Research Article

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Expression profiles of LMAN2 in breast cancer cell lines in hypoxia and normoxia conditions

Hipoksi ve normoksi koşullarında meme kanseri hücre hatlarında LMAN2'nin ifade profilleri

Ceyda Okudu, Gökhan Ağtürk, Elif Kesim, Ayyub Ebrahimi

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Abstract

Purpose: Breast cancer is one of the most common and leading causes of death in women. The mechanism by which breast cancer develops is not fully understood. Understanding the mechanism of initiation and the genes and proteins involved in this process may help us to fight this type of cancer. The LMAN2 gene encodes VIP36, which transports properly folded proteins. The main aim of this study was to investigate the expression of the LMAN2 gene at the molecular level in breast cancer cells with different functional defects. Determining the level of LMAN2 gene expression under hypoxia, known as oxygen deprivation, has a significant impact on tumourigenesis and metastasis, and obtaining new data on the relationship between hypoxia and ER stress was identified as a secondary objective.

Material and methods: In this study, the expression level of the LMAN2 gene will be examined in breast cancer cell lines (SKBR3, MDA-MB-231, MDA-MB-468, MCF-7) and CRL4010 cell line as a control.

Results: LMAN2 gene expression level was evaluated at 48-hour periods by providing normoxic and hypoxic conditions. While the LMAN2 gene expression level in MCF-7, MDA-MB-231, and SK-BR-3 cells is significantly reduced, it was highly expressed in MDA-MB-468 in hypoxic and normoxic conditions. CHOP, HERP, and BiP gene expression levels were significantly higher in MDA-MB-468 under hypoxic conditions like LMAN2 expression.

Conclusion: LMAN2 and other ER stress response elements showed different expression profiles in SK-BR-3, MDA-MB-231, MDA-MB-468, and MCF7 cell lines under hypoxic conditions. Increased expression was found in MCF-7 and MDA-MB-468 cell lines, but decreased expression was detected in SK-BR-3 and MDA-MB-231 cell lines. The underlying reason for this difference is thought to be that the cell lines have different molecular properties, such as triple negative or HER2 (+/-) status.

Keywords: ER stress, hypoxia, LMAN2, breast cancer, apoptosis.

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Öz

Amaç: Meme kanseri kadınlarda en sık görülen ve ölüme neden olan kanser türleri arasındadır. Meme kanserinin başlama mekanizması tam olarak açık değildir. Ortaya çıkış mekanizmasını ve bu süreçte yer alan genleri ve proteinleri anlamak, bu kanser türüyle mücadelemizde yardımcı olabileceğini düşünmekteyiz. LMAN2 geni, doğru katlanmış proteinleri taşıyan VIP36'yı kodlar. Bu çalışmanın temel amacı, çeşitli fonksiyonel bozukluklara sahip meme kanseri hücrelerinde LMAN2 geninin ekspresyonunu moleküler düzeyde incelemektir. Oksijen eksikliği koşulları olarak bilinen hipoksi altında LMAN2 gen ekspresyon düzeyinin saptanması, tümörigenez ve metastaz üzerinde önemli bir etkiye sahiptir ve hipoksi ile ER stresi arasındaki ilişki hakkında yeni veriler elde edilmesi de ikincil bir hedef olarak belirlenmiştir.

Gereç ve yöntem: Bu çalışmada, meme kanseri hücre hatlarında (SKBR3, MDA-MB-231, MDA-MB-468, MCF-7) ve kontrol olarak CRL4010 hücre hattında LMAN2 geninin ifade düzeyi incelendi.

Bulgular: LMAN2 gen ekspresyon düzeyi normoksik ve hipoksik koşullar sağlanarak 48 saatlik periyotta değerlendirilmiştir. MCF-7, MDA-MB-231 ve SK-BR-3 hücrelerinde LMAN2 gen ekspresyon düzeyi önemli ölçüde azalırken, MDA-MB-468'de hipoksik ve normoksik koşullarda yüksek düzeyde eksprese edildi. CHOP, HERP ve BiP gen ekspresyon seviyeleri, LMAN2 ekspresyonu gibi hipoksik koşullar altında MDA-MB-468'de önemli ölçüde daha yüksekti.

Sonuç: LMAN2 ve diğer ER stres yanıt elemanları hipoksik koşullar altında SK-BR-3, MDA-MB-231, MDA-MB-468 ve MCF7 hücre hatlarında farklı ekspresyon profilleri göstermiştir. MCF-7 ve MDA-MB-468 hücre

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hatlarında artmış ekspresyon bulunurken, SK-BR-3 ve MDA-MB-231 hücre hatlarında azalmış ekspresyon tespit edilmiştir. Bu farklılığın altında yatan nedenin, hücre hatlarının üçlü negatif veya HER2 (+/-) durumu gibi farklı moleküler özelliklere sahip olması olduğu düşünülmektedir.

Anahtar kelimeler: ER stres, hipoksi, LMAN2, meme kanseri, apoptoz.

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Introduction

Breast cancer (BC) has a significant socioeconomic impact due to its high incidence worldwide. Recent studies have shown that significant advancements in cancer prevention and treatment, as well as a better understanding of cancer biology, have been made thanks to the rapidly evolving technologies of today. In developed nations, cancer-related fatalities continue to be common and account for about 25% of all deaths [1]. BC is in the first place for cancer diagnoses in the United States, and despite lots of studies on this, it has the second place of mortality in this list after lung cancer [2]. BC is a heterogeneous disease with a wide range of histological and molecular characteristics caused by genetic, epigenetic, and transcriptomic changes, as well as a range of clinical manifestations, therapeutic responses, and formations. The diagnosis, prognosis, and treatment of BC are all impacted by this phenotypic variation [3]. It is not entirely clear how breast cancer begins [4]. Additionally, hormonal pathways affect breast cancer. Each evaluated molecular pathway enters an even more complex biological process with the effect of hormones. For this reason, elucidating the molecular infrastructure of breast cancer is becoming more and more important. With breast cancer cell lines with different characteristics (with different hormones and receptors active), it will be more possible to understand this complex mechanism and move on to clinical studies.

