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EXPRESSION OF UBIQUITIN SPECIFIC PEPTIDASE AND *ATG7* GENES IN U87 GLIOMA CELLS UPON GLUTAMINE DEPRIVATION

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We have studied the effect of glutamine deprivation on the expression of genes encoding for ubiquitin specific peptidases (USP) and ubiquitin activating enzyme E1-like protein/autophagy related 7 (GSA7/ATG7) in U87 glioma cells in relation to inhibition of inositol requiring enzyme-1 (IRE1). It was shown that exposure of control glioma cells (transfected by empty vector) upon glutamine deprivation led to suppression of USP1 and ATG7 mRNA expression and up-regulated USP25 mRNA. At the same time, glutamine deprivation did not significantly change USP4, USP10, USP14, and USP22 gene expressions in these cells. Inhibition of IRE1 signaling enzyme function in U87 glioma cells increased effect of glutamine deprivation on the expression of USP1 gene and introduced sensitivity of USP4 and USP14 genes to this condition. Therefore, glutamine deprivation affected the expression level of most studied genes in gene specific manner in relation to the functional activity of IRE1 enzyme, a central mediator of endoplasmic reticulum stress, which controls cell proliferation and tumor growth.

Key words: mRNA expression, USPs, ATG7, glutamine deprivation, IRE1 inhibition, U87 glioma cells.

Malignant gliomas are highly aggressive tumors with very poor prognosis and to date there is no efficient treatment available. The moderate efficacy of conventional clinical approaches therefore underlines the need for new therapeutic strategies. Glutamine is important to glioma development and a more agressive behaviour [1-4]. However, mechanisms whereby cancer cells regulate glutamine catabolism remain largely unknown [4-6]. A better knowledge of tumor responses to glutamine deprivation condition is required to elaborate new therapeutical strategies of cell sensibilization, based on the blockade of survival mechanisms.

Ubiquitin is a highly conserved protein involved in regulation of intracellular protein breakdown, cell cycle regulation, chromatin remodeling, and stress response. It is released from degraded proteins by disassembly of the polyubiquitin chains, which is mediated by ubiquitin-specific proteases, members of the ubiquitin-specific processing family of proteases for deubiquitination of proteins [7-9]. E3 ubiquitin ligases and deubiquitylases play an important role in cancer [7, 10, 11]. Our previous results demonstrated possible interaction/cross-talk between unfolding protein response signaling and ubiquitin system during adjustment to episodes of hypoxia during tumor development [12]. Ubiquitin specific peptidases (USPs) and ubiquitin activating enzyme E1-like protein/autophagy related 7 (GSA7/ATG7) are involved in cancer cells survival and progression [13-15]. USP1 and USP7 are responsible for deubiquitination of mono-ubiquitinated PCNA (proliferating cell nuclear antigen), which activates error-prone DNA polymerases and controls an oxidative-stress-induced mutagenesis in human cells [13]. Decreased levels of USP1 in cancer cells have been implicated in lung and glioblastoma tumors growth and progression [14, 16]. There is data that serine phosphorylation is critical for the activity of USP1 and its interaction with WD40-repeat protein UAF1; while two nuclear localization signals

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in USP1 mediate nuclear import of the USP1/UAF1 complex [17].

Ubiquitin specific peptidase 4 function is important during tumorigenesis because this deubiquitinating enzyme has a key role in the regulation of TP53 and TGFβ signaling and is also a positive regulator of the WNT/β-catenin signaling [18-20]. Deubiquitinating enzyme UPS10 suppresses the proliferation and growth of cancer cells through stabilization of p53 protein [21]. Additional anti-tumorigenic effect of USP10 achieved by antagonizing c-MYC activity through stabilization of a tumor suppressor SIRT6 [22]. In agreement, microRNA-191 mediated lower protein level of USP10 has been demonstrated to promote pancreatic cancer progression [21]. It was shown that USP14 is a tumor promoting peptidase, its phosphorylation and activation by Akt not only regulates the ubiquitin-proteasome system, but also promotes tumor progression through regulation of cellular proliferation and apoptosis of cancer cells [23]. Inhibition of USP14 could be used as potential anti-cancer therapeutic strategy. USP22 protease has been demonstrated to participate in regulation of the cell cycle progression in many cancer cell types [24, 25]. This enzyme removes ubiquitin from histones, thus regulating gene transcription [26]. It is interesting to note that deubiquitinating enzyme USP25 is involved in endoplasmic reticulum (ER)-associated degradation (ERAD) of misfolded/anomalous proteins [27]. USP25 counteracts ubiquitination of ERAD substrates by the ubiquitin ligase HRD1, rescuing them from degradation by the proteasome [27]. USP25 is a novel TRiC interacting protein that is catalyzed deubiquitination of the TRiC protein and stabilized this chaperonin, thereby reducing accumulation of misfolded protein aggregates [28].

