IRE-1α regulates expression of ubiquitin specific peptidases during hypoxic response in U87 glioma cells

Abstract: IRE-1α (inositol requiring enzyme-1α), the most evolutionarily conserved of the endoplasmic reticulum stress signaling pathways, is highly implicated in sustaining the proliferation of glioma cells and subsequent tumor growth, which is decreased by the inhibition of IRE-1α. To explore the IRE-1α mediated regulation of ubiquitin system in glioma cells, the expression of a subset of ubiquitin specific peptidases (USP) and of ubiquitin activating enzyme E1-like protein/autophagy related 7 (GSA7/ATG7) genes was studied, during hypoxic stress in wild type and U87 glioma cells with inhibited IRE-1α. Hypoxic treatment of wild type glioma cells leads to the up-regulation of USP25 and the concomitant down-regulation of USP1, USP10, USP14, and GSA7 genes. USP4 and USP22 genes expression did not significantly change with hypoxic treatment. Inhibition of IRE-1α activity led to up-regulation of USP1, USP4, USP10, USP22, and USP25, while USP14 and GSA7 genes were down-regulated. Therefore, IRE-1α activity modifies substrate-targeting specificity to proteasome during hypoxic stress, which in turn can affect cell survival. Inhibition of IRE-1α correlates directly with deregulation of ubiquitin specific peptidases and GSA7 in a fashion that ultimately slows tumor growth.

Keywords: IRE-1α, USP, GSA7, U87 glioma cells, hypoxia

1 Introduction

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 [1-3]. E3 ubiquitin ligases and deubiquitylases play an important role in cancer [2,4,5]. Our previous results demonstrated possible interaction/cross-talk between unfolded protein response signaling and ubiquitin system during adjustment to episodes of hypoxia during tumor development [6]. We previously demonstrated that inhibition of IRE-1α gene function in U87 glioma cells induces the expression of TP53 and USP7 genes, but decreases the expression of ubiquitin ligase MDM2 gene and that USP7, which deubiquitinates TP53 and MDM2, and possibly induces TP53-dependent cell growth repression and apoptosis. In this study, we investigated the effect of low IRE-1α (inositol requiring enzyme-1α) activity on the expression of a subset of ubiquitin specific peptidases (USP), and ubiquitin activating enzyme E1-like protein/autophagy related 7 (GSA7/ATG7) which have been previously demonstrated to be involved in cancer cells survival and progression.

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mediate nuclear import of the USP1/UAF1 complex [10,11].

USP4 is a deubiquitinating enzyme with key roles in the regulation of TP53 and TGFβ signaling. USP4 is also a positive regulator of the WNT/β-catenin signaling. Therefore, USP4 function can be important during tumorigenesis [12-14]. Deubiquitinating enzyme USP10 suppresses the proliferation and growth of cancer cells through stabilization of p53 protein [15]. Additional antitumorigenic effect of USP10 is achieved by antagonizing c-Myc activity through stabilization of a tumor suppressor SIRT6 [16]. In agreement, microRNA-191 mediated lower protein level of USP10 has been demonstrated to promote pancreatic cancer progression [15].

USP22 protease has been demonstrated to participate in regulation of the cell cycle progression in many cancer cell types [17,18]. USP22 removes ubiquitin from histones, thus regulating gene transcription [19]. Deubiquitinating enzyme USP25 is involved in ERAD (endoplasmic reticulum (ER)-associated degradation) of misfolded/anomalous proteins [20]. USP25 counteracts ubiquitination of ERAD substrates by the ubiquitin ligase HRD1, rescuing them from degradation by the proteasome [20]. USP25 is a novel TRiC interacting protein that catalyzes deubiquitination of the TRiC protein and stabilizes this chaperonin, thereby reducing accumulation of misfolded protein aggregates [21].

USP4 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 [22,23]. Inhibition of USP4 could be used as potential anticancer therapeutic strategy [24]. 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 [21].

