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# SIGNIFICANCE OF FERRITIN EXPRESSION IN FORMATION OF MALIGNANT PHENOTYPE OF HUMAN BREAST CANCER CELLS

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***Aim:*** **The aim of our study is to investigate the disorders of ferritin functioning in breast cancer (BC) cells of different molecular subtype.** ***Materials and Methods:*** **The cell lines used in the analysis include T47D, MCF-7, MDA-MB-231, MDA-MB-468, MCF-10A, and 184A1. Ferritin heavy chains (FTH) expression was studied by immunohistochemical method. "Free iron" content and superoxide dismutase (SOD) activity were determined by means of EPR spectroscopy. Reactive oxygen species (ROS) level and peculiarities of microRNA expression in studied cell lines were evaluated using flow cytometry and PCR analysis, respectively.** ***Results:*** **It has been demonstrated that FTH expression directly correlates with proliferative activity of cells of both luminal ($r = 0.51$) and basal subtypes ($r = 0.25$), inversely correlates with expression of steroid hormones in cells of basal subtype (ER: $r = -0.46$; PR: $r = -0.44$) and does not depend on tumorigenic activity of both subtypes of studied cells ($r = 0.12$ and $r = 0.9$). Obtained data are the evidence that cells of luminal subtype B (MCF-7 cell line) and basal subtype (MDA-MB-231 and MDA-MB-468 cell lines) with high proliferative activity contain the highest level of free iron ($2.9 \pm 0.19 \cdot 10^{16}$ and $3.0 \pm 0.22 \cdot 10^{16}$) that can be consequence of intensive use of this element by cells, which actively divide and grow. Along with it, in cell of lines of basal subtype MDA-MB-231 and MDA-MB-468, high level of FTH ($254 \pm 2.3$ and $270 \pm 1.9$) is being detected in consequence of increase of level of free iron, ROS ($11.3 \pm 1.05$ and $7.27 \pm 0.26$) and SOD ($9.4 \pm 0.24$ and $8.5 \pm 0.18$) as well as decrease of expression of microRNA 200b. In contrast, cells of luminal subtype B of MCF-7 line were distinguished by high expression of microRNA 200b and low ferritin level ($125 \pm 2.7$).** ***Conclusion:*** **Obtained data demonstrate that tumor cells, which are referred to different molecular subtypes, are characterized by changes in system of support of balance of intercellular iron and certain associations of studied factors.**

***Key Words:*** **breast cancer, cell lines, luminal subtype, basal subtype, ferritin, "free iron", ROS, SOD, miRNA 200b.**

Breast cancer (BC) occupies the first place in cancer-related morbidity among female population in Ukraine [1]. In recent years, prognosis of BC clinical course is based on determination of its molecular subtypes namely, luminal A, luminal B, basal or "triple negative", "HER2/neu-positive". These subtypes are characterized by different response to the therapy, differential course and prognosis of the disease [2–4]. Despite wide application of mentioned classification in clinical practice, it has series of essential disadvantages, since BC within the limits of even one subtype is quite heterogeneous disease both by molecular phenotype and clinical course [5, 6]. Last statement can be confirmed by results of our previous studies, according to which biomolecular markers which characterize metastatic potential and invasive activity of BC cells of certain molecular subtype, have been studied [7].

It is known iron plays an important role in pathogenesis of many diseases, including cancer [8]. Iron-containing proteins including heme-containing proteins of respiratory chain and ribonucleotide reductase are involved in wide range of biological processes: support of respiratory function, DNA synthesis, cellular death and oxidative stress [8–10]. However, "free" iron promotes the formation of dangerous reactive oxygen species (ROS) in cells [11]. Thus, delicate balance between useful and harmful action of iron determines survival and functioning of cells. One of the proteins, which control this balance, is iron-containing protein ferritin.

Ferritin is macromolecular complex, which consists of 24 subunits of two types: heavy (H) and light (L) [12]. Each organ or tissue, depending on physiological functions, contains certain quantity of H- and L-subunits in ferritin [13], and ferritin heavy chain (FTH) content is increased in blood serum of BC patients before clinical signs of BC. Therefore, FTH determination can be used for determination of risk among patients when carrying out screening tests [14].

The main function of ferritin is intracellular deposition of iron (4500 atoms of iron per ferritin molecule) in soluble, nontoxic and physiologically available for organism form [15–17]. Moreover, H-subunit of ferritin acts as peroxidase, i.e. oxidizes iron with formation of such side products as ROS, for instance, $H_2O_2$, which, as it has been stated above, has extremely dangerous consequences for cell [18].