The ubiquitin activating enzyme E1-like protein (GSA7), which is also known as autophagy related 7 (ATG7), is an essential component of autophagic machinery and a multifunctional protein, which mediates inhibition of cell proliferation and activation of apoptosis through induction of cellular senescence [29-32]. Thus, autophagy inhibition by Baf A1 or knockdown of ATG7 or ATG12 induced cytotoxicity in multiple human bladder cell lines. Induction of apoptosis was found in cells with autophagy inhibition [32]. Whereas ATG7 loss leads to the expected decrease in autophagic flux, it also results in endoplasmic reticulum stress, accumulation of dysfunctional mitochondria, oxidative stress, activation of AMPK, and a marked decrease in protein synthetic capacity [33]. Moreover, knockdown of either of the key autophagic genes, ATG5 or ATG7, impacted on CASP8 activation and cell death induction, highlighting the crucial role of autophagy in the activation of this novel endoplasmic reticulum stress-induced death pathway [34]. The endoplasmic reticulum stress is responsible for enhanced cancer cell proliferation and IRE1 knockdown by a dominant-negative construct of IRE1 (dnIRE) resulted in a significant anti-proliferative effect on glioma growth [35, 36].

The rapid growth of solid tumors generates micro-environmental changes in association to hypoxia, nutrient deprivation and acidosis, which promote neovascularisation, cell survival and proliferation [37-40]. Glucose and glutamine are substrates for glycolysis and glutaminolysis, which are important for tumor progression through regulation of the cell cycle at distinct stages [1-5]. The activation of glycolysis and glutaminolysis in cancer cells is tightly regulated by the action of two ubiquitin ligases, which control the transient appearance and metabolic activity of the glycolysis-promoting enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) and glutaminase 1 (GLS1), the first enzyme in glutaminolysis [1, 4, 6]. The activation of endoplasmic reticulum stress is indispensable for tumor growth as it facilitates adaptation to stressful environmental conditions [40]. IRE1 is the most evolutionary conserved sensor that responds to protein misfolding with a highly tuned program aimed to either resolve the stress or direct the cell towards apoptosis in case stress becomes too severe, which makes it a key regulator of cell life and death processes [35, 40]. Recently, we have shown that glutamine deprivation affects the expression of proliferation related genes in U87 glioma cells and that IRE1 knockdown modifies glutamine deprivation effects on these genes expression possibly contributing to suppression of glioma cells proliferation [41]. Previously, we have shown that most USPs are regulated by IRE-1α signaling and hypoxia as well as glucose deprivation [42-44], but the precise mechanism of the exhibited by USP7 anti-proliferative effect is not clear yet.

The aim of this study was investigation of the effect of glutamine deprivation condition on the expression of a subset of genes encoding ubiquitin specific peptidases and of ubiquitin activating enzyme E1-like protein/autophagy related 7 in glioma cells in relation to inhibition of signaling enzyme IRE1

with hopes of elucidating its mechanistic part in the development and progression of certain cancers and the contribution to unfolding protein response.

