# EXPERIMENTAL ARTICLES

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# OVEREXPRESSION OF DOMINANT-NEGATIVE IRE1 ENZYME IN H1299-shE6AP CELLS INCREASES HEAT SHOCK ELEMENT-DEPENDENT TRANSCRIPTION

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To investigate IRE1-dependent branch of endoplasmic reticulum stress pathway in various cancer cells we created cDNA-constructs for expression of dominant-negative inositol-requiring enzyme 1 IRE1 and cytosolic domain of IRE1 fused on a C-terminus with c-Myc and 6xHis tags. The non-small-cell lung carcinoma cells H1299-shE6AP were transfected with these constructs. Using anti-c-Myc antibodies we demonstrated effective, dose-dependent expression of dominant-negative and cytosolic IRE1 proteins. In order to investigate IRE1-mediated, heat shock element-dependent transcription, the cells were further transfected with a reporter construct containing heat shock element. We observed that overexpression of dnIRE1 in H1299-shE6AP cells led to significant induction of heat shock element-dependent transcription. This observation may reflect the induction of heat shock genes, which contribute to cellular adaptation to inhibition of native IRE1, a key sensory-signaling enzyme of endoplasmic reticulum stress pathway, which suppresses cancer cell proliferative capacities and alternates the expression of numerous genes, including many transcription factors.

***Key words:*** endoplasmic reticulum stress, IRE1 enzyme, recombinant protein expression, heat shock element, luciferase reporter assay.

Endoplasmic reticulum (ER) stress signaling pathway is activated as an adaptive response to accumulation of misfolded proteins in its lumen, which might be caused by a handful of factors including increase in protein synthesis, oxidative stress, perturbations of calcium homeostasis, etc. [1, 2]. In mammals three transmembrane stress sensors, namely PERK (double stranded RNA activated protein kinase, PRK-like ER kinase, IRE1/ERN1 (inositol-requiring enzyme 1, endoplasmic reticulum to nucleus signaling 1) and ATF6 (activating transcription factor 6) are responsible for downstream signaling during endoplasmic reticulum stress [3, 4]. Among those IRE1 is a dominant sensory-signaling enzyme, conserved through different groups of organisms including green plants and yeast [5]. IRE1 protein consists of a sensory domain, which is localized in the lumen, a transmembrane part and a cytoplasmic domain with two distinct enzymatic activities: kinase and endoribonuclease [4]. The main function of a kinase domain is IRE1 autophosphorylation, which in turn leads to its dimerization and subsequent activation of endoribonuclease. Activated IRE1 endoribonuclease performs a unique cytosolic splicing of transcription factor XBP1 (X-box binding protein 1) mRNA, as well as specific degradation of a subset of mRNAs [6–8]. Taken together these enzymatic activities contribute to stress alleviation and restoration of cellular homeostasis [5, 8, 9].

For glioma and lung adenocarcinoma it was shown that IRE1 knockdown results in suppression of tumor growth due to alterations in expression of numerous pro- and anti-angiogenic genes, tumor suppressors, cyclins and transcription factors [10–16]. IRE1 is considered to be a promising target for new chemotherapeutic agents, especially in case of aggressive cancers, such as glioma, where surgery still remains a poor therapeutic option [17].

One of the approaches used to inhibit IRE1 function in living cell is a dominant-negative cDNA-constructs strategy, based on the mammalian expression vector systems, such as pcDNA3.1 [4, 10, 11]. In this case, a plasmid DNA, used for transfection, includes a modified cDNA sequence of IRE1 gene, which codes a protein lacking one of or both enzymatic activities [11, 18]. In U87 glioma cells, which express dominant-negative IRE1 (dnIRE1), the downregulation of its phosphorylated form was demonstrated along with an absence of transcription factor XBP1 (X-box binding protein 1) splicing, confirming the inhibition of both kinase and endoribonuclease activities of this bifunctional enzyme [18, 19]. Advantages of this method include the possibility of selection of clones with stable IRE1 knockdown and, in comparison to RNA interference, exclusion of possible off-target effects [11, 18, 20]. Still the detection of modified IRE1 forms, as well as purification of recombinant proteins depends on specific antibodies, which increases the complexity and cost of experimental procedures.

