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Expression of Genes Encoded 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase and 6-phosphofructo-1-kinase in U87 Glioma Cells with ERN1 Loss of Function: Hypoxic Regulation

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ABSTRACT

The endoplasmic reticulum–nuclei-1 (ERN1) sensing and signaling enzyme mediates a set of complex intracellular signaling events known as the unfolded protein response. We have studied the effect of hypoxia on the expression of genes encoded different 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB1, PFKFB2, PFKFB3 and PFKFB4) and 6-phosphofructo-1-kinase (PFKL, PFKM and PFKP) as well as lactate dehydrogenase (LDHA) in glioma U87 cells and its subline with suppressed function of ERN1 signaling enzyme. It was shown that blockade a function of ERN1 enzyme, the key endoplasmic reticulum stress sensor, leads to an increase in the expression levels of PFKFB1, PFKFB2, PFKFB3 and PFKFB4 mRNA, being more significant for PFKFB4 and PFKFB2. Moreover, the expression level of PFKL and PFKP as well as LDHA mRNA also increases in cells with ERN1 loss of function, being more significant for PFKL. At the same time, the expression level of PFKM mRNA significantly decreases at this experimental condition. Exposure cells under hypoxic conditions leads to an increase of the expression level of PFKFB3 and PFKFB4 mRNA both in control glioma cells and cells with ERN1 loss of function, being more significant for PFKFB4. Moreover, the blockade of ERN1 signaling enzyme function decreases the effect of hypoxia on the expression level of both PFKFB3 and PFKFB4 mRNA. At the same time, the expression level of PFKFB1 and PFKFB2 mRNA does not change significantly in control glioma cells; however, in cells with ERN1 loss of function decreases PFKFB2 mRNA only. It was also shown that increased expression of PFKFB3 and PFKFB4 under hypoxic conditions correlates with strong induction of PFKL expression in control glioma cells only. The expression level of PFKP as well as LDHA mRNA increases in both cell types. Thus, results of this study clearly demonstrate that blockade of ERN1 signaling enzyme function leads to glycolysis activation mainly via increased expression of PFKFB4 and PFKL and eliminates effect of hypoxia on PFKL expression as well as suppresses its effect on the expression of PFKFB3 and PFKFB4 and decreases PFKFB2 mRNA level. It is possible that expression level of some PFKFB and PFK1 genes in normoxic and hypoxic conditions in glioma cells is mediated by ERN1 signaling system of endoplasmic reticulum stress.

Keywords: mRNA expression, PFKFB1, PFKFB2, PFKFB3, PFKFB4, PFK1, glioma cells, endoplasmic reticulum–nuclei-1 (ERN1), hypoxia

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INTRODUCTION

The endoplasmic reticulum is a key organelle in the cellular response to ischemia, hypoxia, and some chemicals which activate a complex set of signaling pathways named the unfolded protein response. This adaptive response is activated upon the accumulation of misfolded proteins in the endoplasmic reticulum and is mediated by three endoplasmic reticulum-resident sensors named PERK (PRK-like ER kinase), ERN1 (Endoplasmic Reticulum – Nuclei-1) also known as IRE-1 (Inositol Requiring Enzyme1) and ATF6 (Activating Transcription Factor 6), however, ERN1 is the dominant sensor [1–6]. Activation of the unfolded protein response tends to limit the *de novo* entry of proteins in to the endoplasmic reticulum and facilitate both the endoplasmic reticulum protein folding and degradation to adapt cells for survival or, alternatively, to enter cell death programs through endoplasmic reticulum-associated machineries [7, 8]. As such, it participates in the early cellular response to the accumulation of misfolded proteins in the lumen of the endoplasmic reticulum, occurring under both physiological and pathological conditions.

Two distinct catalytic domains of the bifunctional signaling enzyme ERN1 were identified: a serine/threonine kinase and an endoribonuclease which contribute to this enzyme signalling. The ERN1-associated kinase activity autophosphorylates and dimerizes this enzyme, leading to the activation of its endoribonuclease domain, initiation of the pre-XBP1 (X-box binding protein 1) mRNA splicing, and degradation of a specific subset of mRNA [9–11]. Mature XBP1 mRNA splice variant (XBP1s) encodes a transcription factor that has different C-terminal amino acid sequence and stimulates the expression of hundreds of unfolded protein response-specific genes [1, 9–12]. XBP1 is a unique basic-region leucine zipper transcription factor whose active form is generated by a non-conventional splicing reaction upon disruption of homeostasis in the endoplasmic reticulum and activation of the unfolded protein response. XBP1, first identified as a key regulator of major histocompatibility complex class II gene expression in B cells, represents the most conserved signaling component of unfolded protein response and is also critical for cell fate determination in response to the endoplasmic reticulum stress.

Moreover, the growing tumor requires the endoplasmic reticulum stress as well as hypoxia and ischemia for own neovascularization and growth and the complete blockade of ERN1 signal transduction pathway has anti-tumor effects [4, 8, 13, 14]. The endoplasmic reticulum stress response-signalling pathway is linked to the neovascularization process, tumor growth and differentiation as well as cell death processes [12, 15, 16]. There is data that endoplasmic reticulum stress response-signaling pathway is involved in osteoblast differentiation induced by BMP2 as well as in induction of apoptosis by N-acetyl cysteine and penicillamine [17, 18]. Thus, the blockade of the main unfolded protein response sensor ERN1 is important in studying the role of ERN1 signalling pathways in tumor progression, especially in malignant gliomas [19]; it is important in developing a new understanding concerning molecular mechanisms of malignant tumors progression in relation to ischemia/hypoxia and it can help define the best targets for the design of specific inhibitors that could act as potent antitumor drugs.

