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DISSEMINATED TUMOR CELLS AND ENHANCED LEVEL OF SOME CYTOKINES IN BONE MARROW AND PERIPHERAL BLOOD OF BREAST CANCER PATIENTS AS PREDICTIVE FACTORS OF TUMOR PROGRESSION

N. Semesiuk[1], A. Zhylchuk[2], N. Bezdenezhnykh[1], A. Lykhova[1], A. Vorontsova[1], V. Zhylchuk[2,3], Yu. Kudryavets[1,]*

[1]R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Kyiv 03022, Ukraine
[2]Rivne Region Oncology Hospital, Rivne 33001, Ukraine
[3]Danylo Halytsky Lviv National Medical University, Lviv 79010, Ukraine

Background: **In recent years, the presence of disseminated tumor cells (DTC) in bone marrow (BM) of patients with breast cancer (BC) is considered as an important clinical feature of the distribution process. However, relapse often occurs in spite of the negative results of the bone marrow cytology. This suggests the need to find additional signs of the possibility for predict the recurrence.** ***Aim:*** **to detect DTC in BM and determinate cytokine status of peripheral blood (PB) and BM of primary BC patients for prognosis of tumor recurrence.** ***Patients and methods:*** **72 BC patients with histologically proven diagnosis were enrolled into study. 31 patients with progression of disease and 41 patients with clinical stabilization (conditional remission) were included to "progression" and "remission" group respectively. This division of BC patients was conditional and was made during the 3 years study. The presence of DTC in BM was detected by immunocytochemical analysis. Plasma levels of TNF-α, M-CSF, IFN-α were defined by bioassay tests. Plasma levels of IL-6, TGF-β1 and VEGF were determined by ELISA. BM and PB BC patients were obtained before treatment.** ***Results:*** **In our study DTC in samples of BM were detected in 50% BC patients of progression group. It was found that most significant addition markers of tumor progression with presence of DTC in BM are the levels of cytokines such as TNF in BM and PB, CSF-1 and IL-6 in PB and endogenous IFN in BM and PB of BC patients. In patients of disease "progression" group the levels of TNF in BM were increased by 45.8% ($p < 0.01$), the levels of CSF-1 and IL-6 in PB were increased more than by 70–80% ($p < 0.05$).** ***Conclusion:*** **Comprehensive detection DTC in BM and identification the level of TNF, IFN, CSF-1, IL-6 in PB and BM of BC patients could be one of the ways for prognosis the metastatic process and correction antitumor individualized therapy.**

Key Words: **breast cancer, bone marrow, disseminated tumor cells, tumor necrosis factor α, interferon-α, macrophage colony stimulating factor, interleukin 6, transforming growth factor β1, vascular endothelial growth factor.**

Breast cancer (BC) is the most frequent type of cancer in women. Despite the improvement in detection and treatment, mortality level reaches ≈30% of newly diagnosed cases [1]. In most cases, death results from the dissemination of cancer cells through lymphatic or circulatory system and the development of distant metastases. The presence of circulating tumor cells (CTC) in PB and disseminated tumor cells (DTC) in BM BC patients has been proven to have clinical relevance [2]. It is now possible to detect CTCs in the PB and DTCs in the BM by using immunocytochemical analysis which include staining with monoclonal antibodies against epithelial or tumor-associated antigens and molecular methods which are based on detection specific nucleotide sequences (real-time PCR, reverse transcription — PCR) [3]. Identification of these cells has a crucial value in the disease course and it's progression and in monitoring of chemotherapy. However, in many cases progression of disease as activation minimal residual disease (MRD) can occur without detection of cytokeratin-positive DTC in BM. Based on the fact that cancer is a systemic process the level of certain cytokines associated with tumor growth, may serve as an additional important factors for prediction BC recurrence. Very important role in tumor progression plays microenvironment both the tumor and target-organ for the development of distant metastases, which consists of the cancer cells (for microenvironment of tumor) and the surrounding component (different type of cells: fibroblasts, endothelial cells, pericytes, immune cells, etc.; different soluble factors: cytokines, chemokines). Cytokines which may affect the cancer progression are tumor necrosis factor-α (TNF-α), colony stimulating factors (such as M-CSF (CSF-1)), interleukin 6 (IL-6), interferon (IFN), vascular endothelial growth factor (VEGF) and transforming growth factor β1 (TGF-β1) and others. In BC progression special role play inflammatory mediators like TNF-α and IL-6. It was found that TNF-α is expressed at very low incidence in normal breast epithelial cells but it incidence is significantly elevated in tumor cells which suggest progression-related roles TNF-α in BC [4]. Moreover TNF possess anticancer and pro-cancerous effects and it's role in cancer is ambiguous.

For other proinflammatory cytokine, IL-6, is that produces multifunctional effects among which most important are regulation of immune reactions, hema-

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Correspondence: E-mail: kudryavets@mail.ru
Abbreviations used: BC – breast cancer; BM – bone marrow; CTC – circulating tumor cells; DTC – disseminated tumor cells; EMT – epithelial-mesenchymal transition; IFN – interferon; IL-6 – interleukin 6; M-CSF (CSF-1) – macrophage colony stimulating factor; PB – peripheral blood; TGF-β1 – transforming growth factor β1; TNF – tumor necrosis factor; VEGF – vascular endothelial growth factor.

topoesis and inflammatory state. IL-6 has been shown to be associated with tumor progression and metastasis including inhibition of cancer cells apoptosis and stimulation of angiogenesis and DTC adhesion. Furthermore, this cytokine is considered as a potential prognostic factor of cancer disease [5]. Many publications have demonstrated that IL-6 is one among major factors upregulating and modulating cancer-mediated bone destruction [6].