One of the most intriguing aspects of cancer cell biology is the cross-talk between different signaling pathways. For instance, a number of heat shock proteins are known to be involved in the ER stress response. When ER folding capacity is exceeded, molecules of chaperone BiP/GRP78 dissociate from sensory domains of IRE1, PERK and ATF6, which leads to their activation [21]. It was shown, that stressful conditions result in increased BiP/GRP78 expression in glioma cells regardless of IRE1-XBP1 branch of ER stress [14]. Activation of transcription factor ATF6 results in increased expression of ER chaperones GRP78 and GRP94 [21]. In contrast, induction of two HSP40 (heat shock protein-40)-like proteins Erdj4 and $p58^{IPK}$ upon ER stress seems to be mostly XBP1-dependent [22]. Along with other regulatory elements, such as XBP1 of ATF6 binding sites, promoters of *HSP* genes contain the so-called heat shock elements (HSE), various arrays of inverted repeats of the pentameric sequence nGAAn which are responsible for binding of heat shock transcription factors under conditions of thermal stress [23]. Up to date, it was largely unknown for mammalian cells whether disruption of certain branches of ER stress pathway results in compensatory induction of heat shock response.

The aim of this work was creation of improved cDNA-constructs of IRE1 with C-terminal c-Myc and 6xHis tags for investigation of the IRE1-dependent branch of endoplasmic reticulum stress pathway. Using original dnIRE1 expression construct in combination with HSE-containing luciferase reporter we studied the effect of dnIRE1 overexpression on the HSE-dependent transcription in a subline of non-small cell lung carcinoma H1299-shE6AP [24]. It was shown, that overexpression of dominant-negative IRE1 led to significant induction of HSE-dependent transcription.

## Materials and Methods

*Creation of genetic constructs pcDNA4+dnIRE1 and pcDNA4+cytIRE1*

Genetic constructs pcDNA4+dnIRE1 and pcDNA4+cytIRE1 were based on the vector pcDNA4-Myc/His-A (Invitrogen, USA). The vector was linearized simultaneously with two restriction enzymes HindIII and XbaI (NEB, USA). Coding sequence of dominant-negative IRE1 (dnIRE1) was obtained via PCR with forward 5′-GAGAAGCTTCCTCGCCATGCCGG-3′ and reverse 5′-GACTCTAGAGTCTTG-TTCCAGGGAG-3′primers, which include recognition sites for HindIII and XbaI restriction enzymes, respectively. Coding sequence of cytoplasmic domain of IRE1 (cytIRE1) was obtained via PCR with forward 5′-CATAAGCTTTCCCCTGAGCATGCAT-3′ primer with HindIII recognition site and reverse 5′-CTTCTAGAGAGGGCGTCTGGAG-3′ primer with XbaI recognition site. Introduction of restriction enzyme recognition sites into primer sequences was done in order to enable sticky end directional cloning. As a PCR template we used a construct with a full length IRE1 cDNA sequence in pcDNA3.1 vector (kindly provided by prof. M. Moenner, University of Bordeaux-1, France). Obtained fragments were ligated into a linearized vector using T4 DNA ligase (NEB, USA) according to manufacturer's instructions. DH5α *E. coli* cells were heat-shock transformed with pcDNA4+dnIRE1 and pcDNA4+cytIRE1 constructs and subsequently plated on a solid LB medium with 100 μg/mL ampicillin. Nucleotide sequence of created constructs was determined by GATC Biotech (Germany) using T7 and BGH standard sequencing primers.

*Cell culture*

H1299-shE6AP cells (kindly provided by prof. Martin Scheffner, University of Konstanz, Germany, described in [24]) were cultured in DMEM (Gibco, USA) with 10% FBS at 37 °C, 5% $CO_2$.

*Transfection*

For transient expression of dnIRE1 and cytIRE1 H1299-shE6AP cells that reached > 90% confluence were transfected with different combinations of plasmid DNA by lipofection using Lipofectamine 2000 (Invitrogen, USA) according to manufacturer's instructions. Transfection was performed in 6-well polystyrene plates (Greiner bio-one, Germany). For analytical expression of dnIRE1 cells were transfected with plasmid DNA in following combinations: 100 ng pcDNA4+dnIRE1, 300 ng β-galactosidase expression construct [24] 400 ng pcDNA4-Myc/His-A vector (Invitrogen, USA); 500 ng pcDNA4+dnIRE1, 300 ng β-galactosidase expression construct. For analytical expression of cytIRE1 cells were transfected with plasmid DNA in following combinations: 500 ng pcDNA4+cytIRE1, 300 ng β-galactosidase expression construct, 1 500 ng pcDNA4-Myc/His-A vector, 1 000 ng pcDNA4+cytIRE1, 300 ng β-galactosidase expression construct, 1 000 ng pcDNA4-Myc/His-A vector, 2 000 ng pcDNA4+cytIRE1, 300 ng β-galactosidase expression construct.

