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ESTABLISHMENT OF U-251 MG CELL SUBLINES WITH ECTOPIC OVEREXPRESSION OF CHI3L1 mRNA VARIANTS AS A MODEL SYSTEM OF THE ISOFORMS TRIALS IN GLIOMAGENESIS

Advanced technique of long-read RNA sequencing (RNA-seq) enables the characterization of gene expression patterns at isoform-level resolution. Spatial transcriptomics methods combined with long-read RNA-seq revealed differential enrichment of two (long and short) mRNA isoforms of glioma-associated CHI3L1 in the hypoxic vs invasive niches of glioblastomas. ***Aim.*** *Obtaining glioma derived U-251 MG cell sublines producing the full-length and the short variants of CHI3L1 with subsequent characterization.* ***Methods.*** *Cell lentiviral transduction, Western blot and RT-qPCR analysis.* ***Results.*** *We successfully generated the U-251 MG cell sublines producing the full-length and the short variants of CHI3L1; our results support the suppression of CHI3L1-del8ex secretion and demonstrate differential targeting of microenvironment (LOX and GUSB) and EMT program (TGFB1) modulators by CHI3L1 isoforms.*

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Conclusion. *Here we present the cell model for CHI3L1 isoforms trials in gliomagenesis regarding experiments on co-cultivation and/with 3D culturing.*

Keywords: *CHI3L1, glioblastoma, transcriptional isoforms, splicing, differentially expressing genes (DEG), epithelial-mesenchymal transition (EMT).*

Introduction

The *CHI3L1*gene encodes the Chitinase-3-like protein 1 — a secretory mammalian chitinase-like protein (CLPs) legibly affecting cell proliferation, differentiation, epithelial-to-mesenchymal transition (EMT), apoptosis, inflammation, angiogenesis and extracellular tissue remodeling. CHI3L1 is considered a promising marker associated with multiple inflammatory and pathological processes with poor prognostic potential [1]. Both protein and mRNA upregulation of *CHI3L1* in serum or tumor tissues are frequently linked to a wide variety of cancer types including ovarian, prostate and renal carcinomas, myeloid leukemia, small cell lung cancer, and malignant brain gliomas. Also, numerous clinical studies have shown coherence of pathological gene functioning and such chronic diseases as liver inflammation and fibrosis, neuroinflammation, osteo- and rheumatoid arthritis [2]. Under physiological conditions, CHI3L1 is produced by macrophages, neutrophils, synoviocytes, chondrocytes, fibroblast-like cells, and smooth muscle cells [3]. Recklies and team showed that CHI3L1 stimulates the growth of primary human connective tissue cells synergistically with IGF1 activating ERK1/2- and AKT-related signaling cascades [4]. In CHI3L1-induced signaling interaction of cell adhesion molecule CD44 and Galectin-3 (LGALS3), as well as the cooperation of Interleukin-13 receptor subunit alpha-2 (IL-13Rα2) with transmembrane protein 219 (TMEM219), and integrin $\alpha v\beta 3$ with syndecan-1 (SDC1) represents complex receptor platforms for this glycoprotein [2].

In transcriptomics research the designation of differentially expressed genes in various biological conditions is the basis for further functional analysis. The combined use of short- and long-read RNA sequencing (RNA-seq) enables the characterization of gene expression patterns at isoform-level resolution [5], shifting the paradigm from "gene expression" to "isoform expression". At least two spliced and protein coding isoforms of *CHI3L1* are known up to now — the 10-exons containing full-length variant (*CHI3L1*-FL) and the short variant with spliced 8th one (*CHI3L1*-del8ex) [6]. Shi *et al.* showed that CHI3L1-overexpressed glioma U-251 MG cells characterized by enhancing proliferation, however production of CHI3L1-del8ex inhibited growth promoting cell sensitivity to AKT inhibitor MK2206 [7]. Using advanced methods of spatial transcriptomics and long-read sequencing, further research concluded that both long and short mRNA isoforms of *CHI3L1* are differentially enriched in the hypoxic *vs* the invasive niches [8]. Early it was found out that various glioma cell lines (U-251 MG, U87 MG, and T98G) may not only differ by the level of *CHI3L1* expression, but also by the ratio of its isoforms content [9]. Recently, Jung *et al.* discovered that such factors as macrophage differentiation and cell density affect *CHI3L1* splicing pattern. Moreover, they showed that exclusion of the 8th exon altered the folding of signal peptide in protein globule preventing extracellular secretion *vs* full-length glycoprotein [10]. Collectively, these results confirm the importance of *CHI3L1* splicing as one of the potential mechanisms controlling gene/protein functioning regarding involvement in pathology. Highlighting the need to further study the *CHI3L1* gene expression at the isoform level, here we report the results of characterization of U-251 MG cell sublines after ectopic protein production of two CHI3L1 types.

