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Cancer Stem Cells and Somatic Stem Cells as Potential New Drug Targets, and Prognosis Markers, and Therapy Efficacy Predictors in Breast Cancer Treatment †

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Abstract: Breast cancer (BC) is one of the leading causes of cancer death in women. Thus, the search for drug targets, markers of disease prognosis, and more efficient treatment options is relevant. We have conducted a pilot study including patients with luminal B stage breast cancer IIA-IIIB (T1-3N0-3M0). The control group consisted of healthy women. Presence and frequency of various populations of cancer stem cells (CSC) and somatic stem cells were assessed in the blood, breast tumor tissue, and normal breast tissue. Our results suggest that patients with BC can be divided into two distinct groups based on the frequency of aldehyde dehydrogenase positive cells ($ALDH1^+$ cells) in the blood ($ALDH1^{hi}$ and $ALDH1^{ow}$). In the $ALDH1^{hi}$ cells group, the tumor is dominated by epithelial tumor cells $CD44^+CD24^{low}$, $CD326^+CD44^+CD24^-$ and $CD326^-CD49f^+$, in the $ALDH1^{low}$ cells group, the CSCs of mesenchymal origin ($CD44^+CD24^-$) and epithelial tumor cells ($CD227^+CD44^+CD24^-$ and $CD44^+CD24^-CD49f^+$). *In vitro* CSCs of the $ALDH1^{low}$ cells group expressing CD326 showed high resistance to cytostatics, $CD227^+$ CSCs of the $ALDH1^{hi}$ cells group are sensitive to cytostatics. Populations of epithelial progenitor cells of healthy mammary gland were revealed in normal breast tissue of patients with BC from both groups. The cells were associated with a positive effect of chemotherapy and remission in BC patients.

Keywords: breast cancer; ALDH1; cancer stem cells; somatic stem cells; biomarkers

1. Introduction

Despite advances in diagnosis and treatment, breast cancer (BC) remains the leading cause of cancer death in women [1]. More than 1 million new cases of the disease are registered annually [2]. After the diagnosis of BC the most important is the prognosis of complications and the choice of optimal drug therapy. [3]. "Traditional" prognostic measures are the definition of metastases in the lymph

nodes, the size of the tumor and the type of differentiation of tumor cells [1,3]. When searching for tumor markers, much attention is paid to the tumor subtype, which is determined by the presence (or absence) of the estrogen receptor (ER), progesterone receptor (PR), and the protein associated with the human epidermal growth factor receptor (HER2). In addition, the spectrum of prognostic markers includes Ki-67, urokinase plasminogen activator, plasminogen activator inhibitors (PAI-1) [4]. On the immunohistochemical assessment of these antigens, diagnostic tests have been developed to determine the likelihood of disease recurrence (MammaPrint, Prosigna, Oncotype DX, EndoPredict) [4]. However, the definition of these parameters is insufficient for personalized treatment of patients with breast cancer. In this regard, the search for effective prognosis biomarkers and predictors of breast cancer therapy is relevant. For the diagnosis and dynamic control of BC in the blood of patients, circulating tumor cells (CTC) are determined [5]. However, breast cancer progression and metastasis is associated with cancer stem cells (CSC) [5]. At the same time, CSC breast tumors are extremely heterogeneous population [5]. Perhaps CSCs are the key to early diagnosis and personalized treatment of breast cancer.

In this pilot study, we studied CSCs in breast cancer patients in order to find diagnostic biomarkers and predictors of complications. In addition, we have studied somatic stem cells (SSC). Interest in SSC is based on their possible impact on breast cancer. In this regard, the characteristics of the SSC are extremely important in the search for predictors of the effectiveness of the treatment of the disease.

2. Materials and Methods

2.1. Patients

The study included 12 patients with IIA - IIIB (T1–4N0–3M0) breast cancer of the luminal B and triple-negative molecular subtypes aged from 38 to 66 years (average age of 47,4 ± 0,8 years) who received treatment at the Cancer Research Institute of Tomsk NRMC (Tomsk, Russia) from 2017–2018. Imaging of the primary breast lesion was performed by mammography and ultrasonography. An immunohistochemical study was conducted to determine the molecular subtype of the tumor before treatment. The luminal B subtype of breast cancer was defined as ER+, PR+ or -, Ki67 >30%, and all patients with the luminal B subtype were HER2-negative. Some patients showed no expression of ER, PR, and HER2, and they were classified as a triple-negative subtype. Histological diagnosis was confirmed for all samples. Blood samples from 10 healthy women of similar age were used as control.