As a control cells were transfected with 500 ng vector pcDNA4-Myc/His-A together with 300 ng β-galactosidase expression construct.

For luciferase reporter assay cells were transfected with 500 ng pcDNA4+dnIRE1, 1 000 ng 3xHSE-luc construct (kindly provided by prof. Martin Scheffner, University of Konstanz, Germany), 300 ng β-galactosidase expression construct. Control cells were transfected with 500 ng pcDNA4-Myc/His-A vector, 1 000 ng 3xHSE-luc-construct, 300 ng β-galactosidase expression construct.

*Cell lysis and β-galactosidase assay*

Cell lysis was performed 24 hours after transfection in TNN buffer (100 mM Tris-HCl, 100 mM NaCl, 1% NP-40, 1 mM Pefabloc, 1 μg/mL Aprotinin/Leupeptin, 1 mM DTT, pH 8.0). In order to determine the β-galactosidase activity 5 μl of lysate was mixed with 5 μl ortho-nitrophenyl-β-galactoside (4 mg/ml in 100mM $Na_2HPO_4$, pH 7.0 ) and 120 μl buffer Z (100 mM $Na_2HPO_4$, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0) and incubated at 37 °C for 10 min. Measurements were performed at Wallac 1420 multilabel counter (PerkinElmer, USA) at wave length of 405 nm. Obtained values were used for calculation of relative transfection efficiency.

*Western blot*

Cell lysates were normalized with regard to relative transfection efficiency. Protein transfer to PVDF membrane (Millipore, Germany) was performed with wet electroblotter (Bio-Rad, USA) for 90 min at 60 V. After the transfer membrane was incubated in 5% milk powder for 48 hours at 4 °C. Anti-c-Myc mouse monoclonal antibodies (Abcam, UK) in 1:1 000 dilutions were used for detection of recombinant proteins. Blots were developed using WesternBright ECL (Advansta, USA) Signal detection was performed in the imaging system LAS-3000 (Fujifilm, Japan).

*Luciferase reporter assay*

Luciferase activity in lysates was measured using Luciferase Assay system kit (Promega, USA) on Wallac 1420 multilabel counter (PerkinElmer, USA). Measurement results were normalized according to relative transfection efficiency. For statistical analysis of obtained data we performed one sample T-test.

## Results and Discussion

*Genetic constructs pcDNA4+dnIRE1 and pcDNA4+cytIRE1*

Creation of an appropriate dominant-negative form of IRE1 requires a construction of its truncated cDNA sequence, which would code intact N-terminal signal peptide (residues 1-18), sensory (residues 18-443) and transmembrane (residues 444-464) domains, but would lack the full sequences of kinase (residues 571-831) and endoribonuclease (residues 837-963) parts (Fig. 1, *A*). To the contrary, for expression of cytosolic domain of IRE1 protein must be truncated from N-terminus for at least 464 residues. At the same time, for expression of C-terminally tagged proteins it is necessary to maintain the open reading frame and avoid formation of stop codons during the cloning. A 1690 bp long fragment coding a dominant-negative IRE1, which corresponds to amino acid residues 1 to 537 of native IRE1, was obtained via PCR and subsequently digested with restriction enzymes HindIII and XbaI (Fig. 1, *B*). Respective fragment was ligated into pcDNA4-Myc/His-A vector. Similar approach was used to obtain a coding sequence of cytosolic domain of IRE1. A 1557 long PCR product, which corresponds to amino acid residues 488 to 977 of a full length IRE1 was restriction digested and ligated into vector (Fig. 1, *C*).

