Exp Oncol 2017
39, 3, 203–211

Experimental ONCOLOGY

ASSOCIATION OF CD44^{+}CD24$^{-/low}$ WITH MARKERS OF AGGRESSIVENESS AND PLASTICITY OF CELL LINES AND TUMORS OF PATIENTS WITH BREAST CANCER

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***Aim*: To search for additional molecular-biological markers of cancer stem cell (CSC) involved in the development of intra-tumor heterogeneity for the detection of features of the breast cancer (BC) pathogenesis. *Materialts and Methods:* Expression of estrogen receptors (ER), progesterone receptors (PR), Her2/neu, E- and N-cadherin, CD24, CD44, Bcl-2, Bax, Slug, P-gp, glutathione-S-transferase (GST) and metallothionein in cell lines was determined by the immunocytochemical method. Expression of ER, PR, Her2/neu, CD24 and CD44 in the surgical material of BC patients were determined by the immunohistochemical method. The levels of the miRNA were determined using real-time polymerase chain reaction. *Results*: Cells of high-grade malignancy (HGM), MDA-MB-231 and MDA-MB-468 are characterized by high expression of stem cell markers compared to the cells of low-grade malignancy (LGM), T47D and MCF-7: CD44 levels in T47D and MCF-7 cells were in range of 72–79 points, which is significantly lower than in HGM cells ($p < 0.05$). Also, HGM cells with the properties of CSC were characterized by high expression of antiapoptotic proteins, the transcription factor Slug, and low levels of proapoptotic protein Bax ($p < 0.05$) compared to LGM cells. In cells with CSC characteristics an increased expression of transferrin and its receptor, ferritin, fentorin and hepcidin was revealed indicating activation of the endogenous iron metabolism. The characteristic feature of HGM cells with CSC phenotype were the increased levels of oncogenic miR-221, -155 and -10b by 60%, 92% and 78%, respectively, and decreased levels of oncosuppressive miR-29b, -34a and -200b by 8.4 ± 0.3, 4.6 ± 0.2, and 3.4 ± 0.6 times compared to MCF-7 line cells. It has been established that the development of resistance to cytostatics is accompanied by increased aggressiveness of tumor cells, loss of expression of hormonal receptors and acquiring of stem phenotype. In particular, increased expression of P-gp was observed in BC cells during the development of resistance to doxorubicin, of GST during the development of resistance to cisplatin along with increased CD44 expression ($p < 0.05$). We have revealed the relation between the presence of cells with the CSC phenotype (CD44^{+}CD24$^{-/low}$) and clinical and pathological characteristics of BC patients, their survival and BC sensitivity to neoadjuvant therapy ($p > 0.05$). *Conclusions*: The dependence between the expression of CSC markers and the degree of malignancy of tumor cells, development of resistance to cytostatics *in vitro* was established as well as the predictive value of the detection of the CSC for the individual prognosis of the BC course and sensitivity of the tumors to the treatment.**

***Key Words*: cancer stem cells, resistance, breast cancer, aggressiveness.**

The beginning of the XXI century is marked by the intensification of research on molecular genetic, epigenetic and metabolic factors of tumor cell heterogeneity and their plasticity under conditions of cancer progression and therapy of patients. The post-genome era gave impetus to the search for a system of new coordinates for the causes of these processes, the features of the biology of the tumor cell and their participation in the formation of the relationship between the tumor and the body. The importance of individual differences, which underlie the therapeutic problems of modern clinical oncology, became evident.

A prominent arsenal of molecular genetic, immunocytochemical and immunohistochemical methods allowed to identify the key driving force of the initiation and progression of the malignant process. The theory of the Virchow — Kennayma School, which more than a hundred years ago envisioned the possibility of tumor development from the remnants of embryonic stem cells tumors received its confirmation [1–4]. The existing concept of cancer stem cells (CSC) appeared in the mid-1990s of the XX century [5]. Recently, the biology of CSC is the subject of active research, discussion, and hope. The basis for such attention was the accumulated data on their role in the processes of active proliferation, the creation of protective niche and the formation of the latent state of the tumor lesion [6]. Numerous data are published on the involvement of CSC in suppressing the immune response [7], promoting metastasis [6], development of resistance to chemotherapy [8], and the occurrence of relapses [9]. Intensive research is being carried out on the network of signaling cascades of CSC and their intercellular interactions that will allow revealing the nature of the malignant process and determine the current strategy of diagnosis and therapy [10]. The phenotype of CSC is extremely diverse and can vary both within the intra-tumoral and inter-tumoral lesions, therefore, the definition of a network of markers and factors that

Submitted: August 4, 2017.

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Abbreviations used: BC – breast cancer; CP – cisplatin; CSC – cancer stem cell; Dox – doxorubicin; EMT – epithelial-mesenchymal transition; ER – estrogen receptors; GST – glutathione-S-transferase; HGM – high-grade malignancy; LGM – low-grade malignancy; MT – metallothionein; PCR – polymerase chain reaction; P-gp – P-glycoprotein; PR – progesterone receptors.

contribute to the formation of personified the manifestation of the signs of stem tumor phenotype is the main and actual problem of experimental and clinical oncology [2, 11–13].

