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EXPRESSION OF BIOMARKERS RELATED TO CELL ADHESION, METASTASIS AND INVASION OF BREAST CANCER CELL LINES OF DIFFERENT MOLECULAR SUBTYPE

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Aim: **The aim of our study was to determine the complex of molecular genetic markers which are associated with cancer aggressiveness, invasion and metastasis among different molecular subtypes of breast cancer cell lines.** ***Materials and Methods:*** **The cell lines used in the analysis include T47D, MCF-7, MDA-MB-231, MDA-MB-468, MCF-10A and 184A1. Expression of estrogen receptor, progesterone receptor, Her-2/neu and Ki-67 was studied by immunocytochemical method. CD24, CD44 and E-cadherin expression was studied by flow cytometry.** ***Results:*** **We have identified biomarkers which characterize metastatic potential of human breast cancer cells of certain molecular subtypes. It has been demonstrated that low colony forming activity of human breast cancer cells of luminal subtype is accompanied by increased adhesive properties of these cells due to high level of E-cadherin expression, low level of CD44 expression and absence of CD24 expression. High tumorigenicity of cells of basal subtype is connected to weakening of adhesive contacts that is caused by abnormalities of E-cadherin expression, significant increase of CD44 expression and presence of low level of CD24 expression.** ***Conclusion:*** **Our data indicated that changes of correlation between expression of cellular adhesion molecules inside conventional immunohistochemical subtypes reflect significantly wider biological properties of luminal and basal subtypes of human breast cancer.**

Key Words: **breast cancer, cell lines, luminal subtype, basal subtype, adhesion molecules, cancer stem cells, CD44, CD24, E-cadherin.**

Human breast cancer (BC) is one of the main causes of women morbidity and mortality all over the world [1–3]. The prognosis of clinical course of BC and choice of treatment strategy is traditionally based on clinical and morphological factors, such as stage of disease, histological degree of malignancy, menopausal status of patient, etc. [4, 5]. Last years, great amount of researches have been devoted to the study of etiology, genetic factors and molecular mechanisms of BC [6, 7]. However, this problem still remains unsolved. First of all, human BC is quite heterogeneous disease, which includes approximately 20 histological and over 200 molecular-genetic subtypes, which are characterized by specific molecular and biochemical profiles, diverse clinical course and prognosis [8, 9]. On the basis of the results of immunohistochemical tests, taking into account the profile of gene expression, human BC was arranged into 4 subtypes: 1) luminal A (low proliferative activity, low degree of malignancy, estrogen receptor (ER)/ progesterone receptor (PR)-positive, HER2/ neu-negative); 2) luminal B (high proliferative activity, high degree of malignancy, ER/PR-positive, HER2/ neu-negative); 3) basal or "triple negative" (ER/PR-negative, HER2/neu-negative); 4) HER2/neu-positive (ER/PR-negative, high level of expression or amplification of c-erbB2). It has been proven that molecular subtypes of human BC are characterized by different responses to the therapy, differential course and prognosis of disease [10, 11]. Despite the extensive use of this classification in clinical practice, it had many essential faults, including cases of human BC with wrong prognosis according to this classification; absence of homogeneity inside classification, etc. [12]. Besides, the use of this classification doesn't allow prognosticating the risk of disease recurrence, metastatic potential and invasive activity of tumor cells of certain molecular subtype.

Invasion of tumor cells and their further diffusion in organs and systems of organism are the cardinal stages of malignant growth [13, 14]. It is known that the main mechanism of invasion and metastasis is associated with strong destabilization of intercellular contacts of tumor cells [15]. That is why study of changes of cells adhesive properties in development of metastasis is the key issue of oncology and attracts more and more attention of researchers. Several families of adhesion molecules, which physiological function is to connect cells between each other, endothelium and extracellular matrix, have been discovered [16–18]. CAM (cell-adhesion molecules) belong to these families. CAM, especially multistructural and multifunctional adhesion molecule CD44, are significant for intercellular contacts formation [19, 20]. The surface cell protein CD44 is a receptor of hyaluronan (HA) as well as some other ligands, to which belongs osteopontin, collagen of I and IV types, metalloproteinase of extracellular matrix [21, 22]. Another family of transmembrane proteins — cadherins, calcium-dependent adhesive molecules, which determine "zonula adherens" intercellular contacts also play important role in cell adhesion.

