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Biomarkers and potential targets for immune and cellular therapy in triple negative breast cancer

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Summary

Triple negative breast cancer (TNBC) is the most aggressive variant of breast malignancies, being a heterogeneous group with various molecular abnormalities that require differentiated approach to diagnosis and treatment. The article contains current data on modern molecular classifications of triple negative breast cancer and appropriate defects in signaling pathways as well as their assignment to distinct immunological and metabolic biomarkers. The data on the prognostic and predictive role of the tumor molecular biomarkers, as well as on clinically used and cellular therapy approaches and developing targeted drugs are presented, and the prospects for the future research are outlined. We also present the data of our own research concerning evaluation of the prognostic role of cytokines and lymphocyte subpopulations in peripheral blood of the TNBC patients.

Keywords

Breast cancer, triple negative, molecular subtypes, mutational burden, tumor stem cells, circulating tumor cells, cellular microenvironment, lymphocyte subpopulations, interleukins, molecular targets, cellular therapy.

Introduction

Breast cancer (BC) is a malignant disease with heterogeneous biological characteristics and different clinical course. It ranks first in the world (about 25%) in terms of morbidity and mortality among other tumors in women. According to the global cancer database (GLOBOCAN), 34650951 cases of breast cancer were detected in the world in 2020, and 11210413 patients died from this disease [1, 2]. 66,990 new cases of breast cancer were diagnosed in Russian Federation in 2019 (489.6 cases *per* 100,000 people) [3].

Combined chemo- and hormone therapy is, generally, efficient in breast cancer treatment, in terms of overall and disease-free survival. Special advances are achieved in HER-positive tumors using targeted therapy with drugs which suppress the tumor cell growth factors (trastuzumab, herceptin).

A number of protein markers could be used as diagnostic and therapeutic targets in BC, as follows:
1) estrogen receptors (α-subunit, ERα);
2) progesterone receptors (PR);
3) epidermal growth factor receptors of the second type (HER2/new);
4) epidermal growth factor receptors (EGFR);
5) vascular endothelial growth factor (VEGF);
6) cytokeratins (CK5/6, CK14, CK17);
7) nuclear protein reflecting the level of proliferative activity (Ki-67) [4, 5].

Moreover, novel molecular biology approaches, first introduced by Perou et al., using DNA microarray technology, have discerned 4 molecular subtypes of breast cancer, which, in part, corresponded to the previously accepted immunohistochemical (IHC) markers, i.e., luminal A (PR+, ER+, Her-2-), luminal B (PR±, ER+, Her-2+), with Her-2 overexpression (PR-, ER-, Her-2 overexpression), basal-like or triple negative cancer (PR-, ER-, Her-2 -, as well as CK5/6+, CK14+, CK17+, EGFR+). Subsequent works revealed some other molecular variants of breast cancer [6-10].

Basal-like triple-negative breast cancer (TNBC) makes up 12-20% among other histological types, displaying a number of clinicopathological and molecular features that affect treatment strategy. It occurs in women under 50 years of age being characterized by a high recurrence rate, low differentiation levels, and high risk of metastases to parenchymal organs and brain. Molecular defects are often represented by hereditary BRCA (Breast cancer gene) mutations leading to altered DNA repair, thus presuming higher efficiency of DNA-damaging agents, such as platinum drugs and poly-ADP-ribose polymerase (PARP) inhibitors. Moreover, somatic mutations in the P53 gene are detected in 60-80% of cases [11].

The relatively low immunogenicity of this type of tumor seems to be the main obstacle in cellular and immune therapy for breast cancer, compared with many other types of solid malignancies. At the present time, specific TNBC markers are required to determine molecular targets for personalized therapy, e.c., monoclonal antibodies or antigen-oriented immune cells (for example, CAR- T cells). Meanwhile, there exist a lot of molecular markers for diagnostics and therapy [12]. Recent meta-analysis of available data from clinical trials [13] highlighted some potential TNBC biomarkers and therapeutically relevant protein factors on the surface of tumor cells, as well as some blood biomarkers in the patients at different clinical risk. The informative markers of tumor cells were selected, i.e., EGFR, IGF -binding protein, c-Kit, c-Met, and PD-L1. Plasma markers included PIK3CA, pAKT/S6/p4E-BP1, PTEN, ALDH 1 and metabolites of the regulatory pathway PIK3CA/AKT/mTOR, as well as nuclear biomarkers (BRCA1, glucocorticoid receptors, TP53 and Ki-67).

Clinical significance of the TNBC molecular subtypes

To date, several classifications of TNBC have been proposed. They are based on histological signs, characteristic mutations or RNA expression in tumor tissues (Table 1).

The clinically oriented classifications based on the gene expression profiles offer an advanced tool for the disease prognosis and prediction, in addition to the common IHC approaches. E.g., in 2012 Curtis C. et al. developed a classification based on the assessment of the frequency of point mutations and duplications of several genes in 997 primary tumors [14]. The authors identified 10 integrative transcriptional clusters that differ in dominant mode of gene expression. Tumors of the basal-like type, mainly (80% of the cases), have the characteristics of integrative clusters 4 and 10, with pronounced lymphoid infiltration in cases of cluster 4 transcriptional profile, and multiple chromosomal aberrations in the patients with cluster 10 expression [14].

In 2014, Lehmann et al. analyzed the expression profiles of 2188 genes in 587 patients and identified 6 types of tumors that differ in biological properties: basal-like 1, 2 (BL 1, BL 2); mesenchymal (M), mesenchymal-stem (MSL), immunomodulatory (IM), androgen receptor (LAR). The rest of the variants were classified as Unstable Type (UNS). Moreover, the authors divided these triple negative breast cancer cell types using this classification [15].

In 2013, Masuda et al. analyzed the prognostic significance of molecular subtypes of breast cancer [18]. The following conclusions were drawn: 1) molecular subtypes clearly correlate with the rate of complete responses during chemotherapy with anthracycline antibiotics and taxanes (BL1, 52%; BL2, 0%; LAR, 10%; MSL, 23%); 2) molecular subtype is an independent predictor of complete response (p=0.043); 3) molecular subtypes have greater prognostic value compared to PAM 50 (Prediction Analysis of Microarray 50). This parameter tests a sample of the tumor for a group of 50 genes to predict the chance of progression.