The endoplasmic reticulum (ER) is the most significant organelle in eukaryotic cells that is in charge of protein folding. Properly folded proteins in the ER are transported to other organelles or the cell membrane via cargo receptor proteins. LMAN2, also known as the VIP36 protein, is a leptin-type cargo receptor protein involved in this transport. Previous research has shown that gastric, ovarian, and prostate cancers have elevated levels of the LMAN2 gene expression [5]. Additionally, epigenetic changes in neurodegenerative diseases like multiple system atrophy cause a decrease in the control of protein folding. As a result, by facilitating the transport of glycoproteins folded in the ER, this cargo protein regulates the traffic between the ER, golgi, and cell membrane [6]. By ensuring that proteins that are misfolded and have escaped from control in the ER are returned to the ER, it also actively participates in the folding and transport of proteins in the cell [7]. The fact that the protein modifications between LMAN2 and cancer are among the types of diseases caused by the loss of control led us to examine the expression level of this gene in breast cancer. Another factor that causes ER stress is hypoxia, which is defined as a lack of oxygen in the body [8]. Due to insufficient oxygen levels, hypoxia causes protein misfolding, which then causes ER stress and activates the unfolded protein response (UPR) system [9]. ATF6, IRE1, and PERK are activated by BiP, an ER chaperone in the UPR pathway, which binds to misfolded proteins and cleaves them. The cell can enter the apoptosis pathway thanks to the activation of some nucleus-to-ER communication pathways by the released PERK, IRE1, and ATF6 [10].

The LMAN2 gene encodes VIP36, which acts as a cargo receptor protein in the endoplasmic reticulum. This protein, which transports folded proteins from the ER to other organs, is also responsible for bringing misfolded proteins back to the ER. Accumulation of misfolded proteins, which is one of the elements in cancer pathogenesis, may be caused by abnormalities in carrier proteins such as VIP36. This study aimed to reveal subcellular changes in breast cancer cells. The expression level of the LMAN2 gene, which encodes the cargo receptor protein in breast cancer cells, was investigated to reveal which changes at the ER level are associated with cancer formation. Advanced analyses were planned based on the results of this study.

It is aimed at determining the gene expression level of LMAN2 and ER stress elements in hypoxia conditions known as oxygen deficiency, which has an important effect on the formation of tumorigenesis and metastasis in breast cancer.

Materials and method

Culturing cell lines

Cancer cell lines SK-BR-3 (ATCC, HTB-30), MDA-MB-231 (ATCC, HTB-26), MDA-MB-468 (ATCC, HTB-132), and MCF7 (ATCC, HTB-22) and normal breast epithelial cell CRL-4010 (ATCC) as a control were used to determine the LMAN2 gene expression level.

The cells, which were frozen in cryotubes at -196°C in a liquid nitrogen tank, were transferred to 100 mm Petri dishes after thawing and in media containing 10% serum and 1% antibiotics; they were cultured in an incubator at 5% CO_2 and 37°C. DMEM-F12 (Gibco, 11320) medium for MDA-MB-231, MDA-MB-468, MCF-7, and CRL4010; RPMI1640 (Gibco, 11875) medium was used for SK-BR-3. Fetal bovine serum (Gibco, 26140079) was used as a serum, and penicillin/streptomycin (Gibco, 15140122) was used as an antibiotic. The medium was changed every 2-3 days until these cells were fully confluent (80-90%).

Hypoxia conditions

To determine the gene expression level of the LMAN2 in breast cancer cells under hypoxia and normoxia conditions, 21% of the oxygen level was used as normoxia, and 5% oxygen level was used as hypoxia in the incubator with 5% CO_2 and 37°C.

Determination of cell viability in hypoxia conditions

MTT [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide] (Thermo Scientific, M6494) assay was performed to analyze the viability of cells under norm- and hypoxia conditions. After cells reached 80-90% confluency under appropriate conditions, they were counted and seeded in 1000 cells/well in 96-well cell culture plates. Then they cultured in norm- and hypoxia conditions for 24, 48, and 72 hours. At the end of the specified times, MTT solution was added to each well at 0.5 mg/mL final concentration and incubated for 3-4 hours. Then, DMSO (Sigma Aldrich, D2650) was added to the wells and incubated at room temperature for 10 minutes in a dark place to dissolve the formazan crystals formed by MTT. Each well was read with a microplate reader (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer) at a wavelength of 570 nm, and the cytotoxicity level was determined according to the absorbance value read. The viability levels of the cells were calculated by dividing the tested wells by the control condition.

After calculation, all results were normalized with the control cell (CRL-4010 normal mammary epithelial cell) results. The time to stay in hypoxia, which has a 50% cytotoxic effect compared to the control, was accepted as cytotoxic hours.

Determination of gene expression of LMAN2 and ER stress markers in hypoxia conditions

Following the cell viability analysis, $3x10^5$ cells/well of cells were passed into a 6-well plate and incubated for 24 hours under normal conditions. Then, cells were incubated simultaneously for 48 hours under norm- and hypoxia conditions. Following incubation, cells were harvested for total RNA isolation, cDNA synthesis, and quantitative real-time polymerase chain reaction (qRT-PCR) analysis. After 48 hours of incubation, total RNAs were obtained with the total RNA isolation kit (Thermo Scientific, K0731) in line with the manufacturer's recommendations. The purity and concentration analysis of the obtained RNAs was performed using a spectrophotometer (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer). To determine the expression level of the LMAN2 gene, cDNA synthesis using 1 µg of RNA was performed using a commercially available kit (Thermo Scientific, K1621). PCR cycling conditions for cDNA synthesis were 10 min. 25°C, 15 min. 42°C and 5 min. 85°C. The expression level of the obtained cDNAs and the genes whose primer sequences are given in Table 1 were checked. In the study, primers suitable for detecting the mRNA levels of the LMAN2 gene were determined using Primer3 and NCBI databases, and the synthesis of these primers was commercially performed by Sentromer DNA Technologies (Türkiye) as in Table 1.

Table 1. Primers and sequences used in RT-PCR

Target Gene	Primer Sequence
GAPDH	(F) 5' - CGA GAT CCC TCC AAA ATC AA - 3'
	(R) 5' - TTC ACA CCC ATG ACG AAC AT - 3'
LMAN2	(F) 5' - ACA ATG GCT CCC TGT CCT AC - 3'
	(R) 5'- CTC CCG TGA TGT CAA TGC AG - 3'
BIP	(F) 5' - GCT GAG GCT TAT TTG GGA AAG - 3'
	(R) 5' - TTA GGC CAG CAA TAG TTC CAG - 3'
CHOP	(F) 5' - AGA ACC AGG AAA CGG AAA CAG A - 3'
	(R) 5' - TCT CCT TCA TGC GCT GCT TT - 3'
HERP	(F) 5' - GGT TTA AGG CAA AGG GAA GTTC - 3'
	(R) 5' - AAA GCT GAA GCC ACC CAT AG - 3'

GAPDH: glyceraldehyde-3-phosphate dehydrogenase, LMAN2: lectin, mannose binding 2, BiP: Binding immunoglobulin protein CHOP: C/EBP homologous protein, HERP: Homocysteine-induced endoplasmic reticulum protein, F: Forward Primer Sequence R: Reverse Primer Sequence

The GAPDH gene was used as a housekeeping internal control. To evaluate the effect of hypoxia condition on ER stress, the expression levels of BiP, CHOP, and HERP genes belonging to the UPR system activated under ER stress conditions were also examined as positive controls.