A high rate of glycolytic flux, even in the presence of oxygen, is a central metabolic hallmark of neoplastic tumors. The high glucose metabolism of cancer cells is caused by a

combination of hypoxia-responsive transcription factors, activation of oncogenic proteins and the loss of tumor suppressor function. Over-expression of HIF-1α or HIF-2α and MYC, activation of RAS and loss of TP53 and/or other tumor suppressor functions each have been found to stimulate glycolysis in part by activating a family of regulatory bifunctional 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFB) and hexokinases [20–22]. Hexokinase 2 is a key mediator of aerobic glycolysis and promotes tumor growth in human glioblastoma multiforme, the most common malignant brain tumor [22]. The PFKFB enzymes synthesize fructose-2,6-bisphosphate which allosterically activates 6-phosphofructo-1-kinase, a rate-limiting enzyme and essential control point in the glycolytic pathway [23–25]. Different PFKFB are hypoxia responsive enzymes and overexpressed in different cancer tissues [26–29]. Overexpression of these enzymes is an obligatory factor of tumor cell glycolysis [20, 30, 31]. Recently, it was shown that nuclear targeting of 6-phosphofructo-2-kinase-3 increases proliferation via cyclin-dependent kinase [32]. Moreover, E3 ubiquitin ligase APC/C-CDH1 accounts for the Warburg effect by linking glycolysis to cell proliferation mainly via the glycolysis-promoting enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, isoform 3, because PFKFB3 is degraded by the E3 ubiquitin ligase APC/C-CDH1, which also degrades cell-cycle proteins [33]. It was shown in two different cell types (neoplastic and non-neoplastic) that both proliferation and aerobic glycolysis are prevented by overexpression of CDH1 and enhanced by its silencing. Furthermore, activation of glycolysis, which is essential for cell proliferation, in the presence of active CDH1 does not result in proliferation [33]. There is data that ubiquitin ligase SKP1-CUL1-F (SCF)-beta-TrCP also sequentially regulates glycolysis during the cell cycle via PFKFB because this effect occurs only when PFKFB is present or is substituted by the downstream glycolytic enzyme 6-phosphofructo-1-kinase [34]. Besides that, the induction of de novo lipid synthesis from glucose in prostate cancer cells by androgen requires transcriptional up-regulation of PFKFB2 and phosphorylation of PFKFB2 generated by the PI3K/AKT signal pathway to supply the source for lipogenesis [35]. The increased glycolytic flux through the enhanced expression of PFKFB3 gene was observed after interaction of adenosine with macrophage Toll-4 receptor agonists [36]. Thus, the family of PFKFB proteins participates in the control of glucose metabolism via glycolysis as well as in the regulation of the cell cycle, proliferation, apoptosis and invasiveness. Several alternative splice variants for PFKFB2, PFKFB3 and PFKFB4 were identified in normal and tumor cells which possibly have significance in cancer growth [37–42].

Three phosphofructokinase-1 isozymes exist in humans: muscle, liver and platelet, which are encoded by separate genes located at different chromosomes [42]. These isozymes function as subunits of the mammalian tetramer phosphofructokinase, which catalyzes the phosphorylation of D-fructose-6-phosphate to D-fructose-1,6-bisphosphate, a key step in glycolysis. Moreover, posttranslational modification of PFK1 enzyme is an important feature of cancer metabolism and might be the pivotal factor of deregulated glycolytic flux in tumors [43].

The protein encoded by lactate dehydrogenase genes (EC_1.1.1.27) catalyzes the conversion of L-lactate and NAD to pyruvate and NADH in the final step of anaerobic glycolysis. High lactate dehydrogenase is associated with acute adult T-cell leukemia/lymphoma [44]. Moreover, upregulation of lactate dehydrogenase A by ErbB2 through heat shock factor 1 promotes breast cancer cell glycolysis and growth and

knockdown of this enzyme in renal cancer cells results in significant reduction in tumor growth in a xenograft mouse model [45].

Thus, the PFKFB and phosphofructokinase-1 isozyme families as well as lactate dehydrogenase proteins participate not only in the control of glucose metabolism via glycolysis, but also in the regulation of the cell proliferation and tumor growth.

A better understanding of the impact of different PFKFB and PFK1 gene networks regulation on glycolysis and cell cycle control as well as nutrient balance at the molecular, cellular and system levels promises to shed light on the emerging association between different PFKFB, PFK1, proliferation, endoplasmic reticulum stress response and cancer.

The main goal of this work is to study the role of ERN1-signaling pathways in tumor progression by investigating the expression of different variants of different PFKFB and PFK1 in U87 glioma cells and cells with ERN1 loss of function under normal and hypoxic ischemic conditions.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The glioma cell line U87 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) and streptomycin (0,1 mg/ml; Gibco) at 37^{o}C in a 5% CO_2 incubator. In this work we used two sublines of this glioma cell line. One subline was obtained by selection of stable transfected clones with overexpression of vector, which was used for creation of dnERN1. This untreated subline of glioma cells (control glioma cells) was used as control 1 in the study of the effect of hypoxia on the expression level of PFKFB1, PFKFB2, PFKFB3, PFKFB4, PFKL, PFKM, PFKP and LDHA mRNA. Second subline was obtained by selection of stable transfected clones with overexpression of ERN1 dominant/negative constructs (dnERN1) and has suppressed both protein kinase and endoribonuclease activities of this signaling enzyme [13]. The expression level of PFKFB, PFK1 and LDHA genes in these cells was compared with cells, transfected by vector (control 1), but this subline was also used as control 2 for investigation the effect of hypoxia on the expression level of different studied genes under blockade ofthe ERN1 function.

Hypoxic conditions were created in special incubator with 3 % oxygen and 5 % carbon dioxide levels; culture plates were exposed to these conditions for 16 hrs. For glucose or glutamine deprivation the growing medium in culture plates was replaced with a medium without glucose or without glutamine and thus exposed for 16 hrs. The suppression level of ERN1 enzymatic activity in glioma cells that overexpress a dominant-negative construct of endoplasmic reticulum–nuclei-1 (dnERN1) was estimated by analysis of the expression of XBP1 alternative splice variant (XBP1**s**), a key transcription factor in ERN1 signaling, using cells treated by tunicamycin (0,01 mg/ml during 2 hours).

RNA isolation

Total RNA was extracted from different tumor tissues and normal tissue counterparts as described [46]. RNA pellets were washed with 75 % ethanol and dissolved in nuclease-free water.

Reverse Transcription and Quantitative Polymerase Chain Reaction Analysis

The expression levels of PFKFB1, PFKFB2 and its alternative splice variants mRNA were measured in glioma cell line U87 and its subline with a deficiency of endoplasmic reticulum–nuclei-1 by real-time quantitative polymerase chain reaction (PCR) of complementary DNA (cDNA) using „Stratagene Mx 3000P cycler" (USA) and SYBRGreen Mix (AB gene, Great Britain). QuaniTect Reverse Transcription Kit (QIAGEN, Germany) was used for cDNA synthesis as described previously [43]. Polymerase chain reaction was performed in triplicate.