The endoplasmic reticulum (ER) stress is responsible for enhanced cancer cell proliferation and IRE-1α knockdown by a dominant-negative construct of IRE-1α (dnIRE-1α) resulted in a significant anti-proliferative effect on glioma growth [26-29]. 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 [30-33]. The activation of ER stress is indispensable for tumor growth as it facilitates adaptation to stressful environmental conditions [34]. IRE-1α 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 [26,27,32]. Recently, we have shown that hypoxia increases glioma cell proliferation and that inhibition of IRE-1α signaling enzyme abolishes this effect of hypoxia [35].

Previously, we have shown that USP7 is regulated by IRE-1α signaling and hypoxia. Hypoxic regulation of USP7 was found to be independent from IRE-1α activity [6]. The precise mechanism of the exhibited by USP7 anti-proliferative effect is not clear. We hypothesized that anti-proliferative effect of USP7 is mediated through GSA7/ATG7. Indeed, cross-talk/ or final outcome of the activation of multiple stress signaling pathways, such as autophagy (ATG7)/ between ATG7 and UPR was implicated into regulation of cell proliferation, apoptosis and senescence [4,9,13,17,20,21]. In support of our hypothesis, transcriptome analysis of U87 glioma cells expressing the dominant-negative mutant of IRE-1α indicated possible involvement of ubiquitin specific peptidases such as USP1, USP4, USP10, USP14, USP22, and USP25, as well as GSA7/ATG7 protein in IRE-1α mediated control of cell proliferation [29].

Therefore, based on the amalgamation of evidence listed above, the aim of this study was to investigate the possible roles of genes encoding for ubiquitin specific peptidases and GSA7 as they apply to the suppression of glioma cell proliferation via inhibition of IRE-1α, the ER stress signaling enzyme, with hopes of elucidating its mechanistic part in the development and progression of certain cancers and the contribution to UPR.

2 Material and Methods

Reagents. The glioma cell line U87 (HTB-14) was obtained from ATCC (USA) and grown 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), penicillin (100 units/ml; Gibco, USA) and streptomycin (0.1 mg/ml; Gibco) at 37°C in a 5% CO2 incubator. Rabbit polyclonal anti-USP1 antibody (ab108104), anti-USP14 antibody (ab137432), and anti-Apg7 antibody (ab53255) were from Abcam (UK), anti-ACTB (β-actin) antibodies were from Santa Cruz Biotechnology (Santa Cruz, USA) and rabbit polyclonal anti-HIF-1α antibody was from Novus Biologicals, USA.
Cell lines. In this work we used sublines of U87 glioma cells, which were described previously [36]. One subline was obtained by selection of stable transfected clones overexpressing empty vector (pcDNA3.1), which was used for creation of dominant-negative constructs of IRE-1α (dnIRE-1α). This untreated subline of glioma cells was used as a control (control glioma cells) in the study of the effects of inhibition of IRE-1α, in regards to the expression of GSA7 and the UPS family members of interest (Table 1). The second sub-line was obtained by the selection of stable transfected clones overexpressing dnIRE-1α, having suppression of both the protein kinase and endoribonuclease activities of IRE-1α [29]. It has recently been shown that these cells have a low rate of proliferation and do not express spliced XBP1, a key transcription factor in IRE-1α signaling, after induction of endoplasmic reticulum stress by tunicamycin [29,36].

Induction of hypoxia. For creation of hypoxic conditions, the culture plates were placed in a special incubator with 3% oxygen, 5% carbon dioxide, and 92% nitrogen mix and exposed for 16 hrs.

Isolation of RNA. Total RNA was extracted from glioma cells using Trizol reagent according to the manufacturer’s protocol (Invitrogen, USA) as described previously [37]. RNA concentration and spectral characteristics were measured using NanoDrop Spectrophotometer ND1000 (PEQLAB, Biotechnologie GmbH).

Reverse transcription and qPCR analysis. QuantiTect Reverse Transcription Kit (QIAGEN, Germany) was used for cDNA synthesis according to the manufacturer’s protocol. Expression of USP1, USP4, USP10, USP14, USP22, USP25, GSA7, and ACTB mRNA was measured in U87 glioma cells by real-time quantitative polymerase chain reaction using Mx 3000P QPCR (Stratagene, USA) and Applied Biosystems 7500 (Applied Biosystems, USA), and Absolute qPCR SYBRGreen Mix (Thermo Fisher Scientific, ABgene House, UK). Polymerase chain reaction was performed in triplicate using specific primers, which were received from Sigma-Aldrich (Table 1).