RealQ Plus 2x Master Mix Green kit (Ampliqon, A323406) was prepared according to the manufacturer's recommendations and qRT-PCR was performed in 40 cycles in the thermal cycler (Bio-Rad, CFX Connect); 10 sec. 94°C, 30 sec. 55°C, 30 sec. 72°C using a temperature program. The cycle threshold values (threshold cycle/Ct) of the gene for at least two replicates of each sample examined in the study were normalized by GAPDH as the reference gene, and delta cycle threshold (ΔCt) values were calculated. ΔΔCt values were calculated by comparing them to the control group. Relative expression of each gene is calculated by using the formula $2^{-\Delta\Delta Ct}$. The represented data in the figures are the mean of technical and biological replicates ± Standard Deviation (SD).

Statistical analysis of data

All experiments were performed with at least two biological and two technical replicates. Statistical analysis of the data was done using the "SPSS 26 (IBM Inc., Chicago, IL, USA)" package program. Mann-Whitney U and Kruskal Wallis tests were used for data analysis. The statistical significance of the results obtained was evaluated over a *p*-value of 0.05, and values with a *p*-value of ≤0.05 were considered reliable.

This study has been designed as an in vitro and worked on human cell lines. This study does not require ethics committee approval.

Results

LMAN2 gene expression is differentially regulated in breast cancer cell lines

It was determined that LMAN2 gene expression decreased 0.06, 0.09, and 0.11-fold in MCF-7, MDA-MB-231, and SK-BR-3 cells, respectively, when compared to healthy breast cells. It was found that it increased 1.81 fold in MDA-MB-468 (Figure 1). According to the results obtained, LMAN2 is suppressed in MCF-7, MDA-MB-231, and SK-BR-3 breast cancer cell lines under normal conditions, while it is overexpressed in MDA-MD-468 cell lines. When the expression value of LMAN2 in different breast cancer cell lines under normoxia and hypoxia conditions was compared with the expression value in healthy breast cells, no significant result was obtained ($p>0.05$). The gene expression level for LMAN2 in different cell lines or different conditions did not show significant differences compared to the gene expression in CRL4010 under normoxia conditions.

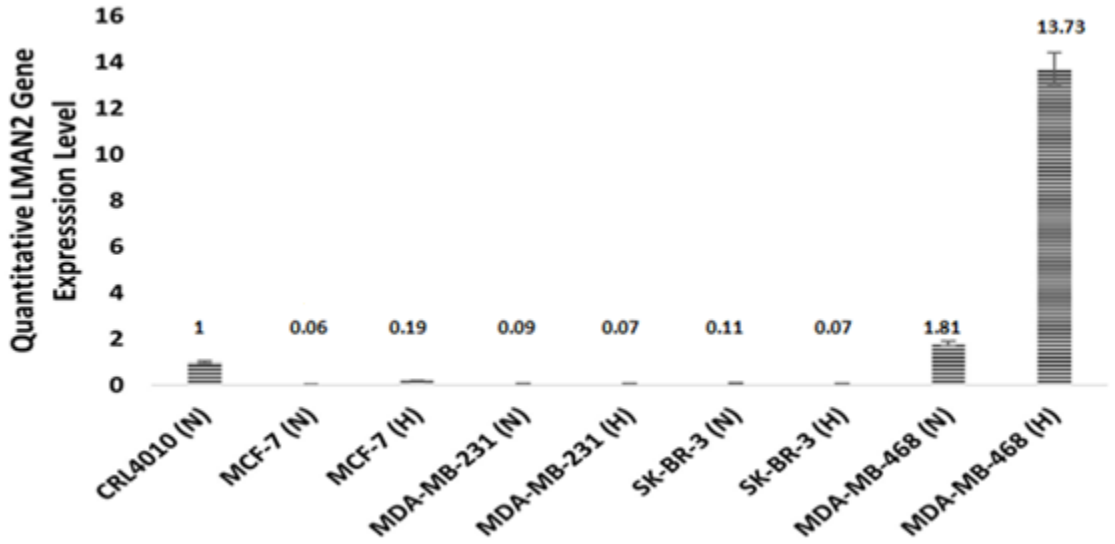

Figure 1. Regulation of LMAN2 gene expression under normoxia and hypoxia conditions

Cells were normalized with CRL4010, normal breast ephitelial cells, N: normoxia, H: hypoxia. LMAN2 gene expression in tumor and normal tissue under hypoxia and normoxia conditions was compared using the Kruskal-Wallis test. *p*=0.508, H=8.259

Hypoxia conditions affect cell viability in breast cancer cell lines

As a result of the cell viability test at the end of 24, 48, and 72 hours, breast cancer cells normalized with healthy breast epithelial cells as a negative control showed different survival patterns under norm- and hypoxia conditions (Figure 2). At the end of 24 hours, cell viability was decreased in MDA-MB-231, MDA-MB-468, and MCF-7 cells, while cell viability increased in SK-BR-3 cells under hypoxia conditions. After 48 hours, a decrease in cell viability was observed in all cancer cells, while no change was observed in the MCF-7 cell line. After 72 hours, a decrease in cell viability was detected in all breast cancer cell lines. Since the 48th-hour findings were statistically significant when the obtained data were analyzed, cells kept in hypoxic conditions for 48 hours were studied to determine gene expression levels.

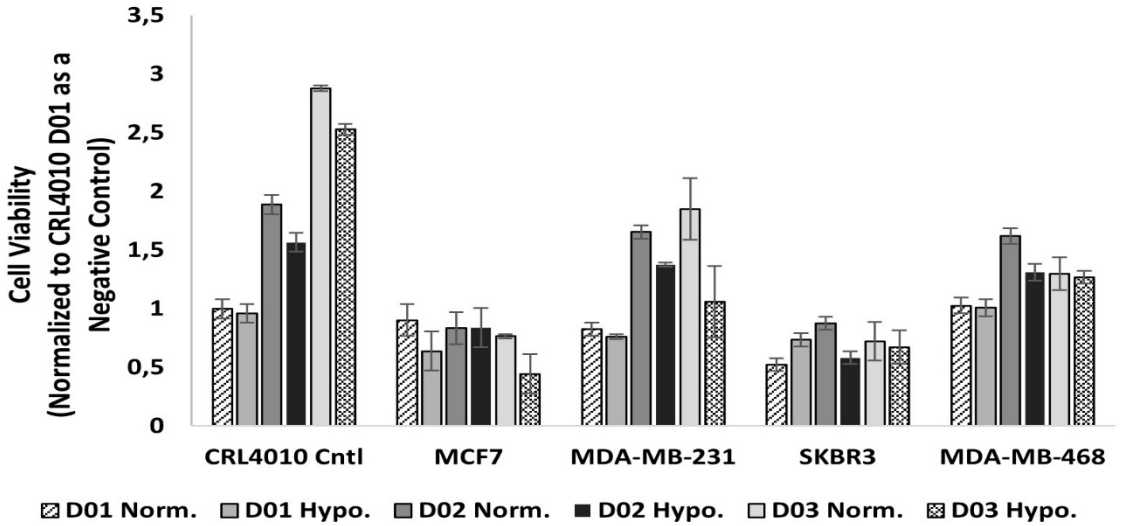

Figure 2. Comparison of cell viability under norm- and hypoxia conditions for 24, 48, and 72 hours