Materials and Methods

Cell lines and culture conditions. Two sublines of U87 glioma cells were used in this study. Cells are growing in high glucose (4.5 g/l) Dulbecco's modified Eagle's minimum essential medium (DMEM; Gibco, Invitrogen, USA) supplemented with glutamine (2 mM), 10% fetal bovine serum (Equitech-Bio, Inc., USA), streptomycin (0.1 mg/ml; Gibco) and penicillin (100 units/ml; Gibco) at 37 °C in a 5% CO_2 incubator. One subline was obtained by selection of stable transfected clones with overexpression of empty vector (pcDNA3.1), which was used for creation of dnIRE1 (dominant-negative constructs of IRE1, bifunctional sensing and signaling enzyme of endoplasmic reticulum stress). This untreated subline of glioma cells (control glioma cells) was used as control in the study of effects of glutamine deprivation on the expression level of *USP1*, *USP4*, *USP10*, *USP22*, *USP25*, and *GSA7* genes. Second subline was obtained by selection of stable transfected clones with overexpression of dnIRE1 and has suppressed both protein kinase and endoribonuclease activities of this enzyme. The expression level of studied genes in these cells was compared with cells, transfected by vector. The subline, which overexpress dnIRE1, was also used as control for investigation the effect of glutamine deprivation condition on the expression level of studied in cells with inhibited signaling enzyme IRE1 function. U87 glial cells clone with dnIRE1 was received by selection at 0.8 mg/ml geneticin (G418) and grown in the presence of this antibiotic at lower concentration (0.4 mg/ml). Glutamine deprivation conditions were created by changing the complete DMEM medium into culture plates on medium without glutamine and plates were exposed to this condition for 16 h.

The suppression level of IRE1 both enzymatic activity in glioma cells that overexpress a dnIRE1 was estimated previously [36] by determining the expression level of XBP1 alternative splice variant (XBP1s), a key transcription factor in IRE1 signaling, using cells treated by tunicamycin (0.01 mg/ml during 2 h). Moreover, the proliferation rate of glioma cells with mutated IRE1 is decreased more than in 2 fold [36]. Thus, the blockade of signaling enzyme IRE1 activity has significant effect on proliferation rate of glioma cells.

RNA isolation. Total RNA was extracted from glioma cells as previously described [43]. The RNA pellets were washed with 75% ethanol and dissolved in nuclease-free water. For additional purification RNA samples were re-precipitated with 95% ethanol and re-dissolved again in nuclease-free water. RNA concentration and spectral characteristics were measured using NanoDrop Spectrophotometer.

Reverse transcription and quantitative PCR analysis. QuaniTect Reverse Transcription Kit (QIAGEN, Hilden, Germany) was used for cDNA synthesis as described previously [43]. The expression level of USP1, USP4, USP10, USP22, USP25, and GSA7 mRNA were measured in glioma cell line U87 and its subline (clone 1C5) by real-time quantitative polymerase chain reaction using "RotorGene RG-3000" qPCR (Corbett Research, Germany) and Absolute qPCR SYBRGreen Mix (Thermo Fisher Scientific, ABgene House, Epsom, Surrey, UK). Polymerase chain reaction was performed in triplicate.

The amplification of cDNA of the ubiquitin specific peptidases 1 (USP1; EC 3.4.19.12) was performed using forward primer (5′-CAGCATGATGCACAGGAAGT-3′) and reverse primer (5′-CCCATTTCCTTTTGGGAGTT-3′). These oligonucleotides correspond to sequences 1347–1366 and 1565–1546 of human USP1 cDNA (GenBank accession number NM_003368). The size of amplified fragment is 219 bp. For amplification of the USP4 (EC 3.4.19.12) cDNA we used next primers: forward 5′-CTTATTGACAGCCGGTGGTT-3′ and reverse 5′-GTTTATTCCACGCCTCGGTA-3′. The nucleotide sequences of these primers correspond to sequences 185-204 and 389-370 of human USP4 cDNA (GenBank accession number NM_003363). The size of amplified fragment is 205 bp. The amplification of cDNA of the ubiquitin specific peptidases 10 (USP10; EC 3.4.19.12) was performed using forward primer (5′-AGAGTGCATCACCTCCTGCT-3′) and reverse primer (5′-GATCCTCTGAAACCGGAACA-3′). These oligonucleotides correspond to sequences 1216-1235 and 1434-1415 of human USP10 cDNA (GenBank accession number NM_001272075). The size of amplified fragment is 219 bp. For amplification of the USP14 (EC 3.4.19.12) cDNA we used next primers: forward 5′-CGTTCTGTGCCTGAACTCAA-3′ and reverse 5′-TTCACCTTTCTCGGCAAACT-3′. The nucleotide sequences of these primers correspond to sequences 586-605 and 789-770 of human USP14 cDNA (GenBank accession number NM_005151). The size of amplified fragment