For amplification of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-1 cDNA we used sequence 5'– GTTTACCAGCTCGAGGCAAG –3' as forward primer and 5'– AAAACCGCAACATGACCTTC –3' as reverse primer. The nucleotide sequences of these primers correspond to sequences 217 – 236 and 460 – 441 of human PFKFB1 cDNA (GenBank accession number NM_002625). The size of amplified fragment is 244 bp.

The amplification of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-2 cDNA was performed using forward (5'– GACATCTGAAGAGCTGCCATG –3') and reverse (5'– CCAATCCAGTTGAGGTAGCG –3') primers. The nucleotide sequences of these primers correspond to sequences 92 – 112 and 306 – 287 of human PFKFB2 cDNA (GenBank accession number NM_006212). The size of amplified fragment is 215 bp.

The amplification of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5'– CTTGTCGCTGATCAAGGTGA –3' and reverse – 5'– TTCTGCTCCTCCACGAACTT –3'. The nucleotide sequences of these primers correspond to sequences 1011-1030 and 1253-1234 of human PFKFB3 cDNA (GenBank accession number NM_004566). The size of amplified fragment is 243 bp.

Two other primers were used for real time RCR analysis of the expression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-4 cDNA: forward – 5'– GGAGTTCAATGTTGGCCAGT –3' and reverse – 5'– TCAGGATCCACACAGATGGA –3'. The nucleotide sequences of these primers correspond to sequences 230 – 249 and 505 – 486 of human PFKFB4 cDNA (GenBank accession number NM_004567). The size of amplified fragment is 276 bp.

For amplification of phosphofructokinaase-1 (ATP:D-fructose-6-phosphate-1-phosphotransferase; PFK1) liver type (PFKL) cDNA we used sequence 5'– GGAGCTTCGAGAACAACTGG –3' as forward primer and 5'– CTGTGTGTCCATGGGAGATG –3' as reverse primer. The nucleotide sequences of these primers correspond to sequences

1186 – 1205 and 1353 – 1334 of human PFKL cDNA (GenBank accession number NM_002626). The size of amplified fragment is 168 bp.

The amplification of the muscle isoform of phosphofructokinaase-1 (PFKM) cDNA was performed using forward (5'– AGAGCGTTTCGATGATGCTT –3') and reverse (5'– CAGTGCCAATGGTCATATCG –3') primers. The nucleotide sequences of these primers correspond to sequences 397 – 416 and 720 – 701 of human PFKM cDNA (GenBank accession number NM_000289). The size of amplified fragment is 324 bp.

For amplification of the platelet isoform of phosphofructokinaase-1 (PFKP) cDNA we used sequence 5'– GCTCCATTCTTGGGACAAAA –3' as forward primer and 5'– GATAGTGTTCAGGGCGGTGT –3' as reverse primer. The nucleotide sequences of these primers correspond to sequences 1497 – 1516 and 1753 – 1734 of human PFKP cDNA (GenBank accession number NM_002627). The size of amplified fragment is 257 bp.

The amplification of lactate dehydrogenase-A (LDHA; cell proliferation-inducing gene 19 protein) cDNA was performed using forward primer (5'– ACGTCAGCAAGAGGGAGAAA –3') and reverse primer (5'– CGCTTCCAATAACACGGTTT –3'). These oligonucleotides correspond to sequences 566 – 585 and 756 – 737 of human LDHA cDNA (GenBank accession number NM_005566). The size of amplified fragment is 191 bp.

The amplification of beta-actin (ACTB) cDNA was performed using forward - 5'– CGTACCACTGGCATCGTGAT –3' and reverse - 5'– GTGTTGGCGTACAGGTCTTT –3' primers. These primers nucleotide sequences correspond to 747 – 766 and 980 – 961 of human *ACTB* cDNA (GenBank accession number NM_001101). The expression of ACTB mRNA was used as control of analyzed RNA quantity. The primers were received from "Sigma" (USA).

The amplification of XBP1 cDNA was performed using HotStarTaq Master Mix Kit ("QIAGEN", Germany), "MasterCycler Personal" ("Eppendorf", Germany) and primers: forward - 5'– GGAGTTAAGACAGCGCTTGG –3' and reverse - 5'– TCACCCCTCCAGAACATCTC –3'. The nucleotide sequences of these primers correspond to sequences 441 – 460 and 608 – 589 of XBP1 mRNA (GenBank accession number NM_005080). The size of amplified fragment is 168 bp for non spliced variant and 142 bp for alternative splice variant (XBP1s). The phosphorylated isoform ERN1 was measured by Western analysis using IRE1p, phosphorylated (Ser724), US Biological.

An analysis of quantitative PCR was performed using special computer program "Differential expression calculator" and statistic analysis – in Excel program. The amplified DNA fragments were analyzed on a 2 % agarose gel and that visualized by 5x Sight DNA Stain (EUROMEDEA).

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RESULTS

Characterization of U87 Glioma Cells That Overexpress a Dominant-Negative Construct of ERN1

To investigate the expression of different PFKFB and PFK1 genes that encode different isoforms of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and 6-phosphofructo-1-kinase as well as the involvement of endoplasmic reticulum stress signaling system in the effect of hypoxia on the expression of these genes we have used the human glioma cell line U87 and a variant of these cells with ERN1 signaling enzyme loss of function. The level of suppression of the enzymatic activity of ERN1 was estimated by analysis of the expression of transcription factor XBP1 and its splice variant (XBP1**s**) in U87 glioma cells that overexpress a dominant-negative construct of ERN1 as compared to control glioma cells transfected with a vector. As shown in figure 1, inductor of endoplasmic reticulum stress tunicamycin (0,01 mg/ml) strongly induces the expression of phosphorylated (Ser724) ERN1 and alternative splicing of XBP1, but in control glioma cells only, while having no effect on this process in transfected by dnERN1 subline glioma cells.