E-cadherin is a 120 kDa glycoprotein, which (the same as CD44s) consists of extracellular, transmembrane and cytoplasmic domains. Cytoplasmic domain is able to bind cytoskeleton proteins through catenins,

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Abbreviations used: BC – breast cancer; CAM – cell-adhesion molecules; CSC – cancer stem cells; ER – estrogen receptor; FBS – fetal bovine serum; HA – hyaluronan; PR – progesterone receptor.

forming with the last ones cadherin-catenin complexes, which connect E-cadherin with other membrane proteins [23, 24]. Complex of CD44 — E-cadherin — catenins takes part in complicated process of intracellular signal transfer (Wnt/catenin signaling pathway), intracellular integration, differentiation, inflammation, morphogenesis in normal and pathology [25, 26]. Another one molecule of adhesion, which is associated with increase of tumor cells invasion, is N-cadherin [27, 28]. Though the acquired data by different research groups argue, abnormality of intercellular adhesion is observed in many malignant tumors [18]. In particular, the loss or decrease of E-cadherin expression is observed in series of tumors of epithelial origin, often in low-differentiated or with high metastatic potential [29]. The high N-cadherin expression in carcinomas of mammary gland correlates with the stage of invasion in consequence of N-cadherin-mediated interactions with stroma cells [27, 30]. The abnormality of CD44 expression has been determined in tumor cells of human BC and correlates with high metastatic potential [31]. Besides, last years, more evidences of existence of particular subpopulation of tumor cells, which belong to cancer stem cells (CSC), on which, in turn, depends tumor growth, recurrences of disease and metastasis development as well as sensitivity of tumor to cytostatics, have appeared [32]. According to the research data, the marker of BC CSC is $CD44^{+}CD24^{-/low}$ phenotype. Cells with such phenotype are representing small population of primary human BC, but they are associated with self-renewal and significant tumorigenic potential [33]. So, now there are indubitable evidences, which prove significance of participation of molecules of intercellular adhesion and CSC in invasion and metastasis of human BC. However, the majority of these studies are devoted to the investigation of the only particular indicators of intercellular adhesion or presence of CSC that doesn't allow defining the role of synchronicity and/or many-vector indicators, which characterize invasive and metastatic peculiarities of human BC cells. Moreover, the role of these biomarkers in prognosis of clinical course and formation of invasive and metastatic potential of human BC cells of certain molecular subtype is still not finally clarified.

The aim of the paper, therefore, is to determine the complex of molecular-genetic markers, which are associated with aggression of clinical course, invasion and metastasis inside molecular subtypes of human BC.

MATERIALS AND METHODS

Cell lines, cell culture and reagents. The study was carried out on the 6 cell lines of human BC of different histogenesis: MCF-10 A and 184A1 — immortalized cells of normal mammary gland, MCF-7 — invasive duct carcinoma of mammary gland, T47D — metastatic duct carcinoma of mammary gland, MDA-MB-231 and MDA-MB-468 — metastatic adenocarcinoma of mammary gland. 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.