Cntl: control, Norm.: normoxia, Hypo.: hypoxia, D01: 24 hours, D02: 48 hours, D03: 72 hours

LMAN2 gene expression under hypoxia is differentially regulated in breast cancer cell lines

In determining the gene expression level under hypoxia conditions, the 48-hour hypoxia period was taken into account according to the cell viability analysis. It was observed that the LMAN2 gene expression level increased 1.28-fold in healthy breast epithelial cells under hypoxia conditions compared to normoxia conditions. CHOP gene expression, which is the other ER stress-regulating gene, was suppressed 0.91-fold, and on the other hand, HERP and BiP gene expression levels increased 1.41 and 1.89-fold, respectively (Figure 3). Mann-Whitney U test was performed to compare gene expression levels in hypoxia and normoxia conditions in the CRL4010 cell line. There were no statistically significant results (p=0.487).

Moreover, there were different LMAN2 gene expression levels in studied cancer cell lines. The LMAN2 expression level was increased 2.95 and 7.57-fold, respectively, in MCF-7 and MDA-MB-468 cell lines under hypoxia in comparison with control cell lines in normoxic conditions. However, it was found that this gene expression was downregulated in MDA-MB-231 and SK-BR-3 cell lines 0.75 and 0.65-fold, respectively (Figure 4). When these data were compared statistically with the Mann-Whitney U test, the increase in the LMAN2 gene only in the MCF-7 cell line among the 4 cell lines was found to be statistically significant (p=0.037).

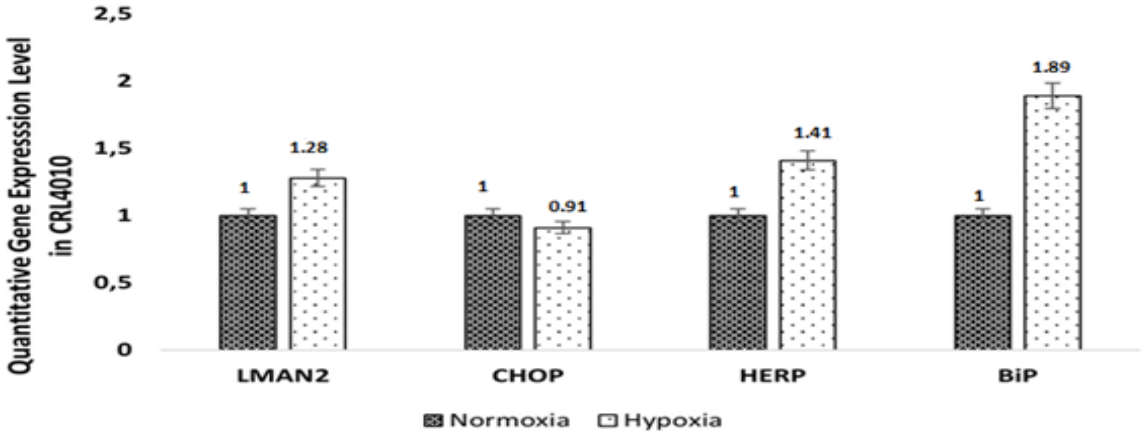

Figure 3. Gene expression levels of genes regulating LMAN2 and ER stress in healthy mammary epithelial cells under 48 hours hypoxia conditions

LMAN2: lectin, mannose binding 2, BiP: Binding immunoglobulin protein, CHOP: C/EBP homologous protein, HERP: Homocysteine-induced endoplasmic reticulum protein. Mann-Whitney U test was performed to compare gene expression levels in hypoxia and normoxia conditions in the CRL4010 cell line. p=0.487, U=3.000

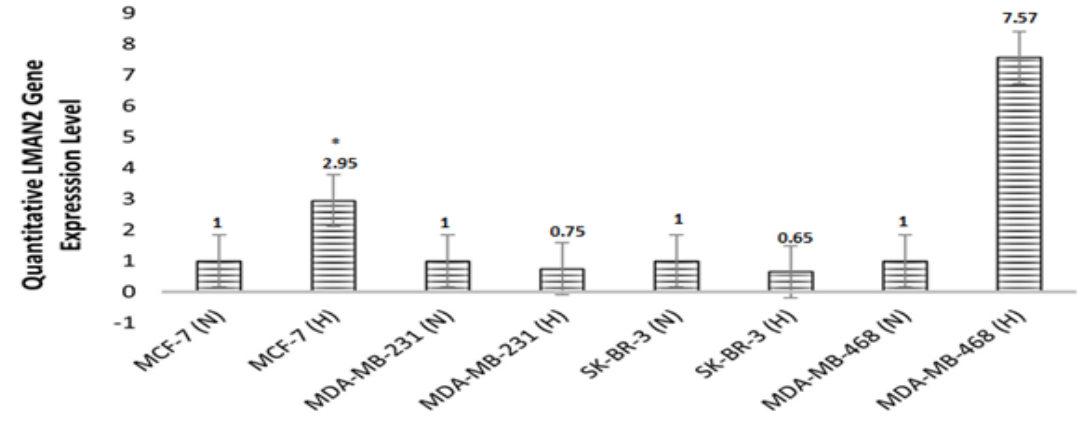

Figure 4. The gene expression level of LMAN2 gene expression at 48 hours hypoxia condition

Cancer cells normalized with normoxia levels. Comparison according to cell line in hypoxia and normoxia conditions was made using the Mann-Whitney U test. N: normoxia, H: hypoxia, *: statistically significant p=0.037 U=0.000

The fact that hypoxia is a factor triggering ER stress and the increased LMAN2 expression in healthy cells suggests that the LMAN2 gene is involved in the ER stress response. In addition, the difference in gene expression of the LMAN2 gene in cancer cells under hypoxia is thought to be due to the difference in the histopathological characteristics of these cell lines. When the CHOP gene expression level, which is one of the ER stress response elements, was normalized with CRL-4010 under hypoxia conditions, it was observed that it increased 9.45-fold in MDA-MB-468 cells and 1.18-fold in MDA-MB-231. On the other hand, it was determined that it decreased 0.12 and 0.10-fold in MCF-7 and SK-BR-3 cells, respectively (Figure 5). When the expression value of CHOP in different breast cancer cell lines under normoxia and hypoxia conditions was compared with the expression value in healthy breast cells, no significant result was obtained (p>0.05).