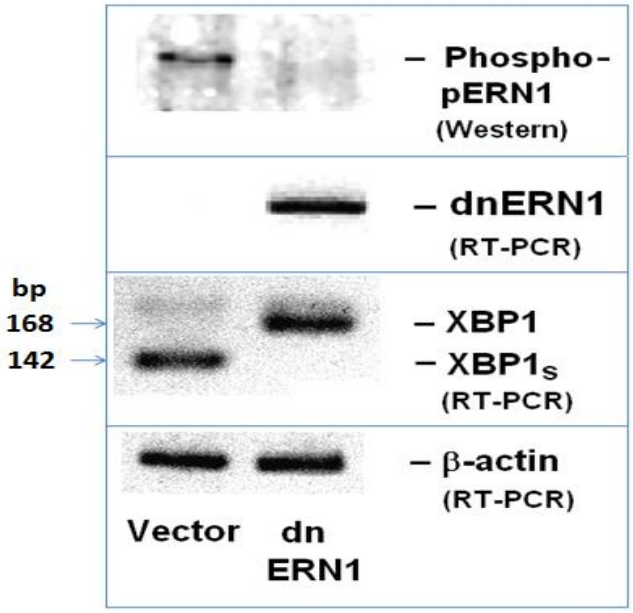

Fig. 1. Expression level of dominant-negative ERN1 (dnERN1) transcript as well as transcription factor XBP1 and its alternative splice variant (XBP1s) in glioma cell line U87 stable transfected with vector (Vector; control cells) and its subline with blockade of signaling enzyme endoplasmic reticulum–nuclei-1 stable transfected with dnERN1 measured by reverse-transcriptase-mediated polymerase chain reaction (RT-PCR). The phosphorylated isoform ERN1 was measured by Western analysis using IRE1p, phosphorylated (Ser724).

Expression of Different PFKFB Genes Glioma Cell Line U87 with ERN1 Loss of Function: Effect of Hypoxia

We have found that PFKFB1, PFKFB2, PFKFB3 and PFKFB4 mRNAs are expressed in the human glioma cell line U87 and the level of their expression is dependent on signaling enzyme ERN1 function. As shown in figure 2, the level of PFKFB1 mRNA expression increases by 61 % in glioma cells, deficient in the function of signaling enzyme ERN1, as compared to

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control cells. PFKFB2 mRNA expression level also increases in cells with signaling enzyme ERN1 loss of function by 78 %, as compared to control glioma cells. We have also found that PFKFB3 and PFKFB4 genes are expressed in the human glioma cell line U87 and the levels of its expression are mainly depend from ERN1 signaling enzyme function (figure 2). The expression level of PFKFB4 mRNA in glioma cells, deficient in signaling enzyme ERN1, increases more significantly as compared with the control value then PFKFB3.

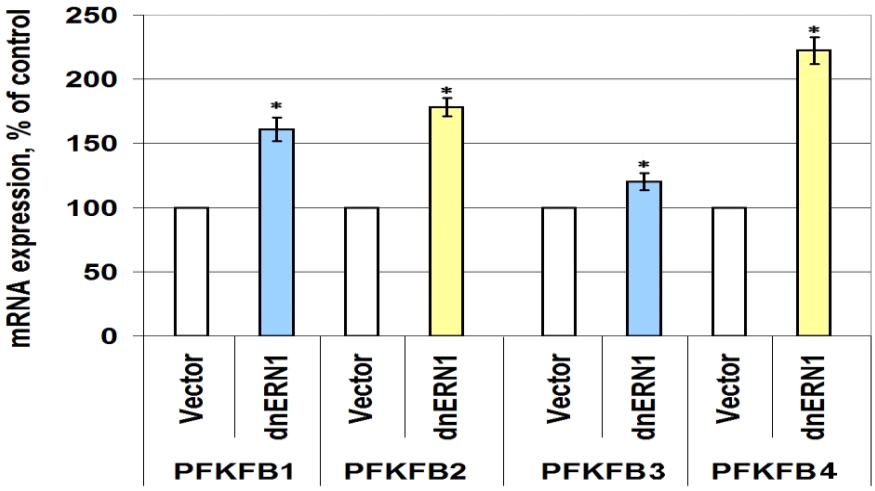

Fig. 2. Effect of blockade of signaling enzyme ERN1 on the expression levels of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-1, -2, -3 and -4 (PFKFB1, PFKFB2, PFKFB3 and PFKFB4) mRNA in glioma cell line U87 (Vector = control) and its subline with suppressed activity of ERN1 (dnERN1) measured by real-time quantitative polymerase chain reaction. Values of the expression of these mRNA were normalized to the expression of beta-actin mRNA and represent as percent of control (100 %); ***n*** = 4; ***** – P < 0.05 as compared to control.

Exposure of cells to hypoxia for 16 hrs does not change significantly the expression level of PFKFB1 mRNA both in control glioma cells and cells with blockade of ERN1 enzyme function (figure 3A). At the same time, hypoxia does not change significantly the expression level of PFKFB2 mRNA in control glioma cells but decreases (-36 %) in glioma cells with signaling enzyme ERN1 loss of function (figure 3B).