is 204 bp. The amplification of cDNA of the USP22 (EC 3.4.19.12) was performed using forward primer (5′-TGGAAATAATCGCCAAGGAG-3′) and reverse primer (5′-GAAGAAGTCCCGCAGAAGTG-3′). These oligonucleotides correspond to sequences 575-594 and 816-797 of human USP22 cDNA (GenBank accession number NM_015276). The size of amplified fragment is 242 bp. For amplification of the USP25 (EC 3.4.19.12) cDNA we used next primers: forward 5′-GGCACATAACGGAGGAAGAA-3′ and reverse 5′-AGCTTGGCCTTCGTGAACTA-3′. The nucleotide sequences of these primers correspond to sequences 1982-2001 and 2178-2159 of human USP25 cDNA (GenBank accession number NM_001283041). The size of amplified fragment is 197 bp. The amplification of the ubiquitin activating enzyme E1-like protein/autophagy related 7 (GSA7/ATG7) cDNA was performed using forward primer (5′-TGAGCCTCCAACCTCTCTTG-3′) and reverse primer (5′-AGATCTCAGCAGCTTGGGTT-3′). These oligonucleotides correspond to sequences 1956-1975 and 2200-2181 of human GSA7 cDNA (GenBank accession number NM_006395). The size of amplified fragment is 245 bp. The amplification of the beta-actin (ACTB) cDNA was performed using forward – 5′-GGACTTCGAGCAAGAGATGG-3′ and reverse – 5′-AGCACTGTGTTGGCGTACAG-3′ primers. These primers nucleotide sequences correspond to 747-766 and 980-961 of human ACTB cDNA (GenBank accession number NM_001101). The size of amplified fragment is 234 bp. The expression of β-actin mRNA was used as control of analyzed RNA quantity.

The primers were received from Sigma-Aldrich (St. Louis, MO, USA). The quality of amplification products was analyzed by melting curves and by electrophoresis using 2% agarose gel. An analysis of quantitative PCR was performed using special computer program “Differential Expression Calculator”. The values of USP1, USP4, USP10, USP22, USP25, and GSA7 mRNA expressions were normalized to the expression of beta-actin mRNA and represented as percent of control 1 (100%).

Statistical analysis. All values are expressed as mean ± SEM from triplicate measurements performed in 4 independent experiments. Statistical analysis was performed according to Student’s *t*-test using Excel program as described previously [45].

Results and Discussion

To determine if glutamine deprivation regulates the genes of interest through the IRE1 branch of endoplasmic reticulum stress response, we investigated the effect of glutamine deprivation condition on the expression of genes encoding USP1, USP4, USP10, USP22, USP25, and GSA7 in two sublines of U87 glioma cells in relation to inhibition of IRE1 signaling enzyme, which is a major component of the unfolded protein response. As shown in Fig. 1, the exposure of control glioma cells (transfected by empty vector) upon glutamine deprivation condition leads to small but statistically significant suppression of USP1 and GSA7 mRNA expression (-13 and -19%, correspondingly) as compared to control glioma cells. At the same time, the expression level of four other genes of USPs (and *USP22*) does not change significantly in control glioma cells treated by glutamine deprivation, but *USP25* gene expression is up-regulated (+22%) under this experimental condition as compared to control glioma cells (Fig. 1).

In glioma cells without functional activity of signaling enzyme IRE1 the expression of USP1, USP4, USP14, and GSA7 mRNA is down-regulated (-32, -20, -14, and -22%, correspondingly) upon glutamine deprivation (Fig. 2). Next we studied the effect of inhibition of IRE1 signaling enzyme on the expression of *USP10*, *USP22*, and *USP25* genes in glioma cells treated by glutamine deprivation.