Another group of biological factors are CSFs which play a significant role in many biological processes such as immunomodulation, in acute inflammatory reactions carrying out the function of autocrine and paracrine regulators of growth and spread of tumors and it's using in antitumor therapy should be careful analyzed. Granulocyte- and granulocyte-macrophage colony-stimulating factors (G- and GM-CSF) are known to be powerful regulators that govern the proliferation, differentiation and maturation hematopoetic progenitors and also enhance the effector functions of mature neutrophils and have already been applied in clinical trials. Particular attention is attracted M-CSF (CSF-1) and its receptor (CSF-1R). These functional ligand-receptor pair involve in the development of the normal mammary gland during pregnancy and lactation, and were found to have an important role in biology of BC (aberrant expression of CSF-1 and its receptor correlates with invasiveness of tumor cells and unfavorable clinical prognosis) [7]. The regulation of expression of CSF-1 and CSF-1R occurs autocrine and paracrine mechanisms involving tumor cells [8].

Another player in the complex process of progression of the disease is TGF-β. TGF-β is a multifunctional regulator of cell growth, apoptosis, differentiation and migration and exists in three isoforms. One of them — TGF-β1 — is one of the most potent metastatic inducers [9]. In normal mammary gland development TGF-β is a great factor which regulates branching morphogenesis and differentiation by acting on both epithelial and stromal cells [10]. In BC this cytokine play a biphasic role: acts as a tumor suppressor at early stages of disease and as a tumor promoter by eliciting an epithelial-mesenchymal transition (EMT) [11].

Vascular endothelial growth factor (VEGF) is a potent angiogenic cytokine in normal tissues [12] and tumors stimulating endothelial cell proliferation *in vitro* and inducing angiogenesis *in vivo* [13]. VEGF is overexpressed in BC tissue when compared to normal breast tissue [14] and levels of VEGF in these tissues correlate with disease-free and overall survival. A soluble form of VEGF (VEGF121) is detectable in the circulation and studies have demonstrated elevated serum levels of VEGF in patients with solid tumours including uterine, ovarian, colorectal, lung and brain cancers [15–17] leading some to hypothesise that serum VEGF measurement may have predictive value for the progression of disease in patients with solid tumors [18].

Finally, cytokine which stands on the other side of its effects from the already described factors. IFN-α is a protein with a wide range of actions like antiviral, antimicrobial, antiproliferative, antitumorogenic, immunomodulating, antiangiogenic. The nature of IFN belong to the tissue hormones which are characterized polipotentnional action: induce resistance of cells to wide range of viruses, inhibit cell division, modify surface properties of normal and tumor cells, modify cell phenotype resulting in a reversal transformation [19–21]. Moreover, IFN is active inhibitor of EMT [22].

Despite the fact that detection DTC in BM is not always accompanied by the progression of the disease, very important and appropriate is to determine the cytokine profile of BM and PB in BC patients to identify possible risk of activation of dormant process and correction antitumor individualized therapy.

MATERIALS AND METHODS

Patients. This study included 72 BC patients Rivne Region Oncology Hospital (Rivne, Ukraine) and 14 healthy donors. The mean age of the subjects was 52.1 ± 1.89 years (range 36–69 years). BC patients were divided in two groups: 1) with progression of disease (31 patients) and 2) with clinical stabilization (conditional remission) (41 patients) according to the definition of the WHO Expert Committee of the terms "progression" of cancer and "remission". This division of BC patients was conditional and was made during the 3 years study. The term "progression" of cancer means the emergence of new foci of cancer or increases all or some foci to 25% or more. The term "remission" means disappearance of symptoms of disease. In oncology there is a concept of complete and partial remission. The complete remission of the disease means disappearance of symptoms and features which are defined by standard laboratory, clinical and instrumental studies. The partial remission means preservation some of features of disease. In the general sense remission of cancer should be interpreted as the absence of recurrence within five years after the active phase of therapy. In our case remission of disease interpreted as the absence of recurrence within three years after the active phase of therapy and terms as "conditional remission".

Receptor status of steroid hormone and the level of HER-2/neu expression in tumor cells were investigated in all patients without exception before treatment through trepan biopsy breast tumors. But establishment of correlation between the levels of cytokines and receptor status was not our task. All BC patients before treatment were randomized by TNM classification, age, histological structure of the tumor, and steroid hormone receptor status and level of HER-2/neu expression in tumor cells.

In this study, BC patients consisted of only II (45 patients) and III (27 patients) stages. They were informed about the survey and provided consent to the use of the material for research purposes. The study was carried out with approval of the local ethics committee. The BM aspirates (3–4 ml) was carried out from sternum with an incision in the skin in the sampling area to avoid contamination by epithelial cells prior

treatment. Blood samples (5–9 ml) were obtained by venipuncture. BM and PB BC patients were obtained before treatment.

For the plasma preparation, venous blood and aspirates of BM were collected into a test tube with EDTA (Sente-Lab, Ukraine). The plasma sample were centrifuged (10 min, 8000 rpm, +4 °C) (Microspin, Eppendorf, USA) to quantitatively remove residual platelets as possible source of different cytokines and stored frozen at –20 °C.

Mononuclear cells (MC) were isolated with Ficoll-Hypaque density gradient LSM 1077 (PAA, Austria) centrifugation at 1,500 rpm for 20 min. MCs were washed three times with RPMI-1640. Cytospins were prepared from aliquots of MC cells ($1 \cdot 10^6$), were dried up and stored at –20 °C.