For many decades, the formation of the main properties of malignant tumors was thought to be associated with the accumulation of genetic changes and disorders in the network of their signaling cascades, which promote the activation of cell proliferation, invasion and metastasis [14, 15]. The results were concentrated around two major theories of the development of malignant neoplasms. Thus, according to the clonal (stochastic) theory, tumor cells can transform any somatic cells, and tumor progression occurs as a result of the appearance of clones that benefit from survival due to oncogenic mutations and/or epigenetic modifications. However, the existing concept of multiple mutations in somatic cells, which deepen the differentiation and loss of their normal phenotype, has become too simplistic model of carcinogenesis [2, 16, 17].

According to the hierarchical theory, the tumor develops from the stem cells, which in the tumor microenvironment lose vertical system of legitimate self-control and transformed into a limited subpopulation, which became a central and inexhaustible element of tumor development and metastatic potential. Biology of CSC is a key factor in the development, plasticity, and progression of the disease. Supporters of hierarchical model argue that only 0.00001–1% of these cells can provide awakening of the tumor from latent state and restore the progression of the disease [17]. It is believed that such cells are capable of asymmetric division, which allows continuous replenishment of the CSC and the generation of a pool of daughter cells that form the tumor mass [3].

The dynamic variability of the microenvironment of the CSCs and the unbalanced network of signaling cascades contribute to the formation of numerous subclones within which each cell differs from one another by the structure of the genome, the nature of transcripts, proteomic elements, etc. [14, 18–20]. Subclones with numerous molecular defects in the conditions of the intra-tumoral environment become the source of the heterogeneous pool of cells that can selectively support oncogenesis, have multiple targets, and exhibit variable sensitivity to the action of cytostatics.

A large number of experimental studies and reviews is devoted to the identification of surface antigens of CSC [21–25]. However, it has been proven that most markers are not stable and depend on the individual characteristics, as well as vary at different stages of the tumor process. Therefore, our research is aimed at finding markers and factors for the inter- and intra-tumor features of CSC that are involved in the formation of a heterogeneous cell pool and contribute to the pathogenesis of breast cancer (BC). Attention was focused on the search for molecular-biological indexes that could characterize additional features of the CSC phenotype and to reveal the mechanisms of their participation in the variability and plasticity of malignant cells.

MATERIALS AND METHODS

Cell lines and drug treatment. The studies were performed *in vitro* on 6 human BC cell lines: T47D — metastatic breast ductal carcinoma; MDA-MB-231 and MDA-MB-468 — metastatic breast adenocarcinoma; MCF-7 — invasive breast ductal carcinoma, MCF-7/CP, MCF-7/Dox — its variants, resistant to cisplatin (CP) or doxorubicin (Dox), respectively.

T47D cells were cultured in RPMI-1640 medium (Sigma, USA) supplemented with bovine insulin (0.2 U/ml) and 10% fetal bovine serum (FBS). MCF-7 cells were grown in DMEM (Sigma, USA) supplemented with recombinant human insulin (0.01 mg/ml) and 10% FBS. MDA-MB-231 and MDA-MB-468 cells were cultured in Leibovitz's L-15 medium (Sigma, USA) supplemented with 10% FBS. All cultures were grown on glass cover slips in humidified atmosphere with 5% CO_2 at 37 °C. The cell lines were obtained from the Bank of Cell Lines from Human and Animal Tissues of the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology (IEPOR) of the National Academy of Sciences (NAS) of Ukraine.

The peculiarities of phenotype were evaluated taking into account receptor status, proliferation activity and invasive properties [26]. MDA-MB-231 and MDA-MB-468 cells were considered highly malignant (absence of steroid hormone receptors, high invasive potential and low adhesive properties). T47D and MCF-7 cells were considered low malignant (high expression of estrogen and progesterone receptors, low invasive activity).

The resistant variants MCF-7/Dox and MCF-7/CP were originated by growing parental MCF-7 cells with rising concentrations of CP (from 0.01 to 6 μg/ml) or Dox (from 0.1 to 32 μg/ml), respectively. CP and Dox were added twice a week after reseeding. Every 2 months, cell survival rate was analyzed by MTT assay. IC_{50} values for MCF-7 and MCF-7/CP cells were 0.25 and 1 μg/ml of CP, respectively, and for MCF-7 and MCF-7/Dox cells — 0.5 and 8 μg/ml of Dox, respectively. Therefore, MCF-7/CP were 4 times as much resistant to the cytotoxic effect of CP and MCF-7/Dox cells were 16 times as much resistant to the cytotoxic effect of Dox as parental MCF-7 cells.