As shown in Fig. 2, glutamine deprivation does not significantly change the level of *USP10* and *USP22* genes expression in glioma cells without IRE1 signaling enzyme function in comparison with corresponding control cells. At the same time, the expression level of *USP25* gene is increased (+17%) in dnIRE1 glioma cells treated by glutamine deprivation (Fig. 2). Therefore, inhibition of IRE1 signaling enzyme function in U87 glioma cells by dnIRE1 does not change significantly the sensitivity of *USP10*, *USP22*, and *ATG7* gene expression to glutamine deprivation and introduces sensitivity of *USP4* and *USP14* gene expressions to this experimental condition, as shown in Fig. 3 and 4.

Thus, inhibition of IRE1 modifies the sensitivity of *USP1*, *USP4* and *USP14* gene expressions to glutamine deprivation in U87 glioma cells. As shown in Fig. 3, the suppression of *USP1* gene expression by

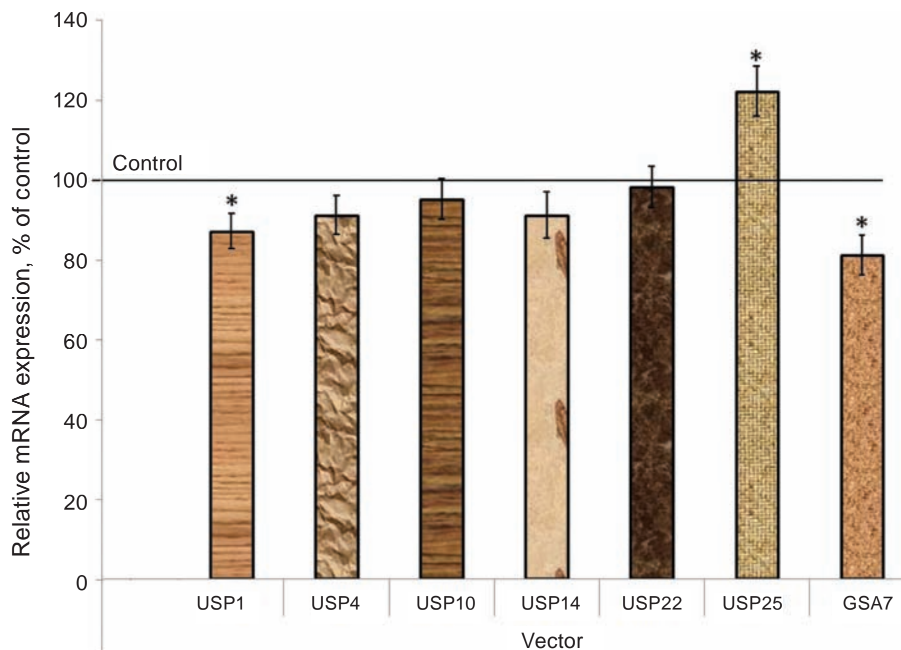

Fig. 1. Effect of glutamine deprivation (16 h) on the expression level of USPs and GSA7 mRNA in control U87 glioma cells stable transfected with vector (Vector) and cells with inhibited function of signaling enzyme IRE1 (dnIRE1) measured by qPCR. Values of these mRNA expressions were normalized to β-actin mRNA and represented as percent of control 1 (100%); mean ± SEM; n = 4