Materials and Methods

Cell culture and conditions

Obtained from the cell bank of the Nucleic Acid Biosynthesis Laboratory (IMBG NASU), human

glioblastoma cells U-251 MG, as well as 293T cells were cultured in high glucose DMEM or Neurobasal™ Medium (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% FBS, and 1% antibiotic-antimycotic solution (100 U/mL penicillin, 100 μg/mL streptomycin) in a humidified atmosphere containing 5% CO_2 at 37 °C.

CHI3L1 isoforms cloning into lentiviral vector and U-251 MG cells transduction

Lentiviral transfer vector pCDH-CMV-MCS-EF1-copGFP (System Biosciences, Mountain View, CA, USA) and psPAX2, pMD2.G packaging plasmids were kindly provided by Dr. D. Trono (Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland) [11]. Sequences of full-length *CHI3L1* (1185 bp)and *CHI3L1-del8ex* (1002 bp) were taken from PCR with specific primers and cDNA, obtained after reverse transcription of U-87 MG cells mRNA, which express both isoforms endogenously, and cloned into the transfer vector using XbaI and BamHI endonuclease restriction sites. The lentiviral transduction protocol was the same as in our previous study [12].

Western blot analysis

For the investigation of recombinant proteins production, parental U-251 MG cells and infected derivatives were seeded into T25 flasks and allowed to grow to near-confluence state under standard culturing. Then, total cell lysates (between 30 and 50 μg of total protein) were mixed with 4 × Laemmli sample buffer, boiled, proteins resolved by 10% SDS-PAGE, and transferred into a nitrocellulose membrane. Next, membranes were blocked for 1 hr at room temperature with 5% powdered skim milk in TBS with 0.05% Triton X-100 (TBST), reacted with mouse Anti-Chitinase-3-like protein 1 (YKL-40) Antibody, clone mAY (Sigma-Aldrich, MABC196) or Mouse monoclonal Anti-β-Actin (ACTB) Antibody (Sigma-Aldrich, A1978-100UL) at 4 °C overnight, and incubated with Anti-Mouse IgG (H+L), HRP Conjugate (Promega, W4021) secondary antibody for 1 hr at room temperature with shaking. Blots were developed with an ECL detection system. Chemiluminescence was observed using a Biorad ChemiDoc XRS+ Imaging System and a Biorad Image Lab Software v.6.0.1.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cells using a NucleoSpin RNA isolation kit (Macherey-Nagel, Germany, 740955). RNA purity and concentration were measured by the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). For RT-qPCR, 1.5—2 μg of total RNA was converted into complementary cDNA with Oligo(dT)18 Primer and Maxima H Minus Reverse Transcriptase (Thermo Fisher Scientific, SO132 and EP0751, correspondingly). Fivefold diluted cDNA and gene specific primers were mixed with HOT FIREPol® EvaGreen® qPCR Mix Plus (no ROX) 5× mix (Solis BioDyne, Estonia) following the manufacturer's recommendations with annealing at 60 °C for 20 sec, and RT-qPCR was performed using CFX96 RT-PCR Detection System (Bio-Rad, USA) with Bio-Rad CFX Manager 3.1. software. Gene expression was normalized to *RPLP0* and *GAPDH* (geometric mean) and quantified using the $2^{-\Delta\Delta CT}$ method. The primer pairs used in analysis are indicated in Table.