Immunocytochemical assay. The cells were fixed on cover slips (in triplicate for each sample) in ice-cold methanol: acetone (1:1) at −20 °C for 120 min and incubated with 1% bovine serum albumin solution for 20 min. For immunocytochemical assay, primary anti-CD24 (clone SN3B) (NeoMarkers, USA); anti-Bcl-2 (clone 124), anti-Bax (Polyclonal Rabbit Anti-Human Antibody) (DakoCytomation, Denmark); anti-CD44/HCAM (clone 156–3C11), anti-N-Cadherin (clone CD235) (Diagnostic BioSystems, USA); anti-E-cadherin (clone NCH-38), anti-P-glycoprotein (clone C219), anti-Slug (clone 2H5) (ThermoScientific, USA); anti-transferrin receptor 1 (clone BS1620) (Bioworld Technology, USA), anti-transferrin (clone ab82411), anti-ferritin light chain (clone ab69090), anti-hepcidin (clone ab30760), anti-metallothionein

(clone ab12228), anti-glutathione-S-transferase (clone ab18183), anti-ferroportin (clone ab78066; Abcam) (Abcam, USA); anti-ferritin heavy chain (clone GTX62020) (Gene Tex, Bioworld Technology, USA). UltraVision LP Detection System (Lab Vision, Thermo Scientific, USA) and DAB Quanto (Thermo Scientific) were used according to the instructions of the manufacturers. When the immunocytochemical reaction was completed, the cells were stained with hematoxylin by Mayer and placed in Faramount Aqueous Mounting Medium (DakoCytomation, Denmark). Results were analyzed by light microscopy (× 1000, 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 marker expression [27]. The level of studied markers expression was assigned as follows: low — from 0 to 100 H-Score points, medium — from 100 to 200 H-Score points, and high — from 200 to 300 H-Score points.

Total RNA isolation. Total RNA extraction was performed, using NucleoSpin® miRNA (MACHEREY-NAGEL GmbH & Co. KG, Germany). Concentration of RNA was measured, using NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA). The purity of isolated RNA was controlled, analyzing the ratio of OD at 260/280 nm. RNA was dissolved in TE buffer and stored at –20 C.

Single-stranded cDNA was synthesized from 100 ng of total RNA, using TaqMan® MicroRNA Kit for reverse transcription.

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Preparation of reverse transcription reaction mix was performed according to manufacturer's protocol. Reverse transcription was performed at a "Tertsik" thermal cycler (DNA Tehnologiya, Russian Federation). qRT-PCR was performed on Applied Biosystems 7900HT Fast Real-Time PCR System using TaqMan® MicroRNA primers and manufacturer's protocol.

Small nucleolar RNA RNU48 was used as an endogenous control for normalization of miRNA expression. Relative expression of the studied miRNAs was identified by comparative Ct method [28]. Experiments were performed in triplicates for each line, and PCR was performed three times for each sample. Expression differences between the studied miRNA levels relative to control were calculated by the formula:

$$Fold\ change = 2^{-\Delta\Delta Ct}\ [28],$$

where ΔCt (target — control) is equal to the difference between threshold cycles for miRNA (target) and the threshold cycle for RNU48 (control) (ΔCt (target — control) = Ct target – Ct control). ΔΔCt = ΔCt (experiment) – ΔCt (control).

Characteristics of the clinical material. The *ex vivo* study is based on the examination of clinical and morphological parameters of 291 patients with BC. Tumor samples were stored in the clinical database of the Department of Monitoring Tumor Process and Therapy Design at the R.E. Kavetsky IEPOR of the NAS of Ukraine.

A retrospective analysis of the features of CSC markers expression and the study of the dependence of their expression on the clinical morphological parameters of BC and survival rates of patients were carried out on the material of 143 BC patients with I–II stages, who received special treatment in Kyiv City Clinical Cancer Center during 2005–2007 (Table 1).

Histological type of tumors was verified on histological sections of tumors' paraffin blocks (staining with hematoxylin and eosin) according to the WHO International Histological Classification (2006). Depending on clinical indications, patients underwent organ-saving surgeries or radical Madden mastectomies, and adjuvant polychemotherapy (CAF or AC schemes with 21 day interval, the number of courses varied from 4 to 6), according to the approved in Ukraine Standards of Treatment of Breast Cancer Patients. Postoperative radiation therapy was performed on the postoperative scar, axillary, parasternal and supraclavicular regions; the single focal dose was 2 Gy, and the total focal dose — 40 Gy.

General clinical description of 143 BC patients with stage I–II is presented in Table 1. The number of BC patients with stage I was 26.6%, with stage II — 73.4%. Patients' age varied from 34 to 70 years, mean age was 52.1 ± 4.4 years. According to the results of complex examination of patients (X-ray, ultrasound, etc.), metastases in regional lymph nodes (N1–3) were detected in 29.4% cases, distant metastases were not detected. The morphological study has determined infiltrating ductal BC (69.2%) more often, than lobular BC (30.8%). More often moderate differentiation of BC (49.0%) as compared with high and low differentiation (28.0 and 23.0%, correspondingly) was observed.