Immunocytochemical assay. The cells grown up on cover slips were fixed in ice-cold methanol: acetone (1:1) at -20 °C for 120 min with washes in PBS and incubated with 1% BSA solution for 20 min. Primary monoclonal antibodies anti-ER, anti-PR and anti-HER2/neu (all supplied by DakoCytomation, Denmark), anti-CD24 (NeoMarkers, USA), anti-CD44 (Diagnostic BioSystems, USA), anti -E-cadherin (Thermo Scientific, USA), anti-CD325 (N-Cadherin, BioLegends, CA) and anti-Ki-67 (DakoCytomation, Denmark)) were diluted in blocking buffer and incubated at room temperature for 1 h followed by incubation with UltraVision LP Detection System (Lab Vision, Thermo Scientific) for 10 and 15 min, after washing, the immune reaction was visualized with DAB Quanto (Thermo Scientific). When immunocytochemical reaction was completed, the cells were stained with hematoxylin solution according to Mayer for 10–15 s and placed in Faramount Aqueous Mounting Medium (DakoCytomation, Denmark). The acquisition of results was made by light microscopy (×1000, oil immersion) with 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 [34].

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Colony-forming assay. Colony-forming ability of the cells was analyzed by their growth in semisoft agar. Cell suspension (2×10^3/dish) in RPMI1640 or DMEM medium/0.33% agar (DIFCO) was placed at the surface of basal 0.5% agar medium. The cells were incubated at standart conditions for 2 weeks, then stained using neutral red stain for 3–4 h. The number of stained colonies with the number of cells > 30 cells/colony was counted.

Flow cytometry. Surface expression of cell adhesion molecules and tumor CSC markers were detected by flow cytometry using primary antibodies against CD24, CD44 and E-cadherin (Abcam, USA). A rabbit anti-mouse IgG(F(ab`)2 fragments) antibody conjugated with FITC was used as a secondary reagent. Analysis was conducted with a Coulter Epics XL (Beckman Coulter, USA), using supplied software (System II, USA).

Statistical analysis. Statistical processing of the obtained results was carried out using STATISTICA 6.0 program. 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

At the first stage of research, we have determined receptor status, expression of HER2/ neu protein and proliferative activity of cells to define molecular subtype of investigated BC cell lines. It has been determined that metastatic ductal breast carcinoma cells (T47D) and invasive ductal breast carcinoma cells (MCF-7) belong to the luminal subtype of human BC because they are characterized by presence of steroid hormone receptors and absence of epidermal growth factor receptor HER2/neu (Table 1). It should be mentioned that proliferative activity of T47D cell line is low, while proliferative activity of MCF-7 cells, in contrast, is high. According to this, we ascribed these lines to luminal A and luminal B subtypes, respectively (Table 1). The cells of all other studied lines (2 lines of metastatic adenocarcinoma — MDA-MB-231 and MDA-MB-468, and 2 lines of immortalized cells of normal mammary gland MCF-10A and 184A1) were characterized by absence of steroid hormones receptors and HER2/neu expression, therefore, we have referred them to basal subtype or "triple negative" BC. It should be mentioned that proliferative activity and histological origin of basal subtype cells, as well as luminal, widely varied (Table 1). Besides, high proliferative activity is determined in lines MDA-MB-231 and MDA-MB-468, and moderate one — in cells MCF-10A and 184A1.

Table 1. BC cell lines: histologic origin, molecular subtypes, antigen expression and colony formation

Cell line	Histologic origin	Molecular subtype	ER	PR	HER2/ neu	Ki-67	% of colonies
T47D	Metastatic ductal breast carcinoma	Luminal A	+	+	-	Low	8.1±0.5
MCF-7	Invasive ductal breast carcinoma	Luminal B	+	+	-	High	8.7±0.3
MDA-MB 231	Metastatic breast adenocarcinoma	Basal	-	-	-	High	19.2±1.1
MDA-MB 468	Metastatic breast adenocarcinoma	Basal	-	-	-	High	34.0±2.3
MCF 10 A	Immortalised normal breast cells	Basal	-	-	-	Moderate	24.0±1.2
184 A 1	Immortalised normal breast cells	Basal	-	-	-	Moderate	23.5±0.9