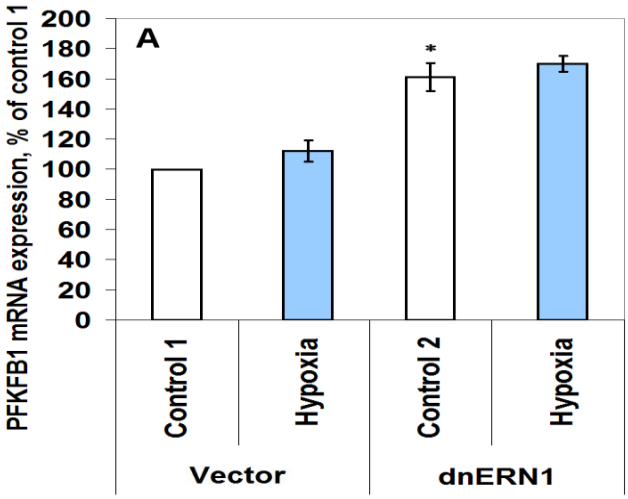

Fig. 3A

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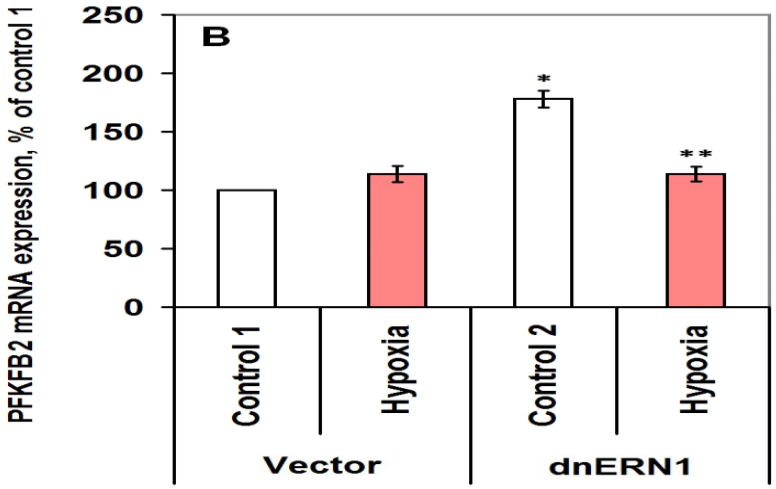

Fig. 3B

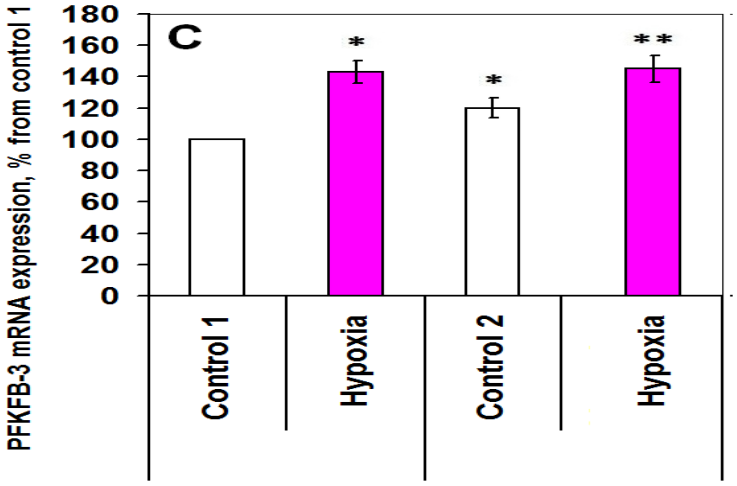

Fig. 3C

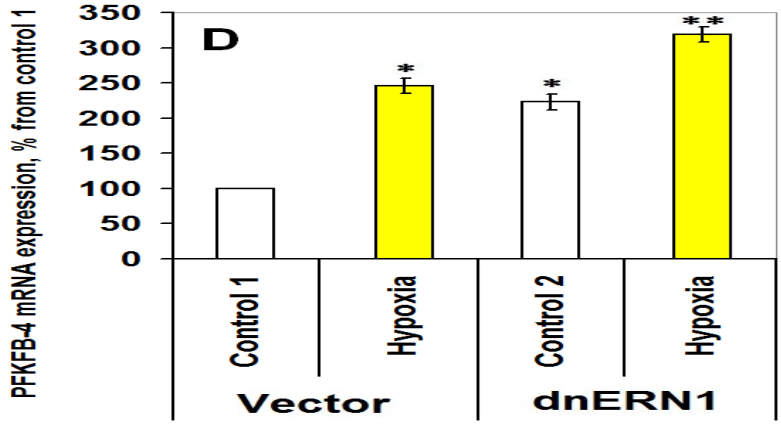

Fig. 3D

Fig. 3. Effect of hypoxia on the expression of PFKFB1 (A), PFKFB2 (B), PFKFB3 (C) and PFKFB4 (D) mRNA in glioma cell line U87 (control 1) and its subline with blockade of signaling enzyme endoplasmic reticulum–nuclei-1 (dnERN1) activity (control 2) measured by quantitative polymerase chain reaction. Values of PFKFB1 mRNA expression were normalized to beta-actin mRNA expression and represent as percent for control 1 (100 %); ***n*** = 4; * - P < 0.05 as compared to control 1; ** - P < 0.05 as compared to control 2.

It was also shown that exposure of U87 glioma cells to hypoxia leads to an increase of PFKFB3 and PFKFB4 mRNA expression level, but in glioma cells with suppressed function of the signaling enzyme ERN1 effect of hypoxia was significantly less: +43 % and 246 %,

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respectively, in control glioma cells, as compared to control 1, and +21 % and 43 %, respectively, in ERN1 loss of function cells, as compared to control 2. (figure 3C and 3D). Thus, hypoxia responsibility of PFKFB4 mRNA expression was much higher than PFKFB3.

Expression of Different Phosphofructo-1-Kinase and LDHA Genes Glioma Cell Line U87 with ERN1 Loss Of Function: Effect Of Hypoxia

In this study, we have investigated the expression of different genes that encode 6-phosphofructo-1-kinase and LDHA proteins in human glioma cell line U87 as well as the involvement of the endoplasmic reticulum stress signaling system in the effect of hypoxia. It was found that PFKL, PFKM, PFKP and LDHA genes are expressed in this glioma cell line and the levels of its expression are mainly depend from ERN1 signaling enzyme function. As shown in figure 4, the expression level of PFKL, PFKP and LDHA mRNA significantly increases in glioma cells, deficient in signaling enzyme ERN1 function, compared to control value: +176, +72 and +31 %, respectively. However, PFKM mRNA expression level decreases (-43 %) in glioma cells with signalling enzyme ERN1 loss of function as compared to control glioma cells (figure 4).