Table 1. Clinical characteristics of patients with BC of stage I–II

Index	Number of patients	
	n	%
Total number of patients	143	100
Age of patients (years)		
Average	52.1 ± 4.4	
Range	34–70	
Menstrual function		
Preserved	51	35.7
Menopause	92	64.3
BC stage by TNM		
I	38	26.6
II	105	73.4
Metastases in regional lymph nodes (Category N)		
N0	101	70.6
N1–N3	42	29.4
Histopathology of BC		
Infiltrative ductal carcinoma	99	69.2
Infiltrative lobular cancer	44	30.8
Differentiation grade of BC		
G1 (high)	40	28.0
G2 (moderate)	70	49.0
G3 (low)	33	23.0
Molecular subtype of BC		
Luminal A	56	39.2
Luminal B	34	23.8
Her2/neu-positive	31	21.7
Basal	22	15.3

Analysis of the results of immunohistochemical study of estrogen receptors (ER), progesterone receptors (PR) and Her2/neu expression evidenced luminal A subtype in 39.2% cases, luminal B subtype — in 23.8%, Her2/neu-positive subtype — in 21.7% and basal subtype — in 15.3% cases (see Table 1).

The study of the association of expression of CSC markers and the effectiveness of neoadjuvant chemotherapy was performed on the material of 148 BC patients with II–III stages, who received special treatment in Kyiv City Clinical Cancer Center during 2013–2015. Tumor stage was determined according to the TNM classification (6th edition, 2002). The histological type of the resected tumors was verified upon morphological study (hematoxylin and eosin staining) according to the International Histological Classification of the World Health Organization (2006). All patients were treated with NACT. The course included 2–6 cycles of chemotherapy by FAC, AC scheme with 21 day-intervals. NACT efficacy was evaluated every 2 cycles by mammography according to RECIST criteria [29, 30]. Depending on the degree of clinical effect of NACT (according to RECIST criteria) all patients were distributed into 2 groups. The 1st group included 71 BC patients who have demonstrated a positive response to the NACT: complete regression was observed in 10 patients, partial regression — in 68 patients. 2nd group was formed of 65 women with BC resistant to the treatment, including 56 patients with stabilization of tumor growth and 21 patients with BC progression in the setting of NACT. All patients were informed and agreed to the use of biopsy material for research purposes.

The clinical characteristics of 148 patients with BC of stage II–III are shown in Table 2. According to the clinical data, the age of patients ranged from 28 to 72 years, mean age was 51.2 ± 6.4 years. The majority of patients (56.0%) were at menopause, the menstrual function was preserved in 44.0% of patients. The number of patients with BC of stage II was 67 (45.3%), of stage III — 81 patients (54.7%). Upon comprehensive examination (X-ray, ultrasound, laboratory) conducted before treatment, metastases (N1–3) in regional lymph nodes were found in 113 patients (76.3%).

Table 2. Clinical characteristics of patients with BC of stage II–III

Index	Number of patients	
	n	%
Total number of patients	148	100
Age of patients (years)		
Average	51.2 ± 6.4	
Range	28–72	
Menstrual function		
Preserved	65	44.0
Menopause	83	56.0
BC stage by TNM		
II	67	45.3
III	81	54.7
Metastases in regional lymph nodes (category N)		
N0	35	23.7
N1–N3	113	76.3
Histopathology of BC		
Infiltrative ductal carcinoma	111	75.0
Infiltrative lobular cancer	37	25.0
Differentiation grade of BC		
G1 (high)	37	25.0
G2 (moderate)	72	48.6
G3 (low)	39	26.4
Molecular subtype of BC		
Luminal A	68	45.9
Luminal B	32	21.6
Her2/neu-positive	14	9.5
Basal	34	23.0

The distribution of patients by histological type of BC showed that most patients had infiltrative ductal carcinoma (75.0%) of moderate differentiation (48.6%). The greatest incidence was registered for luminal A subtype — 45.9%. Incidence of luminal B, Her2/neu-positive and basal subtypes of BC was 21.6; 9.5 and 23.0% respectively.

Immunohistochemical assay. Expression of ER, PR, Her2/neu, proliferative activity marker (Ki-67), CD24 and CD44 in tumor cells were studied on paraffin sections (4–5 microns) of biopsy and operation material. As the primary antibodies used were the same as in the immunocytochemical study. To visualize the reaction, EnVision System kit (Dako LSAB2 system, Denmark) was used according to the manufacturer's recommendations. The sections were stained with Mayer's hematoxylin. The expression of molecular markers was evaluated by a semiquantitative method. Analysis of the results was performed using optical microscopy (×100, oil immersion ×900) using the classical method of H-Score [27].

Statistical analysis. STATISTICA 6.0 computer program (StatSoft Inc., USA) was used for statistical processing of the obtained results. Differences between the average values were compared with the use of Student's *t*-test; correlation analysis was performed using Pearson correlation coefficient. Differences were considered as significant with the probability not less than 95% ($p < 0.05$).