An analysis of quantitative PCR was performed using a special Differential Expression Calculator. Quantitative expression of ubiquitin specific peptidase and ubiquitin activating enzyme E1-like protein genes was normalized to beta-actin and presented as a percent of the control (100%).

XBP1 splicing. For analysis of XBP1 splicing we amplified of cDNA using forward (5’–ggagttaagacagcgcctgg–3’) and reverse (5’–tcacctccctacgaatctc–3’) primers and HotStarTaq Master Mix Kit (“QIAGEN”, Germany) in “MasterCycler Personal” (“Eppendorf”, Germany). These primers correspond to nucleotide sequences 441 – 460 and 608 – 589 of XBP1 mRNA (GenBank accession number NM_005080). The size of amplified fragment is 168 bp.

| Gene symbol | Gene name | Primer’s sequence | Primers position | GenBank accession number |
|-------------|-----------|------------------|------------------|-------------------------|
| USP1        | Ubiquitin specific peptidase 1 | F: 5'-cagcatgatgcacaggaagt R: 5’-cccatctccttggagtt | 1347–1366 | NM_003368 |
| USP4        | Ubiquitin specific peptidase 4 (Proto-Oncogene) | F: 5’-cttatgtacagccccgttggt T: 5’-ggttattccacgcctcggtga | 185–204 389–370 | NM_003363 |
| USP10       | Ubiquitin specific peptidase 10 | F: 5’-cagaaactcagagcctagga R: 5’-agacctgtcctactttctca | 1216–1235 1434–1415 | NM_001272075 |
| USP14       | Ubiquitin specific peptidase 14 (IRNA-guanine transglycosylase) | F: 5’-ctgtctgtgcctgaactcaa T: 5’-ttcactctttctccggcaact | 586–605 789–770 | NM_005151 |
| USP22       | Ubiquitin specific peptidase 22 (ubiquitin thioesterase 22) | F: 5’-ggagaaatctccgaagcctgg R: 5’-gagaagtcgcaagactgg | 575–594 816–797 | NM_0015276 |
| USP25       | Ubiquitin specific peptidase 25 | F: 5’-ggccataaacgagaaaggaa T: 5’-agctttgccctcgtgaacta | 1982–2001 2178–2159 | NM_001283041 |
| GSA7        | Ubiquitin activating enzyme E1-like protein (Autophagy Related 7) | F: 5’-tgagctctcacaactccttg T: 5’-agactctcagcctgggtt | 1956–1975 2200–2181 | NM_006395 |
| ACTB        | beta-actin | F: 5’-ggacctcgcagaagatgg T: 5’-acactgtgcttgctgacag | 747–766 980–961 | NM_001101 |
for non-spliced variant and 142 bp for alternative splice variant (XBP1s).

**Western blot analysis.** Cell extracts were prepared as described Andrews and Faller [38] with minor modifications. Cells were incubated at 37°C before harvesting under normoxic or hypoxic conditions. Subconfluent cells were lysed at 4°C with 10 mM HEPES–KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 5 mM NaF, protease inhibitors (P8340; Sigma), and 0.5% Nonident NP-40. The cells were allowed to sit in ice for 10 min and shortly resuspended. The cytosolic fraction was obtained by centrifugation for 1 min at 4000 rpm. The pellet was resuspended in cold nuclear extract isolation buffer (20 mM HEPES-KOH pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 2 mM EDTA, 420 mM NaCl, 0.5 mM dithiothreitol, and protease inhibitors P8340 and incubated on ice for 20 min. The nuclear extract was removed by centrifugation for 1 min at 13,000 rpm, and supernatant fraction was stored at -70°C. Western blot analysis was mainly performed as described previously [39]. After migration on SDS-PAGE, proteins were transferred to a nitrocellulose membrane by a semi-dry blotting system. The membrane was blocked to reduce nonspecific binding by incubation in blocking buffer (5% milk, 10 mM Tris-HCl, pH 8.0, 1250 mM NaCl and 0.05% Tween 20) and probed using antibodies against USB1, USB14, GSA7, HIF1α, and β-actin proteins. Primary antibodies were revealed with a secondary peroxidase-conjugated anti-rabbit, anti-goat or anti-mouse IgG) with a dilution of 1:2,000 and detected by the enhanced chemiluminescence’s (ECL) Western blotting detection reagents (Amersham Biosciences). Actin was used for normalization.