According to the data of literature, cancer development is accompanied with reprogramming of iron metabolism in different ways that finally causes accumulation of iron directly in tumor tissue and cells of its microenvironment [14]. In particular, levels of iron and ferritin in BC biopsy material are 5–6 times higher than in benign tumors. It has been shown that ferritin content in blood serum is directly proportional to the content of iron and is increased in the cases of ovarian, prostate, testicular, pancreatic and hepatocellular cancer, lymphogranulomatosis, acute leukemia, etc. [16]. Also, it has been determined that high serum ferritin in BC patients correlates with disease stage [19]. Our previous *in vitro* studies have

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*Abbreviations used*: BC – breast cancer; ER – estrogen receptor; FTH – ferritin heavy chain; PR – progesterone receptor; ROS – reactive oxygen species; SOD – superoxide dismutase.

shown that the highest ferritin level and significant decrease of microRNA200b expression are observed in human BC cells, which are characterized by aggressive mesenchymal phenotype [20]. Thus, the data evidence on significance of ferritin for occurrence and progression of BC.

The aim of present research was to study peculiarities of disorders of ferritin functioning in BC cells of different molecular subtype.

## MATERIALS AND METHODS

***Cell lines, cell culture and reagents.*** In the study, 6 BC cell lines (T47D, MCF-7, MDA-MB-231, MDA-MB-468, MCF-10A and 184A1) were used.

T47D cells were cultured in RPMI-1640 medium (Sigma), supplemented with 0.2 U/ml of bovine insulin and 10% fetal bovine serum (FBS). MCF-7 cells were cultured in Eagle's Minimum Essential Medium (Sigma), supplemented with 0.01 mg/ml of human recombinant insulin and 10% FBS. MDA-MB-231 and MDA-MB-468 cells were cultured in Leibovitz's L-15 medium (Sigma), supplemented with 10% FBS. MCF-10A cells were cultured in MEBM medium (Lonza), supplemented with 100 ng/ml cholera toxin. 184A1 cells were cultured in MEBM medium (Lonza), supplemented with 0.005 mg/ml transferrin and 1 ng/ml cholera toxin. Cells were received from the Bank of the Cell Lines from Human and Animal Tissues of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology NAS of Ukraine.

***Immunocytochemical assay.*** The cells grown up of cover slips were fixed in ice-cold methanol: acetone (1:1) at –20 °C for 120 min and incubated with 1% BSA solution for 20 min. For immunocytochemistry, primary monoclonal antibodies anti-FTH (1:150; anti-FTH (GTX62020; GeneTex, Irvine, CA, USA)), UltraVision LP Detection System (Lab Vision, Thermo Scientific) and DAB Quanto (Thermo Scientific) were used according to the instructions of the manufacturers. When immunocytochemical reaction was completed, the cells were stained with hematoxylin by Mayer and placed in Faramount Aqueous Mounting Medium (DakoCytomation, Denmark). The acquisition of results was made by light microscopy (x1000, oil immersion) with the use of classical H-Score method:

$$S = 1 \cdot N_{1+} + 2 \cdot N_{2+} + 3 \cdot N_{3+},$$

where S — "H-Score" index, $N_{1+}$, $N_{2+}$ and $N_{3+}$ — number of cells with low, medium or high expression of the marker [21]. Low level of studied markers expression — from 0 to 100 H-scores, medium level — from 100 to 200 H-scores and high level — from 200 to 300 H-scores.

***Low-temperature Fe(III) EPR.*** After 24 h of culturing, the cells were scrapped, washed in PBS, centrifuged at 1000 g for 10 min at 4 °C and the pellet was re-suspended in PBS. The suspension containing $2 \cdot 10^6$ cells was transferred into EPR tubes and immediately frozen in liquid nitrogen. The level of free iron was determined by a low-temperature EPR method [22]. Briefly, samples were maintained at –196 °C during recording of the spectra using a finger Dewar filled with liquid nitrogen. The following parameters were used for the low-temperature EPR: sweep width 1525 G; frequency 9.15 GHz; microwave power 40 mW; modulation amplitude 10.0 G and modulation frequency 100 kHz. The g-value was calculated using the standard formula:

$$g = h\nu/\beta H,$$

where h — Planck's constant, ν — frequency, β — Bohr magneton and H — external magnetic field at resonance.

