

## EFFECT OF HYPOXIA ON THE EXPRESSION OF GENES THAT ENCODE SOME IGFBP AND CCN PROTEINS IN U87 GLIOMA CELLS DEPENDS ON IRE1 SIGNALING

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*We have studied hypoxic regulation of the expression of different insulin-like growth factor binding protein genes in U87 glioma cells in relation to inhibition of IRE1 (inositol requiring enzyme-1), a central mediator of endoplasmic reticulum stress, which controls cell proliferation and tumor growth. We have demonstrated that hypoxia leads to up-regulation of the expression of IGFBP6, IGFBP7, IGFBP10/CYR61, WISPI, and WISP2 genes and down-regulation – of IGFBP9/NOV gene at the mRNA level in control glioma cells, being more significant changes for IGFBP10/CYR61 and WISP2 genes. At the same time, inhibition of IRE1 modifies the effect of hypoxia on the expression of all studied genes: eliminates sensitivity to hypoxia the expression of IGFBP7 and IGFBP9/NOV genes, suppresses effect of hypoxia on IGFBP6, IGFBP10/CYR61, and WISP2 genes, and slightly enhances hypoxic regulation of WISPI gene expression in glioma cells. We have also demonstrated that the expression of all studied genes in glioma cells is regulated by IRE1 signaling enzyme upon normoxic condition, because inhibition of IRE1 significantly up-regulates IGFBP7, IGFBP10/CYR61, WISPI, and WISP2 genes and down-regulates IGFBP6 and IGFBP9/NOV genes as compared to control glioma cells. The present study demonstrates that hypoxia, which contributes to tumor growth, affects all studied IGFBP and WISP gene expressions and that inhibition of IRE1 preferentially abolishes or suppresses the hypoxic regulation of these gene expressions and thus possibly contributes to slower glioma growth. Moreover, inhibition of IRE1, which correlates with suppression of cell proliferation and glioma growth, is down-regulated expression of pro-proliferative IGFBP genes, attesting to the fact that endoplasmic reticulum stress is a necessary component of malignant tumor growth.*

**Key words:** mRNA expression, endoplasmic reticulum stress, IGFBP6, IGFBP7, IGFBP9/NOV, IGFBP10/CYR61, WISPI, WISP2, IRE1 inhibition, hypoxia, glioma cells.

Insulin-like growth factor binding proteins (IGFBPs) contain insulin-like growth factor (IGF) binding domain and play an important role in the regulation of numerous metabolic and proliferative processes mainly through interaction with IGF1 and IGF2, their cell surface receptors as well as insulin receptor, alter the half-life of the IGFs, modifying their biological activity. It is well known that insulin-like growth factors and the signal transduction networks play important roles in tumorigenesis and metastasis as well as in metabolic diseases [1, 2]. IGFBPs participate in endoplasmic reticulum stress, which is an important factor of tumor growth, insulin resistance, and obesity [3-5]. It is interesting to note that there is the cross talk between IGF and insulin receptor signaling pathways at the receptor

level or at downstream signaling level. This cross talk significantly changed a variety of cancers as a result of insulin receptor isoform. A formation of hybrid receptor isoforms between receptors for IGF1 and insulin, which are sensitive to the stimulation of all three IGF axis ligands, as well as hybrid receptors of IGF1/insulin receptor with other tyrosine kinase potentiate the transformation of cells, tumorigenesis, and tumor neovascularization [6].

The IGFBPs have different affinity for insulin-like growth factors. They bind and regulate the availability of both insulin-like growth factors and inhibit or stimulate the growth promoting effects of the IGFs through IGF/INS receptors and through other signaling pathways and regulate cell proliferation and survival as well as angiogenesis and cancer

cell migration. Moreover, both negative and positive correlations between levels of IGF-1/IGF-1-R and clinical outcomes in head and neck cancer have been reported [10]. IGFBPs are now understood to have many actions beyond their endocrine role in IGF transport [6]. IGFBPs also function in the cells and extracellular matrix to regulate cell proliferation and survival and are involved in tumor development, progression and resistance to treatment. Because they interact with many proteins, in addition to their canonical ligands (IGF1 and IGF2), they play an important role in the regulation of various processes including transcription [6, 7]. Thus, IGFBP6 preferentially binds to IGF2 and also has IGF-independent effects, including inhibition of angiogenesis and promotion of cancer cell migration [8]. Moreover, IGFBP6 regulate cell proliferation and apoptosis: down-regulation of this gene expression leads to inhibition of cell proliferation and to increased apoptotic cell death. It is interesting to note that apoptosis can be also regulated through IGF1 receptor; however, there is data that IGFBP6 can also suppress the cellular proliferation [9].

IGFBP7, which is also known as insulin-like growth factor binding protein-related protein 1 (IGFBPRP1), belongs to the IGFBP family whose members have a conserved structural homology. It has a low affinity for IGFs and a high affinity for insulin, suggesting that IGFBP7 may have a biological function distinct from other members of the IGFBP family. IGFBP7 has diverse biological functions, regulating cell proliferation, apoptosis and senescence; it may also play a key role in vascular biology [6]. It elicits its biological effects by both insulin/IGF-dependent and -independent mechanisms. It has also been shown that IGFBP7 can bind to unoccupied IGF1 receptor, block its activation by insulin-like growth factors and suppress downstream signaling, thereby inhibiting protein synthesis, cell growth, and survival [10]. Increasing evidence suggests that IGFBP7 acts as a tumor suppressor [6].