Bioassay tests. Detection of the level of cytokines TNF, IFN and CSF-1 and it's biological activity in the BM and blood samples BC patients was determined by titration system in cell lines L929a, MDBK and M-NFS-60 respectively. The results of experiments were obtained by staining with dyes (MTT, sulforodamine B or crystal violet) and recorded using spectrometer (Labsystems Multiskan PLUS, Finland) at a wavelength of 540 nm.

L929a cells were cultured in RPMI-1640 medium (PAA, Austria) with 10% newborn bovine serum (NBS) (Sigma, USA) at 37 °C and 5% of CO_2. The cells were seeded in 96-well plate in concentration $1.5 \cdot 10^4$ per well and cultivated 24 h at 37 °C and 5% of CO_2. After that experimental samples (plasma of bone marrow or of peripheral blood) and standard of TNF (GF023 Recombinant Human TNF-alpha (Chemicon International, USA)) were titrated [23].

M-NFS-60 cells were cultured in RPMI-1640 medium (PAA, Austria) with 10% fetal bovine serum (FBS) (Sigma, USA) and 10% supplement at 37 °C and 5% of CO_2. First of all, the experimental samples (plasma of bone marrow or of peripheral blood) and standard of CSF-1 (Neupogen, B2013, $30 \cdot 10^6$ (Hoffmann-La Roche, Switzerland)) were titrated and than the cells were seeded in 96-well plate in concentration $4.5 \cdot 10^4$ per well and cultivated 60 h at 37 °C and 5% of CO_2 [24].

MDBK cells were cultured in DMEM medium (PAA, Austria) with 10% NBS (Sigma, USA) at 37 °C and 5% of CO_2. The cells were seeded in 96-well plate in concentration $1,5 \cdot 10^4$ per well and cultivated 24 h at 37 °C and 5% of CO_2. After that, experimental samples (bone marrow and peripheral blood) and Standard of IFN (2nd WHO International Standard 1999 Interferon alpha 2b Human rDNA, 95/566, 70,000 IU per ampoule (USA)) were titrated [25].

MTT-test. The concentration of M-CSF was assayed using standard MTT-colometric with 3-[4,5-dimethylthiasol-2-1]-2,5-diohenyltetrasolium bromide (Sigma, USA) [26].

Sulforodamine B test. The concentration of M-CSF was assayed using standard Sulforodamine B test (Sigma, USA) [27].

Crystal violet test. Assessment of the proliferative activity of the cells after 48 h of incubation with test samples performed in the colorimetric crystal violet test (Sigma, USA) [28].

Immunocytochemical analysis. Three slides of cytospins were incubated in hydrogen peroxide block to reduce nonspecific background staining due to endogenous peroxidase [29] and than were incubated with primary pancytokeratin monoclonal mouse anti-human cytokeratin antibodies clones AE1/AE3 (Dako, Germany). Immune complex formed by secondary anti-mouse antibodies were revealed by EnVision+System-HRP Labelled Polymer (Dako, Germany) and the slides were counterstained with hematoxylin to study nuclear morphology [30].

Anti-mouse IgG HRP labelled antibodies (Dako, Germany) were used as negative control.

ELISA assay. The level of TGF-beta1 was analyzed by the receptor-based Quantikine ELISA kit (Demeditec, Germany), according to the manufacturers instruction. The absorbance at 450 nm was detected by the microplate reader (Labsystems Multiskan Plus, Finland). Concentrations were calculated from the constructed linear curve. The level of VEGF was analyzed by the ELISA Development kit (R&D Systems, USA).

Statistics. The regression analysis was used to study any possible correlation among the different parameters (Statistica 7.0). All values were expressed as mean±S.E.

RESULTS AND DISCUSSION

Detection of DTC in BM BC patients. In our study using immunocytochemical analysis with anti-pancytokeratin monoclonal antibody we have shown that 50% BC patients was positive in DTC in samples of BM and consist the group of progression (Fig. 1). Yet, in many cases progression of tumor growth is not accompanied by the appearance of DTC in BM. This can be attributed not only to the loss of some markers of tumor cells, but also with the technical problem of low probability of detection of these cells in the BM aspirate. Due to the lack of statistical significance of the detection of tumor cells in the BM, for a more reliable prognosis of BC attempts to use other additional markers to improve the predictive value of tumor cells detection in the BM. In particular, were used a series of specific markers in the primary tumor or micrometastases: p53, Ki-67, TopoII, EGF-R, HER2/neu, hormone receptors, and others [31, 32].

Currently detection of cytokeratin-positive DTC in BM has already become one of the most important features for predicting the course of cancer (indicator for the prognosis of the tumor progression) [33–35] and was associated with a significantly higher risk of recurrence and disease-specific death [36]. During the last years the number of single markers that have been evaluated for DTC detection has considerably increased.