The study by Burstein et al. (2015) aimed at modifying the criteria and clarifying the number of molecular subtypes in triple negative breast cancer in accordance with expression profiles of 80 genes [16]. The workers have identified 4 molecular subgroups determined by overexpression of different genes, and specific biomarkers were shown for each of them: 1) luminal AR (LAR): androgen receptors, mucin (MUC 1); 2) mesenchymal (MES): IGF -1, ADRB 2, EDBRB, PTGER 3/4, PTGFR, PTGFRA; 3) basal-like immunosuppressive (BLIS): VTCN 1; 4) basal-like immunoactivated (BLIA): CTLA-4. The subgroups proved to be predictive for the relapse-free (p=0.019) and tumor-specific survival (p=0.07). The group-specific biomarkers can be considered as targets in the development of treatment for triple-negative breast cancer [17].

At the same time, it should be noted that the molecular typing of breast cancer tissues do not always correlate with spectrum and amount of appropriate proteins, i.e, with results of immunohistochemical studies. Hence, the existing classifications require further improvement.

Altered signaling pathways in breast cancer stem cells

Over recent decades, there has been increasing evidence that the characteristics of cancer stem cells (CSC) may determine high risk of metastases and drug resistance. Hence, the CSCs are one of the promising biomarkers for TNBC prognosis. Their quantitative and functional evaluation may inform about degree of tumor aggressiveness, whereas defective signaling pathways could be affected by targeted therapy.

Compared to other tumors, the TNBC clinical samples and cell lines show much higher contents of the cells with a CD 44+/CD 24-/ phenotype and high ALDH 1 expression. Clinical studies have shown that the expression of CD 44+/CD 24-/ is associated with decreased efficacy of chemotherapy, high incidence of distant metastasis, lymph node involvement, and recurrence, whereas ALDH 1 is an independent prognostic factor for long-term treatment outcomes. Detectable markers of the epithelial-mesenchymal transition

Table 1. Molecular classifications of triple negative breast cancer

Reference	Molecular subtype	Cellular and gene alterations
Curtis C., 2012 [14]	Integrative cluster 1	Translocations 17q23/20q
	Integrative cluster 2	Translocations 11q13/14
	Integrative cluster 3	Genome instability
	Integrative cluster 4	Absence CAN (Copy Number Aberrations)
	Integrative cluster 5	Amplification ERBB2
	Integrative cluster 6	Translocation 8p12
	Integrative cluster 7	Insertion 16p/deletion16q, Amplification 8q
	Integrative cluster 8	Insertion 1q/deletion 16q
	Integrative cluster 9	Translocation 8q/Amplification 20q
	Integrative cluster 10	deletion 5q/Insertion 8p, 10p, 12p
Lehmann B.D., 2014 [15]	Basal-like 1 (BL1)	Cell adhesion, differentiation, epithelial-mesenchymal transition. Mutations of TP 53 genes; PTEN ; RB 1; PIK 3CA. _
	Basal-like -2 (BL2)	Similar to mesenchymal-like. Cell adhesion, differentiation. Hyperexpression of EGFR, PDGF, activation of inositol-phosphate metabolism, low proliferative index, overexpression of angiogene genes. Mutations in the BRCA 1 genes; TP 53; BRAF; HRAS; KRAS; PIK 3CA; _ NF 1.2; PDGFRA; CDKN 2 A.
	Mesenchymal-like	Activation of signaling pathways associated with immune response generation (CTLA -4, IL -2, IL - 7), processing and antigen presentation. Mutations of TP 53 genes; RB 1; BRAF; A.P.C.; HUWE 1; NFKB 1 A.
	Mesenchymal –stem	Activation of androgen receptor synthesis, porphyrin metabolism, steroid synthesis. Mutations in the PIK3CA genes; TP 53 ; PTEN; RB 1.
	Immunomodulatory	Cell adhesion, differentiation, epithelial-mesenchymal transition. Mutations of TP 53 genes; PTEN ; RB 1; PIK 3CA. _
	Androgen receptor	Similar to mesenchymal-like. Cell adhesion, differentiation. Hyperexpression of EGFR, PDGF, activation of inositol-phosphate metabolism, low proliferative index, overexpression of angiogene genes. Mutations in the BRCA 1 genes; TP 53; BRAF; HRAS; KRAS; PIK 3CA; _ NF 1.2; PDGFRA; CDKN 2 A.
Burstein M.D., 2015 [16]	Luminal -AR (LAR)	Activation of expression of androgen, estrogen, prolactin, ERBB 4 receptors.
	Mesenchymal (MES)	Activation of cell cycle gene expression.
	Basal-like immunosuppressive (BLIS)	Suppression of gene expression of T-, B-lymphocytes, natural killers.
	Basal-like immunoactivated (BLIA)	Activation of gene expression of T-, B-lymphocytes, natural killers.
Liu Y.R., 2016 [17]	Immunomodulatory (IM)	Overexpression of cell components enabling cytokine-receptor interaction in T-, B -lymphocytes. Increased expression of chemokines and x? receptors, as well as NF - kB . Overexpression of distinct mRNAs (LOC 100653210, LOC 100653245, IGHV 3-20, IGHV 4-31, IGHJ 1, IGKV 3-7).
	Luminal - AR (LAR)	Activated biosynthesis of steroid hormones, porphyrins and PPAR (peroxisome receptors). Activation of gene expression for distinct mRNAs: TRIM 2, SDR 16 C 5, C 1 QTNF 3, KRT 17, SERPINBS, TFAP 2 B, FAR 2, CYP 39 A 1, KIAA 1467, EDDM 3 B.
	Mesenchymal (MES)	Activation of the epithelial-mesenchymal transition, extracellular matrix-receptor interactions. Activation of the expression of TGF components - β signaling pathway, adipocytokine signaling pathways, as well as signaling pathways associated with growth factors. Activation of express c and mRNA: SELP, CNN 1, ADH 1 B.
	Basal-like immunosuppressive (BLIS)	Multiple mitoses, activation of DNA replication and repair. Reduced efficiency of components of innate and adaptive immunity. Defects in the T-cell receptor. RNASE6, MS4A6A, MTBP, FGFR2, BARD1 overexpression.