The CNN family of regulatory protein represents IGFBP9/CCN3/NOV (nephroblastoma overexpressed) and IGFBP10/CCN1/CYR61 (cysteine-rich angiogenic inducer 61), which play a role in cell growth regulation and are involved in angiogenesis, inflammation and matrix remodeling [11]. The WNT1 inducible signaling pathway (WISP) proteins WISP1/CCN4 and WISP2/CCN5 are downstream in the WNT1 signaling pathway and are also relevant to malignant transformation, cancer cell surviving,

invasion and motility [12-14]. Furthermore, the elevated levels of WISP1/CCN4 and CYR61/CCN1 in primary breast cancers were associated with more advanced features [15].

The endoplasmic reticulum is a dynamic intracellular structure with exquisite sensitivity to alterations in homeostasis and provides stringent quality control systems to ensure that only correctly folded proteins transit to the Golgi [16]. Accumulation of the unfolded or misfolded proteins induces a set of complex intracellular signaling events in the endoplasmic reticulum and restores homeostasis or activates cell death [17, 18]. Multiple studies have clarified the link between cancer and endoplasmic reticulum stress, which controls different processes, including cell proliferation and surviving, as well as circadian rhythms [19-21]. IRE1 is a central mediator of the unfolded protein response and an important component of tumor growth, and its blockade leads to a suppression of tumor growth through down-regulation of the angiogenesis and proliferation processes [22, 23]. It contributes to the expression profile of many regulatory genes resulting in proliferation, angiogenesis, and apoptosis [3, 17, 24]. IRE1 has two enzymatic activities: kinase, which is responsible for autophosphorylation and some gene expressions, and endoribonuclease, which is responsible for degradation of a specific subset of mRNA and alternative splicing of the XBP1 (X-box binding protein 1) transcription factor mRNA for control of the expression of unfolded protein response-specific genes [25, 26].

The endoplasmic reticulum stress response-signaling pathway is tightly associated with hypoxia and linked to the neovascularization, tumor growth and cell death processes as well as to suppression of insulin receptor signaling through activation of c-Jun N-terminal kinase (JNK) and subsequent serine phosphorylation of IRS1 [27, 28]. The endoplasmic reticulum has an essential position as a signal integrator in the cell and is instrumental in the different phases of tumor progression because the signaling pathways elicited by endoplasmic reticulum stress sensors have connections with metabolic pathways and with other plasma membrane receptor signaling networks [17, 19].

Ablation of IRE1 function has been shown to result in a significant anti-proliferative effect in glioma growth through down-regulation of prevalent pro-angiogenic factors and up-regulation of anti-angiogenic genes as well as by modification of hypoxic

regulation of these genes [24, 29]. However, the executive mechanisms of the exhibited anti-proliferative effects of IRE1 inhibition are not yet known. It is possible that this anti-proliferative effect is also realized through mediation by IFGBPs and its receptors signaling, which are integrated into the unfolded protein response signaling pathways, to regulate cell proliferation and surviving [8, 10, 11]. Possible involvement of insulin-like growth factor binding proteins and CCN family proteins such as IGFBP6, IGFBP7, IGFBP9/NOV, IGFBP10/CYR61, WISP1, and WISP2 was made evidently pertinent through transcriptomic analysis of U87 glioma cells expressing the dominant-negative mutant of IRE1 [23].

The main goal of this study was to investigate the role of expression of genes encoding the insulin-like growth binding proteins with different affinity for IGF proteins (IGFBP6, IGFBP7, IGFBP9/NOV/CCN3, IGFBP10/CYR61/CCN1, WISP1/CCN4, and WISP2/CCN5) in U87 glioma cell line and its subline with IRE1 loss of function upon hypoxia for evaluation of its possible significance in the control of tumor growth through IRE1 mediated endoplasmic reticulum stress signaling.

### Materials and Methods

*Cell Lines and Culture Conditions.* 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% CO<sub>2</sub> incubator.

In this work we used sublines of U87 glioma cells, which were described previously [23, 24]. One subline was obtained by selection of stable transfected clones with overexpression of vector pcDNA3.1, which was used for creation of dn-IRE1. This untreated subline of glioma cells (control glioma cells) was used as control 1 in the study of the effect of glucose deprivation on the expression level of different *IGFBP* and related genes. The second subline was obtained by selection of stable transfected clone with overexpression of IRE1 dominant/negative construct (dn-IRE1) and has suppressed both protein kinase and endoribonuclease activities of this signaling enzyme (clone 1C5) [23]. The expression level of studied genes in these cells was compared with cells, transfected by vector (control 1), but this

subline was also used as control 2 for investigation of the effect of glucose deprivation on gene expressions under blockade of both enzymatic activities of IRE1. The efficiency of IRE1 suppression in this glioma cell subline was estimated previously [23, 24] by determining the expression level of the XBP1 alternative splice variant, a key transcription factor in the IRE1 signaling, and the level of the phosphorylated isoform IRE1, using cells treated by tunicamycin (0.01 mg/ml during 2 h). The both sublines of glioma cells used in this study are grown with the addition of geneticin (G418), while these cells carried empty vector pcDNA3.1 or dn-IRE1 construct.

For hypoxia culture plates were exposed in a special chamber with 3% oxygen, 92% nitrogen, and 5% carbon dioxide levels for 16 h.

*RNA isolation.* Total RNA was extracted from glioma cells using Trizol reagent according to manufacturer protocols (Invitrogen, USA) as described previously [24]. 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.