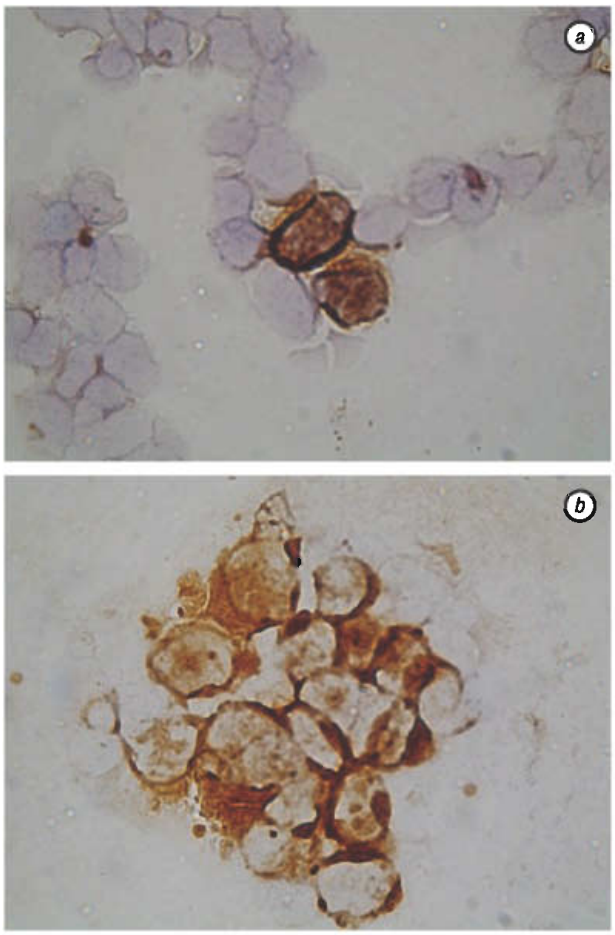

Fig. 1. Cytokeratin-positive DTC in BM BC patients (×100)

An ideal marker should be universally, but uniquely expressed on all BC cells. Regarding to epithelial tumors, cytokeratins have become the markers of choice for DTC detection by immunocytochemical analysis which is still a gold standard for the DTC detection. They are expressed at various level and compositions in all epithelial tumors, but rarely in other tissues [37]. However, the problem is complicated by the fact that DTC are phenotypically heterogeneous and often lose a number of epithelial features and require the most aggressive characteristics in the result of the EMT [34]. Moreover for survival and proliferation of these cells it is not enough only the intrinsic characteristics, but also the microenvironment in which they operate. Tumor cells that have extravasated into different organs need to establish a permissive niche that allows them to proliferate and give rise. Tumor microenvironment components play a critical role in the communication with the tumor cells through the secretion of a large, ever-increasing number of cytokines, chemokines, and growth factors (VEGF, EGF, IGF-1, SDF1, IL-6, IL-8, TGFβ, OPN, or FGF) that have been found to promote tumor progression, affecting tumor cell proliferation, invasion and angiogenesis [38]. In this case, the BM constitutes an unique microenvironment for cancer cells in three specific aspects. The first one that the BM actively recruits CTC where they find a sanctuary rich in growth factors and cytokines that promote their proliferation and survival. When in the BM, tumor cells profoundly affect the homeostasis of the bone and the balance between osteogenesis and osteolysis. As a consequence, growth and survival factors normally sequestered into the bone matrix are released, further fueling cancer progression. The second, tumor cells actively recruit BM-derived precursor cells into their own microenvironment. The third, BM-derived cells can home in distant organs, where they form niches that attract CTC. This creates favorable conditions for the further progression of the disease [39]. All mentioned above define the purpose of our study.

In our work, we focused on the cytokine profile of BC patients, because it is not excluded that increased level of some cytokines may serve as the additional factor of prediction progression of tumor growth, because certain of cytokines create the microenvironmental conditions for the establishment of distant metastases from a primary BC. Therefore, a search of new comprehensive prognostic algorithm, based on simultaneous detection of DTC in BM and cytokine profile in BC patients, should promote for advances in development of personalized therapeutic approaches.

Identification the level of biological activity complex of cytokines in BM and PB samples BC patients. Most tumor markers have a low sensitivity in early stages of disease. It would be great to have tumor markers that reliably predict tumor recurrence independently of or together with TNM stage. Most promising in this respect is to determine the level of certain cytokines associated with tumor progression and which may serve as additional factors of disease progression.

TNF. BC provides a typical example of an inflammation-linked malignant disease. Breast tumors are enriched with inflammatory constituents, including cells that are polarized to the tumor-promoting phenotype, and soluble factors. Cumulative findings of a large number of studies indicate that many of the inflammatory components present in the tumor microenvironment actively support BC development and progression [4]. Nowadays has been investigated a numerous markers for breast cancer: ErbB2 oncogene (Her-2/neu), TOP2A, CA 15-3, carcinoembryonic antigen (CEA), BR 27.29, cytokeratin 19 [1, 40–42]. But insufficient attention is given to the roles of certain inflammatory cytokines such as TNF, IL-6 and IFN, and CSF-1 which are important part of microenvironment of tumor.

Our investigation has shown that the level of TNF in PB samples BC patients was increased in all cases and was higher than 50 pg/ml. This cytokine is absent or it's level was not increased more than 10 pg/ml in healthy human (in BM and PB). The level of TNF (higher than 150 pg/ml) in patients of progression group was increased by 36.8% cases and mean level was 138.6 ± 19.7 pg/ml whereas level of this cytokine in patients of remission group was 81.9 ± 4.9 pg/ml. The level of this cytokine more than 150 pg/ml in PB was met in 8.7% BC patients remission group what was in 4.9 times less than in patients of progression group. Moreover, the mean level of TNF in pa-

tients of progression group was in 1.44 times greater than that in patients other group. Regarding the level of this factor in patients II and III stages of disease in progression group, it was in 1.1 and 1.7 times higher respectively with this in patients the same stages in remission group. The highest level of TNF was observed in patients of III stages of disease in progression group in comparison with patients of III stages of disease in remission group (Fig. 2) ($p < 0.001$)

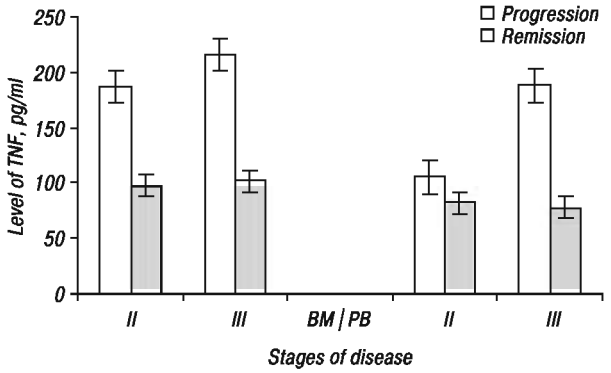

Fig. 2. The level of TNF in BM and PB in different stages of disease of BC patients ($p < 0.001$)