This was a pilot investigation. Informed consent was obtained from all individual participants included in the study. All procedures performed in the studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

2.2. Design of Investigation

Blood samples were obtained from patients one day before surgery (Figure 1). Breast tumor and normal breast tissue were obtained from patients on the day of surgery.

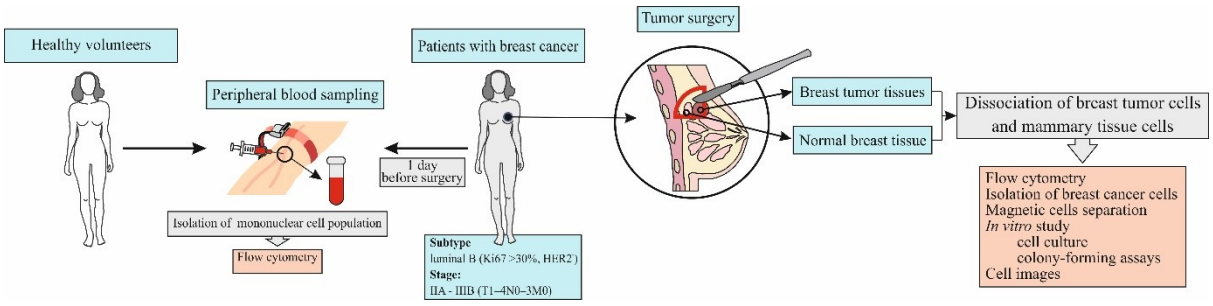

Figure 1. The experimental design of investigation of patients and volunteers.

2.3. Isolation of Blood Mononuclear Cells

We used the Lympholyte-H (CEDARLANE, Netherlands) protocol for the elimination of erythrocytes and dead cells from human blood and receiving mononuclear cells.

2.4. Dissociation of Breast Cancer Cells and Mammary Tissue Cells

Breast cancer was dissociated according to the technical protocol supplied by StemCell Technologies (StemCell Technologies, Canada, Vancouver). These procedures have been optimized to dissociate human mammary tissue or breast cancer tissue to a single-cell suspension for use in cell separation, flow cytometry or progenitor cell assays.

2.5. Flow Cytometry

As the instruction of the manufacturer, Aldehyde Dehydrogenase-Based Cell Detection Kit (StemCell Technologies, Canada, Vancouver) was used to determine ALDH1 enzymatic activity in blood, breast tumor, and normal breast tissue.

The following antibodies were used for cell surface staining of mononuclear cells derived from tumors of patients: PE-Cy7-conjugated anti-CD24, APC-conjugated anti-CD44, APC-H7-conjugated anti-CD45, PE-conjugated anti-CD49f, FITC-conjugated anti-CD227 and PerCP-Cy5-conjugated anti-CD326 (BD Biosciences, USA). The following isotype control groups were used: PerCPCy5.5 IgG1, APC IgG2b, APC-H7 IgG2b, PE IgG2a, FITC IgG1, and PE-Cy7 IgG1. All stained cells were analyzed using a FACSCanto II (Becton Dickinson, San Jose, CA, USA) with the FACS Diva software program.

2.6. Isolation of Breast Cancer Cells

The $CD326^+$ and $CD227^+$ cell fractions were isolated using the EasySep™ Human CD326 (StemCell Technologies, Catalog #18356) or CD227 (StemCell Technologies, Catalog #18359) Positive Selection Kit according to the technical protocol supplied by StemCell Technologies (StemCell Technologies, Canada, Vancouver). The EasySep™ Cell Separation was evaluated by flow cytometry.

2.7. In Vitro Tumor Study

Figure 2 was shown the experimental design of investigation *in vitro*.