Statistical analysis

A two-sided unpaired t-test and ANOVA with Tukey's post-hoc test were used to calculate the significance values (GraphPad Prism 8.0.1 software, USA). Data showing p-values of *P < 0.05, **P < 0.01, and ***P < 0.001 were considered significant.

Results and discussion

A lentiviral vector with fluorescent reporter (copGFP) was used to obtain glioblastoma U-251

Primers

№	Genes	Primers' names	The primers sequences 3' → 5'	Product size (bp)
1	NM_001276.4 Chitinase 3 like 1 (CHI3L1)	CHI3L1-F CHI3L1-R	GGACCCTTGCCTACTATGAGAT GTACTGCACCTTGCTTTTGAC	146
2	XM_047442847.1 PREDICTED: Chitinase 3 like 1 (CHI3L1) / (CHI3L1-d8ex)	CHI3L1-d8f6 CHI3L1-R	GTCCTGACAGATTCAACAACACAATC GTACTGCACCTTGCTTTTGAC	149
3	NM_004000.3 Chitinase 3 like 2 (CHI3L2)	CHI3L2-F CHI3L2-R	TCAAAACCAAGAATCCCAAAC ATCAGCACAGTGAAATGAGT	214
4	NM_033131.4 Wnt family member 3A (WN3A)	WNT3a_FW WNT3a_RV	GTGTTCCACTGGTGCTGCTA TTTAGGTGGGAGTCCTGCTC	156
5	NM_001128128.3Zinc finger E-box binding homeobox 1 (ZEB1)	ZEB1_for ZEB1_rev	GATTCTACACCGCCCAAAAA AAGCGCTTTCCACATTTGTC	245
6	NM_001244438.2 Arginase 1 (ARG1)	ARG1-Fw ARG1-Rv	CCATCTTTCACACCAGCTACTG CTGCTGTGTTCACTGTTCGAG	178
7	NM_003255.5 TIMP metallopeptidase inhibitor 2 (TIMP2)	TIMP2-Fw TIMP2-Rv	AAGCGGTCAGTGAGAAGGAAG GGGGCCGTGTAGATAAACTCTAT	136
8	NM_001040058.2 Secreted phosphoprotein 1 (SPP1)	SPP1-Fw SPP1-Rv	GAAGTTTCGCAGACCTGACAT GTATGCACCATTCAACTCCTCG	91
9	NM_000660.7 Transforming growth factor beta 1 (TGFB1)	TGFB1-Fw TGFB1-Rv	CTAATGGTGGAAACCCACAACG TATCGCCAGGAATTGTTGCTG	209
10	NM_002317.7 Lysyl oxidase (LOX)	LOX- F1rt LOX- R1rt	CACAGGACATCATGCGTATGC CCACTTCAGAACACCAGGCAC	102
11	NM_005576.4 Lysyl oxidase like 1 (LOXL1)	LOXL1-F1rt LOXL1-R1rt	GCTATGACACCTACAATGCGGA GACCTGTGTAGTGAATGTTGCATCT	162
12	NM_002318.3 Lysyl oxidase like 2 (LOXL2)	LOXL2-Fw LOXL2-Rv	AGGACATTCGGATTCGAGCC CTTCCTCCGTGAGGCAAAC	215
13	NM_004530.6 Matrix metallopeptidase 2 (MMP2)	MMP2-Fw MMP2-Rv	CCAGAATACCATCGAGACCATG ATGATCCCAGCGGCCAAAGTT	265
14	NM_001530.4 Hypoxia inducible factor 1 subunit alpha (HIF1A),	Hif1a Hif1a	CCACTGCCACCACTGATGAA GTGAGGCTGTCCGACTTTGA	156
15	NM_005985.4 Snail family transcriptional repressor 1 (SNAI1)	SNAIL1-rtF2 SNAIL1-rtR2	TGCCCTCAAGATGCACATCCGA-3 GGGACAGGAGAAGGGCTTCTC	133
16	NM_003106.4 SRY-box transcription factor 2 (SOX2)	SOX2-F1rt SOX2-R1rt	GGGGAAAGTAGTTTGCTGCC CGCCGCCGATGATTGTTATT	131
17	NM_006617.2 Nestin (NES)	NESTIN-F1rt NESTIN-R1rt	CAAGACTTCCCTCAGCTTTCAG AGGTGTCTCAAGGGTAGCAG	142
18	NM_002701.6 POU class 5 homeobox 1 (POU5F1)(OCT3/4)	OCT3-4-F1 OCT3-4-R1	CGAACCAGTATCGAGAACCG AGAGAGACATGGCACTCACATC	136
19	NM_000610.4 CD44 molecule (IN blood group) (CD44),	CD44-F1rt CD44-R1rt	CCATCTGTGCAGCAAACAACA TTCAGGTGGAGCTGAAGCATT	95
20	NM_000181.4 Glucuronidase beta (GUSB)	GUSB-F1rt GUSB-R1rt	GAAAATACGTGGTTGGAGAGCTCATT CCGAGTGAAGATCCCCTTTTTA	101
21	NM_001002.4 Ribosomal protein lateral stalk subunit P0 (RPLP0)	RPLPO-for RPLPO-rev	GCAATGTTGCCAGTGTCTGT GCCTTGACCTTTTCAGCAAG	142
22	NM_002046.7 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	GAPDH-F GAPDH-R	GTCTCCTCTGACTTCAACAGC ACCACCCTGTTGCTGTAGCCAA	131