When CHOP gene expression was normalized with the expression level of each cancer cell line under normoxia conditions, it was suppressed 0.94, 0.59, 0.38-fold in MCF-7, MDA-MB-231, and SK-BR-3 cells, respectively. On the contrary, it was observed that it increased 3.36-fold in the MDA-MB-468 cell lines (Figure 6). This change in CHOP gene expression in different breast cancer cell lines was not found to be statistically significant (p>0.05).

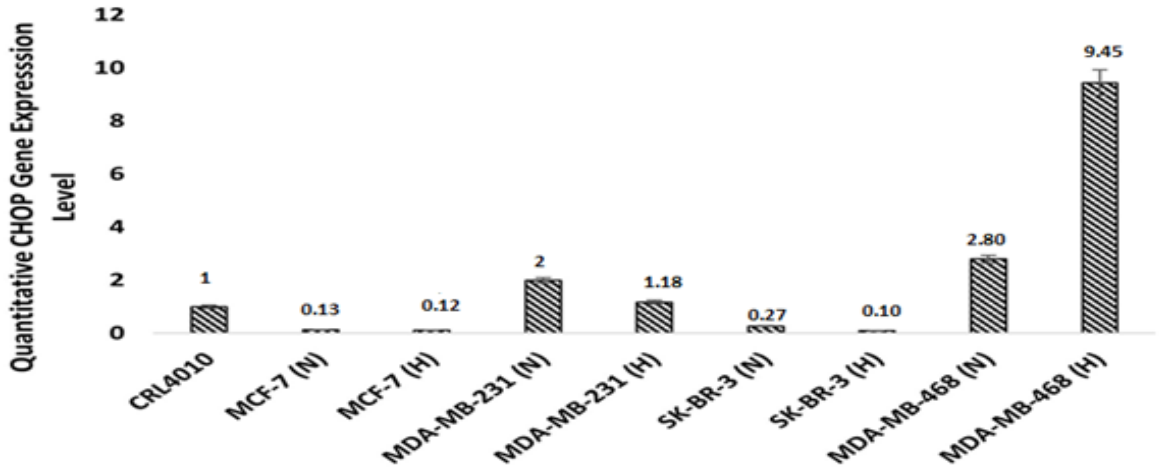

Figure 5. Regulation of CHOP gene expression under normoxia and hypoxia conditions

Cells were normalized with CRL4010, normal breast ephitelial cells, N: normoxia, H: hypoxia
CHOP gene expression in tumor and normal tissue under hypoxia and normoxia conditions was compared using the Kruskal-Wallis test. p=0.738, H=6.024

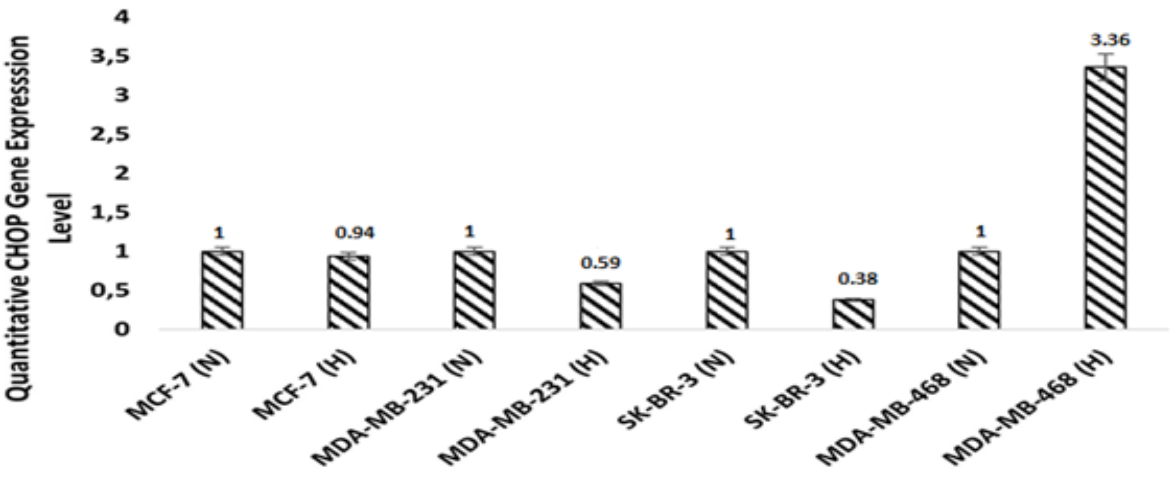

Figure 6. The gene expression level of CHOP gene expression at 48 hours hypoxia condition

Cancer cells normalized with normoxia levels, Comparison according to cell line in hypoxia and normoxia conditions was made using the Mann-Whitney U test. N: normoxia, H: hypoxia, p=0.487, U=3.000

It was also observed that the BiP gene expression level, which is one of the other ER stress response elements, increased 19.84 times in MDA-MB-468 cells and 3.41 times in MDA-MB-231 when normalized with CRL4010 under hypoxia conditions. It was determined that it decreased 0.35 and 0.93 in MCF-7 and SK-BR-3 cells, respectively (Figure 7). When the expression value of BiP in different breast cancer cell lines under normoxia and hypoxia conditions was compared with the expression value in healthy breast cells, no significant result was obtained (p>0.05). When BiP gene expression was normalized with the expression level of each cancer cell under normoxic conditions, it increased 2.04, 17.51, and 1.34-fold in MCF-7, MDA-MB-468, and SK-BR-3 cells, respectively, and in the other hand, it was found to be suppressed 0.35-fold in MDA-MB-231 cell lines (Figure 8). The increase in BiP gene only in the SK-BR-3 cell line was found to be statistically significant with the Mann-Whitney U test (p=0.037).