***Measurement of intracellular ROS.*** CM-H2DCFDA, a lipid soluble membrane permeable dye upon entering cells undergoes deacetylation by intracellular esterases and forms the more hydrophilic, nonfluorescent dye dichlorodihydrofluorescein (DCFH2). This is subsequently oxidized by ROS with formation of a highly fluorescent oxidation product, Dichlorofluorescein (DCF). The generated fluorescence is directly proportional to the amount of ROS. Fluorescence was analyzed by flow cytometry. After centrifugation (1500 rpm for 5 min) cells were resuspended in PBS, incubated for 30 min at 37 °C with CM-H2DCFDA (10 mM) for measurement of ROS. Positive control with 25 μM $H_2O_2$ was also made (data not presented). Cells were masked then and analysed by FC. Fluorescence was acquired in the log mode and expressed as geometrical mean fluorescence channel (GMean). Acquisition was performed on 10,000 gated events.

***Measurement of SOD.*** SOD activity was detected as described earlier [23]. Briefly, cells were homogenized in glass homogenizer with 2 ml of 0.1 M PBS. After centrifugation at 3000 rpm for 20 min supernatant was analyzed for SOD activity using radiospectrometer EPR-1307 at room temperature as described in [23].

***MicroRNA expression analysis.*** Total RNA extraction from the cells was performed using a commercial kit "Rhibo-zol" (Amplisense, Russia) as per the manufacturer's protocol. Concentration of isolated RNA was determined using spectrophotometer "NanoDrop 2000c" (ThermoScientific, USA). Purity of isolated RNA is controlled by E260/E280 ratio. RNA then was dissolved in TE buffer and stored at –20 °C. RNA was reverse transcribed to cDNA with gene specific primers according to the TaqMan MicroRNA Assay protocol (PE Applied Biosystem) using the TaqMan MicroRNA Reverse Transcription Kit. Real-time PCR was performed using an Applied Biosystems 7500 real-time PCR instrument equipped with a 96-well reaction block. The 20 μl PCR mix included 5 μl RT product, 10 μl TaqMan Universal PCR master mix, and 1 μl of primers from TaqMan MicroRNA Assay and was performed for 15 s at 95 °C and 1 min at 60 °C for 40 cycles followed by the thermal denaturation protocol. The threshold cycle (Ct) was determined using default threshold settings. The Ct value is defined as the fractional cycle number at which the fluorescence passesthe fixed threshold. All experiments were done in triplicates and repeated three

times. RNU48 miRNA was used as an internal control to normalize RNA input in thereal-time RT-PCR assay. The expression of microRNA relative to RNU48 miRNA was determined using the $2^{-\Delta CT}$ method.

***Statistical analysis.*** Statistical processing of the obtained results was carried out with the help of mathematical program of medical and biological statistics STATISTICA 6.0. Calculation and comparison of the significance of differences between the average values was carried out with usage of Student's *t*-criterion; correlation analysis was carried out using the Pearson correlation coefficient. Significant were considered the differences with the probability not less than 95% ($p < 0.05$).

## RESULTS AND DISCUSSION

Immunohistochemical study of FTH expression has shown its different expression levels in BC cell lines (Table 1). The highest FTH expression (2–3 times higher compared with cells of luminal type) has been detected in basal subtype cell lines, especially in MDA-MB 231 and MDA-MB 468 cells. Low and medium FTH expression have been observed in cells of luminal subtype. Correlation analysis allowed us to determine inverse dependence of FTH expression on receptor status in basal subtype cells (Table 2).

**Table1.** Features of expression of FTH, level of "free iron" content, ROS and SOD activity in BC cells of different molecular subtype

| Cellular lines | Molecular subtype | Level of expression of FTH, H-score | Quantity of paramagnetic centers of "free iron", spins/ml | Level of ROS generation, G/Mean | SOD activity, U/ml |
|---|---|---|---|---|---|
| T 47 D | Luminal A | 94±1.6 | $0.47±0.1·10^{16}$ | 2.71±0.45 | 5.8±0.18 |
| MCF-7 | Luminal B | 125±2.7 | $0.56±0.1·10^{16}$ | 3.1±0.29 | 6.9±0.13 |
| MDA-MB 231 | Basal subtype | 254±2.3 | $2.9±0.19·10^{16}$ | 11.3±1.05 | 9.4±0.24 |
| MDA-MB 468 | Basal subtype | 270±1.9 | $3.0±0.22·10^{16}$ | 7.27 ±0.26 | 8.5±0.18 |
| MCF-10 A | Basal subtype | 223±1.8 | $1.0±0.3·10^{16}$ | 3.9±0.15 | 6.5±0.31 |
| 184 A 1 | Basal subtype | 206±1.9 | $1.4±0.35·10^{16}$ | 4.94±0.22 | 7.3±0.11 |