**Small interfering RNA knockdown.** For small interfering RNA (siRNA) knockdown experiments, U87 cells were plated at a density of 10⁵ cells per well in six-well plates. Small interfering RNA against human IRE-1α (sc-40705) and non-targeting siRNA were from Santa Cruz Biotechnology. Transfection was performed for 48 h using lipofectamine RNAiMAX (Invitrogen) in accordance with the manufacturer’s protocol, with siRNA at a final concentration of 100 nM.

**Statistical analysis** was performed using OriginPro 7.5 software. All values were expressed as mean ± SEM from triplicate measurements performed in 4 independent experiments. Comparison of two means was performed by the use of two-tailed Student’s t-test as described previously [40]. P < 0.05 was considered significant in all cases.

**Ethical approval:** The conducted research is not related to either human or animals use.

## 3 Results

### 3.1 Inhibition of IRE-1α affects the expression of ubiquitin specific peptidase and ubiquitin activating enzyme E1-like protein genes in U87 glioma cells

Expression of ubiquitin specific peptidase genes was studied in U87 glioma cell using quantitative PCR. In order to test the effect of IRE-1α inhibition on expression level of the ubiquitin specific peptidase as well as ubiquitin activating enzyme E1-like protein of interest in relation to the control of cell proliferation and apoptosis, we used U87 glioma cell sub-line constitutively expressing the dominant-negative mutant of IRE-1α, dnIRE-1α, lacking both endoribonuclease and kinase activities [44,45].

Figure 1 and Table 2 demonstrate that inhibition of IRE-1α gene function in U87 glioma cells by dnIRE-1 leads to the up-regulation of USP1 and USP4 mRNA (+70% and +59%, respectively) as well as USP10 mRNA (+31%). Moreover, the expression level of USP22 and USP25 genes is also increased (+34% and +73%, respectively). At the same time, the expression of USP14 gene, which encoded enzyme with both ubiquitin specific peptidase and tRNA-guanine transglycosylase activities, is down-regulated (-32%) in glioma cells with inhibited IRE-1α signaling enzyme activity (Figure 1 and Table 2).

Thereafter, we tested the impact of IRE-1α inhibition on the modulation of expression levels of ubiquitin activating enzyme E1-like protein (GSA7), which also known as ATG7 (autophagy related 7). As shown in Figure 1 and Table 2, inhibition of IRE-1 by dnIRE-1α has a suppressive effect on the expression of GSA7 gene (-49%).

We have also shown that suppression of IRE-1α mRNA by specific to IRE-1α siRNA in U87 glioma cells leads to slightly smaller but similar changes in the expression of USP1, USP4, USP10, USP14, USP22, USP25, and GSA7 genes as was shown in experiments with dnIRE-1α (Table 2). Furthermore, as shown in Figure 3, the level of USP1 protein in glioma cells with a deficiency of IRE-1α measured by Western blot analysis is increased, but USP14 and GSA7/ATG7 protein levels are down-regulated at this experimental condition. Therefore, these changes in USP1, USP14, and GSA7/ATG7 protein quantities are congruent with the levels of respective mRNAs.