The level of TNF in BM was increased by 45.8% (more than 150 pg/ml) cases of BC patients of progression group and mean level was 198.7 ± 25.3 pg/ml compared with this level of TNF in patients of remission group 97.7 ± 8.8 pg/ml which was in 1.6 times less than in patients of progression group. Regarding the level of this factor in patients II and III stages of disease in progression group, it was in 1.9 and 2.1 times higher respectively with this in patients of the same stages in remission group. The level of TNF in BM from II to III stages of disease in patients of progression group only increases (see Fig. 2) ($p < 0.001$). Interestingly, the level of TNF in BM BC patients with DTC in BM was 360.0 ± 34.2 pg/ml and without DTC in BM was 225.0 ± 23.7 pg/ml ($p < 0.05$).

TNF is a proinflammatory cytokine which has been found to have pro-cancerous and antitumor effects [43, 44]. In our laboratory was established that TNF is an active modulator of metastatic potential tumor cells. *In vitro* assays were showed that interaction TNF and tumor cells accompanies by intensification and inhibition of ability the cells to metastasis depending on their type, concentration of this cytokine and duration of TNF action. Additionally, *in vivo* entering of TNF was created conditions to stimulation of process metastasis in early stages: increases adhesive properties of endothelial cells and damages the endothelial layer of blood capillaries [45, 46]. Hematogeneous metastasis can occur via a cascade of circulating tumor cell adhesion events to the endothelial lining of the vasculature. Interestingly, the pro-inflammatory cytokines (such IL-6 and TNF-α) play an important role in potentiating the inflammatory cascade, are significantly elevated in metastatic BC patients. Blood plasma triggers an adhesive phenotypic switch of BC cells and pro-inflammatory cytokines IL-6 and TNF-α induce adhesive recruitment of BC cells [47]. This demonstrates the importance of determining the level of this cytokine in the PB and BM patients as an additional prognostic marker of BC progression in combination with detection DTC in BM [48]. Moreover the expression of TNF is minimal in normal breast epithelial cells and is simultaneously acquired by the cells one malignant transformation has taken place. Besides TNF induce EMT properties in the tumor cells [4].

This suggests that investigation of the level of TNF in BM and PB BC patients might be an additional prognostic factor of tumor recurrence.

IL-6. IL-6 is the one of wide array of cytokines which is secreted by the breast tumors. The level of IL-6 increase with tumor grade. Increased serum level IL-6 has been demonstrated in BC patients compared to normal donors and correlates with advanced breast tumor grade and increased number of metastatic sites [49]. Moreover, IL-6 promotes BC cell growth and EMT in BC cells [50].

Our investigation has shown that level of IL-6 in PB BC patients in progression group was 1.2 times higher (the mean level — 352.27 ± 19.45 pg/ml) compared with this in patients of remission group (the mean level — 289.71 ± 24.7 pg/ml). The level of IL-6 in PB healthy donors was 49.5 ± 17.5 pg/ml.

According to the receiving data we can conclude that the identification IL-6 level in PB BC patients is not as significant as TNF level is.

CSF-1. Elevation of biological activity CSF-1 especially in serum PB PC patients is associated with poor prognosis of these patients. In our study we was found that biological activity of CSF-1 in PB was increased by 81.3% cases BC patients of progression group (428.8 ± 50.2 U/ml) and in patients remission group was 27.8% (217.7 ± 30.2 U/ml) ($p < 0.01$). But the level of this factor in samples of BM BC patients was not depended on the clinical stage of disease and average level in patients of progression and remission group was 548.8 ± 30.6 U/ml and 499.0 ±39.4 U/ml respectively (Fig. 3). This increased level of CSF-1 in BM can be explained by its sufficient amount in BM in healthy human (range 200–320 U/ml).

It is known that CSF-1 plays a significant role in many biological processes such as immunomodulation, in acute inflammatory reactions carrying out the function of autocrine and paracrine regulators of growth and spread of tumors. A specific and sensitive biomarker that indicates the presence of BC is highly desirable, yet available markers are of limited value. CSF-1 is involved in mammary gland development and mediates BC progression. Earlier work indicated correlation of serum CSF-1 with BC staging, and a recent report suggests that CSF-1 is a potential breast cancer marker, however the data reported so far await validation [51, 52]. Moreover CSF is a component of therapy BC patients but insufficient attention is given to the fact that this factor can be produced by different type of cells including cancer cells and its level may be high without additional external input in the treatment. Despite this special attention should

be given to determine the level of this factor especially in the plasma of blood in BC patients.

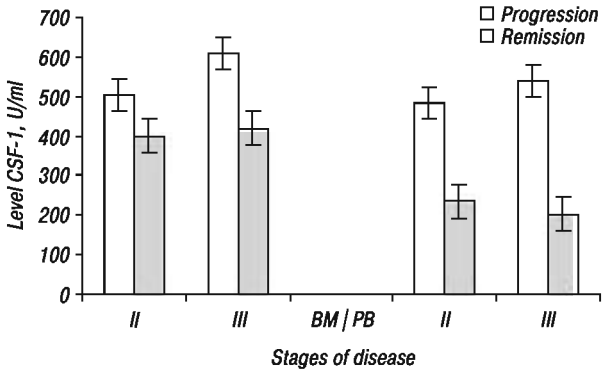

Fig. 3. The level of CSF-1 in BM and PB in different stages of disease of BC patients ($p < 0.01$)

TGF-β1. The role of TGF-beta in suppression or promotion of tumor growth is controversial and described in numerous papers [9–11, 53, 54]. Moreover, the elevated level of this cytokine was found in various pathologies including BC [55, 56].