RESULTS AND DISCUSSION

Hypotheses of tumorigenesis and heterogeneity of BC with the participation of the CSC have great biological and clinical significance and are actively discussed in the modern scientific literature. In particular, in 2003, for the first time, surface antigens of $CD44^+CD24^-$ on tumor-forming cells of BC were detected [31]. Their population is less than 0.1–1% of the tumor mass [32]. High levels of expression of the epithelial specific antigen (ESA^+), CD44 marker ($CD44^+$) and the absence or low expression of CD24 ($CD24^{-/low}$) were detected on the surface of these cells. The surface cellular protein CD44 is a receptor of hyaluronan, as well as some other ligands, which include osteopontin, collagen I and IV types, metalloproteinases of the extracellular matrix [33]. The interaction of CD44 with its ligand (hyaluronan) leads to activation of a number of intracellular signaling pathways that promote cell survival. Subsequently, numerous studies confirmed the high tumorigenic activity of $CD44^+CD24^{-/low}$ cells isolated from a variety of biological material (primary cultures and stable cell lines *in vitro*, biopsy, operation material, primary and serial xenografts of tumor tissue of the mammary gland) [34, 35]. According to data from own studies concerning the markers of CSC, the expression of CD24 was noted only in three lines of BC cells of low and high-grade malignancy: T47D (55.0 ± 3.1 points), MDA-MB-231 (34.0 ± 2.6 points), and MDA-MB-468 (153.0 ± 4.1 points) (Fig. 1).

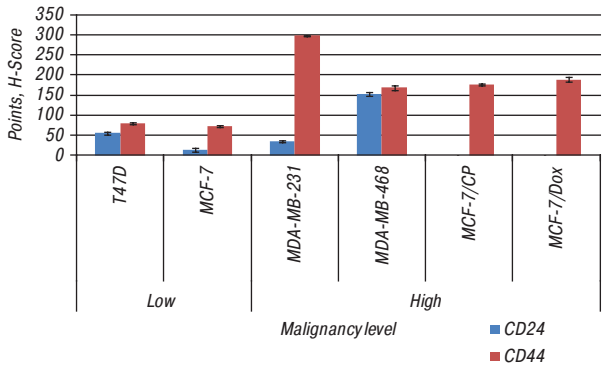

Fig. 1. Expression of CSC markers in human BC cells of varying degrees of malignancy and sensitivity to cytostatics (H-score, points)

Instead, the expression of CD44 was established in all BC lines, but its degree was different. On average, it was in the range of 72–79 points in the lines of low malignant cells, which is significantly less than in high malignant cells, which also showed the variability of the expression of this marker on average from 167 to 298 points. The highest level of CD44 expression was observed in malignant MDA-MB-231 cells (298.0 ± 0.7 points) compared to other lines with aggressive phenotype ($p < 0.05$). As can be seen from the data presented in Fig. 1, the development of the phenotype of drug resistance to CP and Dox in MCF-7 cells is accompanied by an increase in the number of cells with the CSC phenotype. This is evidenced by a decrease in the number of cells that are positive for expression of CD44 (2.4 and 2.6 times) in MCF-7/CP and MCF-7/Dox sublines, respectively. Consequently, among all the examined cells, low levels of stemness were determined in the low-malignant MCF-7 line, while the high stemness level, according to the findings, was observed in a population of MDA-MB-468 highly malignant cells and cells resistant to cytostatics. It has been shown that the level of CSC markers expression directly correlates with the proliferative activity of low- and highly malignant BC cells (r = 0.54).

A little later C. Ginestier et al. [36] found that more significant biochemical marker of CSC can be a level of activity of aldehyde dehydrogenase 1 (ALDH1), which provides cell resistance to cytotoxic drugs. The high expression of ALDH1 correlates with the aggressiveness of the BC course and resistance to chemotherapy [32, 37, 38]. In general, it is known that in the cells with the CSC phenotype high activity enzymes of phase I and II of xenobiotics metabolism is observed. In particular, high expression of ATP-binding transporters of xenobiotics, including ABCG2, ensures the withdrawal of many drugs (taxanes, topoisomerase inhibitors, antimetabolites) from cells, while high activity of various enzymes inactivating anticancer drugs, including ALDH, NAT, glutathione-S-transferase (GST), provides detoxification of cyclophosphamide, Dox, paclitaxel, etc. [39–41].

According to our data, an increase of the stemness during the development of drug resistance is accompanied by changes in the expression of P-glycoprotein (P-gp), GST and metallothioneins (MT). As can be seen from the data shown in Fig. 2, upon the development of resistance to Dox expression of proteins of I phase xenobiotics metabolism (ABC-transporter — P-gp) increased by 16.5 times, and upon the development of resistance to CP expression of proteins of I phase xenobiotics metabolism, including GST, increased by 30 times. Formation of phenotype of resistance to CP and Dox is also associated with a reduction in the number of cells positive for expression of MT (by 80.4 and 84.8%, respectively).