*Reverse transcription and quantitative PCR analysis.* QuantiTect Reverse Transcription Kit (QIAGEN, Germany) was used for cDNA synthesis according to manufacturer protocol. The expression level of insulin-like growth factor binding proteins (IGFBP6, IGFBP7, IGFBP9/NOV, and IGFBP10/CYR61) and WNT1 inducible signaling pathway proteins (WISP1 and WISP2) mRNAs as well as ACTB mRNA were measured in U87 glioma cells by real-time quantitative polymerase chain reaction using Mx 3000P QPCR (Stratagene, 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, USA.

For amplification of *IGFBP6* cDNA we used forward (5'-GCTGTTGCAGAGGAGAATCC-3') and reverse (5'-GGTAGAAGCCTCGATGGTCA-3') primers. The nucleotide sequences of these primers correspond to sequences 397–416 and 655–636 of human *IGFBP6* cDNA (GenBank accession number NM\_002178). The size of amplified fragment is 259 bp. The amplification of *IGFBP7* cDNA for real time RCR analysis was performed using two oligonucleotide primers: forward – 5'-AGCTGTGAGGT-CATCGGAAT-3' and reverse – 5'-TATAGCTCG-

GCACCTTCACC–3'. The nucleotide sequences of these primers correspond to sequences 572–591 and 882–863 of human *IGFBP7* cDNA (GenBank accession number NM\_001553). The size of amplified fragment is 311 bp.

The amplification of *IGFBP9/NOV/CCN3* cDNA for real time RCR analysis was performed using two oligonucleotide primers: forward – 5'–GCGAAGAAAGTCTCGTTTGG–3' and reverse – 5'–ACACCAGACAGCATGAGCAG–3'. The nucleotide sequences of these primers correspond to sequences 176–195 and 420–401 of human *IGFBP9* cDNA (GenBank accession number NM\_002514). The size of amplified fragment is 245 bp. For amplification of *IGFBP10/CYR61/CCN1* cDNA we used forward (5'–CTCCCTGTTTTTGGAAATGGA–3') and reverse (5'–TGGTCTTGCTGCATTTCTTG–3') primers. The nucleotide sequences of these primers correspond to sequences 852–871 and 1092–1073 of human *IGFBP10* cDNA (GenBank accession number NM\_001554). The size of amplified fragment is 241 bp.

The amplification of WNT1 inducible signaling pathway protein 1 (*WISP1*), also known as *CCN4*, cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5'–GACTTTACCCCAGCTCCACT–3' and reverse – 5'–GTAGTCACAGTAGAGGCCCC–3'. The nucleotide sequences of these primers correspond to sequences 203–222 and 415–396 of human *WISP1* cDNA (GenBank accession number NM\_003882). The size of amplified fragment is 213 bp. For amplification of *WISP2/CCN5* cDNA we used forward (5'–CTGTATCGGGAAGGGGAGAC–3' and reverse (5'–GGGAAGAGACAAGGCCAGAA–3') primers. The nucleotide sequences of these primers correspond to sequences 463–482 and 709–690 of human *WISP2* cDNA (GenBank accession number NM\_003881). The size of amplified fragment is 247 bp.

The amplification of beta-actin (*ACTB*) cDNA was performed using forward – 5'–GGACTTCGAGCAAGAGATGG–3' and reverse – 5'–AGCACTGTGTTGGCGTACAG–3' primers. These primer 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 beta-actin mRNA was used as control of analyzed RNA quantity. The primers were received from Sigma-Aldrich (St. Louis, MO, USA).

Quantitative PCR analysis was performed using a special computer program "Differential expression calculator". The values of *IGFBP6*, *IGFBP7*, *IGFBP9*, *IGFBP10*, *WISP1*, *WISP2* and *ACTB* gene expressions were normalized to the expression of beta-actin mRNA and represent as percent of control (100%). All values are expressed as mean  $\pm$  SEM from triplicate measurements performed in 4 independent experiments. The amplified DNA fragments were also analyzed on a 2% agarose gel and that visualized by SYBR\* Safe DNA Gel Stain (Life Technologies, Carlsbad, CA, USA).

*Statistical analysis.* Statistical analysis was performed according to Student's *t*-test using Excel program as described previously [31]. All values are expressed as mean  $\pm$  SEM from triplicate measurements performed in 4 independent experiments.

## Results and Discussion

To determine if hypoxia affects the expression of a subset of genes encoding for different insulin-like growth factor binding proteins through the IRE1 branch of endoplasmic reticulum stress response, we investigated the effect of hypoxic condition on mRNA expression levels of different members of *IGFBP* and *CCN* families, which can participate in the regulation of glioma growth through insulin-like growth factor receptors as well as through other signaling pathways and mechanisms.

To investigate a possible role of endoplasmic reticulum stress signaling mediated by signaling enzyme IRE1 in the expression of insulin-like growth factor binding protein gene *IGFBP6* and its sensitivity to hypoxia we studied the effect of hypoxic condition on this gene expression in glioma cells with functional IRE1 (control glioma cells) and cells without both enzymatic activities of this signaling enzyme. As shown in Fig. 1, the expression level of *IGFBP6* mRNA is significantly up-regulated by hypoxia in both types of glioma cells: +39% in control glioma cells in comparison with the control 1 and +18% in cells with suppressed function of signaling enzyme IRE1 as compared to control 2. At the same time, the inhibition of IRE1 by dnIRE1 strongly down-regulated the expression level of *IGFBP6* mRNA (2.2-fold) in glioma cells in comparison with the control cells (Fig. 1).