Our study has shown that the level of TGF-β1 in BM of BC patients was increased in all cases independent from the group: the mean level in BM in patients of progression group was 17.0 ± 1.5 ng/ml and of remission group — 17.6 ± 2.3 ng/ml. It is level increased from stage to stage in BM in BC patients of progression group: II stage of disease — 14.88 ± 1.55 ng/ml, III stage of disease — 19.18 ± 7.6 ng/ml. The mean level of this cytokine in PB in patients of progression group was 17.14 ± 3.15 ng/ml and didn't much vary in patients in different stages: II stage — 20.1 ± 4.4 ng/ml, III stage — 19.92 ± 7.7 ng/ml. But the mean level of TGF-β1 in PB BC patients of remission group was in 1.6 times (10.78 ± 1.9 ng/ml) less than this one in PB in patients of progression group and was in 1.65 times higher compared with level in PB of healthy donors (6.5 ± 0.7 ng/ml).

In another study we have shown that postoperative elevation of TGF-β1-like activity in PB BC patients was associated with positive prognosis of disease [57]. In that case, TGF-β1-like activity was revealed in bioassay test using sensitive cell line.

In this our study the level of TGF-β1 in BM was also more higher in BC patients but significant correlation was not revealed between the level of this factor and prognosis of disease. We need to say that TGF-β1 levels in these last cases were detected by ELISA. This analysis is sensitive to anyone of proteins which react with antibodies against TGF-β1 but for some reasons these proteins, probably, do not always have full biological activity. Furthermore, may be absolutely not these proteins induce biological TGF-β1-like activity, which determine in TGF-β1-sensitive tissue culture.

It is obvious that the discrepancies in the obtained results could be caused by differences in the method of laboratory analysis (the use of bioassays for TGF-β1 rather than immunoassays).

Assuming that TGF-β inhibits tumor growth in it's initial stages, but it may increase the migration and metastatic potential of cells by inducing EMT in late stages. This process is accompanied not only with increased metastasis, but also the formation of drug-resistant subpopulations of tumor cells [58].

Thus our results correlate with literature data, however, to consider the TGF-β1 as an additional risk factor of activation of the tumor process required further research.

VEGF. The role of VEGF in oncogenesis and metastasis had already been established long year ago [59]. Angiogenesis, primarily regulated by VEGF, is a critical event in tumor progression and metastasis. Tumor cells release VEGF in response to oxygen and nutrients deprivation that in turn stimulates the formation of new vessels and promotes tumor growth and dissemination. Besides its role in new blood vessels, VEGF has been also shown to stimulate the proliferation of tumor cells. Indeed, VEGF enhanced the proliferation and the migration of breast cancer cells [60].

The level of VEGF we have investigated only in BM of BC patients. So, it is level was much higher in all cases independently of their clinical status and increased from stage to stage in patients as a group of progression and remission. The level of cytokine was 336.15 ± 45.7 pg/ml and 316 ± 56.1 pg/ml in progression and remission group respectively. The evidence suggests that the level of this cytokine should be continued to analyze in patients BC. The serum level of VEGF in BC patients depends on the degree of tumor spread to regional lymph nodes (RLN): increase in the number of affected metastases RLN accompanied by significant ($p < 0.001$) increased accumulation of VEGF in the serum [61]. Moreover, IFN prevents the growth of VEGF levels in BC patients.

IFN. IFN is the regulator of homeostasis in organism and is the modifier of phenotype of tumor cells which reveals in inhibition of proliferative potential of cells in long-term action of IFN, increases of sensitivity of cells to cytostatic agents of different nature and changes of phenotype of tumor cells [62]. It is known that IFN has antimutagenic properties, intensification the apoptosis in tumor cells inducing by different factors, inhibits the motility of tumor cells and expression oncogenes in these cells [45].

IFN in BM samples patients of progression group was revealed in 21.7% cases and mean level was consisted 11.4 ± 3.1 U/ml but in remission group was composed 24.7 ± 3.4 U/ml. In PB samples BC patients of remission group the level IFN was 18.8 ± 2.5 U/ml in 85.1% cases compared with these level in progression group (50% cases) — 10.2 ± 1.3 U/ml. Our study has shown that IFN is correlated with stage of disease, and it's level and the frequency of detection are higher in remission group (Fig. 4) ($p < 0.02$). In our investigation IFN in BC patients was to carried out full safety functions. The titer of IFN and the frequency of it detection directly correlated with positive prognosis of disease. Thus, the presence of IFN in blood of BC patients can be considered as a natural anti-tumor response. This fact indicates that endogenous IFN

plays a significant role in the inhibition of metastasis altering phenotype properties of tumor cells as previously shown in studies *in vitro* [22].

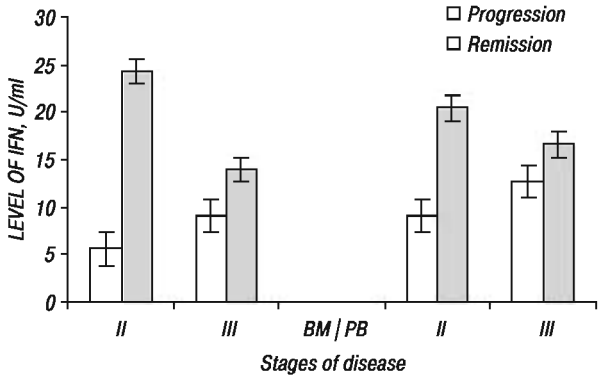

Fig. 4. The level of IFN in BM and PB in different stages of disease of BC patients ($p < 0.02$)

The obtained results allow conclude that the determination of complex prognostic factors such as the cytokine status of PB and BM of BC patients especially levels of pro-inflammatory cytokines TNF and IL-6, growth factors like CSF-1 and IFN simultaneously with the detection of DTC in BM of these patients play a major role in the prediction of metastatic process activation. This set of prognostic factors allows identifying groups at higher risk of cancer recurrence and making corrections in the therapeutic regimen for each patient.