**Table 2.** Coefficients of correlation between expression of FTH and other studied indexes of BC cells of different molecular subtypes

| Indexes | Value of correlation coefficient | |
|---|---|---|
| | Luminal type | Basal type |
| FTH/ER | −0.12 | −0.46* |
| FTH/PR | −0.13 | −0.44* |
| FTH/Ki-67 | 0.51* | 0.25* |
| FTH/colony formation | 0.12 | 0.09 |
| FTH/number of paramagnetic centers of "free iron" | 0.44* | 0.39* |
| FTH/level of ROS generation | 0.32* | 0.22* |
| FTH/activity of SOD | 0.36 * | 0.14* |
| FTH/colony formation | 0.12 | 0.09 |
| FTH/number of paramagnetic centers of "free iron" | 0.44* | 0.39* |
| FTH/activity of SOD | 0.36* | 0.14* |

*Notes*: ER – estrogen receptor; PR – progesterone receptor; *p < 0.05.

Since we have demonstrated in previous studies that cells of luminal and basal subtypes of BC are essentially different by tumorigenic properties *in vitro*, and their proliferative activity varies within wide limits, we have compared FTH expression and these indexes [7]. It has been determined that level of FTH expression correlates with proliferative activity of BC cells of both luminal ($r = 0.51$) and basal ($r = 0.25$) subtypes. It has been demonstrated that level of FTH expression does not correlated with tumorigenic activity of cells of both studied subtypes (Table 2). Thus, we have determined that cells of luminal and basal BC are essentially different by FTH expression, which within the limits of particular subtypes is associated with cell proliferative activity.

It is known that the main function of ferritin is deposition of iron ions, i.e. its expression level correlates with content of "free iron" in tumor cells [6, 7]. Indeed, the highest "free iron" content among studied BC cell lines has been observed in basal molecular subtype cell lines MDA-MB 231and MDA-MB468 and constituted $2.9 \pm 0.19 \cdot 10^{16}$ spins/ml and $3.0 \pm 0.22 \cdot 10^{16}$ spins/ml, respectively. We emphasize that these cells are characterized with highest FTH expression, lack of steroid hormone receptors, high proliferative potential and tumorigenicity.

In cells of T47D and MCF-7 lines, luminal A and B molecular subtypes, content of "free iron" was reliably lower — $0.47 \pm 0.1 \cdot 10^{16}$ spins/ml and $0.56 \pm 0.1 \cdot 10^{16}$ spins/ml, respectively ($p < 0.05$). When analyzing features of cells of basal molecular subtype MCF-10 A and 180A1, it should be mentioned that for these cells moderate proliferative activity and, along with it, medium FTH expression, insignificant content of "free iron" and high colony forming are typical. Analysis of dependencies of FTH expression on content of free iron allowed us to determine correlation of these indexes in cells of both studied subtypes (Table 2).

Next, we have compared ROS and SOD indexes as integral representation of the "free iron" level in BC cells of different molecular subtypes. As it is shown in Table 1, in cells luminal A (T47D) and B (MCF-7) molecular subtypes, the lowest levels of ROS generation and SOD activity has been observed. ROS content and SOD activity in cells of luminal subtype was associated with low expression of FTH, content of "free iron" and proliferative activity of cells (Table 2).

In BC cells of basal subtype, medium and high levels of ROS and SOD activity have been detected (Table 1). The highest indexes of ROS and SOD, which were associated with high "free iron" content, FTH expression, proliferative activity of cells have been determined in MDA-MB 231 and MDA-MB 468 cells of basal molecular subtype (Table 2).

Thus, we have determined that there is a relation between FTH expression, content of free iron, ROS and SOD in BC cells of different molecular subtype.