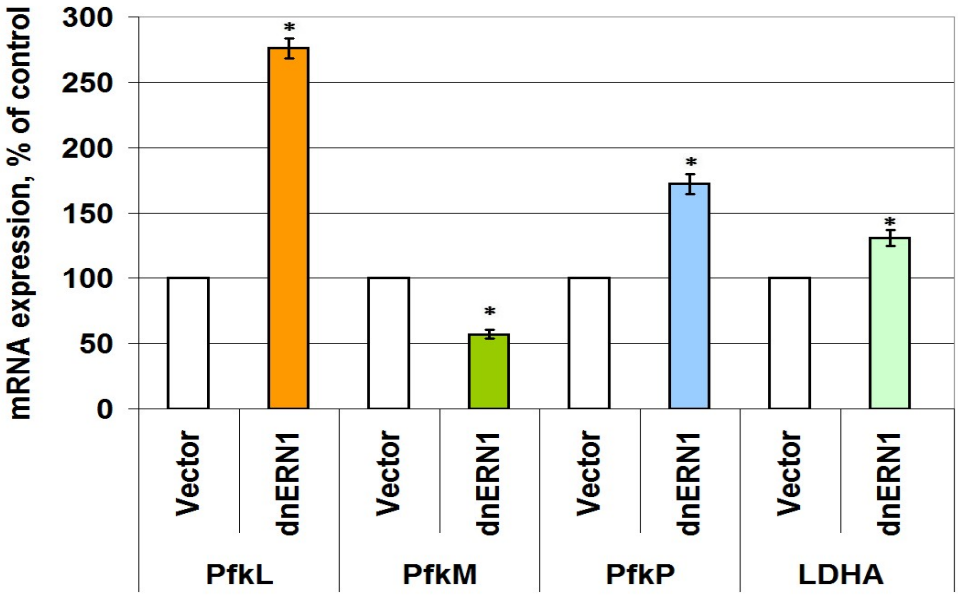

Fig. 4. Effect of blockade of signaling enzyme ERN1 on the expression levels of 6-phosphofructo-1-kinase L, M and P (PFKL, PFKM and PFKP) and LDHA mRNA in glioma cell line U87 (Vector = control) and its subline with suppressed activity of ERN1 (dnERN1) measured by real-time quantitative polymerase chain reaction. Values of the expression of these mRNA were normalized to the expression of beta-actin mRNA and represent as percent of control (100 %); ***n*** = 4; ***** – $P < 0.05$ as compared to control.

Exposure of glioma cells to hypoxia for 16 hrs leads to an increase the expression level of PFKL (19 fold), PFKP (2.5 fold) and LDHA (+31 %) mRNA in control glioma cells, as compared to control 1, but to decrease the expression level of PFKM (figure 5A, 5B, 5C and 5D). At the same time, in glioma cells with suppressed function of the signaling enzyme ERN1 effect of hypoxia on the expression level of PFKL and PFKM was not observed, as compared to control 2 (figure 5A and 5B). At the same time, hypoxia increases the expression level of PFKP and LDHA mRNA in glioma cells with ERN1 loss of function (+44 and +23 %, respectively); however, effect of hypoxia on PFKP expression was more significant in control glioma cells (figure 5C and 5D).

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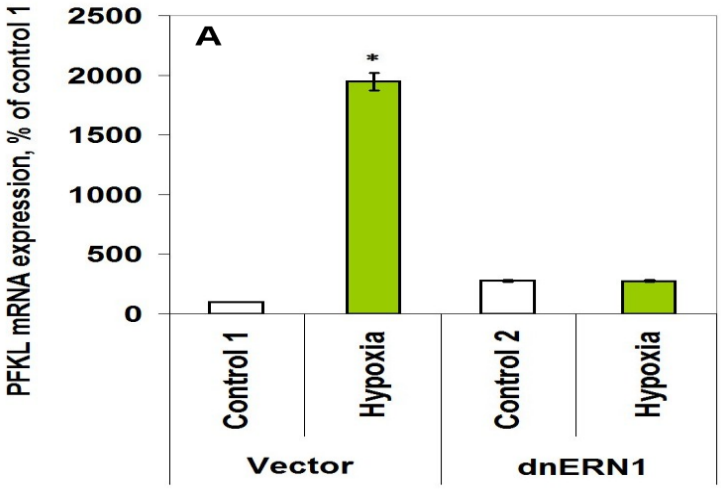

Fig. 5A

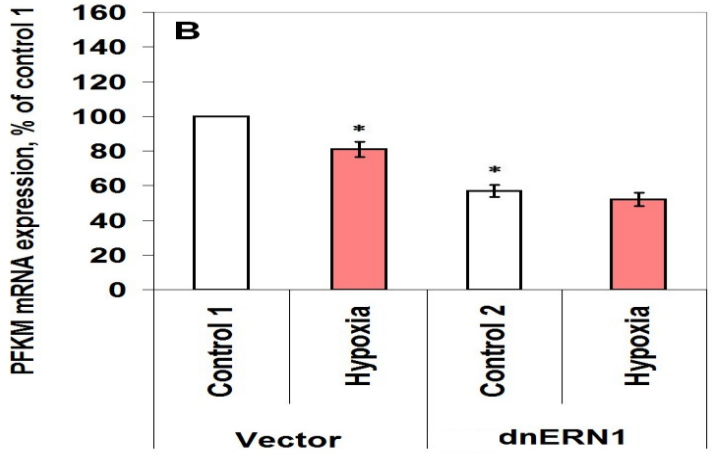

Fig. 5B

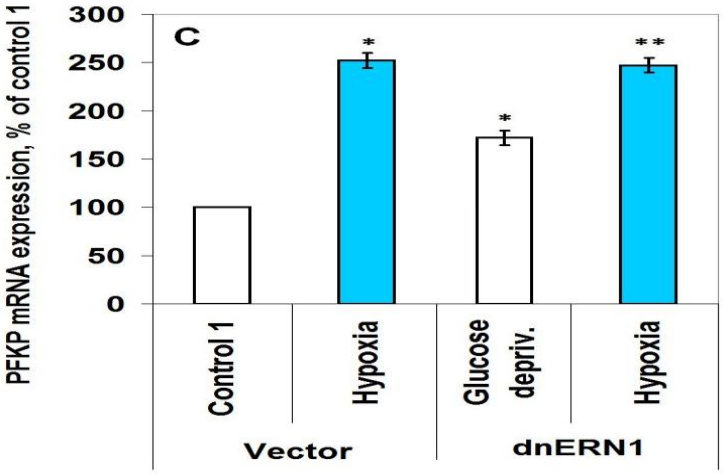

Fig. 5C

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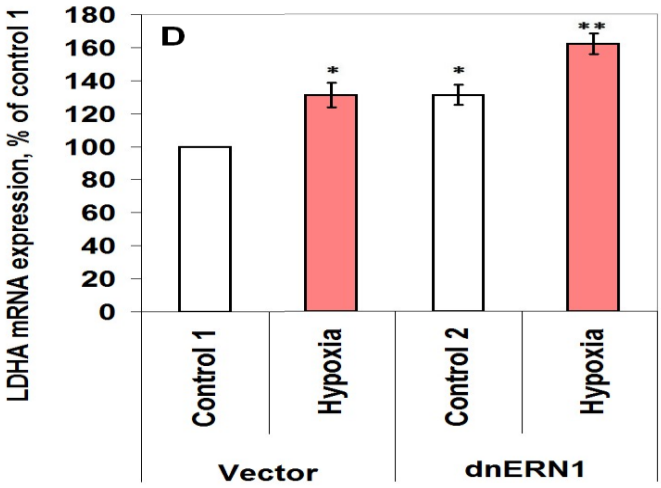