We next tested the sensitivity of *IGFBP7* gene expressions to hypoxia and whether IRE1 also participates in the hypoxic regulation of this gene. We

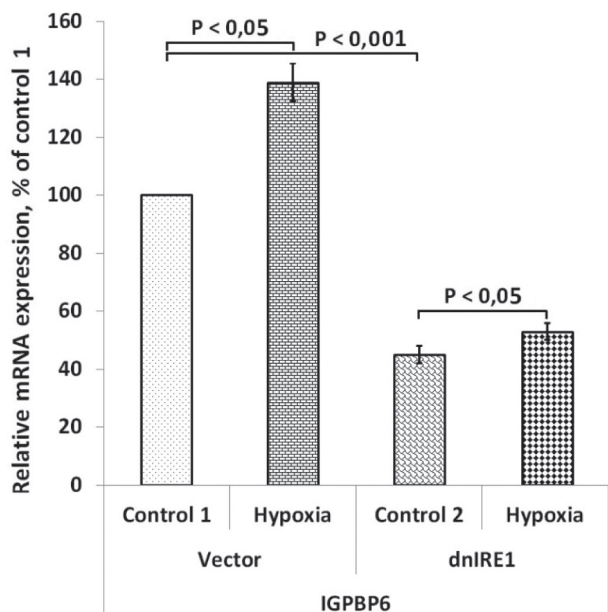


Fig. 1. Effect of hypoxia on the expression level of insulin-like growth factor binding protein 6 (IGFBP6) mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dn-IRE1 (dn-IRE1) measured by qPCR. Values of IGFBP6 mRNA expressions were normalized to  $\beta$ -actin mRNA level and represented as percent of control 1 (100%);  $n = 4$

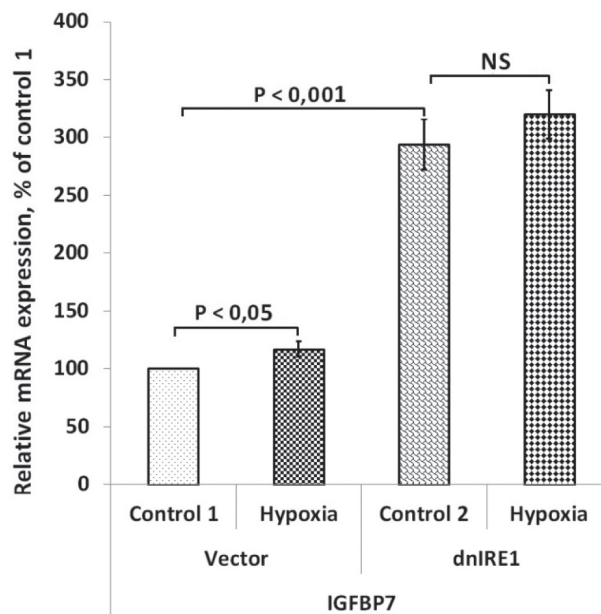


Fig. 2. Effect of hypoxia on the expression level of insulin-like growth factor binding protein 7 (IGFBP7) mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dn-IRE1 (dn-IRE1) measured by qPCR. Values of IGFBP7 mRNA expressions were normalized to  $\beta$ -actin mRNA level and represented as percent of control 1 (100%);  $n = 4$

have found that the expression of *IGFBP7* gene is also up-regulated by hypoxia in control glioma cells (+17%), but cells without IRE1 signaling enzyme function are resistant to hypoxia in comparison with the corresponding control (control 2) (Fig. 2). At the same time, the inhibition of IRE1 in glioma cells strongly up-regulated the expression of *IGFBP7* gene upon normoxic condition as compared to control 1 (2.9-fold; Fig. 2).

Next we investigated the cysteine-rich regulatory proteins *IGFBP9/NOV/CCN3* and *IGFBP10/CYR61/CCN1*, which have insulin-like growth factor binding domain and are members of CCN family proteins, associate with the extracellular matrix and play an important role in cancer development by regulation of angiogenesis, cell migration and proliferation. We tested how hypoxia affects these insulin-like growth factor binding protein genes and how IRE1 inhibition modifies the effect of hypoxia on the expression of *IGFBP9/NOV* and *IGFBP10/CYR61* genes. In Fig. 3 and 4 is shown the expression of *IGFBP9/NOV* and *IGFBP10/CYR61* genes, which are sensitive to hypoxia, but their expression changed in diverse ways. Thus, hypoxia decreases

the expression of *IGFBP9/NOV* gene (-39%) and induces *IGFBP10/CYR61* gene expression (+67%). However, the inhibition of IRE1 eliminates the effect of hypoxia on *IGFBP9/NOV* gene expression in glioma cells, but expression of *IGFBP10/CYR61* gene is also increased (+44%) in these glioma cells (Fig. 3 and 4). Thereafter, we studied the effect of inhibition of IRE1 enzyme function by dn-IRE1 on the expression of *IGFBP9/NOV* gene in glioma cells upon normoxic condition. As shown in Fig. 3, the expression of *IGFBP9/NOV* gene is strongly down-regulated (4.8-fold) by inhibition of IRE1 signaling enzyme. At the same time, the expression of *IGFBP10/CYR61* gene significantly increased (1.5-fold) in these glioma cells in comparison with control glioma cells. Thus, the inhibition of IRE1 signaling enzyme function affects the expression level of *IGFBP9/NOV* and *IGFBP10/CYR61* mRNAs in gene-specific manner.