It is known that BC malignancy grade is accompanied with certain epigenomic changes in tumor cells [3]. For evaluation of role of epigenomic component in determination of FTH expression in cells of studied lines, we have analyzed features of microRNA expression, which participates in regulation of expression of this protein. Since in previous studies we have shown that miR-200b plays important role in post-transcriptional regulation of ferritin expression (FTH is a target of miR-200b) [20], we have focus our attention on study of miR-200b expression. As one may see in the Figure, the highest level of miR-200b expression has been observed in T47D and MCF-7 cells of luminal subtype. In 184A1 and MCF10A cells of basal subtype, microRNA 200b expression level reliably decreased compared with control and cells

of luminal subtype. The lowest indexes of microRNA 200b expression have been determined in BC cells of basal subtype (MDA-MB-231 and MDA-MB-468). The obtained data demonstrate that there is a dependence between FTH expression and microRNA, which participates in regulation of this protein.

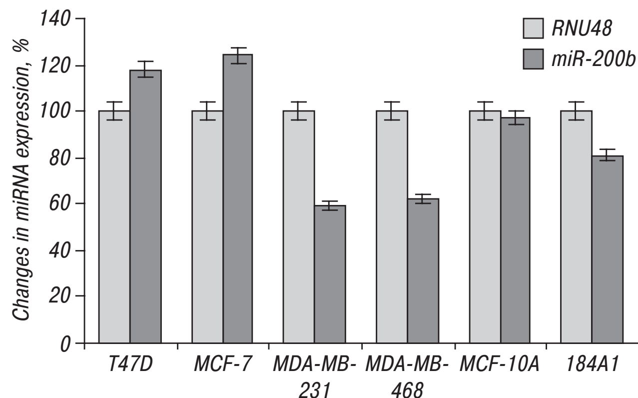


**Figure.** MicroRNA 200b expression in BC cells of different molecular subtype

So, our data show that there are certain correlations between FTH expression and such indexes of BC malignancy as proliferation rate, receptor status and colony forming activity (Table 2). In particular, FTH expression directly correlates with proliferative activity of BC cells of both luminal and basal subtypes, inversely correlates with expression of steroid hormones in cells of basal subtype and does not depend on tumorigenic activity of both subtypes. The cells of luminal subtype B of MCF-7 line and basal subtype of MDA-MB-231 and MDA-MB-468 lines with high proliferative activity contain the highest level of free iron that can be consequence of intensive use of this element by cells, which actively divide and grow. In basal subtype MDA-MB-231 and MDA-MB-468 cell lines, high levels of FTH, ROS and SOD are detected, as well as decreased expression of microRNA 200b. In contrast, cells of luminal subtype B MCF-7 line were distinguished by high expression of microRNA 200b and low ferritin level, what evidences that certain molecular subtypes of BC are characterized by differences in regulatory mechanisms of storage and accumulation of endogenous iron.

Moreover, among four studied lines of basal subtypes, two subtypes can be distinguished by determined indexes: 1) cells of MDA-MB 231 and MDA-MB 468 lines are characterized by high proliferative activity and tumorigenicity *in vitro*, high level of FTH expression, content of "free iron", ROS and SOD; 2) cells of MCF-10 A and 184A1 lines are characterized by moderate proliferative activity and tumorigenicity, medium content of FTH, high content of "free iron", moderate levels of ROS and SOD activity.

The role of iron metabolism in tumor development is one of the fundamental problems of modern experimental oncology [8, 10, 24]. Such studies are of special interest in the context of BC, since hormonal status of woman is tightly associated with significant shifts of iron content in organism. Both deficiency and proficiency of iron can have negative consequences for health. It is known that in developed countries, 20% of women have iron deficiency that is an additional factor, which causes increase of VEGF concentration and, as the result, activation of angiogenesis in women of premenopausal period [25]. Also, there is a synergism of iron and estrogen metabolism disorders in BC occurrence [26–28]. In the process of malignant transformation the excess of iron contributes to ROS formation causing DNA damage. At the same time, estrogen can act as additional substrate in these reactions due to adjunction of hydroxyl group and formation of catechol estrogen, which is one of the factors of hormonal carcinogenesis.

We have analyzed certain mechanisms involved in iron metabolism in BC cells of different molecular subtypes. Our data demonstrate that BC cells, which are referred to different molecular subtypes, are characterized by shifts in intercellular iron balance associated of altered indexes mentioned above. Obtained data are in accordance with the results of our previous studies concerning significant increase of FTH in BC cells of aggressive mesenchymal subtype and in BC cells with phenotype of drug resistance to cisplatin and doxorubicin [20, 30–31]. So, disorders of FTH expression may reflect biological properties of luminal and basal BC subtypes and play a role in clinical course of breast cancer BC.

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