Fig. 5D

Fig. 5. Effect of hypoxia on the expression of PFKL (A), PFKM (B), PFKP (C) and LDHA (D) mRNA in glioma cell line U87 (control 1) and its subline with blockade of signaling enzyme endoplasmic reticulum–nuclei-1 (dnERN1) activity (control 2) measured by quantitative polymerase chain reaction. Values of different PFK1 and LDHA mRNA expression were normalized to beta-actin mRNA expression and represent as percent for control 1 (100 %); ***n*** = 4; ***** - P < 0.05 as compared to control 1; ****** - P < 0.05 as compared to control 2.

DISCUSSION

It has been known that the glycolysis and tumor growth processes are linked to the endoplasmic reticulum stress and its sensing and signal transduction pathways and ERN1 pathway, in particular, because the complete blockade of this signaling enzyme activity had anti-tumor effects [14, 16, 19]. Moreover, there is data that growing tumor requires ischemia and hypoxia which initiate the endoplasmic reticulum stress for own neovascularization and growth, for apoptosis inhibition [18, 19]. It is known that some PFKFB enzymes are components of the endoplasmic reticulum stress system; they participate in proliferation processes [21, 32, 33, 38].

In this study we have shown that blockade of ERN1, the key endoplasmic reticulum stress sensor, changes the expression levels of all PFKFB isoform (PFKFB1, PFKFB2, PFKFB3 and PFKFB4), which play an important role in the control of glycolysis and tumor growth [29, 35], but more strongly PFKFB4 and PFKFB2. This data agrees with idea that some PFKFB participate in endoplasmic reticulum stress signaling and are the important components of IRE-1 signaling [21, 32, 33]. It is possible that increased expression of PFKFB1, PFKFB2 and, especially, PFKFB4 in ERN1 knockdown glioma cells is responsible, at least partly, for suppression of these cells proliferation [14], because there are many alternative splice variants of PFKFB with different properties and significance in the regulation of glycolysis and proliferation and some of them can really suppress tumor cell growth [37- 41, 47]. Really, detailed molecular mechanisms of PFKFB expression at the level of its alternative splicing in cells with suppressed function of signaling enzyme ERN1 in connection with its proliferation capacity have not been elucidated and warrant further study.

We also studied the expression of several genes that encode phosphofructokinase-1 and lactate dehydrogenase proteins in glioma cells with ERN1 knockdown for the purpose of evaluating the dependence of these gene expressions upon the ERN1 signaling enzyme function, being more significant for PFKL. It was shown that the expression level of PFKL, PFKP and LDHA increases in glioma cells without ERN1 signaling enzyme function. This data is completely correlates with enhanced invasiveness of these cells [26, 28]. It is well known that phosphofructokinase-1 isoenzymes and lactate dehydrogenase participate in glycolysis and proliferation and are the components of endoplasmic reticulum stress system, but its activity controls by different mechanisms, not only ERN1 signaling system [16, 45].

Results of this investigation demonstrated that different phosphofructokinase-1 genes have significant difference in hypoxia responsibility. More strong induction of the expression was shown for PFKL and much less for PFKP mRNA in control glioma cells; however, a blockade of the functional activity of signaling enzyme endoplasmic reticulum–nuclei-1 leads to a complete reduction of hypoxic effect on the expression of PFKL and PFKM genes and reduce effect of hypoxia on the expression of PFKP gene in glioma cells. These results correlate with data concerning biological significance of different phosphofructokinase-1 proteins and lactate dehydrogenase in tumor growth [13, 16, 43]. This data demonstrates that effect of hypoxia on the expression of most phosphofructokinase-1 as well as PFKFB genes mediates by ERN1 signaling system, at least partly, because blockade of the activity of ERN1 signaling enzyme by dnERN1 construct which completely suppresses the formation of alternative splice variant of XBP1 and phosphorylation of ERN1 as well as the main biological function of ERN1, significantly reduces effect of hypoxia.

Thus, results of this study clearly demonstrate that the expression level of PFKFB1, PFKFB2, PFKFB3 and PFKFB4 as well as PFKL, PFKP and LDHA mRNA increases in glioma cells with ERN1 signaling enzyme loss of function and that hypoxic conditions also lead to increase the expression level of most of these PFKFB and 6-phosphofructo-1-kinase mRNA but predominantly in control glioma cells. It is possible that decrease of the expression level of PFKFB2 genes in hypoxic conditions is also mediated by ERN1 signaling system because it present in cells with ERN1 loss of function and miss in control glioma cells. Moreover, an increased expression of PFKFB3 and PFKFB4 under hypoxic conditions correlates with strong induction of PFKL expression, but in control glioma cells only. The expression level of PFKP as well as LDHA mRNA also increases in both cell types under hypoxia; however, suppression the function of ERN1 enzyme reduces effect of hypoxia on PFKFP expression. Our results clearly demonstrate that blockade of ERN1 signaling enzyme function leads to glycolysis activation mainly via increased expression of PFKFB4 and PFKL and eliminates effect of hypoxia on PFKL expression as well as suppresses its effect on the expression of PFKFB3 and PFKFB4 and decreases PFKFB2 mRNA level. However, the molecular mechanisms underlying these seemingly mutually exclusive behaviors have not been elucidated. This provides a rationale for the molecular analysis of expression signatures of different related genes in glioma cells which control the tumor growth and glycolysis for a comprehensive approach of these complex mechanisms.

The major finding reported here is that the expression of different PFKFB and 6-phosphofructo-1-kinase genes dependents on the function of ERN1 signaling enzyme – both

in normal and hypoxic conditions and possible participates in glioma cell proliferation and tumor growth via regulation of various signaling pathways. However, the detailed molecular mechanisms of regulation of genes encoding different PFKFB and 6-phosphofructo-1-kinase by ERN1 signaling system under hypoxic stress conditions is complex and warrants further study.