According to the data [35, 36], proliferative activity of tumor is one of the most important indicators of its biological aggressiveness that determines prognosis of the clinical course, probability of metastasis and sensitivity to antitumor therapy [36]. It is known that luminal A type of human BC is mostly characterized by ductal histological type with low proliferative activity and favorable course. In contrast, luminal B type of human BC represents the group of hormone-sensitive tumors with high proliferative activity, which is associated with unfavorable prognosis. The overall survival of patients with luminal B type doesn't exceed rates of survival of patients with basal type of tumor. Also it has been defined that high proliferative activity of tumors of patients with human BC of luminal B and basal subtypes, from the one side, stipulates high sensitivity to antitumor therapy, from the other side — aggressive clinical course and low survival rate of patients.

Increase of proliferative activity usually correlates with decrease of migration abilities and invasiveness of tumor cells *in vitro* [37]. Taking into account that ability of colony-formation in semi-soft agarized medium *in vitro* possess only transformed cells, and number of colonies correlates with their malignancy and is feature of tumorigenicity of cells *in vitro* [39], following series of experiments was aimed to study the colony-forming ability of cells of human BC of different molecular subtypes (Fig. 1).

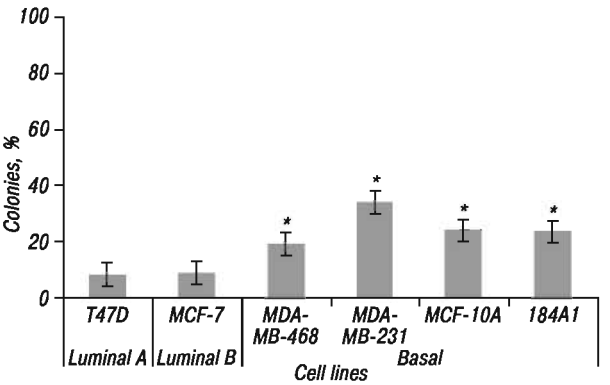

Fig. 1. Colony formation of BC cell lines with different molecular subptypes. *p < 0.05

As one may see in Fig. 1, the lowest number of colonies has been detected in cells of luminal subtype lines (T47D and MCF-7) compared to the cells of basal subtype. Among cell lines of basal subtype the highest percentage of colonies was observed in MDA-MB-231 cells. The percentage of colonies, formed by MCF-10A and 184A1 cells, exceeded the percentage of colonies, which were formed by MDA-MB-468 cells (by 10% less compared to MCF-10A and by 10.5% less compared to 184A1) (Fig. 1, Table 1). At the same time, we have determined the absence of relation between proliferative and invasive activity of cells of human BC of different molecular subtypes (Table 1). The cells of luminal A and B subtypes were characterized by low colony forming activity, and proliferative activity of T47D cells was low, while proliferative activity of MCF-7 cells, in contrast, was high. Studied BC cells of basal subtype did not show any dependence of proliferation level on their colony forming activity (Table 1). The obtained data showed the evidence that determination of proliferative activity of tumor cells can not be used for prognosis of aggressiveness of human BC and probability of metastasis development.

Many studies have shown correlations between colony formation potential of tumor cells and activity of cellular adhesion molecules. For clarification of above-mentioned distinctions between studied cells in their colony-formation ability we performed series

of experiments to determine their molecular profile according the adhesion markers.

We found that cells of luminal subtype were characterized by high levels of E-cadherin expression (Table 2). At the same time, cells of basal type demonstrated significant variability of expression of this adhesion molecule. As it may be seen from data in Table 2, high level of E-cadherin expression was observed in MDA-MB-468 cells, which is characterized by the lowest tumorigenicity among the cells of basal subtype. The medium level of E-cadherin expression was detected in MCF-10A and 184A1 cells, which showed moderate colony formation activity. At the same time, cells with the highest colony-forming ability, MDA-MB-231, were characterized by low expression of E-cadherin. So, we have defined correlative dependence of level of E-cadherin expression with tumorigenicity of human BC cells of both luminal ($r = 0.46$ and $r = 0.38$ for Luminal A and B subtypes, respectively, $p < 0.05$) and basal ($r = 0.42$, $p < 0.05$) subtypes (Table 2).