In conclusion, we have demonstrated that the IRE-1α signaling enzyme participates in the fine-tuning of mRNA levels of a subset of the ubiquitin specific peptidase as well as ubiquitin activating enzyme E1-like protein genes, which are important for control of cell proliferation and apoptosis.
isoenzymes of ubiquitin specific peptidase as well as ubiquitin activating enzyme E1-like protein, which can mediate both the protein ubiquitination and cellular senescence. As shown in Figure 2A and Table 2, hypoxic exposure of control glioma cells (transfected by empty vector pcDNA3.1) leads to suppression of USP1 mRNA expression (-36%). In glioma cells containing dnIRE-1α, the effect of hypoxia on this mRNA expression was similar (-33%), indicating down-regulation of this gene expression by hypoxia in IRE-1α-independent manner.

Table 2. Summary of changes in the expression of ubiquitin specific peptidase genes upon inhibition of IRE-1α by dnIRE-1α and specific to IRE-1α siRNA

| Tested condition                   | dnIRE-1α-cells versus control U87 glioma cells | IRE-1α siRNA-cells versus control-siRNA U87 glioma cells |
|-----------------------------------|-----------------------------------------------|---------------------------------------------------------|
| Gene                              |                                               |                                                         |
| USP1                              | Up 70%                                        | Up 47%                                                  |
| USP4                              | Up 59%                                        | Up 42%                                                  |
| USP10                             | Up 31%                                        | Up 18%                                                  |
| USP14                             | Down 32%                                      | Down 22%                                                 |
| USP22                             | Up 34%                                        | Up 15%                                                  |
| USP25                             | Up 73%                                        | Up 42%                                                  |
| GSA7/ATG7                         | Down 49%                                      | Down 38%                                                 |

3.2 Hypoxia regulates expression of ubiquitin specific peptidase and ubiquitin activating enzyme E1-like protein genes in U87 glioma cells in IRE-1α-dependent as well as IRE-1α-independent manner

To determine if hypoxia regulates the genes of interest through the IRE-1α branch of ER stress response, we investigated the effect of hypoxic conditions (3% oxygen – 16 hrs) on the mRNA expression levels of different
(Figure 2A and Table 2).

Additionally, we found that expression of gene encoding for USP4 does not change significantly upon hypoxic treatment in control glioma cells as well as in cells with inhibited IRE-1α signaling enzyme activity, indicating resistance of this gene expression to hypoxia independently from IRE-1α inhibition (Figure 2B and Table 2). As shown in Figure 2C and Table 2, hypoxia significantly down-regulates the expression of USP10 mRNA both in control glioma cells (34%) and cells without activity of signaling enzyme IRE-1α (-29%), indicating that inhibition of the IRE-1α slightly decreases the effect of hypoxia on USP10 gene expression. Hypoxic regulation of the USP14 gene is similar to that of the USP1 gene: exposure of both control glioma cells and cells with inhibited activity of IRE-1α enzyme leads to down-regulation of USP14 gene expression (-21% and -25%, respectively), indicating that hypoxic down-regulation of this gene expression is independent from IRE-1α signaling (Figure 2D and Table 2).

At the same time, hypoxia does not significantly change the expression of USP22 mRNA in control glioma cells, but inhibition of IRE-1α signaling introduces small but statistically significant down-regulation of this gene expression by hypoxia (Figure 2E and Table 2). It was also shown that USP25 mRNA expression is up-regulated in both types of used glioma cells upon the induction of hypoxia, but inhibition of IRE-1α does not significantly change the expression of USP22 mRNA both in normoxic and hypoxic conditions (Figure 6).

As shown in Figure 2F and Table 2, GSA7/ATG7 gene expression is significantly down-regulated by hypoxia both in control glioma cells (35%) and cells overexpressing dnIRE-1α (-49%), indicating that IRE-1α participates in hypoxic regulation of this gene expression because inhibition of the IRE-1α enhances the effect of hypoxia on GSA7 gene expression.

As shown in Figure 4, inhibition of IRE-1α signaling does not significantly change the effect of hypoxia on the expression of USP1, USP4, USP14, and USP25 genes in glioma cells, but modifies hypoxic regulation of USP10 and GSA7 genes expression in diverse ways and introduces sensitivity to hypoxia the expression of USP22 gene.