We next studied the effect of hypoxia on the expression of WNT1 inducible signaling pathway proteins (*WISP1* and *WISP2*), which have IGF binding domain and are members of CCN family proteins as well as *IGFBP9/NOV/CCN3* and *IGFBP10/CYR61/CCN1*. As shown in Fig. 5, the expression of *WISP1/*

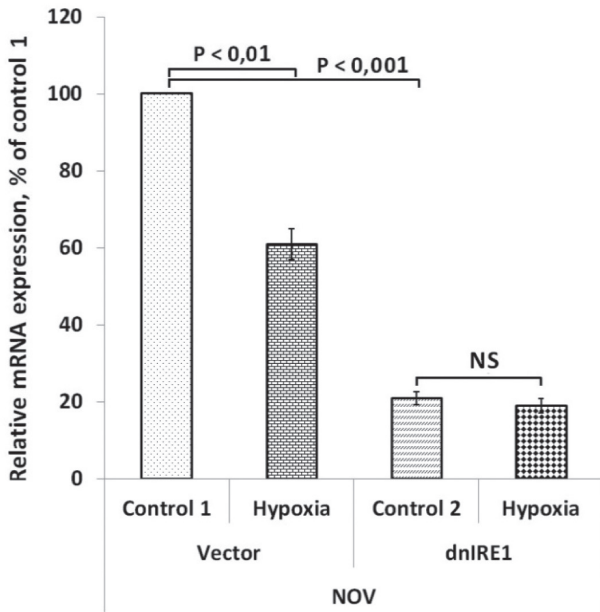


Fig. 3. Effect of hypoxia on the expression level of insulin-like growth factor binding protein 9 (IGFBP9), also known as NOV (nephroblastoma overexpressed) and CCN3 (CCN family member 3), mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dn-IRE1 (dn-IRE1) measured by qPCR. Values of IGFBP9 mRNA expressions were normalized to  $\beta$ -actin mRNA level and represented as percent of control 1 (100%);  $n = 4$

CCN4 gene at mRNA level is up-regulated by hypoxia both in cells with native IRE1 and cells with inhibited function of IRE1 signaling enzyme: +45% in control glioma cells and +68% in cells with a deficiency of IRE1 enzyme function. We have also shown that the inhibition of IRE1 signaling enzyme leads to strong up-regulation of WISP1/CCN4 gene expression (8.4-fold) in glioma cells upon normoxic condition (Fig. 5).

Significant up-regulation was also shown for the expression of WISP2/CCN5 gene upon hypoxia in both types of glioma cells: 2.6-fold in control glioma cells and 1.8-fold in cells without IRE1 enzyme function as compared to corresponding controls (Fig. 6). Moreover, the inhibition of IRE1 is strongly up-regulated expression of WISP2/CCN5 gene at mRNA level in glioma cells upon normoxic condition (3.6-fold) as compared to control 1.

Additionally, we analyzed the effect of hypoxic condition on the expression level of IGFBP6, IGFBP10/CYR61/CCN1, WISP1/CCN4, and WISP2/

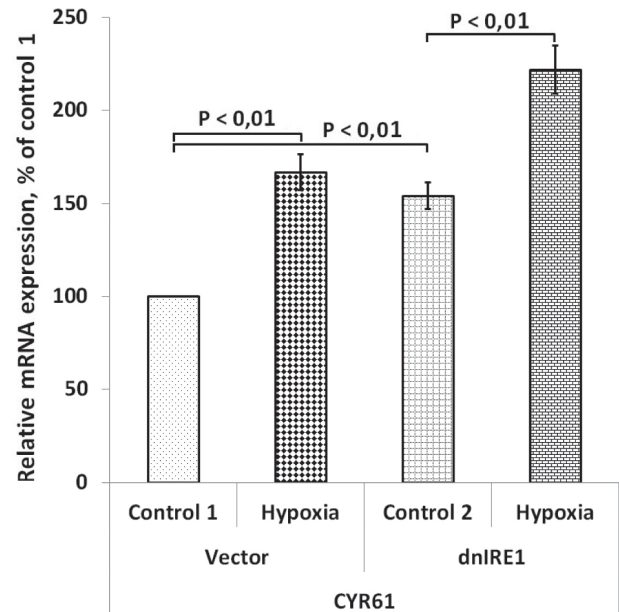


Fig. 4. Effect of hypoxia on the expression of insulin-like growth factor binding protein 10 (IGFBP10), also known as CYR61 (cysteine-rich angiogenic inducer 61) and CCN1 (CCN family member 1), mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dn-IRE1 (dn-IRE1) measured by qPCR. The values of IGFBP10 mRNA expressions were normalized to  $\beta$ -actin mRNA level and represented as percent of control 1 (100%);  $n = 4$

CCN5 mRNA in glioma cells with intact and inhibited IRE1 enzyme function in conditions when both controls (control 1 and control 2) are established as 100% to more precisely clarify the difference in the sensitivity of these gene expressions to hypoxia in respect to inhibition of IRE1. As shown in Fig. 7, there are statistically significant differences in the expression levels of IGFBP6, IGFBP10/CYR61, WISP1, and WISP2 mRNA in control glioma cells and cells without IRE1 function exposure upon hypoxia: 2.17-fold for IGFBP6, 1.52-fold for IGFBP10/CYR61, 1.51-fold for WISP1, and 2.02-fold for WISP2.