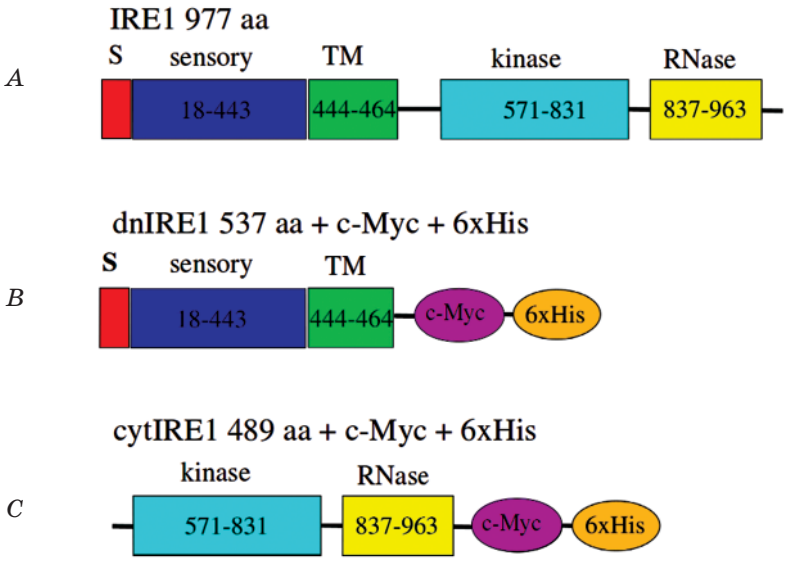


*Fig. 1.* **Schematic representation of expected protein products:**
*A* — domain structure of native IRE1;
*B* — dominant-negative form of IRE1 (dnIRE1) with C-terminal c-Myc and 6xHis tags;
*C* — cytosolic domain of IRE1 (cytIRE1) with C-terminal c-Myc and 6xHis tags.
*S* — N-terminal signal peptide, *TM* — transmembrane region, RNase — endoribonuclease

Sequencing results have shown that the sequences of the fragments were as expected and open reading frames remained intact, thus fusing the desired IRE1 parts with C-terminal tags.

*Analytical expression of IRE1 cytosolic domain and dominant-negative IRE1 in H1299-shE6AP cells*

In order to determine, whether the created constructs are suitable for expression of desired protein products, we transfected H1299-shE6AP cells with different amounts of pcDNA4+dnIRE1 and pcDNA4+cytIRE1 plasmids and performed the Western blot analysis with anti-c-Myc antibody. Our data show, that dominant-negative IRE1 is expressed as a protein product of expected size (Fig. 2). For both dnIRE1 and cytIRE1 the amount of protein depends on the quantity of plasmid, used for transfection (Fig. 2, Fig. 3). Surprisingly, in case of cytIRE1 two fragments of similar size were detected (Fig. 3).

Interestingly, a similar picture was earlier observed by different authors, who also utilized different cloning strategies. For instance, two bands were detected by Wang and co-authors after expressing a cytoplasmic domain of murine IRE1, fused N-terminally with GAL4 and C-terminally tagged with c-Myc epitope [25]. However, they did not attempt to explain their observation. Uemura and co-authors expressed a C-terminally HA-tagged cytoplasmic domain of IRE1 (residues 469-977) and observed two distinct protein products on the blot [26]. The authors suggest that the bands represent an autophosphorylated and non-phosphorylated forms of IRE1 cytoplasmic domain, supporting their suggestion by the fact, that a kinase-dead cytoplasmic part of IRE1 forms a single band on the blot. Moreover, they demonstrate that the resulting recombinant protein is able to catalyze the XBP1 mRNA splicing [26]. Thus, we can assume that pcDNA4+cytIRE1 construct is suitable for expression of a catalytically active cytoplasmic domain of IRE1. Notably, no second band was observed in case of dnIRE1, providing additional evidence for its dominant-negative character (Fig. 2).