We also studied the effect of hypoxia on the levels of USP1, USP14, and GSA7/ATG7 proteins in control glioma cells and cells with a deficiency of IRE-1α by Western blot analysis. As shown in Figure 3, hypoxia decreases the levels of all these proteins both in control glioma cells and cells with IRE-1α deficiency and observed changes in USP1, USP14, and GSA7/ATG7 protein levels upon hypoxia are congruent with hypoxia induced changes in respective mRNA levels.

Next, we studied effect of hypoxia on HIF-1α protein level in control glioma cells and cells with a deficiency of IRE-1α by Western blot. Although, the basal level of HIF1 is down-regulated in cells with a deficiency of IRE-1α, hypoxia strongly increases the level of this protein both in control glioma cells and dnIRE-1α cells (Figure 5). The splice variant of transcription factor XBPI was not detectable neither in control glioma cells nor not in cells with a deficiency of IRE-1α, both in normoxic and hypoxic conditions (Figure 6).

Thus, investigation the effect of hypoxia on the expression of ubiquitin specific peptidase as well as ubiquitin activating enzyme E1-like protein genes demonstrates that hypoxia affects the expression of most studied genes. Furthermore, inhibition of IRE-1α modifies the effect of hypoxia on USP10, USP22, and GSA7 gene expressions, indicating that IRE-1, signaling enzyme of ER stress, participates in hypoxic regulation of these genes expression.

4 Discussion

This study has demonstrated that inhibition of both the endoribonuclease and kinase activities of the IRE-1α signaling enzyme in U87 glioma cells causes an increase in the levels of most ubiquitin specific peptidase genes (Table 2), which are known as deubiquitinating enzymes and participate in the control of cell proliferation, apoptosis, and metastasis. The changes observed in the expression of USP1, USP4, USP10, USP14, USP22, and USP25 genes in glioma cells upon inhibition of IRE-1α, signaling enzyme of ER stress, associate with slower cell proliferation in cells harboring dnIRE-1α, attesting to the fact that endoplasmic reticulum stress is a necessary component of malignant tumor growth and cell survival [29,33,41]. These results are confirmed using alternative complementary approaches, such as siRNA specific to IRE-1α. In particular, suppression of IRE-1α mRNA by specific to IRE-1α siRNA in U87 glioma cells leads to similar changes in the expression of USP1, USP4, USP10, USP14, USP22, USP25, and GSA7 genes as was shown in experiments with dnIRE-1α (Table 2). However, effect of IRE-1α siRNA was slightly smaller versus effect of dnIRE-1α. It is possible that stronger effect of dnIRE-1α on
Figure 2. Effect of hypoxia (3% oxygen – 16h) on the expression level of USP1 (A), USP4 (B), USP10 (C), USP14 (D), USP22 (E), USP25 (F), and GSA7/ATG7 (G) mRNA (by qPCR) in glioma cells with a deficiency of IRE-1α (dnIRE-1α). mRNA expressions values were normalized to beta-actin mRNA expression and presented as percent of control 1 (control glioma cells transfected by vector, 100%); mean ± SEM; n = 4.
Figure 2. Effect of hypoxia (3% oxygen – 16h) on the expression level of USP1 (A), USP4 (B), USP10 (C), USP14 (D), USP22 (E), USP25 (F), and GSA7/ATG7 (G) mRNA (by qPCR) in glioma cells with a deficiency of IRE-1α (dnIRE-1α). mRNA expressions values were normalized to beta-actin mRNA expression and presented as percent of control 1 (control glioma cells transfected by vector, 100%); mean ± SEM; n = 4.
Figure 3. Effect of hypoxia (3% oxygen – 16h) on protein level of USP1, USP14, and GSA7/ATG7 in glioma cells with a deficiency of IRE-1α (dnIRE-1α) measured by Western blot. ACTB protein level was used to ensure equal loading of the samples.

Figure 4. Comparative effect of hypoxia (3% oxygen – 16h) on the expression level of USP1, USP4, USP10, USP14, USP22, USP25, and GSA7 mRNA cells with a deficiency of IRE-1α (dnIRE-1α). mRNA expressions values were normalized to beta-actin mRNA expression and presented as percent of corresponding controls (vector as well as dnIRE-1α), 100%; mean ± SEM; n = 4.