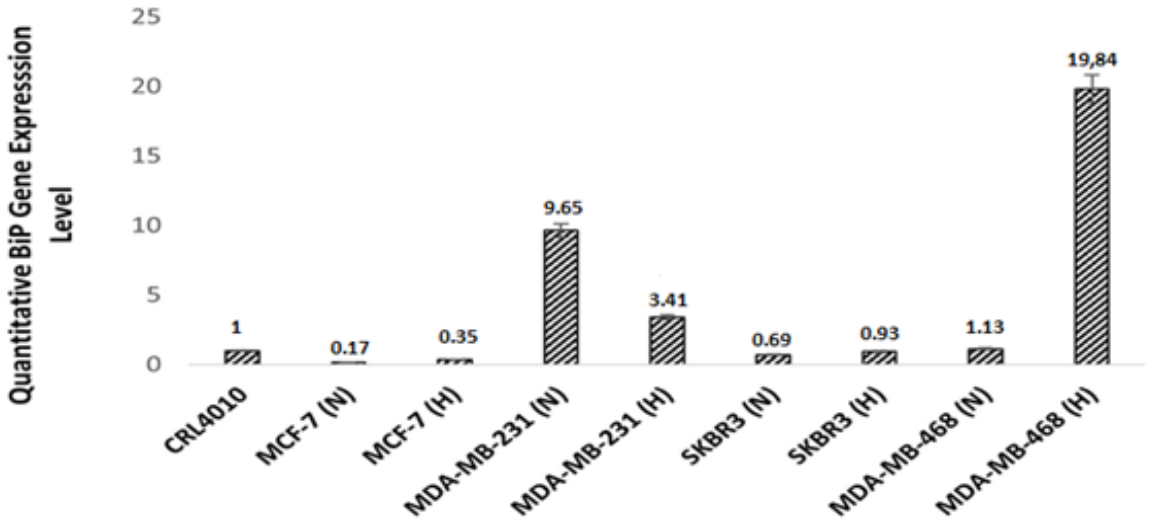

Figure 7. Regulation of BiP gene expression under normoxia and hypoxia conditions

Cells were normalized with CRL4010, normal breast ephitelial cells, N: normoxia, H: hypoxia
BiP gene expression in tumor and normal tissue under hypoxia and normoxia conditions was compared using the Kruskal-Wallis test. p=0.234, H=11.643

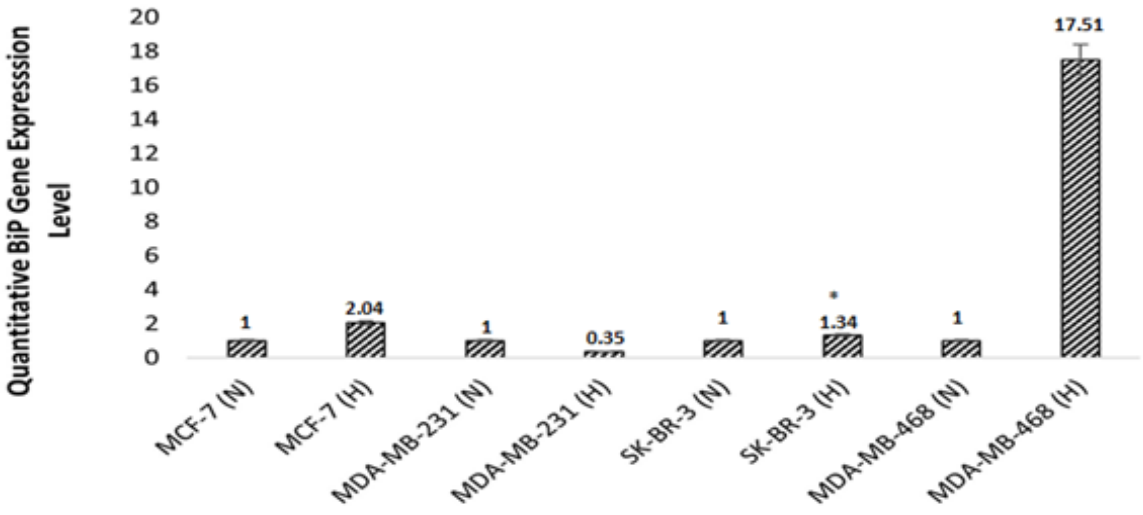

Figure 8. The gene expression level of BiP gene expression at 48 h hypoxia condition

Cancer cells normalized with normoxia levels, Comparison according to cell line in hypoxia and normoxia conditions was made using the Mann-Whitney U test. N: normoxia, H: hypoxia, p=0.487, U=3.000 for MCF-7, MDA-MB-231 and MDA-MB-468; p=0.037, U=0.000 for SK-BR-3

When HERP gene expression level, another ER stress response element, was normalized with CRL-4010 under hypoxia conditions, it was observed that it increased 13.45 times in MDA-MB-468 cells and 1.41 times in MDA-MB-231. It was determined that it decrease 0.24 and 0.23-fold in MCF-7 and SK-BR-3 cells, respectively (Figure 9). The gene expression level for HERP in different cell lines or different conditions did not show significant differences compared to the gene expression in CRL4010 under normoxia conditions (p>0.05).

On the other hand, when HERP gene expression was normalized with the expression level of each cancer cell under normoxic conditions, it was increased by 3.46 and 1.04 times in MDA-MB-468 and MDA-MB-231 cells, respectively, moreover, in MCF-7 and SK-BR-3 cells, it was found to be suppressed 0.97 and 0.77, respectively (Figure 10). The decrease in HERP gene only in the SK-BR-3 cell line was found to be statistically significant with the Mann-Whitney U test (p=0.037).

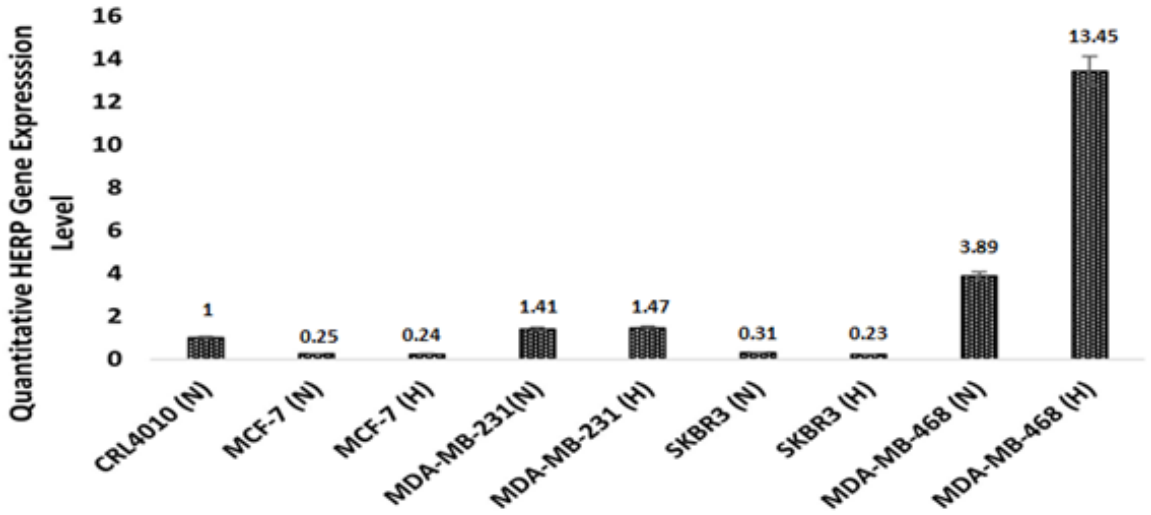

Figure 9. Regulation of HERP gene expression in normoxia and hypoxia conditions

Cells were normalized with CRL4010, normal breast ephitelial cells, N: normoxia, H: hypoxia
HERP gene expression in tumor and normal tissue under hypoxia and normoxia conditions was compared using the Kruskal-Wallis test. p=0.847, H=4.853