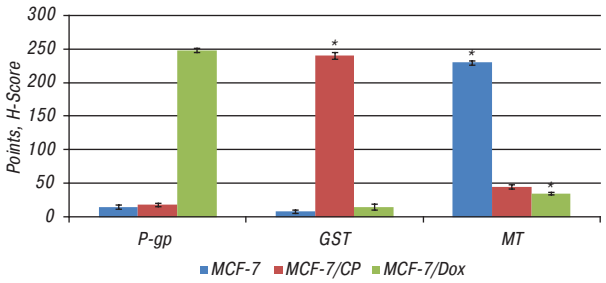

Fig. 2. Expression of proteins of xenobiotic metabolism in the BC cell lines of varying degrees of sensitivity to cytostatics (H-score, points). *$p < 0.05$ compared with MCF-7 cell line

A network of signaling cascades, which constrains the CSC from the active phase of differentiation, is widely studied. In particular, inhibition of one of the elements of Wnt1 kinase cascade associated with membrane glycoprotein of BC cell line results in loss of stem properties [42]. Significant role in formation of pathways regulating cell proliferation, differentiation, apoptosis and epithelial-mesenchymal transition (EMT) belongs to a conservative intracellular cascade Notch, which is activated by the interaction of the transmembrane ligand from Jagged family (Jagged 1 and 2) and Delta family with receptors Notch 1 and 4 [43]. The family of Notch transmembrane proteins includes extracellular sequences — the domains EGF and DSL, which take an active part in lateral inhibition and embryogenesis [44]. It is known that the first step in the activation of EMT is the rupture of various bonds between epithelial cells, which helps the latter to acquire the mesenchymal features and increases their migration and metastatic activity [45–49]. Transmembrane structures involved in cell adhesion — cadherins — are members of the family of calcium-dependent adhesion molecules, which are involved in intercellular contacts such as zona adherence. The cytoplasmic domain of E-cadherin is able to bind to cytosolic protein via catenins, forming the cadherin-catenin complexes that bind E-cadherin to other membrane proteins [50, 51]. The complex of CD44–E-cadherin-catenin is involved in the complex signaling process in the cell (Wnt/catenin signaling pathway), in intracellular integration, differentiation, inflammation, morphogenesis in normal state and pathology [48]. It is known that the transcription factor Slug is capable to suppress the expression of E-cadherin, by binding to its promoter, promoting an increase in the migration properties of cells. Another molecule of adhesion associated with an increased level of invasion of tumor cells is N-cadherin [52, 53]. There-

fore, the studied by us proteins of the family of cadherins and the Slug transcription factor, which may be associated with the Notch signaling, allow us to expand our understanding of the network of cascades involved in the regulation of CSC. In particular, the highest expression rates for E-cadherin were observed in low-grade malignant BC lines T47D and MCF-7 (251.0 ± 5.0 points and 268.0 ± 4.6 points, $p > 0.05$, respectively) (Fig. 3).

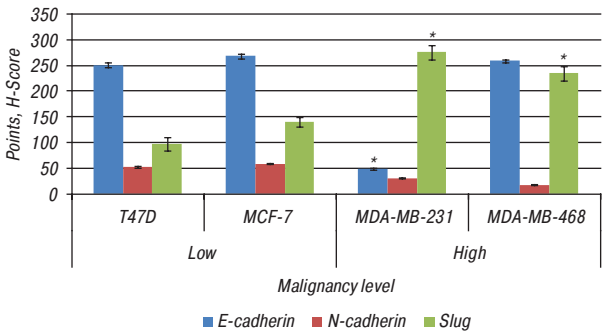

Fig. 3. Indexes of expression of markers of intercellular adhesion and transcription factor in BC cell lines of different degrees of malignancy (H-score, points). *$p < 0.05$ compared with cells of low degree of malignancy

At the same time, in cells of high-grade malignancy significant variability of expression of this molecule of intercellular adhesion was revealed. Thus, in the MDA-MB-468 line it was 259.0 ± 2.5 points, while in the MDA-MB-231 line with a high level of stemness, it was less than 57.0 ± 2.7 points. According to the data obtained (Fig. 3), high-grade malignant lines also differed in the elevated levels of expression of the Slug transcription factor. The highest expression rates for this molecular marker were observed in the MDA-MB-231 line with a high level of stemness (276.0 ± 8.4 points). It should be noted that most cell lines of varying degrees of malignancy are characterized by low levels of N-cadherin expression, which is likely to indicate that this intercellular adhesion molecule is not involved in the formation of a stem phenotype. Thus, the data obtained by us show the key role of the molecules of the cadherin complex and the transcription factor Slug in the activation of stem cells. The loss of epithelial properties by the cells in the process of dedifferentiation upon EMT can be one of the ways for the acquisition of the CSC phenotype by tumor cells. Preventing the loss of epithelial dedifferentiation, in particular the level of E-cadherin, may be one of the priority treatment approaches for patients with BC.

However, the main problem of modern clinical oncology remains the formation of resistance to medical therapy, and every step in understanding the mechanisms of this phenomenon can bring us closer to overcoming one of the most complex medical and biological problems of our time [1, 54–56]. The study of the plasticity of CSCs associated with EMT and the change in the apoptotic program of cells and the factors of their epigenetic modeling was the subject of our further research, since CSCs, through conservative resources, survive on the background of chemotherapy and restore a heterogeneous population of tumor cells. First, this happens at the expense of CSCs, which divide slowly in the G0 phase of the cell cycle [17, 40, 57]. Secondly, but equally important, the resistance to apoptosis may be manifested by changing the level of expression of pro- and anti-apoptotic proteins [58]. This is confirmed by data from our studies, according to which high-grade malignant BC cell lines with high levels of stemness are characterized by an increase in the expression of the anti-apoptotic protein Bcl-2 and a decrease in the level of proapoptotic protein Bax by more than 1.86 and 2.4 times, respectively, in comparison with MCF-7 cells (Fig. 4).