(EMT) combined with high CSC concentration are also associated with resistance to chemotherapy and, in particular, to PARP inhibitors [19].

Self-renewal of malignant stem cells and other features providing invasiveness, resistance to therapy, and high metastatic potential, are associated with hyperactivation of several key signaling pathways, e.g., Notch, Wnt/β-catenin, Hedgehog, STAT 3. Thus, the Notch signaling cascade includes a family of transmembrane ligands and their receptors, which are critical for the processes of cell proliferation and differentiation. Disturbances of this cascade are detected in patients with lung cancer, prostate cancer, colorectal cancer, breast cancer and leukemia, thus regarded as prospective targets for anticancer drugs [20, 21].

To date, a lot of experimental and clinical data has been obtained confirming that dysregulation of the classical Wnt/β-catenin signaling pathway leads to increased incidence of distant metastases. The members of non-canonical Wnt-signaling pathway (FZD6 and FZD8) are also associated with aggressive behavior of the tumor and its chemoresistance [22, 23]. These molecules are considered potential targets for the newly developed drugs [24].

HH (Hedgehog) is a signaling pathway that promotes self-renewal of the CSC population. The HH family includes three secretory ligands: SHH (Sonic), expressed in embryonic cells; IHH (Indian), found predominantly in hematopoietic stem cells; DHH (Desert) found in cells of the peripheral nervous system and testicles. Overexpression of HH components (SHH, GLI 1/2, SMO) is associated with tumor invasion, angiogenesis, and chemoresistance and, therefore, with poor clinical prognosis. The components of this signaling pathway, especially SMO and GLI, are considered targets for the novel anticancer drugs [25].

TGF-β is a member of the cytokine superfamily, which includes more than 30 functionally related growth factors, including 3 TGF-β isoforms (TGF-β1-3) involved in the regulation of cell growth, adhesion, apoptosis, differentiation and immunoregulation. It inhibits the secretion and regulation of the functions of a number of cytokines, including IFN-γ, TNF-α, IL-2. The role of TGF-β in carcinogenesis is to promote proliferation, angiogenesis, metastasis, chemoresistance, immunosuppression. In addition, the presence of TGF-β is critical for CSC. TGF-β is secreted by the cells from tumor microenvironment which supports CSC population, and, in turn, promotes alternative polarization of immunocompetent cell precursors. In clinical practice, overexpression of TGF-β is a marker of chemoresistance and poor prognosis. TGF-β receptors are considered targets for some prospective drugs [26].

JAK/STAT signaling pathway plays an important role in a number of carcinogenesis-associated events, including proliferation, inflammation, and the pathological changes of microenvironment. E.g., JAK is a family of non-receptor tyrosine kinases that includes 4 components: JAK 1, JAK 2, JAK 3 and TYK 2. JAK 1, JAK 2 and TYK 2 are expressed in many cell types, whereas JAK 3 is specific to hematopoietic stem cells. Under the influence of cytokines and growth factors (IL-6, IL-8, TGF-β, IGF, EGF), the JAK/STAT 3 complex is activated causing overexpression of the genes providing synthesis of growth factors and cytokines (TGF-β, IL-6) which stimulate proliferation of TNBC cells. Experimental and clinical studies have shown that expression of IL-6, IL-8, and STAT 3 is associated with poor prognosis and chemoresistance [27-29].

Circulating tumor cells

Circulating tumor cells (CTC) are considered a potential biomarker associated with prognosis, prediction of efficacy, and treatment monitoring in TNBC. CellSearch technology is the conventional approach to CTCs isolation offered by Menarini Silicon Biosystems, based on recognition of EpCAM adhesion molecules [30]. In 2004, Cristofanilli et al. have shown that detection of >5 CTCs *per* 7.5 ml of blood is an independent predictor of overall and relapse-free survival of the patients with metastatic breast cancer. In 2019, the prognostic role of this marker was proven in the study in 1944 TNBC patients stratified into two large groups: indolent, for which standard treatment is adequate, and aggressive course, for which new, including experimental, methods of treatment were required [31].

However, the data on significance of CTC as a prognostic factor in TNBC patients still remain contradictory. E.g., Munzone E. et al., in retrospective analysis of data from 203 patients, showed that the number of CTCs correlated with overall survival, but not with progression-free survival. Meanwhile, in the SWOG study S0500, the CTC scores were found to be predictive of overall survival and predictive of chemotherapy efficacy [32]. At present, the most promising areas of CTC research are their molecular biology characterization, cluster studies, and combined assays with other biomarkers. The study which involved 360 TNBC cases has shown that occurrence of CTC clusters correlated with the median time to progression [33].

Genetic biomarkers

Molecular markers of TNBC include gene mutations affecting DNA repair systems, signaling molecules, growth factors and their receptors, as well as microsatellite instability and general mutation load. Among the mutated or overexpressed genes, specific targets are searched for the recently used and novel immunotherapeutic drugs. A separate group consists of immunological biomarkers. These indexes reflect the state of tumor microenvironment, peripheral immunological components, and tertiary lymphoid structures.

Mutations in genes associated with DNA repair

Finding the relationships between BRCA 1/2 gene mutations and inherited ovarian and breast tumors was a key discovery in clinical oncology, opening up new opportunities for screening and prevention. Detailed studies of appropriate mechanisms has led to the development of new treatment options. BRCA 1 and BRCA 2 are autosomal dominant genes that are critical in DNA repair by homologous recombination (homologus recombination repair, HRR). Mutations of BRCA 1 and BRCA 2 (gBRCAm) occur in a small part of the population (approximately 0.25%), whereas in women with TNBC their frequency varies from 11% to 31%. The risk of developing breast cancer with hereditary BRCA

mutations is 65% and 45%, respectively [34]. These mutations may trigger an alternative DNA repair mechanism, i.e., a non-homologous end joining (NHEJ). This process depends on poly-ADP-ribose polymerase activity (PARP), and its inactivation leads to cell death. Currently, a number of PARP-blocking drugs entered the clinical practice, e.g., Olaparib, Talazoparib, Niraparib, Rucaparib, Veliparib [35].