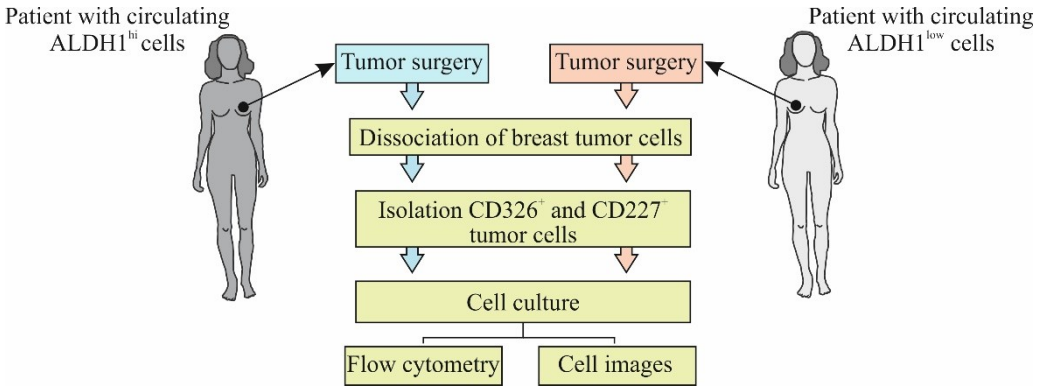

Figure 2. The experimental design of investigation *in vitro*.

Separation-sorted human breast cancer cell concentrations were determined using the cell counter and then seeded at densities of 5×10^5 cells/mL in low adherence 6-well plates. The cultures were maintained in Human EpiCult-C (StemCell Technologies, Canada, Vancouver) supplemented with 5% FBS (StemCell Technologies, Canada, Vancouver) for 24 hours, and then the medium was replaced with serum-free conditions and maintained for an additional 10 days. At the end of the assays, the colonies were enumerated under a microscope. After 10 days, the medium was removed and the plates gently rinsed with PBS. The culture cells were then counted. The procedure was repeated three times. Further,

the sorted cells were seeded and cultured in the presence MammoCult™ (StemCell Technologies, Catalog #05620) supplemented with 0.48 μg/mL freshly dissolved hydrocortisone (StemCell Technologies, Catalog #07904) and 4 μg/mL heparin (StemCell Technologies, Catalog #07980) and 10 ng/mL IL-6 (Sigma, Catalog # SRP3096) to induce greater numbers of mammospheres and tumorspheres, and the cultures were maintained for an additional 7 to 10 days. At the end of the assay, the cells were assessed by flow cytometry and image processing of each well with Cytation™ 3.

Moreover, the $CD227^+$ and $CD326^+$ sorted cell populations were cultured in the presence of 10 ng/mL cytostatics (doxorubicin+docetaxel+cyclophosphamide), after 2 h culture we evaluated apoptosis using flow cytometry and image processing of each well with Cytation™ 3.

2.8. Imaging

Cell images were captured with a Cytation™ 3 Cell Imaging Multi-Mode Reader (BioTek Instruments, Inc., Winooski, VT) configured with DAPI, GFP, and Texas Red light cubes. We used Hoechst 33342 for cell number measurements, or Annexin V-iFluor™ 350 Conjugate and 7-AAD fluorescent probes for apoptosis/ necrosis assessments. Image analysis and quantification was performed using Gen5 Software (BioTek Instruments, Inc., Winooski, VT).

2.9. Statistical Analysis

All statistical analyses were carried out by using SPSS statistical software (version 15.0, SPSS Inc., Chicago, IL, USA). Statistical analysis was performed using the Mann-Whitney U-test. P values <0.05 were considered to indicate statistically significant differences. All quantitative data presented are the mean value and standard error.

3. Results and Discussion

The high heterogeneity of the CSC phenotype in cancer was the reason for the study of various oncoantigens in our study. When studying blood samples from patients with BC, we found an increase in the number of tumor cells with overexpression of CD227 and CD326, as well as CSC of mesenchymal origin ($CD44^+CD24^-$) relative to healthy volunteers (Figure 3). In addition, in BC an increase in the number of $ALDH1^+$ cells circulating in the blood was observed.