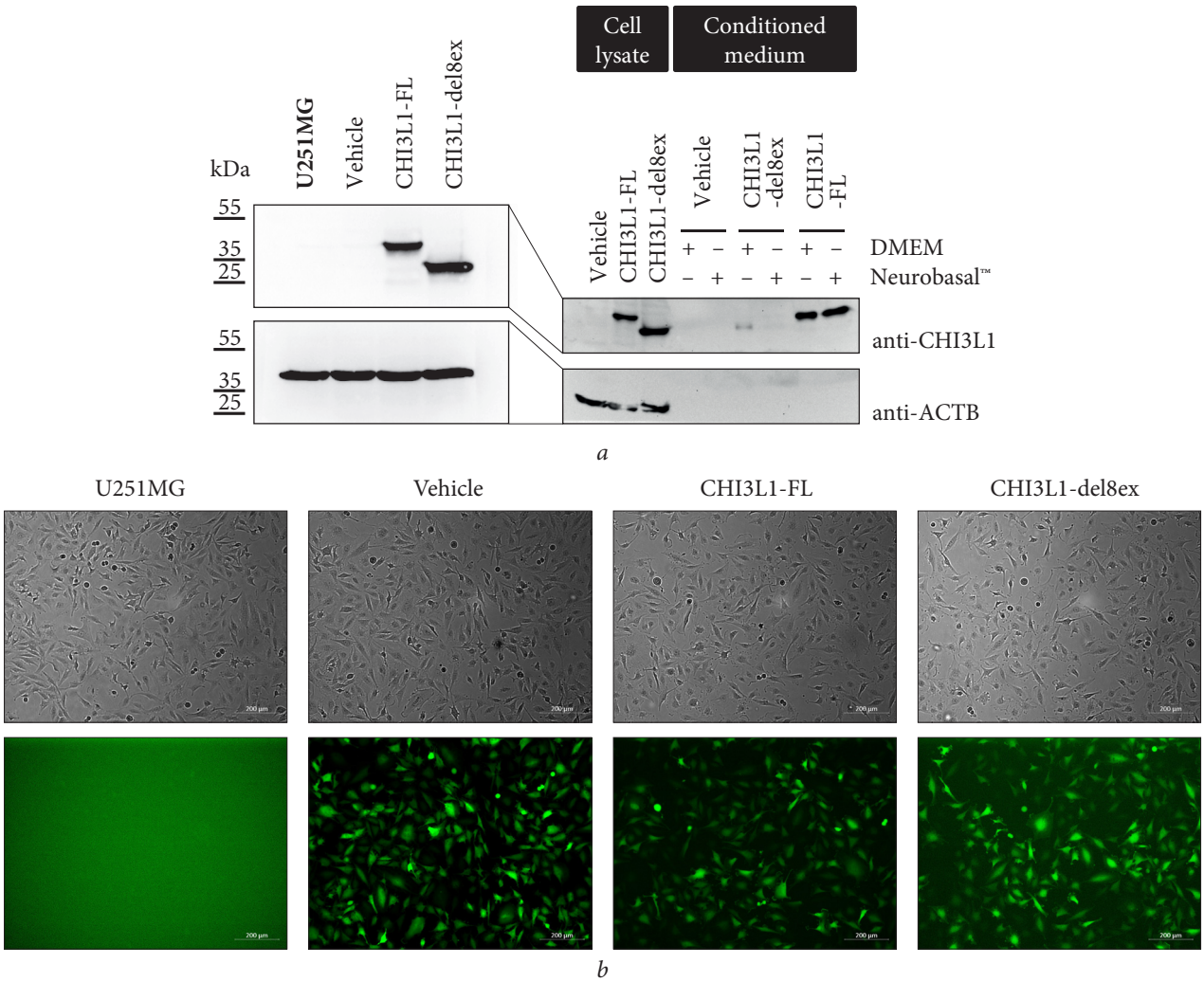

Fig. 1. Overexpression of full-length (–FL) and spliced variant (–del8ex) of *CHI3L1* in glioma-derived U-251 MG cells. *a* — Western blotting of total lysates and conditioned media of U-251 MG cells stably expressing two variants of CHI3L1; *b* — Fluorescence microscopy of reporter copGFP expression in U-251 MG cells stably producing two variants of CHI3L1 after lentiviral transduction. Scale bar: 200 μm

MG sublines with ectopic overexpression of two CHI3L1 variants. This approach will favor the upcoming isoforms trials in experiments on 3D culturing and co-cultivation. U-251 MG glioma cells express *CHI3L1* RNAs at extremely low levels and do not produce Chitinase-3-like protein 1. The infection of cells by *CHI3L1*-FL and *CHI3L1*-del8ex cDNA-containing viral particles resulted in heterogeneous and stably transformed cell sublines regarding each transgene. For clarifying the gene-specific effects, the U-251 MG cells transduced by an empty vector were also obtained as a control cell group (Vehicle) (Fig. 1).

To test the preservation of the native state of CHI3L1 variants we cultured infected cells in two types of media: DMEM and Neurobasal™ Medium, since co-cultivation with cells of neuronal and glial origin is intended. Detection of CHI3L1-FL protein with molecular weight about 42 kDA using specific antibodies in cell lysate and conditioned