In 2015, Domogala P. et al. studied the distribution of 36 mutations in genes involved in homologous recombination. They were found in 22% (35 out of 158) of the patients with TNBC [36], thus suggesting usage of PARP inhibitors and other DNA damaging agents for defects in other genes associated with HRD (homologus recombination deficiency) [37,38]. Microsatellite instability (MSI) is an additional feature of malignant disease progression caused by deficient DNA mismatch repair (dMMR). MSI is associated with highly frequent neoantigen production, thus affecting sensitivity to immunooncological drugs. Some tumor variants (colorectal cancer, endometrial cancer) are characterized by increased MSI rates (20%-30% of the cases).

Immunological markers for TNBC

Mutual interactions between the tumor and host immune system were studied for decades. In the mid-20th century, animal experiments on tumor xenotransplantation showed that effective antitumor immune response is possible only at high levels of tumor-specific antigens. Based on these data, in 1957 M. Burnet formulated the "clonal selection theory" and coined the term "immunological surveillance" [39]. In particular, it was suggested that the transformed cells expressing foreign antigens permanently occur in the body, being normally eliminated by the host immune system. This immune response is similar to classic "immunological surveillance" as described by M. Bernet. Over this period, the tumor cells are recognized and eliminated by factors of innate and adaptive immunity. Both immune cells of tumor microenvironment and peripheral blood may be of prognostic and predictive value. In both cases, quantitative characteristics and ratios of different populations, as well as concentration and production of cytokines (spontaneous and induced) should be assessed.

Lymphocytes and mononuclear cells in peripheral blood

In several studies concerning prognostic cellular markers in TNBC, an assessment was made of lymphocytes or peripheral blood mononuclear cells, as well as the ratios of different leukocyte subpopulations. E.g., a prognostic significance of lymphocytosis, monocytosis, and lymphocyte: monocyte ratio (LMR ≥ 4.7; p <0.001) was shown by He et al. (2016) in 230 patients with local and locally advanced forms of TNBC. Moreover, LMR correlated with tumor size (p <0.005) and disease stage (p=0.013) [40]. Losada B. et al., when studying a group of older BC patients have revealed by univariate analysis that epy platelet-lymphocyte ratio (PLR) is the only independent predictor of disease-free (p=0.04) and overall three-year survival (p=0.03), whereas, among the 3-year survivors (n=69), whereas only ALC has predictive properties in a multivariate analysis at the marginal significance level (p=0.04) [41].

Evaluation of myeloid and lymphoid cell subpopulations, either in peripheral blood, or in tumor microenvironment is a more accurate method for assessing the prognosis. Among lymphoid cells, the role of lymphocytes (CTL, Treg, B-lymphocytes), myeloid cells – monocytes/macrophages (M 1.2), dendritic cells (DC), and suppressor cells of myeloid origin (MDSC) have been studied [42].

Monocyte/macrophage cell lineage

To date, monocytes and macrophages (MFs) are shown to be associated with carcinogenesis in breast cancer, as well as with prognosis and efficiency of various treatment approaches. There are two macrophage subpopulations, M1 and M2, discerned in the tumor microenvironment and peripheral blood of the patients. M1 represents classical activated MFs, that develop from their precursors under the action of lipopolysaccharide, IFN-γ and TNF-α. M2 is the collective name for the macrophages induced *via* IL-4, IL-13, IL-10, TGF-β, Fc receptors, complement and glucocorticoids. M2 are derived from peripheral blood monocytes recruited to the affected site by chemokine ligands (CCL – 2, MCP – 1), colony-stimulating factors (M – CSF, CSF – 1) and vascular endothelial growth factor (VEGF), due to their higher concentration in the areas with low oxygenation. Under the chronic local hypoxia, the macrophages produce hypoxia-induced factors (HIF-1 and HIF-2) which derepress the synthesis of several proteins that increase angiogenic potential (VEGF, bFGF, PDGF), invasive and metastatic ability of tumor cells (MMP, CCL 2, CCL 18). Moreover, they promote arginase (Arg) and IDO expression, thus reducing local contents of arginine and tryptophan, which are essential to the normal functioning of T-lymphocytes and NK cells [43].

Concentration of M2 cells in peripheral blood is significantly higher compared to M1 population, thus correlating with a short relapse-free period in TNBC patients. The M2 macrophages are more common in the blood of patients with distant metastases. The ratio of monocyte subpopulations in TNBC differs from other types of breast cancer, i.e., the alternative polarization variant (CD 14+CD 16+) dominates over the classical one (CD 14hi CD 16-). High concentration of monocytes (CD 14+) is a predictor of good response to high-dose systemic therapy with cyclophosphamide and taxanes [44-46].

Dendritic cells (DCs) comprise a highly specialized subpopulation which performs uptake, processing, and antigen presentation within major MHC I and II histocompatibility complexes, along in combination with co-stimulatory Th molecules (CD 4+), acting with CTL in direct and indirect manner. They are activated by the "danger signals" from the tumor cells, including chemokines and neoantigens. The DC maturation, along with antigen-presenting functions includes expression of costimulatory molecules (CD40, ICAM I, CD80/86, CD 83), secretion of numerous cytokines (IFN-γ, IL-4, IL-5, IL-6, IL-10, IL-13), and migration to the lymph nodes, where the T-cell activation program is launched. In humans, two subpopulations of DC are morphologically and functionally distinguished. I.e., myeloid DC (mDCs) comprise classical DCs of the CD11c+ CD4+ CD45RO+ phenotype expressing MHC I, II which trigger the immune response upon contact with soluble antigens.

Plasmacytoid DCs (pDCs) display the CD11c- CD4+ CD45RA+ CD123+ phenotype and MHC I expression, being reactive for the cell-associated antigens. The DCs in TNBC patients showed reduced expression of cytokines (IL-12), co-stimulatory molecules (CD 80, CD 86), activation markers (HLA-DR), and lower ability to present antigens [47].

There are some controversial data on prognostic and predictive role of DC in the patients with TNBC. Despite conflicting data on the role of dendritic cells, considerable attention is paid to this cell population, in terms of vaccine therapy for cancer, in particular, breast cancer. According to several studies, their high levels may be a favorable prognostic factor for overall survival [48,49]. However, further research is needed to determine their therapeutic potential in TNBC.