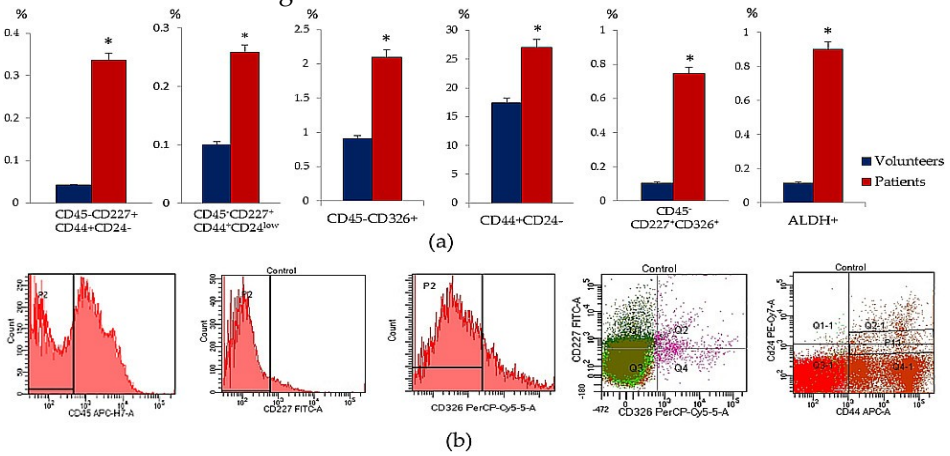

Figure 3. Characterization of epithelial tumor cells and tumor stem cells population isolated from blood of BC patients and healthy volunteers. Cells were analyzed by flow cytometry using antibodies for CD24, CD44, CD45, CD227, CD326, and ALDH1. (**a**) The level of epithelial tumor cells and tumor stem cells blood of BC patients and healthy volunteers; (**b**) Phenotype establishment and qualitative analysis of CD45 (APC-H7), CD227 (FITC), CD326 (PerCP-Cy5.5), CD24 (PE-Cy7), CD44 (APC). * - differences are significant in comparison with the healthy volunteers (P<0.05).

The tumor fraction ALDH1 is widely used as a biomarker of metastatic activity and a determinant of the clinical outcome of breast cancer [6]. The collection of material for biopsy is a rather painful manipulation. In this regard, we turned to reports discussing the prognostic potential of ALDH1+ CTC in patients with BC [6-8]. ALDH expression by circulating tumor cells correlates with poor clinical outcome, metastatic progression and response to therapy in patients with metastatic breast cancer [8]. Another study showed that ALDH1 expression in primary breast tumors correlated with the presence of CTCs and clinical outcome in patients with non-metastatic disease [8]. Objectively, $ALDH1^+$ cells circulating in the blood can be used as diagnostic markers. In our studies, we found that patients with BC differed in the content of ALDH1+ cells in the blood. We identified a group of patients with a significant number of ALDH1+ cells (≥0.9% of all isolated mononuclear cells, group $ALDH1^{hi}$ cells) and a group of patients with a small number of $ALDH1^+$ cells (≤0.9% of all isolated mononuclear cells, group $ALDH1^{low}$ cells) (Figure 4). It is important that the level of $ALDH1^+$ cells in the tumor of patients of $ALDH1^{hi}$ cells group was also higher than that of patients of $ALDH1^{low}$ cells group.