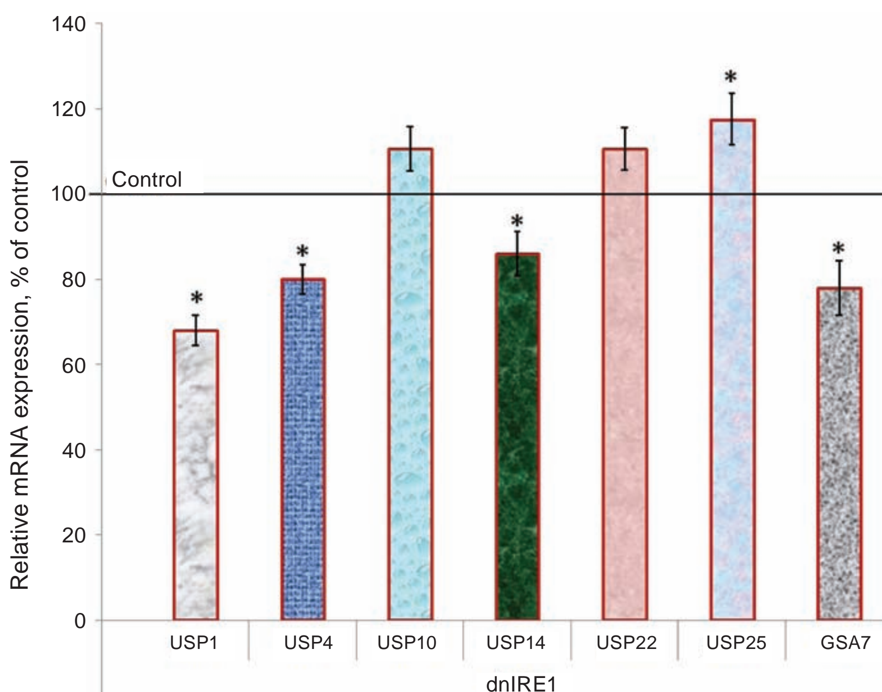

Fig. 2. Effect of glutamine deprivation (16 h) on the expression level of USPs and GSA7 mRNA in control U87 glioma cells stable transfected with vector (Vector) and cells without signaling enzyme IRE1 function (dnIRE1) measured by qPCR. Values of these mRNA expressions were normalized to β-actin mRNA expression and represented as percent of control 1 (100%); mean ± SEM; n = 4

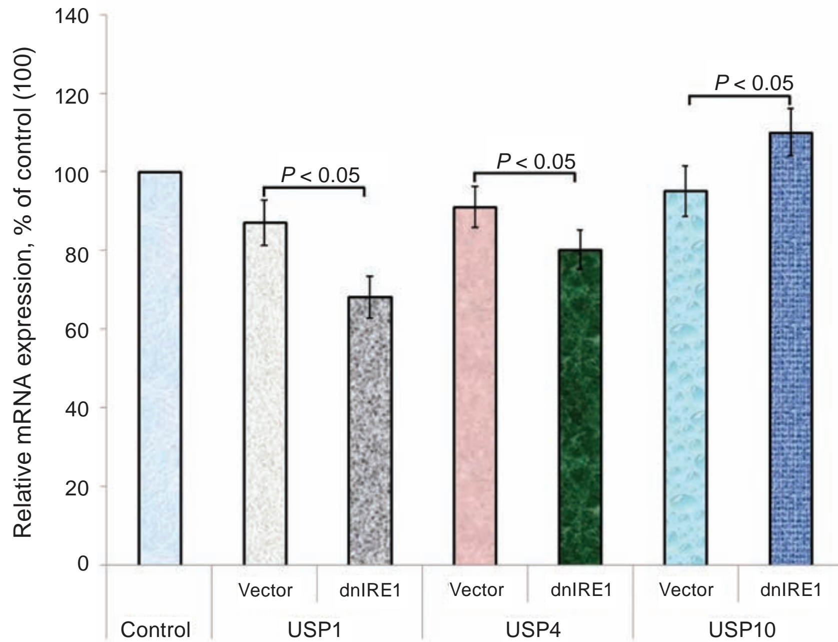

Fig. 3. Comparative effect of glutamine deprivation on the expression level of USP1, USP4, and USP10 mRNA in two types of glioma cells: control cells transfected by vector (Vector) and cells with a deficiency of the signaling enzyme IRE1 (dnIRE1) measured by qPCR. Values of these mRNA expressions were normalized to β-actin mRNA expression and represented as percent of corresponding control (control for both cell types is accepted as 100%); mean ± SEM; n = 4