The populations of natural killer cells (NKs) are formed from a common lymphoid precursor in the bone marrow, from where they further spread to the primary and secondary lymphoid organs, as well as to the lungs, liver, and blood. Two NK subpopulations are identified in humans: $CD56^{bright}$ CD 16- (cytokine-producing) and $CD56^{dim}$ CD16+ (cytotoxic). In addition, there are several groups of NK depending on the degree of maturity, determined by the expression of CD 27 and CD11b surface markers which are not expressed by the immature NKs. In the course of maturation, CD 27 appears first, followed by CD11b. NK with the CD 27+ phenotype show the best ability for cytokine secretion, whereas the NK cells with CD11b+ CD27 phenotype demonstrate maximal cytolytic activity. NK can eliminate cells that do not express MHC I, and this mechanism is used by malignant cells and CSCs to prevent attack by CTLs. Potentially, NK cells are the most effective cells against the tumor, but they may acquire the $CD56^{bright}$ CD16- phenotype under the influence of microenvironmental factors (TGF-β, adenosine), and express pro-angiogenic factors (MMP 9, VEGF), thus increasing the invasive potential, leading to T-cell depletion [59]. Low blood levels of NKs seem to predict low efficacy of neoadjuvant chemotherapy in TNBC. Expression of CD 163 and CXCR 4 in the NK microenvironment is a marker of early relapse [50, 51].

Tumor microenvironment

The study of the tumor microenvironment in TNBC is an important component of assessing the prognosis of the disease. From a clinical point of view, the cellular microenvironment can be assessed both quantitatively and qualitatively, taking into account the population profile, by the presence of a specific "immunological signature". Moreover, it is currently possible to assess the contents and production levels of cytokines by lymphoid cells of peripheral blood and tumor microenvironment. The lymphoid component, which makes up to 50-60% of the stromal volume in all molecular subtypes of TNBC, as a rule, suggests good prognosis and potential sensitivity to immuno-oncological drugs and chemotherapy [52, 53].

In 2020 He L. et al. conducted a meta-analysis of randomized trials with assessment of tumor-infiltrating lymphocytes (TIL) which reflected the results of treatment in 15,676 patients with breast cancer, including 3847 TNBC cases. The results of multivariate analysis showed that any 10% increase in TIL density was associated with increase in overall survival and complete morphological response rates for all molecular subtypes. High TIL density (≥50%) leads to a 2.7-fold increase in the complete response rates in TNB [54].

A similar study was done by Mao et al. [55]. They analyzed data from 25 works (22964 patients) concerning the major TIL subsets: CD 8+, Foxp 3+, PD-1+, γδ T cells, CD3+, CD4+. CD8+ TIL in the infiltrate proved to be a favorable prognostic factor for disease-free and tumor-specific survival in all subgroups. Foxp3+ TILs seem to be a dismal unfavorable prognostic factor for relapse-free and overall survival in all the subgroups except of TNBC. PD-1+ TIL and γδT TILs are poor prognostic factors for overall survival in all subgroups, whereas CD3+ TIL and CD4+ TIL did not show any predictive potential [55]. Thus, in most behavioral studies, the authors conclude that the formation of tertiary lymphoid organs is a favorable prognostic factor for TNBC.

Local and systemic concentrations of cytokines

Cytokines are currently considered universal regulators of homeostasis for many cell types. In TNBC, they are involved in regulation of angiogenesis, arrangement of immunosuppressive networks, tumor metastasis, and metabolic processes associated with obesity, chronic inflammation, and carcinogenesis. Involvement in carcinogenesis enables usage of the cytokines as prognostic markers. Cytokines can be measured in blood or in tumor microenvironment. Their contents, as well as spontaneous and induced production, may be assessed in these samples. IL-1, -6, -8, -10, -11, -17, -19, -20, -23, like as TNF-α; TGF-β, adipokines (leptin, adiponectin) are involved in TNBC carcinogenesis. Many of them have predictive potential (Table 2).

IL-6, 8, 10, TNF-α and TGF-β are the most studied cytokines associated with carcinogenesis and prognosis of TNBC. IL-6 is a cytokine that functionally integrates the immune and neuroendocrine systems, being produced by T cells, macrophages, myocytes, endotheliocytes, fibroblasts, and tumor cells. IL-6 promotes cell proliferation and synthesis of antibodies by B-lymphocytes, CTL proliferation, stimulates the granulocytic hematopoietic lineage, and induces the expression of acute phase proteins in the liver. Overexpression of IL-6 in malignant tumor and increased concentration in peripheral blood is considered an unfavorable prognostic factor in terms of overall and disease-free survival [67-69].

Interleukin-8 (IL -8) belongs to the chemokine family, being produced by the MF and endothelial cells. In the course of carcinogenesis, IL -8 can act as an autocrine growth factor and stimulate angiogenesis. Serum IL -8 is not a favorable prognostic factor for overall and disease-free survival [70-72].

IL-10 is a key regulator of the antitumor immune response. Treg, Th0, Th1, Th2, CTL, monocytes, MF, tumor cells, TAM and NK are the main producers of IL-10 in humans. Maturation by reducing MHC expression II, adhesion molecules and cytokines (IL-12), as well as reducing the sensitivity of receptors that respond to "danger signals". IL-10 inhibits proliferative activity and production of Th 1 cytokines, T-dependent activation of CTL and CD 19 [73]. The main biological effects of TNF - α in carcinogenesis are associated with

Table 2. Prognostic role of functional overexpression in microenvironment, or increased levels of cytokines in blood plasma in patients with triple-negative breast cancer