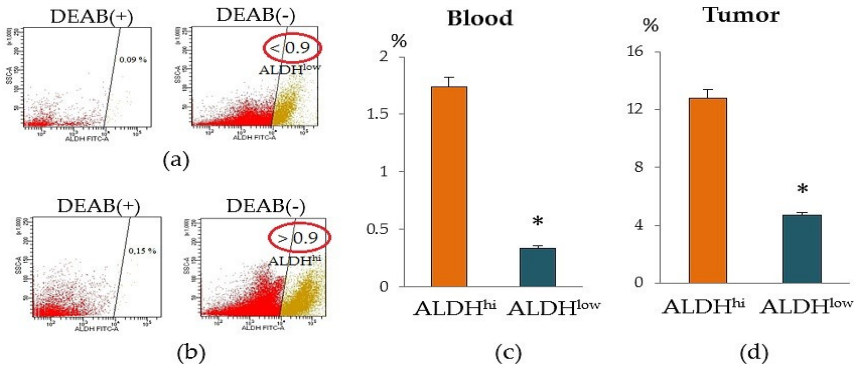

Figure 4. Isolation of circulating $ALDH1^+$ cells from the blood of BC patients. (a) The patients with BC were divided into two distinct groups based on the level of aldehyde dehydrogenase positive cells ($ALDH1^+$ cells) in the blood. (**a**) ALDEFLUOR FACS analysis of the BC patients with ≤0.9 % of all isolated mononuclear cells ($ALDH1^{low}$); (**b**) ALDEFLUOR FACS analysis of the BC patients with ≥0.9 % of all isolated mononuclear cells ($ALDH1^{hi}$); (**c**) The level of circulating $ALDH1^+$ cells; (**d**) The level of $ALDH1^+$ cells in tumor. * - differences are significant in comparison with the $ALDH1^{hi}$ group ($P<0.05$).

In BC metastases in their development go through the mesenchymal and epithelial phases of the metastatic cascade [8]. ALDH1 is involved in the epithelial and more proliferative phase of metastatic tissue colonization. In this regard, the use of ALDH1 as a key biomarker of the metastases risk may be more useful in the diagnosis of advanced breast cancer (epithelial phase) [8]. In patients with an unexpanded disease, which is characterized by an intravascular and more mesenchymal phase of the metastatic cascade, the level of ALDH1 expression is significantly reduced. We found confirmation of this in the present study. According to our data, a patient of the $ALDH1^{hi}$ cells group had metastases in the lungs.

When determining other antigens, we found intergroup differences in other CSCs. The $ALDH1^{hi}$ cells group showed an increased number of epithelial tumor cells $CD44^+CD24^{low}$, $CD326^+CD44^+CD24^-$, and $CD326^-CD49f^+$ (Figure 5). At the same time, in the $ALDH1^{low}$ cells group, the content of CSCs of mesenchymal origin ($CD44^+CD24^-$) and epithelial tumor cells ($CD227^+CD44^+CD24^-$ and $CD44^+CD24^-CD49f^+$) in the tumor prevailed.

Since we see group differences in the content of tumor $CD326^+CD44^+CD24^-$ cells and $CD227^+CD44^+CD24^-$ cells from the tumor of patient A of the $ALDH1^{hi}$ cells group and patient B of the $ALDH^{low}$ cells group, we isolated $CD326^+$ and $CD227^+$ cells, obtained primary cultures and characterized them. At the same time, *in vitro* we evaluated the effects of IL-6 (a factor that stimulates CSC [9]) and cytostatics proposed for chemotherapy (CT) of patients.

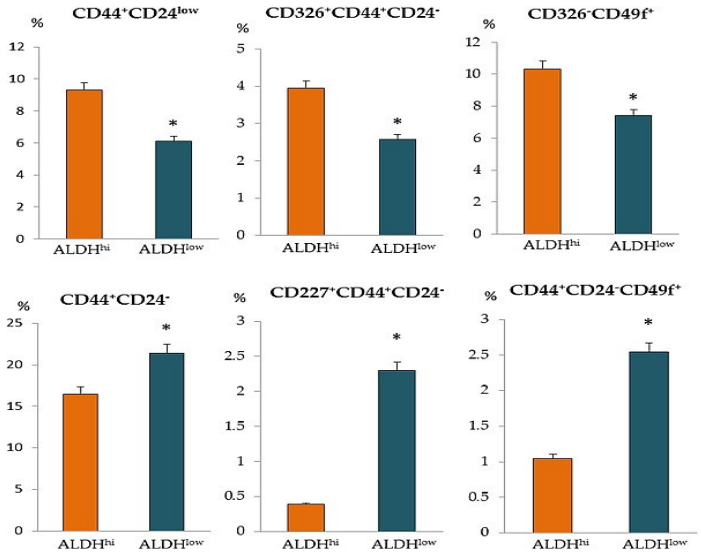

Figure 5. Characterization of epithelial tumor cells and tumor stem cells population isolated from tumor of BC patients. Cells were analyzed by flow cytometry using antibodies for CD24, CD44, CD49f, CD227, CD326. The patients with BC were divided into two distinct groups based on the level of aldehyde dehydrogenase positive cells (ALDH1+ cells) in the blood (≥0.9 % of all isolated mononuclear cells, ALDH1^{hi} and ≤0.9 % of all isolated mononuclear cells, ALDH1^{low}). * - differences are significant in comparison with the ALDH1^{hi} group ($P<0.05$).