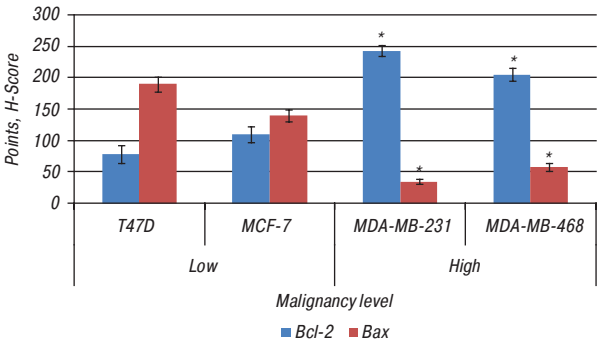

Fig. 4. Indexes of expression of proteins-regulators of apoptosis in BC cells of low- and high-grade malignancy (H-score, points). *$p < 0.05$ compared with cells of low degree of malignancy

Nontraditional but important component of the stem cell phenotype may be markers of iron-containing proteins and miRNAs. Thus, the most pronounced difference in the expression of iron-containing proteins, according to our data, is observed in the BC cell lines of a high degree of malignancy, which are characterized by the expression of CSC markers. In particular, in the T47D and MCF-7 cells, the level of expression of the main proteins of iron metabolism (transferrin and its receptor, ferritin, ferroportin, and hepcidin) was average and did not exceed 140 points (Fig. 5). Instead, in cells of high degree of malignancy, positive for the expression of CSC markers, MDA-MB-231 line, the expression of these proteins was higher and amounted to 200–240 points.

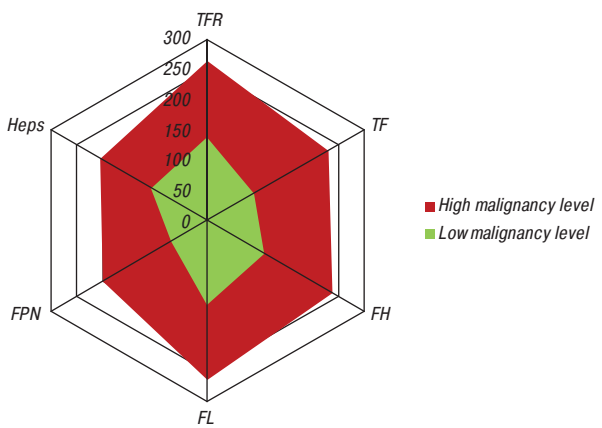

Fig. 5. Indexes of expression of proteins of iron metabolism in low- and high-grade malignant BC cells (H-score, points). TFR — transferrin receptor; TF — transferrin; FH — ferritin heavy chains; FL — ferritin light chains; FPN — ferroportin; Heps — hepcidin

Some differences we have also observed in miRNA expression levels depending on the level of human BC cells stemness of varying degrees of malignancy. It is shown that the characteristic feature of high-grade

malignant cells with stemness features is increased expression of oncogenic miRNAs and reduced expression of antioncogenic miRNA responsible for the passage of the cell cycle, invasive and adhesive properties, proliferative activity and apoptosis (Fig. 6). Thus, in the MDA-MB-231 cell line expression levels of miR-221, -155 and -10b were by 60%, 92% and 78% higher, and the levels of miR-29b, and -200b -34a were 8; 4; 4.6 and 3.4 times lower compared with MCF-7 cells.

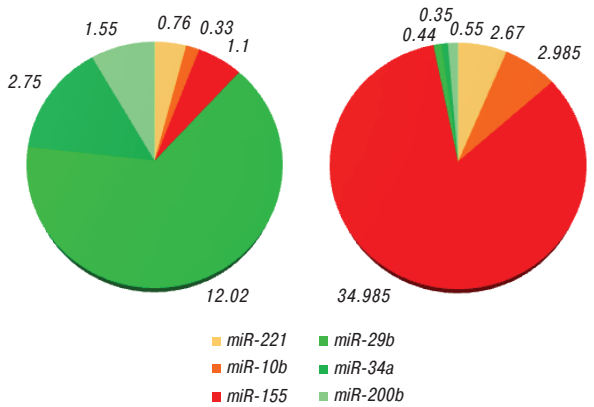

Fig. 6. Indexes of expression of oncogenic and antioncogenic miRNAs in low- and high-grade malignant BC cells, positive for expression of CSC markers (H-score, points)

Thus, the results we received in the *in vitro* system indicate that there are significant correlations between the level of aggressiveness of the BC cells and the level of their stem phenotype.