Cytokine name, group, producing cells	Sample types: microenvironment (M); plasma (P)	Prognostic Role	Effects upon survival
IL-1. Monokines. Monocytes, MF, B – lymphocytes, fibroblasts, endotheliocytes [56, 57]	M, P	Increased invasive potential	No data
IL-6. IL-6 family . Lymphocytes, MF, myocytes, fibroblasts, tumor cells [57-60]	M, P	Predictor of Poor Chemotherapy Effectiveness	Poor overall and disease-free survival prognosis
IL-8. Chemokines. monocytes, MF, lymphocytes, endotheliocytes, neutrophils, fibroblasts, tumor cells [53, 54, 59-61]	M, P	Increased invasiveness and metastatic potential	Poor overall and disease-free survival prognosis
IL-10. IL-10 family . Treg, Th0, Th1, Th2, NK, CD8+, MF, tumor cells [55]	P	Multidirectional influence. Inhibits proliferation by suppressing IL-6. Increases invasive potential at high concentrations	No data
IL-19. IL-10 family. Monocytes, B-lymphocytes. [55]	M	Increases the risk of early relapse	Poor prognosis for disease-free survival
IL-20 (IL-20RA). IL-10 family. Monocytes, keratinocytes [62]	M	Increases invasive potential	Poor overall and disease-free survival prognosis
TNF-α. TNF-α family. Monocytes, MF, neutrophils, CTL, Th 1 [59, 63]	P, M	Multidirectional influence. Predictor of lymph node involvement. Associated with activation of effector cells	No data
TGF-β. Superfamily of growth factors. Family TGF [64-66]	M, P	Predictor of early recurrence and metastasis. Poor prognosis for lymph node metastases	Favorable prognosis in the early stages. Poor prognosis for metastatic disease

the maintenance of the peritumoral inflammation, increased capillary permeability and stimulation of angiogenesis. The role of TNF-α in TNBC is twofold. On the one hand, it promotes EMT, on the other hand, it activates antitumor CTLs [74-76].

Recently, the workers at A.M.Granov Research Centre for Radiology and Surgical Technologies and Pavlov University have performed a pilot study to assess prognostic significance of subpopulations of lymphocytes and cytokines which involved 29 TNBC patients. Before and after neoadjuvant chemotherapy, the amounts of lymphocyte subpopulations and cytokine contents were measured in peripheral blood, as follows: CD3+CD8+ (cytotoxic lymphocytes); CD3+CD4+ (T helpers); CD4+CD8+ (double positive T cells); CD16+CD56+HLADR+ (activated natural killers); CD3+CD16+CD56+ (TNK cells); CD4+CD25+FoxP3 (T-regulatory cells); CD3+HLA DR+ (activated T cells); αβ T cells (alpha/beta T cells); γδ T cells (gamma/delta T cells); interleukin-1β (IL-1); interleukin-2 (IL-2); interleukin-4 (IL-4); interleukin-6 (IL-6); interleukin-8 (IL-8); interleukin-10 (IL-10); interleukin-12 (IL-12); interferon-α (IFN-α); interferon-γ (IFN-γ); tumor necrosis factor-α (TNF-α). The assays were carried out at Laboratory of Immunology, A.M. Nikiforov Center for Emergency and Radiation Medicine (St. Petersburg) using the Cytomics laser flow cytometer FC 500 (BECKMAN COULTER, USA). As a result of multivariate analysis, we have revealed that, among these parameters, the concentrations of T regulatory cells in peripheral blood (CD4+CD25+FoxP3) ($p=0.045$), as well as spontaneous production of IL-6 ($p<0.005$) and IL-10 ($p<0.005$) proved to be independent predictors of early relapse in triple-negative breast cancer.

The use of monoclonal antibodies in TNBC treatment

As already noted, specific tumor target antigens for immunotherapy have not yet been identified in breast cancer. Therefore, in recent years, much attention has been paid to the mobilization of immune surveillance of cells in the microenvironment. For example, many cancers are undergoing extensive clinical trials of immune checkpoint (ICT) inhibitors. These drugs relieve the state of local immunosuppression in these patients and enhance the activity of antitumor immunity. Reviewed by [77] Radoza et al. (2020) provide results from the IMpassion 130 program and other trials where therapy with antibodies to the programmed death receptor or its ligand (PD-1/PD-L1) and paclitaxel was used as first-line therapy for PDL 1-positive metastatic TNBC. Other ICT

trials have used carboplatin or other cytotoxic drugs. The biological meaning of such combined schemes is the death of malignant cells and, which leads to the appearance of neoantigens – additional targets for activated immune cells of the patient. Along with this, within the framework of the generally accepted concept of targeted therapy for malignant neoplasms, programs of clinical trials of monoclonal antibodies are being carried out, against EGFR2 (epidermal growth factor receptor 2).

Potential targets for immunoconjugates of anticancer antibodies

A separate group of biomarkers is regarded as potential targets for a new group of drugs – conjugates of monoclonal antibodies with cytotoxic agents. Monoclonal antibodies bind to the target, and the complex is internalized into the tumor cell, realizing selective cytotoxicity. The target molecule for the conjugate must be overexpressed on the cell surface and have the property of internalization upon interaction with the ligand. Currently, several molecules have been identified in TNBC cells with the following properties: 1) non-metastatic glycoprotein b (GPNMB); 2) surface trophoblastic antigen-2 (Trop -2); 3) zinc-containing transport protein (LIV-1); 4) sialoglycomucin (CA 6).

GPNMB is involved in several processes associated with carcinogenesis, including cell migration, invasion, angiogenesis, and EMT. In addition, it is a biomarker of poor prognosis [90]. GPNMB is a target for Glematumumab vedotin (CDX -011), a conjugate containing a microtubule-destroying chemical agent, monomethyl auristatin E (MMAE), as an effector. Phase II data from the EMERGE study demonstrated that CDX -011 is more effective and less toxic than chemotherapy in TNBC patients with GPNMB overexpression [78, 79].

Trop -2 is a transmembrane glycoprotein involved in the processes of migration and proliferation, which is a target for Saccituzumab govitecan (IMMU -132), which contains a topoisomerase I inhibitor as an active agent. SN -38. Results of a phase II study of 33.3% objective responses in patients with TNBC in the third line of therapy [80, 81].

LIV -1 is involved in the regulation of STAT -3 expression, cell adhesion, and EMT. Preclinical studies have demonstrated the efficacy of Ladiratuzumab vedotin, which binds to the extracellular domain of LIV -1 [82].

CA 6 is selectively expressed on many solid tumor cells. It is a target for SAR 566658, which contains microtubule-destroying DM 4 as an active component [83].

Immune response inhibitors: PD1/PDL

Biomarkers that predict the effectiveness of immunotherapy in patients with TNBC include co-inhibitory molecules – targets of immunooncological drugs, microsatellite instability, mutation load, and tumor-infiltrating lymphocytes.