Figure 5. Effect of hypoxia (3% oxygen – 16h) on protein level of HIF-1α in control glioma cells (Vector) and cells with a deficiency of IRE-1α (dnIRE-1α) measured by Western blot. ACTB protein level was used to ensure equal loading of the samples.
the expression of USP genes in glioma cells is explained by stronger inhibition of IRE-1α function (more than 95%) [36]. Furthermore, the mRNA expression level of USP1, USP14, and GSA7/ATG7 genes in glioma cells with a deficiency of IRE-1α is supported by the protein Western blot analysis data. The changes in USP1, USP14, and GSA7/ATG7 mRNA levels are congruent with levels of respective proteins.

Thus, increased expression level of USP1 may contribute to the suppression of cell proliferation and glioma growth because there is data [8] that decreased USP1 level in lung cancer cells may play an important role in lung cancer progression through a novel USP1-PHLP1-Akt signaling axis. Another ubiquitin specific peptidase, USP4, has variable functions. The expression of this enzyme is increased in glioma cells when IRE-1α is inhibited (Table 2) and increased level of USP4 can be responsible for TNFα-induced apoptosis via deubiquitination of RIP1 (receptor-interacting serine-threonine kinase 1) [42]. Thus, the changes observed in the USP4 gene correlate with slower cell proliferation in cells harboring dnIRE-1α. At the same time, there is also data that USP4 overexpression may contribute to progression of certain tumors [9,13].

We have also demonstrated that expression level of USP10 is also up-regulated in glioma cells when IRE-1α function is inhibited (Table 2). There is data that USP10 protein suppresses the proliferation and growth of cancer cells through stabilizing p53 protein and up-regulation of its activity [15,43], antagonizes c-Myc transcriptional activation through SIRT6 stabilization to suppress tumor formation [16] and that microRNA-191 promotes pancreatic cancer progression by targeting USP10 [15]. Thus, our results that inhibition of IRE-1α via overexpression of dnIRE-1 up-regulates the expression of the USP10 gene agree well with functional role of this deubiquitinating enzyme [15,16,43]. Moreover, these results fall in line with our previous data that indicate the inhibition of IRE-1α up-regulates TP53 gene expression [37]. We have shown that USP14 gene expression is significantly down-regulated in glioma cells upon inhibition of IRE-1 signaling enzyme (Table 2) and, thus, down-regulation of this gene expression should contribute to suppression of glioma cell proliferation and tumor growth because USP14 promotes tumor progression through regulation of cellular proliferation and apoptosis of cancer cells, at least in hepatocellular carcinoma [22].

It is interesting to note that inhibition of IRE-1α via overexpression of dnIRE-1α up-regulates the expression of the USP22 gene in glioma cells. These results agree with data that indicate that USP22 inhibits the invasion capacity of colon cancer cells by suppression of the signal transducer and activator of transcription 3/matrix metalloproteinase 9 (STAT3/MMP9) pathway [19]. At the same time, data is available that USP22 induces epithelial-mesenchymal transition, which play an important role in migration and invasion of the tumor cells [44]. However, it is known that this deubiquitinating enzyme is involved in tumor progression as regulator of the cell cycle and can remove ubiquitinating enzyme from histones, thus regulating gene transcription [17,18]. Moreover, p38 mitogen-activated protein kinase, which plays an important role in stress related transcription and cell cycle regulation, inhibits USP22 transcription in HeLa cells [45].

In this study, we have shown that inhibition of IRE-1 leads to up-regulation of USP25 gene expression in glioma cells, which have decreased proliferation rate but enhanced invasion and metastasis [6]. Our results are consistent with recent data that shows [3] that miRNA-200c, which is involved in carcinogenesis and exerts tumor-suppressive effects for human non-small cell lung cancer, inhibits invasion and metastasis of these cells through the suppression of USP25 expression. At the same time, the expression of this gene is also necessary for ER-associated degradation of unfolded proteins: USP25 counteracts ubiquitination of ERAD substrates by the ubiquitin ligase HRD1, rescuing them from degradation by the proteasome [20]. It is possible that up-regulation of USP25 gene expression upon inhibition of IRE-1 reflects suppressed ER stress signaling in glioma cells.