At the next stage, we analyzed the peculiarities of the expression of CSC markers in tumors of patients with BC and their significance in the prognosis of the disease. We have revealed the dependence of the frequency of tumors with the CSC markers was determined on the molecular subtype: the higher number of tumors with CSC markers was observed in patients with BC of basal subtype in comparison with luminal one ($r = 0.54$, $p < 0.05$). We have found the existence of an associative relation between a higher frequency of tumors with CSC markers in tumors with low differentiation grade compared with high differentiation grade ($p < 0.05$), as well as the close correlation between the frequency of such cells in the primary tumor and the metastases of BC in regional lymph nodes ($r = 0.62$, $p < 0.05$) (Fig. 7).

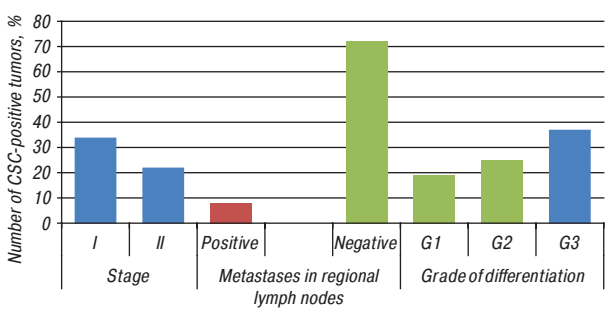

Fig. 7. Distribution of tumors with CSC phenotype depending on clinical and pathological features of BC

We have also established certain patterns in the study of the dependence of the expression of CSC markers in tumor cells from the sensitivity of BC patients to neoadjuvant chemotherapy. The highest percentage of cases positive for expression of CSC markers was registered in a group of patients with tumors resistant to neoadjuvant chemotherapy by the FAC and AC schemes (stabilization or progression of the tumor process according to RECIST criteria). In most tumors (82 and 73%) of patients with luminal A and luminal B subtypes that showed a positive response to treatment, no expression of CSC was revealed. The highest rates of CSC markers expression in tumor cells of 88% and 93% of patients were identified in the basal and Her2/neu-positive BC groups that were resistant to neoadjuvant chemotherapy (Fig. 8).

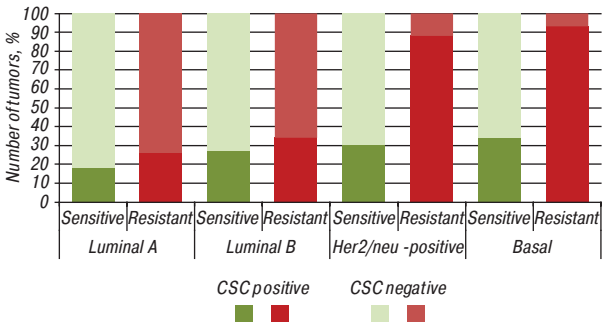

Fig. 8. Distribution of tumors with CSC phenotype depending on the sensitivity of patients with BC to neoadjuvant chemotherapy.

In analyzing the survival rates of patients with BC, it was found that survival of patients with basal BC was significantly higher in the absence of $CD44^{+}CD24^{-/low}$ cells in tumors and, accordingly, less — in the presence of such cells (Log-rank test, $p < 0.05$). In patients with luminal A and luminal B subtypes no significant changes in the survival rate of patients, depending on the presence or absence of $CD44^{+}CD24^{-/low}$ cells (Log-rank test, $p > 0.05$) in tumor cells was revealed. To determine the value of expression indexes of $CD44^{+}/CD24^{-/low}$ CSC markers in tumor cells in patients with BC and to determine their use in clinical practice as a prognostic criterion, a Cox regression analysis was performed. The reliable dependence of the expression of tumor stem cell markers for basal (triple receptor-negative) molecular BC subtype was established, indicating the possibility of using such molecular markers of tumor stem cells as $CD44^{+}$ and $CD24^{-/low}$ as markers for predictive estimation of individual prognosis of patients with basal BC.

Thus, *ex vivo* on clinical material we have confirmed the participation of CSC in the formation of aggressiveness of the BC course and its sensitivity to neoadjuvant chemotherapy.

Consequently, our analysis of the literature and the results of our studies indicate that there is a wide regulatory network and markers of activity of CSC indicating their active role in the pathogenesis of BC. The deepening of knowledge about molecular, genetic and epigenetic links in the formation of the CSC phenotype can be useful in the search for new methods that should be directed to the deviations and elimination of CSC

through the influence on their membrane markers, blockade of the corresponding signaling molecules, modification of epigenetic molecules and regulation of levels of growth factors of the microenvironment. An alternative and promising approach to influence CSC is immunotherapy. The use of antitumor vaccines will promote the activation of T cell immunity and allow recognition of the antigenic components of the stem phenotype in a heterogeneous tumor cell pool.

Sources of Funding:

- NAS of Ukraine Scientific Project (2017–2021) No 2.2.5.411 "Molecular-biological factors of cancer cells heterogeneity and variability of clinical course of hormone sensitive tumors";
- NAS of Ukraine Scientific Project (2017–2019) No 2.2.5.395/1 "Investigation of cancer-associated miRNAs as extratumor predictive breast cancer markers".

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