PD-1 is a co-inhibitory molecule that regulates the functions of components of the innate and adaptive immune response. It is expressed on the surface of T-lymphocytes, B-lymphocytes, MF, monocytes, DC. Under physiological conditions, it contributes to the formation of tolerance to autoantigens; in the tumor microenvironment, it promotes tumor immunological tolerance [84]. The PD-1 ligand (PD-L1) is a transmembrane protein that is expressed both on tumor and immunocompetent cells (T, B-lymphocytes, DC, MF). The interaction of PD-1/PD-L1 leads to deactivation of T-lymphocytes, activation of T-regulatory cells, and persistence of tumor cells [85, 86].

PD-L1 is expressed in 20% of TNBC cases. PD-L1 is expressed in about 10% on tumor cells, and 40-65% on cells of the tumor microenvironment. Expression of PD-L1 on tumor cells is a predictor of a favorable prognosis and a marker of sensitivity to chemotherapy [87]. Expression of PD-L1 on lymphocytes in the microenvironment is a marker of sensitivity to blockers of co-inhibiting molecules [88].

PD-1 and PD-L1 blockers are currently the standard treatment for TNBC. Atezolizumab (anti-PD-L1) was the first approved drug in this group for the treatment of its metastatic forms. The Phase III study IMPASSION 130 evaluated the efficacy and safety of atezolizumab and included 451 participants. The median overall survival in the group where the expression of PD-L1 ≥ 1% on the cells of the lymphoid infiltrate was significantly higher in the group where patients received atezolizumab in combination with nab-paclitaxel (25 and 18 months). To assess the expression of PD-L1 in the study, the test system VENTATA was used [89]. The efficacy and safety of pembrolizumab (anti-PD-1) in previously untreated patients in phase III was assessed in the KEYNOTE -355 protocol. The study included 847 patients who received various chemotherapy regimens (paclitaxel, nab-paclitaxel, platinum drugs and gemcitabine) in combination with placebo or pembrolizumab. Expression of PD-L1 was assessed in points using the 22C3 test system (DAKO PharmaDx), which considered the ratio of PD-L1 on tumor cells, lymphocytes and macrophages to the total number of detected tumor cells, multiplied by 100 (CPS, combined positive score). Significant differences in median relapse-free survival were found only at CPS ≥ 10 (9.7 months in the pembrolizumab group and 5.6 months in the placebo group). PD-1 and PD-L1 in patients with TNBC are associated with prognosis. PD-L1, in addition, plays the role of a predictive factor in relation to the effectiveness of blockers of co-inhibitory molecules [90, 91].

Cellular and immunotherapy of breast cancer

Early attempts of hematopoietic stem cell transplantation (HSCT)

In the 1980s, with the development of cytostatic therapy for solid tumors, it became necessary to maintain and restore hematopoiesis in patients with intensification of chemotherapy regimens. Therefore, methods of bone marrow transplantation taken from the patient himself (autologous BMT) before the start of intensive chemotherapy, in combination with hematopoiesis stimulation factors, were proposed. At the same time, the main problem was the purification of the harvested bone marrow of patients from metastatic

tumor cells. At that time, there were no sufficiently effective immunological markers for the detection of malignant cells and their elimination in transplants. However, the first clinical studies of the 90s according to the use of auto-TKM in breast cancer, an increase in overall and recurrence-free survival was revealed in some patients [92]. However, later these positive results were not confirmed in larger samples and in randomized trials [93].

Subsequently, with the development of transplantation of hematopoietic and immune cells from HLA -compatible donors (allo-HSCT), there were proposals to use allogeneic cells to implement the immune effect "graft-versus-tumor", by analogy with the "graft-versus-leukemia" reaction in oncohematological diseases [94]. A positive effect of allo-HSCT was noted in some patients with solid neoplasms, including breast cancer. In general, the clinical response here was associated with the development of acute and chronic graft-versus-host disease. The authors pointed to low specificity and pronounced undesirable effects in this type of treatment.

The use of individual fractions of donor immune cells (primarily lymphocytes) for the adoptive therapy of solid cancers is considered. However, the authors express doubts about the duration of the therapeutic effects of adoptive immunotherapy [95]. It is possible that adoptive immunotherapy will find its place in combined regimens for the treatment of solid tumors, along with targeted drugs.

Current opportunities of cell therapy of breast cancer

One of the long-standing methods of biotherapy is the use of individual cell-based vaccines that have a therapeutic effect in breast cancer. Their clinical development is in the 2nd-3rd phases. Early work in this area consisted of short-term incubation of the patient's tumor tissues with his lymphocytes/monocytes with the addition of several cytokines to induce the presentation of these antigens and activate the response of immune cells (both T-lymphocytes and macrophages) to tumor antigens, after which these stimulated, the cells were returned to the patient. More modern approaches involve targeting antigens that are expressed mainly in malignant tumors and, to a much lesser extent, on normal cells. Typically, T-lymphocytes targeting these antigens are eliminated by the tolerance system. However, in this clinical situation, cell-based vaccines must be immunogenic enough to activate, among other things, T cells with low affinity for these antigens. Here it becomes expedient to use ICT to activate these cell populations. In addition, there is currently a search for individual mutations in the genome of cancer cells, based on which it is supposed to create cell-based vaccines for specific patients with cancer [96].

A few works [97] consider the possibility of using activated populations of natural killer (NK) cells of malignant killer T cells in oncological diseases. Most often, peripheral blood mononuclear cells are used for this and stimulated with interferon gamma and/or IL-2 for 2-3 days. In particular, Sommaggio et a11. (2020), cytokine-induced killer cells (CECs) in combination with cetuximab (an EGFR inhibitor) showed good antitumor and antimetastatic efficacy in NOD/SCID (NSG) mice with human breast transplants [98].

One of the latest trends is the development of CAR-T cells as a selective means of eliminating malignant cells that carry a specific antigen.