In this work we studied the expression of genes which encoded different insulin-like growth factor binding proteins in glioma cells with inhibition of IRE1 signaling enzyme function upon hypoxia for evaluation of possible significance of these genes in the control of glioma growth through endoplasmic reticulum stress signaling mediated by IRE1 and hypoxia. Investigation of the expression of different

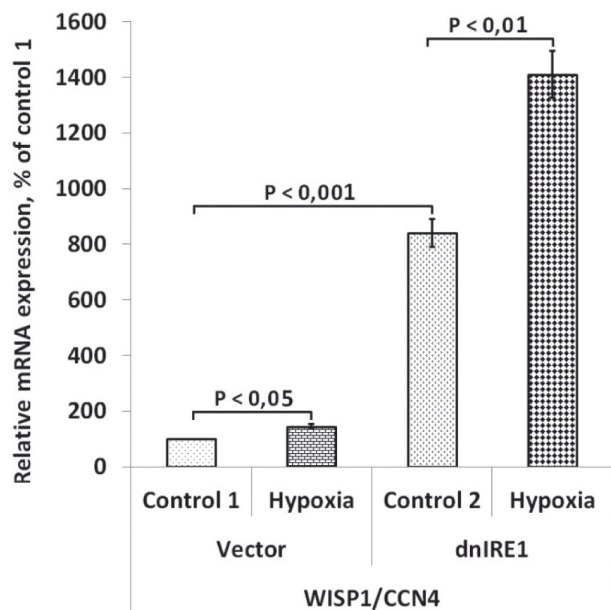


Fig. 5. Effect of hypoxia on the expression of WNT1 inducible signaling pathway protein 1 (WISP1), also known as CCN4 (CCN family member 4), mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dn-IRE1 (dn-IRE1) measured by qPCR. Values of WISP1 mRNA expressions were normalized to  $\beta$ -actin mRNA level and represented as percent of control 1 (100%); n = 4

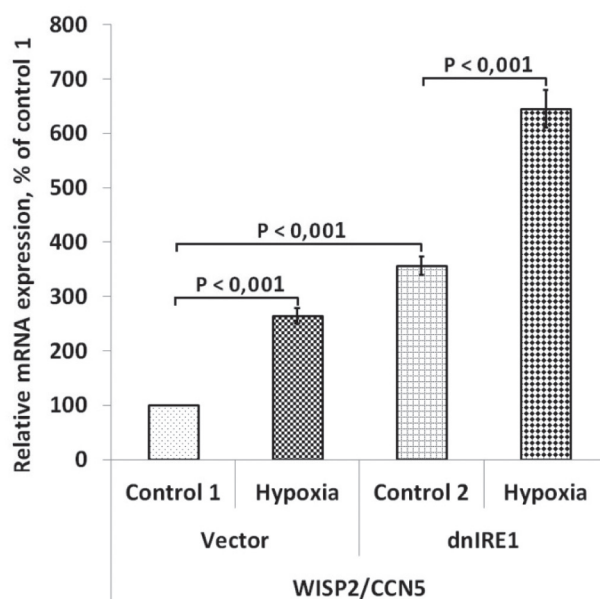


Fig. 6. Effect of hypoxia on the expression of WNT1 inducible signaling pathway protein 2 (WISP2), also known as CCN5 (CCN family member 5), mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dn-IRE1 (dn-IRE1) measured by qPCR. Values of WISP2 mRNA expressions were normalized to  $\beta$ -actin mRNA level and represented as percent of control 1 (100%); n = 4

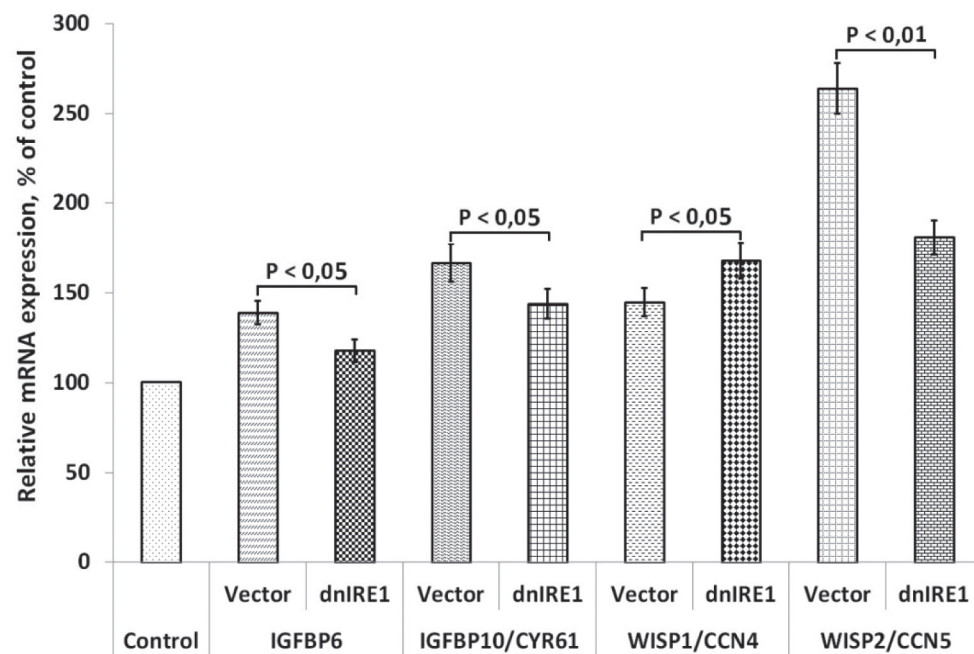


Fig. 7. Inhibition of IRE1 modifies the effect of hypoxia on the expression of IGFBP7, IGFBP10/CYR61, WISP1, and WISP2 mRNA in glioma cells (by qPCR). These mRNA expression values were normalized to  $\beta$ -actin mRNA expression and represented as percent of corresponding control (both controls are taken as 100%); mean  $\pm$  SEM; n = 4

genes of IGFBP and CCN families in glioma cells upon hypoxia in respect of inhibition of IRE1 signaling is very important for understanding the malignant tumor growth mechanisms, because hypoxia as well as nutrient deprivation plays essential role in the control of tumor progression [27, 32]. Thus, our results are consistent with numerous data [22, 30] that hypoxia is associated with malignant progression through the endoplasmic reticulum unfolded protein response, but mechanism how malignant cells cope with potentially lethal metabolic stress induced by hypoxia remains poorly understood.