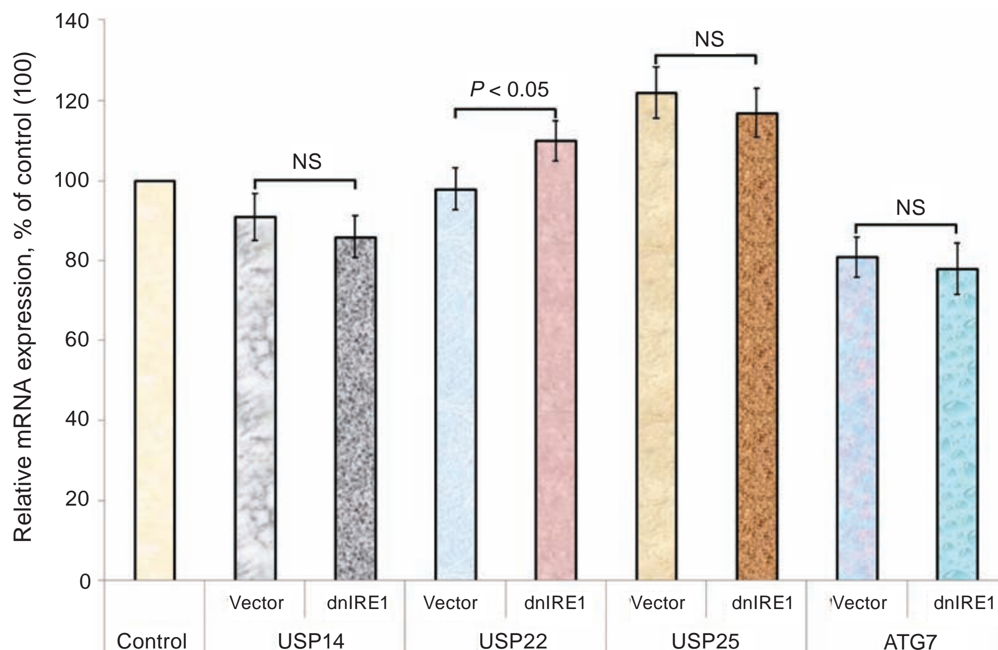

Fig. 4. Comparative effect of glutamine deprivation (16 h) on the expression level of USP14, USP22, USP25, and ATG7/GSA7 mRNA in two types of glioma cells: control cells transfected by vector (Vector) and cells with a deficiency of the signaling enzyme IRE1 (dnIRE1) measured by qPCR. Values of these mRNA expressions were normalized to β-actin mRNA expression and represented as percent of corresponding control (control for both cell types is accepted as 100%); mean ± SEM; n = 4

glutamine deprivation is more significant in glioma cells transfected by dnIRE1 as compared to control glioma cells. Moreover, the expression of *USP4* and *USP14* genes, which is resistant to glutamine deprivation in control glioma cells, is decreased after inhibition of IRE1 signaling enzyme function (Fig. 3 and 4). At the same time, IRE1 knockdown does not change significantly sensitivity of *USP10*, *USP25*, and *GSA7* gene expressions to glutamine deprivation condition in these glioma cells (Fig. 3 and 4).

In this study we have shown that the expression of *USP4*, *USP10*, *USP14*, and *USP22* genes are resistant to glutamine deprivation condition in control (transfected by empty vector) glioma cells because the exposure of cells to glutamine deprivation does not significantly change the level of their expression. In control glioma cells glutamine deprivation affects the expression of *USP1* and *USP25* genes only. It is possible that the resistance of most studied USPs to glutamine deprivation can be associated with important functions of these enzymes in metabolic processes, cell proliferation and surviving [7-9, 13, 14]. Inhibition of IRE1 signaling enzyme function in U87 glioma cells increased effect of glutamine deprivation on the expression of *USP1* gene and introduced sensitivity of *USP4* and *USP14* genes to this condition. A decreased level of USP1, USP4, and USP14 mRNA expression upon glutamine deprivation agrees well with functional role of these enzymes and suppression of glioma cell proliferation, because there is data that USP1 targeting impedes GBM growth and that USP4 and USP14 regulate cellular proliferation and apoptosis [16, 18, 23].