Thus, some authors are considering the possibility of using CAR-T cells against the MAGE-A4 antigen, which is considered a promising target for the treatment of lung cancer and TNBC [99]. The main objective of this work was to select T cells directed against a small MAGE-A4 region recognized by the corresponding HLA-A2 allele. These TCR-T cells with CD4 markers showed a direct selective cytotoxic effect *in vitro* and *in vivo* (in mice with xenografts) against various human malignant tumors expressing the antigen MAGE-A4.

Epidermal growth factor receptor (EGFR) is one of the most promising targets in cell therapy for breast cancer, against which effective T-cell products with a chimeric antigen receptor have been developed. Chinese authors have created EGFR lines using a lentiviral vector. CAR-T cells against TNTC, which was tested on cells *in vitro*. The 3rd-generation drugs caused a pronounced and specific suppression of the growth of tumor cells. At the same time, only minimal toxicity was noted in relation to normal breast cells. The antitumor effect was confirmed and *in vivo* in mice with transplanted human tumors. It is hypothesized that EGFR stimulation CAR T cells cause this population to proliferate and support their growth. Transcriptome studies have shown that the effect of CAR T cells consists in the activation of systems of ќ≥-interferon, granzyme-perforin, and enzymes of apoptosis of tumor cells.

Another possible target antigen for immunotherapy is the so-called ROR1 (tyrosine kinase-like orphan receptor 1). A group of German authors developed ROR 1-specific CARs T cells [100]. Their biological activity was assessed in 3D models of lung and breast tumors based on the corresponding cell lines similar in structure and phenotype to primary tumors. ROR 1- CAR T cells in this model had a pronounced antitumor effect, actively inhabiting the tumor tissue and destroying its cell layers. Thus, the fundamental possibility of bioeffects of these genetically modified T cells under conditions close to the situation *in vivo* was shown.

At the same time, the action of CAR T cells against tumors may be defective. Thus, the suppression of the cellular immune response under the influence of the widespread factor TGF-β is supposed. The already mentioned group of German authors [101] studied the issue of this pronounced immune suppression, and the ways of neutralizing this effect. For this purpose, the lines CD 8 + and CD 4 + were prepared. ROR 1- CAR T cells from healthy blood donors, and their antitumor activity was determined on TNBC cells (MDA-MB-231) *in vitro* and in 3D models. It turned out that adding TGF-β led to decreased viability, cytolytic activity, cytokine production, and ROR 1 – CAR proliferation. T cells in mixed culture with tumor cells. Blockade of the TGF-β receptor with a specific inhibitor SD-208 protected CD 8+ and CD 4+ ROR 1- CAR T cells from this inhibitory effect and maintained the antitumor properties of CAR T cells. Thus, to preserve the effects of CAR T cells may need combined exposure, in particular – and in subsequent testing of these cell products. Among the factors of tumor resistance is called immunosuppression, which may develop with the introduction

of CAR T cells against EGFR, as shown by the same group of authors in experiments on mice with TNBC [102]. This negative effect of CAR T cell therapy is associated with the induction of interferons, suppression of the activity of a number of immune response genes and can be overcome with epigenesis inhibitors (for example, inhibitors of the CDK7 gene).

In addition to the generally accepted cell therapy for oncological diseases, additional means of enhancing the effects of chemotherapy on the tumor are also possible. Thus, it is known that the system of macrophages and other phagocytic cells is able to capture and inactivate most of the drug when it is administered in a free form. To solve this problem, various means are proposed for its microencapsulation and targeted delivery to the tumor tissue. Chinese authors proposed preparations of the so-called "analogs of apoptotic bodies" (AAT) prepared from malignant cells containing CD47 and adhesion molecules [103]. These artificial structures, according to the authors, combine antiphagocytic properties and, at the same time, can be used for more efficient delivery of chemotherapy drugs to the tumor. An increased accumulation of AAT and, accordingly, increased efficiency of encapsulated drugs has been shown in an experimental model of metastasis.

Conclusions

1. The search for new biomarkers of TNBC, as well as the assessment of their prognostic and therapeutic potential, is currently one of the main tasks in the development of effective individualized treatment programs.

2. Several lines of research seem to be the most promising. The first (diagnostic) is associated with the development of "liquid biopsy" technology and evaluation of biomarkers in the blood, including subpopulations of lymphocytes, spontaneous and induced production of cytokines.

3. To address these standardization issues, an international working group on tumor immunological biomarkers has now been established, as well as analytical centers for immunological monitoring, whose tasks include identification, assessment of prognostic and predictive potential, and validation of biomarkers.

4. Another direction is related to the improvement of the technology of 3D tumor models, which allow modeling the microenvironment and selecting the most specific effects on the tumor, in accordance with the individual biomarkers of a given patient.

5. In the field of TNBC cell therapy, the data of numerous clinical trials are gradually accumulating. The results obtained so far make it possible to determine the dosage, the frequency of administration and the possibility of combination with conventional cytostatic anticancer drugs. To date, EGFR may be a suitable target for cellular immunotherapy in TNBC, and appropriate CAR-T cell products may be promising in the future in the clinical setting.

Conflicting interests

Not declared.

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Биологические маркеры тройного негативного рака молочной железы: поиск мишеней для иммунотаргетной и клеточной терапии

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Резюме

Трижды негативный рак молочной железы является одним из наиболее агрессивных. Он представляет собой гетерогенную группу заболеваний с различными молекулярными дефектами, требующими дифференцированного подхода к диагностике и лечению. В статье приведены данные о современных молекулярных классификациях трижды негативного рака молочной железы и дефектах сигнальных путей, а также продемонстрирована их связь с иммунологическими и неиммунологическими биомаркерами. Обобщены данные о прогностической и предсказательной роли молекулярных биомаркеров, существующих и разрабатываемых подходах к разработке таргетных препаратов, для которых они являются мишенями, а также перспективных методах клеточной терапии. Приведены данные собственных исследований, касающиеся оценки прогностической роли цитокинов и субпопуляций лимфоцитов в крови пациентов с трижды негативным раком молочной железы, обозначены перспективы дальнейших исследований.

Ключевые слова

Рак молочной железы, трижды негативный, молекулярные подтипы, мутационная нагрузка, стволовые опухолевые клетки, циркулирующие опухолевые клетки, клеточное микроокружение, субпопуляции лимфоцитов, интерлейкины, молекулярные мишени, клеточная терапия.