The growing tumor requires the endoplasmic reticulum stress as well as hypoxia and nutrient deprivation, which initiate the endoplasmic reticulum stress for own neovascularization and growth, for apoptosis inhibition [23, 30]. Cell proliferation is strongly dependent upon hypoxia and glycolysis because there is the molecular connection between cell cycle progression and the provision of substrates essential for this purpose [30, 33]. The endoplasmic reticulum has an important position as a signal integrator in both normal and malignant cells because the endoplasmic reticulum stress signaling pathways have connections with other plasma membrane receptor signaling networks and with numerous metabolic pathways [19, 30]. It has been known that the complete blockade of the activity of IRE1 signaling enzyme in glioma cells had anti-tumor effects [24, 29].

In this study we have also demonstrated that the expression of all studied genes in glioma cells is regulated by IRE1 signaling enzyme upon normoxic condition, because inhibition of IRE1 significantly up-regulates *IGFBP7*, *IGFBP10/CYR61*, *WISP1* and *WISP2* genes and down-regulates *IGFBP6* and *IGFBP9/NOV* genes as compared to control glioma cells and that these genes participate in IRE1 mediated network of unfolded protein response. It is possible that decreased expression of *IGFBP6* and *IGFBP9/NOV* genes in glioma cells without both enzymatic activities of IRE1 contributes to the suppression of glioma growth from cells with IRE1 knockdown [23, 24] because our results conform to data that *IGFBP6* and *IGFBP9/NOV* have mainly pro-proliferative functions through interaction with different proteins and signaling pathways and are overexpressed in various malignant tumors [6, 11].

The insulin-like growth factor binding proteins bind and regulate the availability of both IGFs with different affinity and inhibit or stimulate the

growth promoting effects of the IGFs through IGF/insulin receptors and through many other signaling pathways and regulate cell proliferation and survival as well as angiogenesis and cancer cell migration [6, 11]. In this study we have shown that inhibition of IRE1 signaling, which is a central mediator of the unfolded protein response and an important component of malignant tumor growth, strongly up-regulated the expression level of *IGFBP7* gene in glioma cells and that this induction of *IGFBP7* possibly contributes to suppression of IRE1 knockdown cell proliferation, because this protein acts as a tumor suppressor [6]. Moreover, the effect of this protein on cell proliferation, apoptosis and senescence is realized through both insulin/IGF-dependent and -independent mechanisms [6].

We also observed significant induction of *IGFBP10/CYR61/CCN1*, *WISP1/CCN4* and *WISP2/CCN5* gene expressions in glioma cells by inhibition of IRE1 signaling enzyme, which supports the IRE1-mediated mechanisms of the expression of both these genes. Furthermore, up-regulation of the expression of these multifunctional proteins in glioma cells after inhibition of IRE1 can also contribute to the suppression of these glioma cells proliferation, because there is data that elevated expression of *WISP-1* and *CYR61* in primary breast cancers associated with more advanced features [14]. It is possible that *IGFBP10/CYR61/CCN1* can suppress hepatocarcinogenesis by inhibiting compensatory proliferation [34], although its role in tumor growth is not well established yet. At the same time, an increased level of *CYR61/CCN1* as well as a decreased level of *NOV/CCN3* possibly has relation to increased metastasis of glioma cells without IRE1 function [40], because *CYR61/CCN1* is responsible for induction of the tumor epithelial-mesenchymal transition, invasion, and metastasis as well as inhibition of *NOV* levels promotes the invasive phenotype of metastatic cancer cells [35]. Furthermore, elevated levels of *WISP1* and *CYR61* in primary breast cancers associated with more advanced features [14]. *WISP2* is a transcriptional repressor of genes associated with the epithelial-mesenchymal transition; it is undetectable in more aggressive breast cancer cells [15]. Consequently, up-regulation of *WISP1* and *WISP2* gene expression in glioma cells after inhibition of IRE1 can also contribute to the suppression of this proliferation of glioma cells.

Thus, the changes observed in the most above studied insulin-like growth factor binding proteins



gene expressions correlate well with slower cell proliferation in cells harboring dn-IRE1, attesting to the fact that endoplasmic reticulum stress is a necessary component of malignant tumor growth, cell survival and metastasis. Moreover, our results demonstrate that hypoxia, which contributes to tumor growth, affects all studied insulin-like growth factor binding protein and WNT1 inducible signaling pathway protein gene expressions and that inhibition of IRE1 preferentially abolishes or suppresses the hypoxic regulation of these gene expressions and thus possibly contributes to slower glioma growth. However, the detailed molecular mechanisms of IRE1-mediated hypoxic regulation of these genes, which have a pivotal role in the control of cell proliferation as well as metastasis, are complex and warrants further investigation.