*Overexpression of dominant-negative IRE1 influences HSE-mediated transcription in H1299-shE6AP cells*

Previously, it was shown that in yeast a constitutive activation of heat shock response (HSR) by overexpression of Hsf1 (heat shock factor 1) is able to rescue growth in IRE1 knockout cells [27]. Moreover, it was demonstrated that in IRE1-deficient yeast cells heat shock response is activated by ER stress, while in wild-type IRE1 cells no ER stress-mediated activation of HSR was observed [27]. In this study we aimed to test, whether overexpression of dnIRE1 alone, with no additional stress induction is sufficient for activation of HSE-dependent transcription in mammalian cells. For this we used an HSE-containing luciferase reporter construct

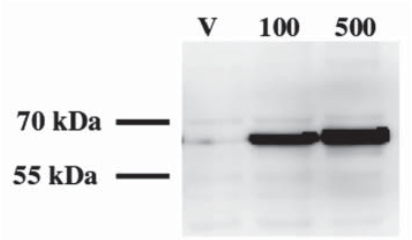


*Fig. 2.* **Western blot analysis of dnIRE1 expression in H1299-shE6AP cells:**
V — cells transfected with empty vector pcDNA-4mycHis4; 100 — cells transfected with 100 ng pcDNA4+dnIRE1; 500 — cells transfected with 500 ng pcDNA4+dnIRE1

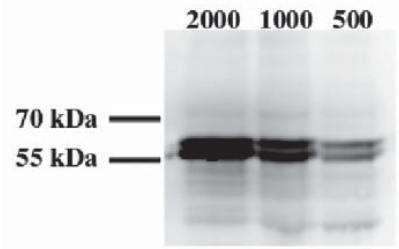


*Fig. 3.* **Western blot analysis of cytIRE1 expression in H1299-shE6AP cells:**
2000 — cells transfected with 2000 ng pcDNA4+cytIRE1; 1000 — cells transfected with 1000 ng pcDNA4+cytIRE1; 500 — cells transfected with 500 ng pcDNA4+cytIRE1

(3xHSE-luc) (Fig. 4). H1299-shE6AP cells were co-transfected with pcDNA4+dnIRE1, 3xHSE-luc and β-galactosidase expression construct. Luciferase activity was measured in cell lysates and normalized according to relative transfection efficiency. We found that overexpression of dominant-negative IRE1 led to more than two-fold increase in HSE-mediated transcription (Fig. 5). These results suggest that inhibition of IRE1 function may lead to heat-shock independent activation of HSR pathway in mammalian cells, which in turn may contribute to restoration of cellular homeostasis.

Overall, as a result of this study we created original expressing constructs for dominant-negative IRE1 and cytoplasmic part of IRE1. The respective recombinant proteins possess C-terminal c-Myc and 6xHis tags, which make their detection easier, and also provide an option for effective affinity purification. Despite promising indirect evidence, the enzymatic activity of cytIRE1 is still to be tested. We were first to demonstrate that overexpression of dominant-negative IRE1 alone with no additional thermal or ER stress leads to activation of HSE-dependent transcription in H1299-shE6AP cells. This might reflect a cellular adaptive response to inhibition of IRE1 activity. An interaction between two pathways: endoplasmic reticulum stress and heat shock response in cancer cells is an interesting and therapeutically relevant topic, which requires further attention.


*Fig. 4.* **Schematic representations of 3xHSE-luc reporter construct:**
*A* — general scheme; *B* — nucleotide sequence of the region containing heat shock response elements (3xHSE — single elements underlined, inverted repeats marked in red)

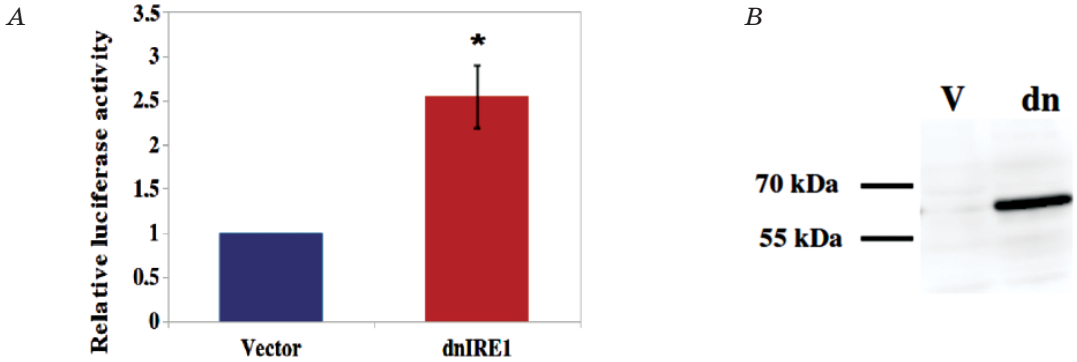


*Fig. 5.* **Effect of dominant-negative IRE1 overexpression on HSE-mediated transcription in H1299-shE6AP cells:**
*A* — relative luciferase activity, * — $P < 0.05$; $n = 4$; *B* — Western blot analysis of dnIRE1 expression:
V — cells transfected with empty vector pcDNA4-Myc/His-A; dn — cells transfected with pcDNA4+dnIRE1