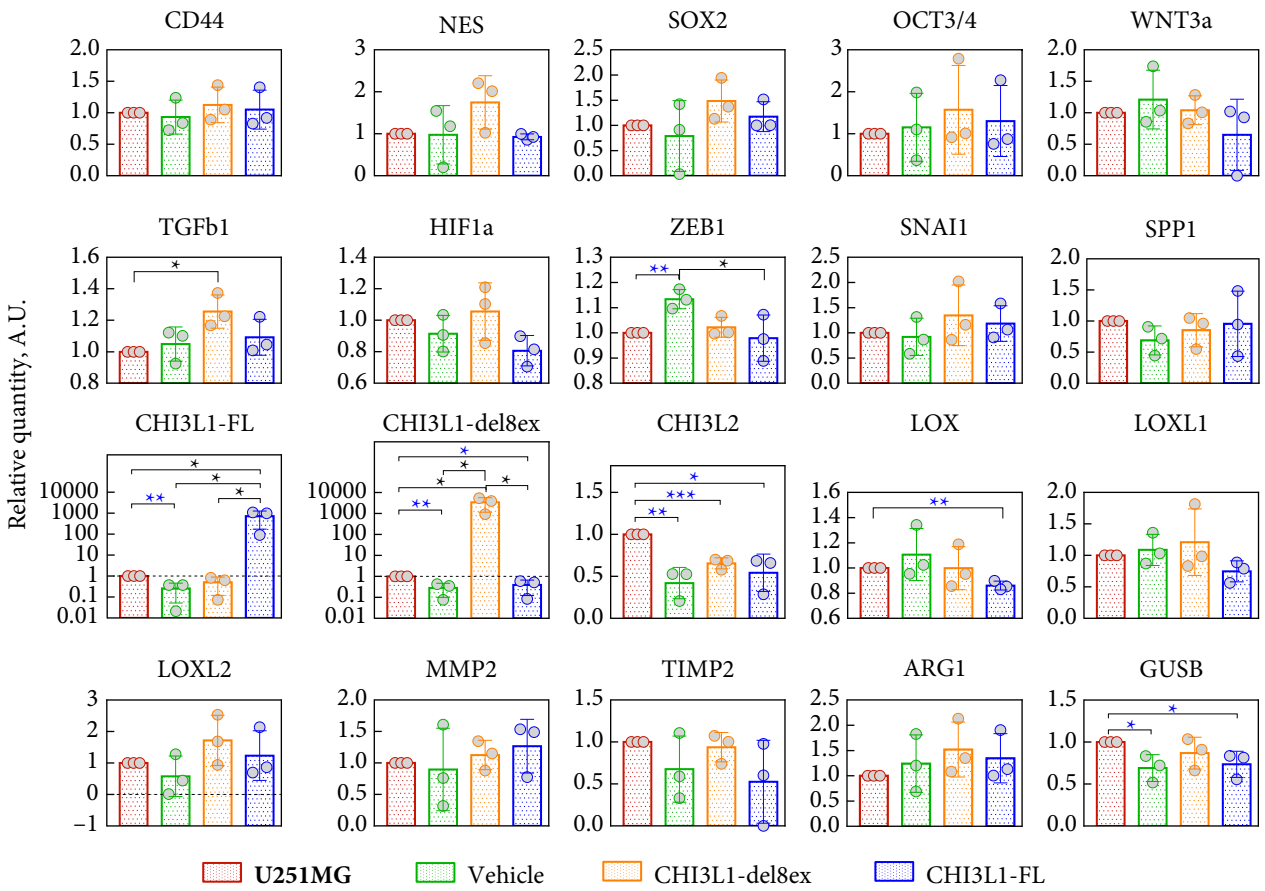

Fig. 2. Relative mRNA expression level of indicated genes assessed by qPCR in U-251 MG cells stably expressing two variants of *CHI3L1*. Results were calculated using the $2^{-\Delta\Delta Ct}$ method and the bar graph shows the mean (±SD) of three individual experiments. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test (black asterisks) and two-tailed unpaired t-test (blue asterisks)

media of both types indicated the functional state of the interested secretory CLP. Remarkably, analysis of "short" CHI3L1 expression pattern regarding intercellular *vs* extracellular secretion revealed its presence dominantly within the cells, though small amounts of CHI3L1-del8ex also was detected in DMEM but not in Neurobasal™ Medium (Fig. 1*a*). Fluorescence microscopy of the reporter-producing cells after transduction showed high efficiency of transgenesis, supporting Western blot results (Fig. 1*b*). In total, we displayed successful establishment of U-251 MG cell sublines producing the full-length and the short variants of CHI3L1. Our results support the role of *CHI3L1* mRNA splicing in suppression of CHI3L1-del8ex secretion, however after all revealed its secretory potential.

To identify the pathways targeted by each *CHI3L1* isoform we performed the mRNA profiling of a set of genes associated with gliomagenesis and preferably with EMT, stemness program and extracellular tissue remodeling by RT-qPCR analysis. The analyzed genes were grouped into three groups: 1) Genes belonging to the program of stemness (*CD44*, *NES*, *SOX2*, *OCT3/4*, *SPP1*) 2) genes of the EMT program (*TGFB1*, *HIF1A*,

ZEB1, SNAIL1, WNT3A, CHI3L1, CHI3L2), 3) genes of the stroma or microenvironment modulation (*LOX, LOXL1, LOXL2, MMP2, TIMP2, ARG1, GUSB*).