Also it should be mentioned that majority of studied cells lines of both luminal and basal subtypes was characterized by low levels of N-cadherin expression that was associated with absence of participation of this adhesion molecule in formation of invasive and metastatic potential of BC cells of both subtypes.

At the same time, it has been showed that cells of luminal subtype were characterized by low level of CD44 expression, while all cell lines of basal subtype showed presence of medium and high level of expression of this protein (Table 2). Expression of CD24 was observed mainly in cells of basal subtype. So, low level of CD24 expression was determined in three cell lines of basal subtype, which were characterized by high invasive properties (MDA-MB-231, MCF-10A and 184A1). Absence of CD24 expression was determined in both luminal subtypes of BC cells (Table 2). In MDA-MB-468 cells with moderate degree of malignancy we have observed moderate level of CD24 expression. So, we have defined the relation of CD44 and CD24 expression with molecular subtypes of human BC and have demonstrated that human BC cells with high tumorigenicity by the in vitro test are characterized by increased level of expression of CD44 (Fig. 2).

Analysis of CSC markers has showed that $CD24^-/CD44^{low}$ phenotype is mostly observed among luminal A and B human BC cells. In three cell lines of basal subtype, which were characterized by high tumorigenic properties, we have defined prevalence of $CD24^{low}/CD44^+$ phenotype. At the same time, $CD24^+/CD44^+$ phenotype, according to the obtained data, was observed in MDA-MB-468 cells (basal subtype with moderate degree of colony-forming ability) (Table 2).

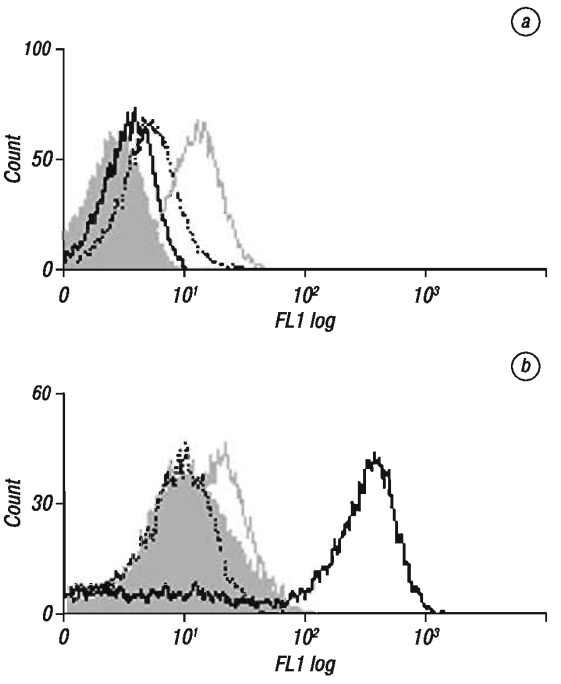

Fig. 2. Phenotype of MDA-MB -231 (*a*) and T47D (*b*) cell line. Filled histogram is isotype control, solid black — E-cadherin, solid gray — CD44, dotted black — CD24

Thus, we have defined that cells of luminal and basal subtypes of human BC could be differentiated according to their adhesive and colony forming properties, while their proliferative activity varies within broad limits. It has been demonstrated that human BC cells of luminal A and luminal B subtypes were characterized by low colony formation activity, absence of CD24 expression, low level of expression of CD44 and N-cadherin and high level of E-cadherin expression. It has been defined that cells of human BC of basal subtype differ by significant variability of colony forming and adhesive characteristics. Besides, in all cell lines of basal type we determined presence of expression of all investigated molecules of cellular adhesion of different intensity degree. It has been demonstrated that high level of malignancy of human BC cells of basal subtype correlated with presence of high level of CD44 expression and low level of CD24 and E-cadherin expression.