We have also shown that inhibition of IRE-1 signaling enzyme leads to down-regulation of ubiquitin activating enzyme E1-like protein (GSA7) gene expression in glioma cells (Table 2). It is possible that down-regulation of GSA7 gene expression may contribute to suppression
of glioma cell proliferation and tumor growth because GSA7 promotes tumor progression through regulation of cellular proliferation and apoptosis of cancer cells, at least in hepatocellular carcinoma [22]. Moreover, GSA7 is a multifunctional protein, which also controls cell proliferation inhibition and apoptosis via cellular senescence [25]. Thus, our results, which demonstrate that inhibition of IRE-1 down-regulates the expression of the GSA7 gene, agree well with data that suppression of GSA7 in the intestinal epithelium decreases tumor growth [46]. Consequently, inhibition of IRE-1α signaling enzyme leads to up-regulation of most enzymes responsible for deubiquitination, but down-regulation of the ubiquitin activating enzyme E1-like protein (GSA7).

In this work, we have studied the hypoxic regulation of a subset of ubiquitin specific peptidase genes to clarify their possible relationship with IRE-1α-mediated endoplasmic reticulum stress. We have shown that hypoxia affects the expression of most USP genes (Figure 2). Furthermore, inhibition of IRE-1α modifies the effect of hypoxia on the expression of USP10, USP22, and GSA7 genes, indicating that IRE-1α, signaling enzyme of ER stress, participates in hypoxic regulation of these genes expression (Figure 4). Thus, hypoxia, as an important factor in tumor growth, suppresses the expression of USP1 and USP10 genes in a way opposite to that of IRE-1α inhibition. However, effect of hypoxia on the expression of USP1 gene does not depend from inhibition of IRE-1α. At the same time, the sensitivity of USP10 gene expression to hypoxia is decreased in cells overexpressed dnIRE-1α. We have also shown that the expression of USP4 and USP22 genes in control glioma cells is resistant to hypoxia, but inhibition of IRE-1 leads to down-regulation of USP22 gene expression upon hypoxia. Moreover, inhibition of IRE-1α enhances suppressive effect of hypoxia on GSA7 gene expression but this hypoxic regulation is additive to that of IRE-1α inhibition.

Thus, hypoxia affects the expression of most studied genes in a gene-specific manner and the hypoxic regulation of some genes depends on IRE-1α function. Consequently, hypoxic regulation of some studied genes has pro-proliferative qualities. Recently, we showed that inhibition of IRE-1α strongly suppresses the expression of the EPAS1 gene in glioma cells, which controls a specific group of hypoxia-responsive genes [47]. Consequently, functional significance of hypoxic regulation of studied genes is not clear yet because there are several regulatory mechanisms of gene expression, which determine a gene-specific character of this regulation as well as its responsibility will regard to IRE-1 signaling [6,30,37,48]. Biological significance of changes in diverse ubiquitin specific peptidases on cell proliferation and protein degradation is possibly related to USP1-PHLPP1-Akt signaling axis, TNFα-induced apoptosis via deubiquitination of RIP1 (USP4), regulation of p53 protein stability and activity, control of STAT3/MMP9 pathway, regulation of the cell cycle and stress related transcription [8,15,17,18,42-45].

In conclusion, our results demonstrate that the majority of the genes studied are both responsive to endoplasmic reticulum stress and hypoxia and potentially contribute to regulation of apoptosis and cell proliferation (Figure 7), but the mechanisms and functional significance of activation or suppression of their expression through IRE-1α inhibition as well as hypoxia are different and warrant further investigation. Thus, the changes observed in the studied genes expression partially agree with slower proliferation rate of glioma cells harboring dnIRE-1, attesting to the fact that targeting the unfolded protein response is viable, perspective approach in the development of cancer therapeutics [27,36,48,49].

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Figure 7. Schematic representation of key results. Green arrow – up-regulation of gene expression; red arrow – down-regulation of gene expression; NS – no significant changes.

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