людини секретується хондроцитами суглобового хряща, синовіоцитами, в легенях і серці. Продемонстровано підвищені рівні YKL-39 в синовіальній рідині пацієнтів з ревматоїдним артритом або остеоартритом, а також при деяких інших захворюваннях і злоякісних пухлинах, зокрема гліобластомах. Цей білок належить до родини глікогідролаз 18 і найбільш структурно схожий з хітиназа 3-подібним білком 1 (CHI3L1, HC gp-39 або YKL-40) та, як показано раніше, сприяє росту синовіальних клітин людини, а також фібробластів шкіри та ембріональних фібробластів легенів. Залежну від дози стимуляцію росту зафіксовано при дії YKL-40 на декілька ліній фібробластів в діапазоні концентрацій від 0.1 до 2 нМ. Ця концентрація схожа з ефективною дозою добре охарактеризованого мітогену – інсуліноподібного фактора росту I. Використання селективних інгібіторів сигнального шляху мітоген-активованої протеїнкінази (MAP кінази) вказує на залучення обох білків, YKL-40 і IGF-I, у фосфорилювання позаклітинних сигнал-регульованих кіназ 1 і 2 (ERK1/ ERK2). Таким чином, YKL-40 ініціює сигнальний каскад, який посилює клітинну проліферацію, і це може свідчити про певну його роль в інгібуванні апоптозу. В цій роботі ми інформуємо, що YKL-39, як і YKL-40, має значно підвищену експресію в гліобластомах, а також активує сигнал-регульовані кінази ERK1/ERK2 в клітинах ембріональної нирки людини (HEK-293) та гліобластоми людини (U87 MG).

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