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## НАДЕКСПРЕСІЯ ДОМІНАНТ-НЕГАТИВНОЇ ФОРМИ ЕНЗИМУ IRE1 У СУБЛІНІЇ КЛІТИН H1299-shE6AP ПОСИЛЮЄ ТРАНСКРИПЦІЮ, ЩО ЗАЛЕЖИТЬ ВІД ЕЛЕМЕНТУ ТЕПЛОВОГО ШОКУ

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Метою роботи було дослідження функції IRE1-залежної гілки сигнального шляху стресу ендоплазматичного ретикулуму в різних пухлинних клітинах. Для цього було створено кДНК-конструкції для експресії домінант-негативної форми ензиму IRE1 — dnIRE1 та цитозольного домену IRE1, злитих на С-кінці із с-Мус епітопом та 6xHis. Цими конструкціями було трансфіковано клітини недрібноклітинної карциноми легень сублінії H1299-shE6AP і за допомогою анти-с-Мус антитіл показано ефективну дозозалежну експресію протеїнів домінант-негативної форми та цитозольного домену IRE1. Для дослідження опосередкованої IRE1-транскрипції, залежної від елементу теплового шоку, клітини були повторно трансфіковані люциферазним репортером, який включав елемент теплового шоку. Встановлено, що надекспресія dnIRE1 у клітинах сублінії H1299-shE6AP призводить до вираженого індукування транскрипції, залежної від елементу теплового шоку. Це може свідчити про посилення експресії генів теплового шоку, які відіграють важливу роль в адаптації цих клітин до пригнічення активності нативного IRE1, ключового сенсорно-сигнального ензиму стресу ендоплазматичного ретикулуму, що знижує здатність пухлинних клітин до проліферації та модифікує експресію численних генів, включаючи велику кількість транскрипційних факторів.

***Ключові слова:*** стрес ендоплазматичного ретикулуму, ензим IRE1, експресія рекомбінантних протеїнів, елемент теплового шоку, метод люциферазного репортера.

## СВЕРХЭКСПРЕССИЯ ДОМИНАНТ-НЕГАТИВНОЙ ФОРМЫ ЭНЗИМА IRE1 В СУБЛИНИИ КЛЕТОК H1299-shE6AP УСИЛИВАЕТ ТРАНСКРИПЦИЮ, ЗАВИСЯЩУЮ ОТ ЭЛЕМЕНТА ТЕПЛОВОГО ШОКА

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Целью работы было исследование функции IRE1-зависимой ветви сигнального пути стресса эндоплазматического ретикулума в разных опухолевых клетках. Для этого были созданы кДНК-конструкции для экспрессии доминант-негативной формы энзима IRE1 и цитозольного домена IRE1, сшитых на С-конце с с-Мус эпитопом и 6xHis. Этими конструкциями были трансфицированы клетки немелкоклеточной карциномы легких сублинии H1299-shE6AP и с помощью анти-с-Мус антител показана эффективная дозозависимая экспрессия протеинов доминант-негативной формы и цитозольного домена IRE1. Для исследования опосредованной IRE1-транскрипции, зависимой от элемента теплового шока, клетки были повторно трансфицированы люциферазным репортером, включающим элемент теплового шока. Установлено, что сверхэкспрессия dnIRE1 в клетках сублинии H1299-shE6AP приводит к выраженной индукции транскрипции, зависимой от элемента теплового шока. Это может свидетельствовать об усилении экспрессии генов теплового шока, играющих важную роль в адаптации клеток к подавлению активности нативного IRE1, ключевого сенсорно-сигнального энзима стресса эндоплазматического ретикулума, который снижает способность опухолевых клеток к пролиферации и модифицирует экспрессию многочисленных генов, включая большое количество транскрипционных факторов.

***Ключевые слова:*** стресс эндоплазматического ретикулума, энзим IRE1, экспрессия рекомбинантных протеинов, элемент теплового шока, метод люциферазного репортера.