CONCLUSIONS

Results of this investigation clearly demonstrate that the expression of different 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and 6-phosphofructo-1-kinase genes as well as LDHA gene in glioma cells depends on the functional activity of signaling enzyme ERN1 and regulates by hypoxia and that this hypoxic regulation mainly depends on ERN1 enzyme. Thus, different PFKFB and 6-phosphofructo-1-kinase isoenzymes possibly participate in cell adaptive response to endoplasmic reticulum stress associated with hypoxia.

REFERENCES

[1] Bouchecareilh M, Higa A, Fribourg S et al. FASEB J 2011; 25(9): 3115-3129.
[2] Badiola N, Penas C, Minano-Molina A et al. Cell Death Dis 2011; 2: E149.
[3] Aragón T, van Anken E, Pincus D et al. Nature 2009; 457(7230): 736 –740.
[4] Bi M, Naczki C, Koritzinsky M et al. EMBO J 2005; 24(19): 3470–3481.
[5] Fels DR and Koumenis C. Cancer Biol Therap 2006; 5(7): 723–728.
[6] Korennykh AV, Egea PF, Korostelev AA et al. Nature 2009; 457(7230): 687–693.
[7] Liu G, Guo H, Guo C et al. Brain Res Bull 2011; 84(1): 94-102.
[8] Romero-Ramirez L, Cao H, Nelson D et al. Cancer Res 2004 ; 64(17): 5943–5947.
[9] Acosta-Alvear D, Zhou Y, Blais A et al. Mol Cell 2007; 27: 53–66.
[10] Luo D., He Y., Zhang H et al. J Biol Chem 2010; 283(18): 11905–11912.
[11] Hollien J, Lin JH, Li Het al. J Cell Biol 2009; 186(3): 323–331.
[12] Han D, Upton J-P, Hagen A et al. Biochem Biophys Res Commun 2008; 365: 777–783.
[13] Denko NC. Reviews Cancer 2008; 8: 705–713.
[14] Moenner M, Pluquet O, Bouchecareilh M and Chevet E. Cancer Res 2007; 67(22): 10631–10634.
[15] Woehlbier U and Hetz C. Trends Biochem Sci 2011; 36(6): 329-337.
[16] Drogat B, Auguste P, Nguyen DT et al. Cancer Res 2007; 67(14): 6700–6707.
[17] Saito A, Ochiai K, Kondo S et al. J Biol Chem 2011; 286(6): 4809–4818.
[18] Guan D, Xu Y, Yang M et al. Mol Carcinogenesis 2010; 49(1): 68–74.
[19] Auf G, Jabouille A, Guérit S et al. Proc Natl Acad Sci USA 2010; 107(35): 15553–15558.
[20] Bartrons R and Caro J. J Bioenergy Biomembr 2007; 39(3): 223–229.
[21] Yalcin A, Telang S, Clem B and Chesney J. Exp Mol Pathol 2009; 86(3): 174–179.
[22] Wolf A, Agnihotri S, Micallef J et al. J Exp Medicine 2011; 208(2): 313–326.
[23] Wu C, Khan SA, Peng L-J and Lange AJ. Advances in Enzyme Regulation 2006; 46: 72–88.
[24] Rider MH, Bertrand L, Vertommen D et al. Biochem J 2004; 381(3): 561–579.
[25] Minchenko DO, Bobarykina AY, Kundieva AV et al. Studia Biologica 2009; 3(3): 123–140.
[26] Minchenko AG, Leshchinsky I, Opentanova IL et al. J Biol Chem 2002; 277(8): 6183–6187.

[27] Minchenko O, Opentanova I and Caro J. FEBS Lett 2003; 554(3): 264–270.
[28] Minchenko OH, Opentanova IL, Minchenko DO et al. FEBS Lett 2004; 576(1): 14–20.
[29] Minchenko OH, Ochiai A, Opentanova IL et al. Biochimie 2005; 87(11): 1005–1010.
[30] Guo X, Xu K, Zhang J et al. J Biol Chem 2010; 285(31): 23711–23720.
[31] Chesney J. Curr Opin Clin Nutr Metab Care 2006; 9(5): 535–539.
[32] Yalcin A, Clem BF, Simmons A et al. J Biol Chem 2009; 284(36): 24223–24232.
[33] Almeida A, Bolaños JP and Moncada S. Proc Natl Acad Sci USA 2010; 107(2): 738–741.
[34] Tudzarova S., Colombo S.L., Stoeber K et al. Proc Natl Acad Sci USA 2011; 108(13): 5278[–5283.
[35] Moon JS, Jin WJ, Kwak JH et al. Biochem J 2011; 433(1): 225-233.
[36] Ruiz-García A., Monsalve E., Novellasdemunt L et al. J Biol Chem 2011; 286(22): 19247–19258.
[37] Kessler R, Bleichert F, Warnke J P and Eschrich K. J Neuro-Oncology 2008; 86(3): 257–264.
[38] Zscharnack K, Kessler R, Bleichert F et al. Neuropathol Appl Neurobiol 2009; 35(6): 566 – 578.
[39] Atsumi T, Nishio T, Niwa H et al. Diabetes 2005; 54(12): 3349–357.
[40] Lypova NM, Minchenko DO, Ratushna OO et al. Ukr Biokhim Zh 2010; 82(1): 90–99.
[41] Minchenko OH, Ogura T, Opentanova IL et al. Mol Cell Biochem 2005; 280(1-2): 227–234.
[42] Yamada S, Nakajima H and Kuehn MR. Biochem Biophys Res Commun 2004; 316(): 580–587.
[43] Smerc A, Sodja E and Legisa M. PLoS ONE 2011; 6(5): E19645.
[44] Beltran B, Quinones P, Morales D et al. Leuk Res 2011; 35(3): 334-339.
[45] Zhao Y.H., Zhou M., Liu H et al. Oncogene 2009; 28(42): 3689–3701.
[46] Minchenko DM, Hubenya OV, Terletsky BM et al. Ukr Biokhim Zh 2011; 83(1): 5–16.
[47] Atsumi T, Chesney J, Metz C et al. Cancer Res 2002 ; 62: 5881–5887.