Ji-Ying Yu and Di Lu, with their teams, showed that in colorectal cancer, CHI3L1, interacting with TGFβ1 and TGFR, enhances tumor growth and migration, and also promotes the development of hepatocellular carcinoma by disrupting the metabolism of lipid peroxidation enzymes.[2, 13]. Our results demonstrate that these findings can be extrapolated on glioma biology since we showed the impact of *CHI3L1* regarding noted targets: overexpression of *CHI3L1*-FL was associated with statistically significant downregulation of Lysyl Oxidase gene (*LOX*),whereas *CHI3L1*-del8ex upregulation was accompanied with elevated expression of *TGFb1* in U-251 MG glioma cells too (Fig. 2.). Also we detect that the *CHI3L1*-FL overexpressed cells display downregulation of *GUSB*, a gene of Glucuronidase Beta, a hydrolase degrading glycosaminoglycans (heparan sulfate and others). That is interesting in the context of the ability of CHI3L1 to interact with glycosaminoglycan heparin and different extracellular matrix components, such as hyaluronic acid and heparan sulfate (HS), and by binding to HS chain of syndecan-1 to induce coordination between syndecan-1 and the integrin αvβ3 [3]. The interaction between *CHI3L1* pathological functioning, moreover in isoform context, and *GUSB* expression in gliomagenesis has not been reported before and must be studied more precisely, as there are pseudogenes of this locus in the human genome. Interestingly, we observed decreasing in endogenous *CHI3L1* (both isoforms) and closely related *CHI3L2* mRNAs content in U-251 MG cells after infection, which highlights their importance in cell response to systemic stress (Fig. 2).

Conclusion

Spatial transcriptomics methods combined with long-read RNA-seq revealed differential enrichment of two (long and short) mRNA isoforms of glioma-associated CHI3L1 in the hypoxic *vs* invasive niches of glioblastomas — the most malignant and aggressive type of primary brain tumor in adults. Along with other findings regarding tumor-suppressive properties of *CHI3L1*-del8ex (short) variant, these results confirm the importance of *CHI3L1* splicing in gliomagenesis. Here we present the cell model for *CHI3L1* study in context of the gene isoforms based on glioma derived U-251 MG cells stably producing the full-length mRNA variant or the short one, revealing some peculiarities in the *CHI3L1*-del8ex secretion mode.

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ОТРИМАННЯ СУБЛІНІЙ КЛІТИН U-251 MG З ЕКТОПІЧНОЮ НАДЕКСПРЕСІЄЮ ДВОХ ВАРІАНТІВ мРНК CHI3L1 ЯК МОДЕЛЬНОЇ СИСТЕМИ ДОСЛІДЖЕННЯ ІЗОФОРМ У ГЛІОМАГЕНЕЗІ

Удосконалена техніка секвенування «long-read» РНК (RNA-seq) дозволяє характеризувати патерни експресії генів з роздільною здатністю на рівні ізоформ. Методи просторової транскриптоміки в поєднанні з «long-read» секвенуванням РНК виявили диференціальне збагачення двох (довгої та короткої) ізоформ мРНК гена *CHI3L1*, асоційованого з гліомою, в гіпоксичних та інвазивних нішах гліобластом. ***Мета.*** Отримання сублініій клітин гліомного походження U-251 MG, що продукують повнорозмірний та короткий варіанти CHI3L1, з подальшою характеристикою. ***Методи.*** Лентивірусна трансдукція клітин U-251 MG, вестерн-блот та RT-qPCR аналіз. ***Результати.*** Ми успішно створили сублінії клітин U-251 MG, що продукують повнорозмірний та короткий варіанти CHI3L1; наші результати підтверджують пригнічення секреції CHI3L1-del8ex та демонструють диференціальне таргетування модуляторів мікрооточення (LOX та GUSB) та програми EMT (TGFB1) ізоформами CHI3L1. ***Висновки.*** Представлено клітинну модель гліоми для дослідження ролі ізоформ CHI3L1 в експериментах із сокультивування та/або 3D-культивування.

***Ключові слова*:** *CHI3L1*, гліобластома, transcriptional isoforms, splicing, differentially expressing genes (DEG), epithelial-mesenchymal transition (EMT).