The ubiquitin activating enzyme E1-like protein/autophagy related 7 (GSA7/ATG7) is a multifunctional protein, which requires for mitophagy and contributes to regulation of mitochondrial quantity and quality by eliminating the mitochondria to a basal level to achieve cellular energy requirements and preventing excess ROS production as well as modulates TP53 activity to regulate cell cycle and survival during metabolic stress [15, 31-33, 45]. We have shown that glutamine deprivation suppresses ATG7 mRNA expression independent of IRE1 knockdown. These results agree well with functional role of this protein and suppression of glioma cell proliferation [15, 46].

Therefore, glutamine deprivation affected the expression level of most studied genes in gene specific manner in relation to the functional activity of IRE1 enzyme, a central mediator of endoplasmic reticulum stress, which controls cell proliferation and tumor growth.

ЕКСПРЕСІЯ ГЕНІВ, СПЕЦИФІЧНИХ ДО УБІКВІТИНУ ПЕПТИДАЗ ТА *ATG7*, У КЛІТИНАХ ГЛІОМИ ЛІНІЇ U87 ЗА ДЕФІЦИТУ ГЛУТАМІНУ

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Вивчали вплив дефіциту глутаміну на експресію генів, що кодують специфічні до убіквітину пептидази (USP) та ензим E1, який активує убіквітин (GSA7) і відомий ще як протеїн 7 (ATG7), у клітинах гліоми лінії U87 за умов пригнічення IRE1 (inositol requiring enzyme-1). Показано, що в контрольних (трансфікованих пустим вектором) клітинах гліоми за дефіциту глутаміну знижувалась експресія мРНК USP1 та ATG7 і підвищувалась мРНК USP25. У той самий час дефіцит глутаміну істотно не змінював експресію генів *USP4*, *USP10*, *USP14* та *USP22* в цих клітинах. Пригнічення функції сигнального ензиму IRE1 у клітинах гліоми лінії U87 посилювало ефект дефіциту глутаміну на експресію гена *USP1* та індукувало чутливість експресії генів *USP4* і *USP14* до цих умов. Таким чином, дефіцит глутаміну геноспецифічно змінює рівень експресії більшості досліджених генів залежно від функціональної активності ензиму IRE1, центрального медіатора стресу ендоплазматичного ретикулума, який контролює процеси проліферації та росту пухлин.

Ключові слова: експресія мРНК, USPs, ATG7, дефіцит глутаміну, пригнічення IRE1, клітини гліоми лінії U87.

ЭКСПРЕССИЯ ГЕНОВ, СПЕЦИФИЧЕСКИХ К УБИКВИТИНУ ПЕПТИДАЗ И *ATG7*, В КЛЕТКАХ ГЛИОМЫ ЛИНИИ U87 ПРИ ДЕФИЦИТЕ ГЛУТАМИНА

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Изучали влияние дефицита глутамина на экспрессию генов, кодирующих специфические к убиквитину пептидазы (USP) и энзим E1, активирующий убиквитин (GSA7) и известный еще как протеин 7 (ATG7), в клетках глиомы линии U87 при угнетении IRE1 (inositol requiring enzyme-1). Показано, что в контрольных (трансфицированных пустым вектором) клетках глиомы при дефиците глутамина снижалась экспрессия мРНК USP1 и ATG7 и увеличивалась мРНК USP25. В то же время, дефицит глутамина существенно не изменял экспрессию генов *USP4*, *USP10*, *USP14* и *USP22* в этих клетках. Угнетение функции сигнального энзима IRE1 в клетках глиомы линии U87 усиливало эффект дефицита глутамина на экспрессию гена *USP1* и индуцировало чувствительность экспрессии генов *USP4* и *USP14* к этим условиям. Таким образом, дефицит глутамина геноспецифически изменяет уровень экспрессии большинства исследованных генов в зависимости от функциональной активности энзима IRE1, центрального медиатора стресса эндоплазматического ретикулума, контролирующего процессы пролиферации и роста опухолей.

Ключевые слова: экспрессия мРНК, USPs, ATG7, дефицит глутамина, угнетение IRE1, клетки глиомы линии U87.

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