Patient B was interested in CD227^{+} cells, patient A is in CD326^{+} cells (Figure 6 a). We found that in patient B, the culture of CD227^{+}cells was characterized by the absence of cells in apoptosis, insignificant clonal activity (mammosphere) and an increase in cell mass during the cultivation cycle (Figure 6 d-f). In culture of CD227^{+} cells of patient B, IL-6 increased the activity of mammosphere formation. After co-cultivation with cytostatics, the number of CD227^{+} cells in apoptosis was 70% of the total (Figure 6 b, c).

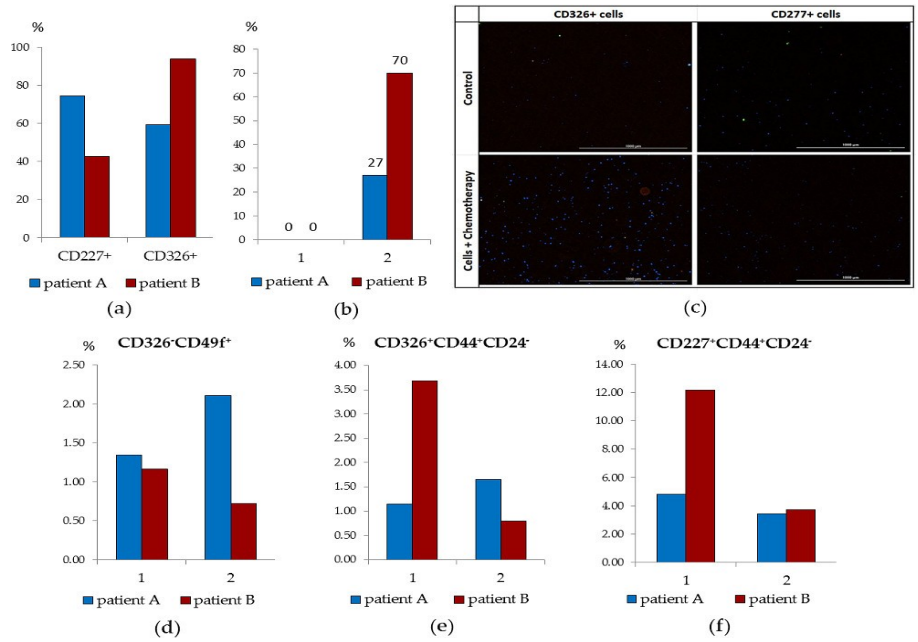

Figure 6. The *in vitro* tumor cell activity from patients A and B. (**a**)The content of tumor stem cells in the CD227^{+} and CD326^{+} cell enriched environment after a cycle of cultivation and IL-6 treatment (% of the initial cell content in each population); (**b**)The count of tumor cells with apoptosis after a cycle of cultivation without cytostatic (1) and with cytostatics (doxorubicin+docetaxel+ cyclophosphamide) (2)

treatment in the CD326$^+$ cell population of patient A and CD227$^+$ cell population of patient B; (**c**) 20x images of CD227$^+$ and CD326$^+$ cells stained with: Hoechst (blue) to identify cell nuclei; Annexin V (green); 7-AAD (red); (Hoechst + Annexin V + 7-AAD) composite image using all three colors. Determination of the percent of cells in apoptosis is made by the ratio of cells counted in green and red channel to total cells counted using blue (DAPI) channel. All scale bars are 1000 μm; (**d**) The count of tumor cells (CD326$^-$CD49$^+$) after a cycle of cultivation without cytostatic (1) and with cytostatics (doxorubicin+docetaxel+cyclophosphamide) (2) treatment in the CD326$^+$ cell population of patient A and CD227$^+$ cell population of patient B; (**e**) The count of tumor cells (CD326$^+$CD44+CD24$^-$) after a cycle of cultivation without cytostatic (1) and with cytostatics (doxorubicin+docetaxel+cyclophosphamide) (2) treatment in the CD326$^+$ cell population of patient A and CD227$^+$ cell population of patient B; (**f**) The count of tumor cells (CD227$^+$CD44$^+$CD24$^-$) after a cycle of cultivation without cytostatic (1) and with cytostatics (doxorubicin+docetaxel+ cyclophosphamide) (2) treatment in the CD326$^+$ cell population of patient A and CD227$^+$ cell population of patient B.