### **ЕФЕКТ ГІПОКСІЇ НА ЕКСПРЕСІЮ ГЕНІВ, ЩО КОДУЮТЬ ДЕЯКІ IGFBP ТА CCN ПРОТЕЇНИ, В КЛІТИНАХ ГЛІОМИ ЛІНІЇ U87 ЗАЛЕЖИТЬ ВІД IRE1 СИГНАЛЮВАННЯ**

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Ми вивчали регуляцію гіпоксією експресії різних генів протеїнів, що зв'язуються із подібними до інсуліну факторами росту, у клітинах гліоми лінії U87 у зв'язку з пригніченням IRE1 (залежного від інозитулу ензиму 1) – центрального медіатора стресу ендоплазматичного ретикулула, який контролює проліферацію клітин та ріст гліоми. Встановлено, що за гіпоксії спостерігається посилення експресії генів *IGFBP6*, *IGFBP7*, *IGFBP10/CYR61*, *WISP1* та *WISP2* і зниження гена – *IGFBP9/NOV* на рівні мРНК у контрольних клітинах гліоми, причому вираженіші зміни виявлено для генів *IGFBP10/CYR61* та *WISP2*. Водночас, пригнічення IRE1 модифікує ефект гіпоксії на експресію всіх досліджених генів: ліквідує чутливість до гіпоксії експресії генів *IGFBP7* та *IGFBP9/NOV*, знижує ефект гіпоксії на *IGFBP6*, *IGFBP10/CYR61* та *WISP2* і злегка посилює регуляцію гіпоксією експресію гена *WISP1* у клітинах

гліоми. Показано, що експресія всіх досліджених генів у клітинах гліоми регулюється сигнальним ферментом IRE1 за умов нормоксії, оскільки пригнічення IRE1 істотно посилює експресію генів *IGFBP7*, *IGFBP10/CYR61*, *WISP1* та *WISP2* і знижує експресію генів *IGFBP6* та *IGFBP9/NOV* порівняно з контрольними клітинами гліоми. Результати цієї роботи продемонстрували, що гіпоксія сприяє росту пухлин, порушує експресію всіх досліджених генів груп *IGFBP* та *WISP* і що пригнічення IRE1 переважно знімає чи знижує регуляцію гіпоксією експресії цих генів і, таким чином, можливо, робить внесок у зниження росту гліоми. Більше того, пригнічення IRE1, що корелює зі зниженням інтенсивності проліферації клітин та росту гліоми, знижує експресію про-проліферативних генів *IGFBP6* та *IGFBP9/NOV*, підтверджуючи той факт, що стрес ендоплазматичного ретикулула є необхідним компонентом росту злоякісних пухлин.

Ключові слова: експресія мРНК, стрес ендоплазматичного ретикулула, *IGFBP6*, *IGFBP7*, *IGFBP9/NOV*, *IGFBP10/CYR61*, *WISP1*, *WISP2*, пригнічення IRE1, гіпоксія, клітини гліоми.

### **ЭФФЕКТ ГИПОКСИИ НА ЭКСПРЕССИЮ ГЕНОВ, КОДИРУЮЩИХ НЕКОТОРЫЕ IGFBP И CCN ПРОТЕИНЫ, В КЛЕТКАХ ГЛИОМЫ ЛИНИИ U87 ЗАВИСИТ ОТ IRE1 СИГНАЛИНГА**

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Мы изучали регуляцию гипоксией экспрессии генов протеинов, которые связываются с подобными инсулину факторами роста, в клетках глиомы линии U87 в связи с угнетением IRE1 (зависимого от инозитола фермента 1) – центрального медиатора стресса эндоплазматического ретикулула, который контролирует пролиферацию клеток и рост глиомы. Установлено, что при гипоксии отмечается усиление экспрессии генов *IGFBP6*, *IGFBP7*, *IGFBP10/CYR61*, *WISP1* и

*WISP2* и снижение гена – *IGFBP9/NOV* на уровне мРНК в контрольных клетках глиомы, причем более выраженные изменения выявлены для генов *IGFBP10/CYR61* и *WISP2*. В то же время, угнетение IRE1 модифицирует эффект гипоксии на экспрессию всех исследованных генов: ликвидирует чувствительность к гипоксии экспрессии генов *IGFBP7* и *IGFBP9/NOV*, снижает эффект гипоксии на *IGFBP6*, *IGFBP10/CYR61* и *WISP2*, слегка усиливает регуляцию гипоксией экспрессии гена *WISP1* в клетках глиомы. Показано, что экспрессия всех исследованных генов в клетках глиомы регулируется сигнальным энзимом IRE1 при нормоксии, поскольку угнетение IRE1 существенно усиливает экспрессию генов *IGFBP7*, *IGFBP10/CYR61*, *WISP1* и *WISP2* и снижает экспрессию генов *IGFBP6* и *IGFBP9/NOV* по сравнению с контрольными клетками глиомы. Результаты этой работы продемонстрировали, что гипоксия содействует росту опухоли, нарушает экспрессию всех исследованных генов групп *IGFBP* и *WISP* и что угнетение IRE1 преимущественно снимает или снижает регуляцию гипоксией экспрессии этих генов и, таким образом, возможно, вносит вклад в снижение роста глиомы. Более того, угнетение IRE1, коррелирующее со снижением интенсивности пролиферации клеток и роста глиомы, снижает экспрессию про-пролиферативных генов *IGFBP6* и *IGFBP9/NOV*, подтверждая тот факт, что стресс эндоплазматического ретикулума является необходимым компонентом роста злокачественных опухолей.

**Ключевые слова:** экспрессия мРНК, стресс эндоплазматического ретикулума, *IGFBP6*, *IGFBP7*, *IGFBP9/NOV*, *IGFBP10/CYR61*, *WISP1*, *WISP2*, угнетение IRE1, гипоксия, клетки глиомы.

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