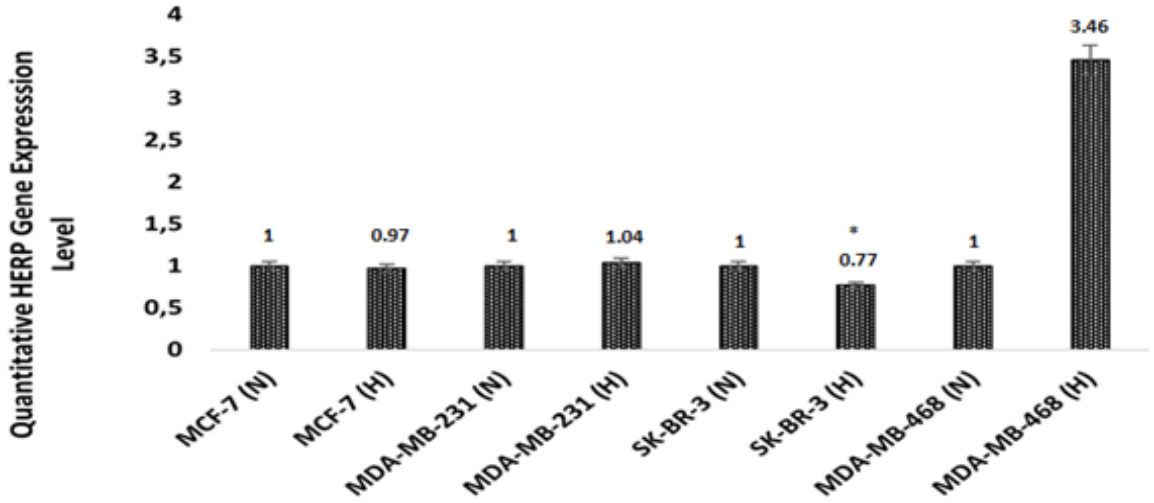

Figure 10. The gene expression level of HERP gene expression at 48 h hypoxia condition

Cancer cells normalized with normoxia levels, Comparison according to cell line in hypoxia and normoxia conditions was made using the Mann-Whitney U test. N: normoxia, H: hypoxia, p=0.487, U=3.000 for MCF-7, MDA-MB-231 and MDA-MB-468; p=0.037, U=0.000 for SK-BR-3

As a result, the gene expression level of LMAN2 differs according to the type of breast cancer under normal conditions, and it also differs according to the cell type in hypoxia conditions. While LMAN2 and BiP overexpressed in the MCF-7 cell line, HERP, and CHOP's gene expression levels were decreased. In the MDA-MB-231 cell line, LMAN2, CHOP, and BiP gene expression levels are decreased while HERP is increased. In SK-BR-3 cells, on the other hand, LMAN2, CHOP, and HERP gene expression levels were increased while BiP *was* downregulated. Interestingly, the expression levels of all genes are excessively increased in MDA-MB-468 cells.

Discussion

The endoplasmic reticulum is the organelle that is involved in the intracellular organization and the realization and control of folding, which is one of the post-translational modifications of proteins. This organelle, which has a unique system, arranges itself by regulating the release of misfolded proteins into or out of the cell. In this system, correctly folded proteins are transported by the cargo receptor protein VIP36. ER stress develops and the cell switches to apoptosis when there is an accumulation of folded proteins inside the cell. Cancer cells, which frequently resist apoptosis, are regulated differently from normal cells in terms of this physiological regulation. For instance, the cancer cell naturally escapes apoptosis and continues to exist in hypoxia, even though hypoxia is one of the conditions that lead to ER stress in the cell and triggers it to undergo apoptosis. In addition, hypoxia studies have shown that the microenvironment both promotes cancer cell metastasis and confers resistance to standard therapeutics [11]. This study aimed to determine how normoxia and hypoxia affect the gene expression level of LMAN2, which produces the VIP36 protein, one of the ER cargo receptors. One of the hypotheses put forward at the beginning of the study, "The pathogenesis of breast cancer under normal conditions is associated with the change in the expression level of the LMAN2 gene" was supported by the detection of different expression levels of LMAN2 in breast cancer cells showing different histopathological features under normoxia conditions. As a result of the experiments, LMAN2 gene expression was suppressed in MCF-7, SK-BR-3, and MDA-MB-231 cell lines. The MCF-7 cell line, which has a better prognosis than the others, has Receptor Tyrosine-protein Kinase erbB-2 (HER2-), Estrogen Receptor (ER +), and Progesterone Receptor (PR +/-) immune profiles and is in the luminal class A. LMAN2 was suppressed in SK-BR-3, which is in the HER2 (+) class, which has a moderate prognosis without ER and PR, and MDA-MB-231, which is known as triple negative with a poor prognosis and in the low claudin class [12]. However, it increased 1.18 times in the MDA-MB-468 cell line, which is also in the basal class with a triple-negative prognosis. However, this increase is very small compared to the decrease in other cell lines. In the studies conducted multiple databases, they found high levels of LMAN2 to be associated with poor prognosis in HER2+ breast carcinoma. They also found that LMAN2 gene expression was regulated at different levels according to the clinicopathological features of breast cancer [13, 14]. When studies on different cancer types were investigated, it was reported that LMAN2 gene expression level increased in the prostate, gastric, and ovarian cancers [6, 10, 15, 16].