The culture of CD326$^+$ cells of patient A withstood three passages and by the end of the cultivation cycle its cell mass significantly increased, under the influence of IL-6 the activity of mammosphere formation increased (Figure 6 a). These culture parameters of patient A were superior to those of patient B. On the other hand, patient A's CD326$^+$ cells were resistant to cytostatics: the number of cells in apoptosis was 14% versus 27% in patient B.

These data made it possible to suggest that when diagnosing breast cancer, it is necessary to determine the level of ALDH1$^+$ cells circulating in the blood and the ratio of CD326$^+$ and CD227$^+$ tumor cells in the tumor. Results on the resistance of CD326$^+$ tumor cells in patients A and B are important. These cells can act as tumor progression factors and inducers of metastases in all groups, regardless of ALDH1 expression. To increase the effectiveness of chemotherapy, we suggest *in vitro* selection of cytostatics. This approach can enhance effective patient care.

In conclusion, it should be noted that in the breast tissue adjacent to the tumor (normal tissue) of patients with BC, different populations of epithelial progenitor cells of healthy mammary gland were revealed (Figure S1). We did not find significant differences in their content between the patients of the ALDH1^{hi} and ALDH1^{low} groups. In all groups, cells were associated with a positive effect of chemotherapy and remission of patients with BC. This data is extremely important. Perhaps, for patients with stable remission, it is necessary to carry out measures aimed at the epithelium and endothelium regeneration of the mammary gland, which, in our opinion, will make it impossible for the disease to recur. However, the study has some limitations. Because it was a short-term single-center study, it has a relatively small sample size, which diminishes the likelihood of generalization. To assess their applicability to a larger population, the results presented here need further validation using multi-center cohorts with large numbers of patients.

4. Conclusions

Patients with IIA-IIIB (T1-4N0-3M0), triple negative BC and HER+ BC are divided into a group with a significant number of ALDH1$^+$ cells and a group with a small number of ALDH1$^+$ cells in the blood and tumors. The composition of tumor CSCs and their activity in patients of the ALDH1^{hi} cells group and the ALDH1^{low} cell group differ. ALDH1 expression level and ratio of tumor cells CD44$^+$CD24^{low}, CD326$^+$CD44$^+$CD24$^-$, CD326$^-$CD49f$^+$, CSC of mesenchymal origin (CD44$^+$CD24$^-$) and epithelial tumor cells (CD227$^+$CD44$^+$CD24$^-$ and CD44$^+$CD24$^-$CD49f$^+$) in tumors can act as personalized diagnostic markers, predictors of complications and the effectiveness of breast cancer treatment in further research.

5. Patents

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Tomsk National Research Medical Center. Informed consent was obtained from all subjects involved in the study.

Supplementary Materials: The following are available online at http://www.xxxxx, Figure S1: Characterization of bipotent precursors of breast cells ($CD326^{low}CD49f^{hi}CD227^{+}$), basal tissue-specific stem cells of the mammary gland ($CD326^{hi}CD49f^{hi}$), precursors of luminal cells ($CD326^{low}CD49f^{low}CD227^{+}$), and precursors of myoepithelial cells ($CD326^{low}CD49f^{+}$) isolated from the breast tissue adjacent to the tumor (normal tissue) of patients with BC of the $ALDH1^{hi}$ and $ALDH1^{low}$ groups.

Author Contributions: "conceptualization, E.G.S.; methodology, O.P., N.E. and A.P.; software, E.P.; validation, O,P. and N.E.; formal analysis, O.P. and E.G.S.; investigation, O.P., N.E., A.P., E.P. and M.Z.; resources, E.S.; data curation, O.P. and E.G.S.; writing—original draft preparation, O.P. and E.G.S.; writing—review and editing, D.W., E.G.S. and A.D.; visualization, A.P. and M.Z.; supervision, E.G.S.; project administration, A.D.; funding acquisition, S.M. and A.K.".

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