So, we have analyzed an expression of biomarkers which characterize metastatic potential and colony forming activity of human BC cells of certain molecular subtypes. It has been demonstrated that low colony forming activity of human BC cells of luminal subtype is accompanied by increased adhesive properties of these cells due to high level of E-cadherin expres-

Table 2. Expression of cell adhesion molecules and CSC markers in BC cell lines with different molecular subtypes

№	Cell line	Molecular subtype	CD24	CD44	E-cadherin	N-cadherin
1	T47D	Luminal A	$0^{3,4,5,6}$	$79 \pm 3.1^{3,4,5,6}$	$251 \pm 5.0^{3,5,6}$	$53 \pm 2.4^{3,4,5,6}$
2	MCF7	Luminal B	$0^{3,4,5,6}$	$72 \pm 2.8^{3,4,5,6}$	$268 \pm 4.6^{3,5,6}$	$59 \pm 1.6^{3,4,5,6}$
3	MDA-MB-231	Basal	$34 \pm 2.6^{1,2,4,5,6}$	$298 \pm 0.7^{1,2,4,5,6}$	$49 \pm 1.3^{1,2,4,5,6}$	$31 \pm 0.7^{1,2,4}$
4	MDA-MB-468	Basal	$153 \pm 4.1^{1,2,3,5,6}$	$167 \pm 5.0^{1,2,3}$	$259 \pm 2.5^{3,5,6}$	$18 \pm 0.8^{1,2,3,5,6}$
5	MCF-10A	Basal	$63 \pm 1.8^{1,2,3,4}$	$174 \pm 4.3^{1,2,3}$	$149 \pm 1.1^{1,2,3,4}$	$31 \pm 1.6^{1,2,4}$
6	184A1	Basal	$51 \pm 2.2^{1,2,3,4}$	$173 \pm 4.9^{1,2,3}$	$158 \pm 1.2^{1,2,3,4}$	$32 \pm 1.1^{1,2,4}$

Note: $^{1,2,3,4,5,6}p < 0.05$ compared to T47D, MCF-7, MDA-MB-231, MDA-MB-468, MCF-10A and 184A1 cells, respectively.

sion, low level of CD44 expression and absence of CD24 expression. High tumotigenicity of cells of basal subtype is connected to weakening of adhesive contacts that is caused by abnormalities of E-cadherin expression, significant increase of CD44 expression and presence of low level of CD24 expression.

It has been proved that prognosis of aggressiveness of clinical course and occurrence of metastasis in basal (phenotype CD24low/CD44$^+$, E-cadherin-) or luminal BC subtypes (phenotype CD24$^-$/CD44^{low}, E-cadherin+) is strongly dependent on expression of adhesion and CSC markers. According to the research data, absence of E-cadherin expression in malignant cells of mammary gland is associated with larger tumor size and development of lymph node metastasis [38]. Presence of CD44 expression directly correlates with metastasis independently of histological type and localization of malignant tumors [39]. Also it was shown that abnormality of E-cadherin expression and methylation of its gene is associated with aggressive clinical course and process of epithelial-mesenchymal transition of tumor cells [40]. Presence of E-cadherin expression in tumors of luminal type points on presence of adhesive properties and is associated with less aggressive tumor behavior and favorable prognosis of clinical course [41].

In conclusion, we have demonstrated that changes of correlation between expression of cellular adhesion molecules inside conventional immunohistochemical subtypes reflect significantly wider biological properties of luminal and basal subtypes of human BC, and play important role in development of metastatic process and aggressiveness of tumors. The obtained data are the evidence of requirement to determine the expression of cellular adhesion molecules and markers of CSC phenotype in tumor cells of BC patients in order to increase effectiveness of antitumor treatment.

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