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REFERENCES

1. **Campos M, Prior C, Warleta F, *et al.*** Phenotypic and genetic characterization of circulating tumor cells by combining immunomagnetic selection and FICTION techniques. J Histochem Cytochem 2008; **56**: 667–75.

2. **Lianidou ES, Mavroudis D, Sotiropoulou G, *et al.*** What's new on circulating tumor cells? A meeting report. Breast Cancer Res 2010; **12**: 307–18.

3. **Ross JS, Slodkowska EA.** Circulating and disseminated tumor cells in the management of breast cancer. Am J Clin Pathol 2009; **132**: 237–45.

4. **Soria G, Ofri-Sharak M, Haas I, *et al.*** Inflammatory mediators in breast cancer: Coordinated expression of TNF-α and IL-1β with CCL2 and CCL5 and effects on epithelial-to-mesenchymal transition. BMC Cancer 2011; **11**: 130–49.

5. **Lukaszewicz M, Mroczko B, Szmitkowski M.** Clinical significance of interleukin-6 (IL-6) as a prognostic factor of cancer disease. Pol Arch Med Wewnetrzhej 2007; **117**: 1–5.

6. **Tawara K, Oxford JT, Jorcyk CL.** Clinical significance of interleukin (IL)-6 in cancer metastasis to bone: potential of anti-IL-6 therapies. Cancer Manag Res 2011; **3**: 177–89.

7. **Sapi E.** The role of CSF-1 in normal physiology of mammary gland and breast cancer: an update. Exp Biol Med 2004; **229**: 1–11.

8. **Patsialou A, Wyckoff J, Wang Y, *et al.*** Invasion of human breast cancer cells *in vivo* requires both paracrine and autocrine loops involving the colony stimulating factor-1 receptor. Cancer Res 2009; **69**: 9498–506.

9. **Perera M, Tsang CS, Distel RJ, *et al.*** TGF-β1 interactome: metastasis and beyond. Cancer Genomics Proteomics 2010; 7: 217–30.

10. **Serra R, Crowley MR.** TGF-beta in mammary gland development and breast cancer. Breast Dis 2003;**18**: 61–73.

11. **Dumont N, Arteaga CL.** Transforming growth factor-β and breast cancer; tumor promoting effects of transforming growth factor-β. Breast Cancer Res 2000; **2**: 125–32.

12. **Hoeben A, Landuyt B, Highley MS, *et al.*** Vascular endothelial growth factor and angiogenesis. Pharm Rev 2004; **56**: 549–80.

13. **Nor JE, Christensen J,. Mooney DJ, *et al.*** Vascular endothelial growth factor (VEGF)-mediatedangiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. Am J Pathol 1999; **154**: 375–84.

14. **Mohamed Ali E, Sheta M, El Mohsen MA.** Elevated serum and tissue VEGF associated with poor outcome in breast cancer patients. Alexandria J Med 2011; **47**: 217–24.

15. **Ferrero S.** Serum and plasma vascular endothelial growth factor levels in testicular cancer patients. Ann Oncol 2004; **15**: 989–90.

16. **Gagne D, Page M, Robitaille G, *et al.*** Levels of vascular endothelial growth factor (VEGF) in serum of patients with endometriosis. Human Reprod 2003; **18**: 1674–80.

17. **Karayiannakis AJ, Syrigos KN, Polychronidis A, *et al.*** Circulating VEGF levels in the serum of gastric cancer patients correlation with pathological variables, patient survival, and tumor surgery. Ann Surg 2002; **236**: 37–42.

18. **Berne GJ, McDowell G, Agarawal R, *et al.*** Serum vascular endothelial growth factor in breast cancer. Anticancer Res 2007; **27**: 3481–8.

19. **Bezdenezhnykh NO, Lukyanova NYu, Lykhova OO, *et al.*** Markers of cell proliferation, differentiation and malignancy of non-small cell lung carcinoma in the long-term IFN exposition. Cytology 2008; **50**: 795–6 (in Russian).

20. **Taniguchi T.** The interferon-alpha/beta system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. Curr Opin Immunol 2002; **1**: 111–6.

21. **Shayna A.** Perforin and interferon-gamma activities independently control tumor initiation, growth and metastasis. Blood 2001; **97**: 192–7.

22. **Kudryavets YuI, Bezdenezhnykh NO, Lykhova OO, *et al.*** The role of interferon as a modifier of epithelial-mesenchymal transition in tumor cells. Exp Oncol 2011; **33**: 178–81.

23. **Lang BA, Silverman ED, Laxer RM, *et al.*** Spontaneuos tumor necrosis factor production in Kawasaki disease. J Pediatrics 1989; **115**: 939–43.

24. **Gascan H.** Response of murine IL3-sensitive cell lines to cytokines of human and murine origin. Lymphokine Res 1989; **8**: 79–84.

25. **Rubinstein S, Familletti PC, Pestka S.** Convenient assay for interferons. J Vir 1981; **37**: 755–8.