As ER stress is induced in hypoxia conditions, increased gene expression of the transcription factor CHOP is observed because this transcription factor induces apoptosis by stimulating pro-apoptotic pathways [17]. However, it is expected that the expression of the CHOP factor, which stimulates the pro-apoptotic pathway, will decrease in cancer cells as it will help them escape from hypoxia apoptosis. In this study, CHOP was suppressed in MCF-7, MDA-MB-231, and SK-BR-3 cell lines under hypoxia conditions. A study conducted in MCF-7 cells showed that CHOP expression was increased by inducing ER stress with different external factors under hypoxia conditions [18]. Furthermore, CHOP was significantly increased in MDA-MB-468 cells. At this stage, the reason for the change in the CHOP gene expression level can be attributed to the molecular infrastructure and clinicopathological features. Under hypoxia conditions, the expression level of the BiP chaperone was increased in cells other than the MDA-MB-231 cell line. In the study, it was determined that BiP expression increased, apoptosis decreased, and migration triggered in hypoxic conditions in breast cancer cell lines [1]. While HERP was suppressed in MCF-7 and SK-BR-3 cells, it was increased in

MDA-MB-231 and MDA-MB-468 cells. HERP expression is one of the enzymes involved in protein degradation that induces ER stress, which is suppressed under hypoxia conditions in cancer cells, as in CHOP [19].

The most important limitation of the study is that it was only studied at the gene level, and studies are aimed to be conducted at the protein level. In addition, new studies are planned to investigate the relationship between LMAN2 and ER stress and apoptosis in more detail.

In conclusion, different expression profiles in cancer cell lines may be due to the molecular infrastructure of breast cancer. Under hypoxic conditions, LMAN2 gene expression was higher in MCF-7 and MDA-MB-468 cells than in MDA-MB-231 and SK-BR-3 cells. According to these findings, the LMAN2 gene is suppressed in cancer cells under normoxic conditions while its expression is increased under hypoxic conditions. There is no information in the literature about the level of LMAN2 under hypoxic conditions. Still, since LMAN2 is involved in this pathway, it can be assumed that its expression should decrease further and increase if it is engaged in the migration-promoting pathway.

In conclusion, hypoxia causes an increase in LMAN2 expression in healthy breast epithelial cells. It might be a protein involved in the ER stress pathway. To support these interpretations, more in-depth research is required.

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References

1. Banach A, Jiang YP, Roth E, Kuscu C, Cao J, Lin RZ. CEMIP upregulates BiP to promote breast cancer cell survival in hypoxia. *Oncotarget*. 2019;10(42):4307-4320. doi:10.18632/oncotarget.27036
2. Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics, 2023. *CA Cancer J Clin*. 2023;73(1):17-48. doi:10.3322/caac.21763
3. Eliyatkın N, Yalçın E, Zengel B, Aktaş S, Vardar E. Molecular Classification of Breast Carcinoma: From Traditional, Old-Fashioned Way to A New Age, and A New Way. *J Breast Health*. 2015;11(2):59-66. doi:10.5152/tjbh.2015.1669
4. Landskron G, De la Fuente M, Thuwajit P, Thuwajit C, Hermoso MA. Chronic inflammation and cytokines in the tumor microenvironment. *J Immunol Res*. 2014;2014:149185. doi:10.1155/2014/149185
5. Bettencourt C, Foti SC, Miki Y, et al. White matter DNA methylation profiling reveals deregulation of HIP1, LMAN2, MOBP, and other loci in multiple system atrophy. *Acta Neuropathol*. 2020;139(1):135-156. doi:10.1007/s00401-019-02074-0
6. Davalieva K, Kiprijanovska S, Maleva Kostovska I, et al. Comparative Proteomics Analysis of Urine Reveals Down-Regulation of Acute Phase Response Signaling and LXR/RXR Activation Pathways in Prostate Cancer. *Proteomes*. 2017;6(1):1. doi:10.3390/proteomes6010001
7. Demiryürek AN, Göktürk Ö, Saracaloglu A, Demiryürek S, Demiryürek AT. Protective effects of verbenalin and (+)-eudesmin against 6-hydroxydopamine-induced oxidative/nitrosative stress in SH-SY5Y cells. *Mol Biol Rep*. 2023;50(1):331-338. doi:10.1007/s11033-022-08039-z
8. Maekawa H, Inagi R. Stress Signal Network between Hypoxia and ER Stress in Chronic Kidney Disease. *Front Physiol*. 2017;8:74. doi:10.3389/fphys.2017.00074
9. Díaz Bulnes P, Saiz ML, López Larrea C, Rodríguez RM. Crosstalk Between Hypoxia and ER Stress Response: A Key Regulator of Macrophage Polarization. *Front Immunol*. 2020;10:2951. doi:10.3389/fimmu.2019.02951
10. Zhou D, Li X, Zhao H, et al. Combining multi-dimensional data to identify a key signature (gene and miRNA) of cisplatin-resistant gastric cancer. *J Cell Biochem*. 2018;119(8):6997-7008. doi:10.1002/jcb.26908
11. Yao X, Li W, Li L, et al. YTHDF1 upregulation mediates hypoxia-dependent breast cancer growth and metastasis through regulating PKM2 to affect glycolysis. *Cell Death Dis*. 2022;13(3):258. doi:10.1038/s41419-022-04711-1

12. Teles RHG, Moralles HF, Cominetti MR. Global trends in nanomedicine research on triple negative breast cancer: a bibliometric analysis. *Int J Nanomedicine*. 2018;13:2321-2336. doi:10.2147/IJN.S164355

13. Zhang D, Ye L, Hu S, Zhu Q, Li C, Zhu C. Comprehensive Analysis of the Expression and Prognostic Value of LMAN2 in HER2+ Breast Cancer. *J Immunol Res*. 2022;2022:7623654. doi:10.1155/2022/7623654

14. Yang G, Höti N, Chen SY, et al. One-Step Enrichment of Intact Glycopeptides From Glycoengineered Chinese Hamster Ovary Cells. *Front Chem*. 2020;8:240. doi:10.3389/fchem.2020.00240

15. Seagle BL, Eng KH, Yeh JY, et al. Discovery of candidate tumor biomarkers for treatment with intraperitoneal chemotherapy for ovarian cancer. *Sci Rep*. 2016;6:21591. doi:10.1038/srep21591

16. Marimuthu A, Subbannayya Y, Sahasrabuddhe NA, et al. SILAC-based quantitative proteomic analysis of gastric cancer secretome. *Proteomics Clin Appl*. 2013;7(5-6):355-366. doi:10.1002/prca.201200069

17. Iurlaro R, Muñoz Pinedo C. Cell death induced by endoplasmic reticulum stress. *FEBS J*. 2016;283(14):2640-2652. doi:10.1111/febs.13598

18. Isohashi F, Endo H, Mukai M, Inoue T, Inoue M. Insulin-like growth factor stimulation increases radiosensitivity of a pancreatic cancer cell line through endoplasmic reticulum stress under hypoxic conditions. *Cancer Sci*. 2008;99(12):2395-2401. doi:10.1111/j.1349-7006.2008.00970.x

19. Hori O, Ichinoda F, Yamaguchi A, et al. Role of Herp in the endoplasmic reticulum stress response. *Genes Cells*. 2004;9(5):457-469. doi:10.1111/j.1356-9597.2004.00735.x