26. **Ni J, Hollander D.** Application of the MTT-assay to functional studies of mouse intestinal intraepithelial lymphocytes. J Clin Lab Anal 1996; **10**: 42–52.

27. **Skenan F, Storeng R, Scudiero D, *et al.*** New colorimetric cytotoxicity assay for anticancer-drug screening. J Nat Cancer Inst 1990; **82**: 1107–12.

28. **Vega-Avila E, Pugsley KM.** An overview of colorimetric assay methods used to assess survival or proliferation of mammalian cells. Proc West Pharmacol Soc 2011; **54**: 10–4.

29. **Li C-Y, Ziesmer SC, Lazcano-Villareal O.** Use of azide and hydrogen peroxide as an inhibitor for endogenous peroxidase in the immunoperoxidase method. J Histochem Cytochem 1987; **35**: 1457–60.

30. **Gluzman DF, Sklyarenko LM, Nadhornaya VA, *et al.*** Diagnostic immunocytochemistry of tumors. Kiev: Morion, 2003: 78–91 (in Russian).

31. **Schindlbeck C, Kampik T, Janni W, *et al.*** Prognostic relevance of disseminated tumor cells in the bone marrow and biological factors of 265 primary breast carcinomas. Breast Cancer Res 2005; 7: 1174–85.

32. **Gerber B, Krause A, Miller H, *et al.*** Simultaneous immunohistochemical detection of tumor cells in lymph nodes and bone marrow aspirates in breast cancer and its correlation with other prognostic factors. J Clin Oncol 2001; **19**: 960–71.

33. **Vincent-Salomon A, Bidard FC, Pierga JY.** Bone marrow micrometatasis in breast cancer: review of detection methods, prognostic impact and biological issues. J Clin Pathol 2008; **61**: 570–6.

34. **Kasimir-Bauer S, Hoffmann O, Wallwiener D, *et al.*** Expression of stem cell and epithelial-mesenchymal transition markers in primary breast cancer patients with circulating tumor cells. Breast Cancer Res 2012; **14**: R15.

35. **Osinsky SP, Gluzman DF, Kleeff J.** Molecular diagnostics of tumor: basic principles and practical application: Monography. Kiev: "DIA". 248 p. (in Russian).

36. **Braun S, Vogl FD, Naume B.** A pooled analysis of bone marrow micrometastasis in breast cancer. N Engl J Med 2005; **353**: 793–802.

37. **Lacroix M.** Significance, detection and markers of disseminated breast cancer cells. Endocrine-Related Cancer 2006; **13**: 1033–67.

38. **Apellaniz-Ruiz M, Castano Z.** Adapt or dye: tumor microenvironment, a powerful regulator of cancer progression. J PostDoc Res 2013; **1**: 1–8.

39. **Chantrain CF, Feron O, Marbaix E, *et al.*** Bone marrow microenvironment and tumor progression. Cancer Microenv 2008; **1**: 23–35.

40. **Duffy MJ.** Serum tumor markers in breast cancer: are they of clinical value? Clinical Chem 2006; **52**: 345–51.

41. **Daskalaki A, Agelaki S, Perraki M, *et al.*** Detection of cytokeratin-19 mRNA-positive cells in the peripheral blood and bone marrow of patients with operable breast cancer. Br J Cancer 2009; **101**: 589–97.

42. **Benoy IH, Elst H, Philips M.** Real-time RT-PCR detection of disseminated tumour cells in bone marrow has superior prognostic significance in comparison with circulating tumour cells in patients with breast cancer. Br J Cancer 2006; **94**: 672–80.

43. **Waterston A, Bower M.** TNF and cancer: good and bad? Cancer Ther 2004; **2**: 131–48.

44. **van Horssen R, Hagen TL, Eggermont AM.** TNF-alpha in cancer treatment: molecular insight, antitumor effects and clinical utility. Oncologist 2006; **11**: 397–408.

45. **Kudryavets YuI.** Interferon and tumor necrosis factor as modifiers of metastasis of malignant tumors. Abstr of Doctoral Thesis. Kyiv 1999: 36 (in Ukrainian).

46. **Kudryavets YuI.** Modifying effect of recombinant tumor necrosis factor in the metastatic potential of tumor cells. Exp Oncol 1990; **12**: 47–50 (in Russian).

47. **Geng Y, Chandrasekaran S, Hsu J-W, *et al.*** Phenotypic switch in blood: effects of pro-inflammatory cytokines on breast cancer cell aggregation and adhesion. PloS One 2013; **8**: e54959.

48. **Semesiuk NI, Zhylchuk AV, Bezdenezhnykh NO, *et al.*** Tumor necrosis factor in peripheral blood and bone marrow of breast cancer patients as a marker of the tumor progression. Oncologiya 2012; **4**: 293–7 (in Ukrainian).

49. **Sullivan NJ.** Interleukin-6 in the breast tumor microenvironment. In: Breast Cancer — Focusing Tumor Microenvironment, Stem cells and Metastasis. M Gunduz (ed.), 2011: 165–75.

50. **Sullivan NJ, Sasser AK, Axe AE, *et al.*** Interleukin-6 induces an epithelial-mesenchymal transition phenotype in human breast cancer cells. Oncogene 2009; **28**: 2940–7.

51. **Aharinejad S, Thomas A, Singer C.** Significance of serum colony-stimulating factor-1 as a breast cancer marker. J Clin Oncol 2009; **27**: 15s (suppl; abstr 11071).

52. **Lin EY, Nguyen AV, Russell RG, *et al.*** Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med 2001; **193**: 727–40.

53. **Buijs TJ, Stayrook KR, Guise TA.** TGF-β in the bone microenvironment: role in breast cancer metastasis. Cancer Microenv 2011; **4**: 261–81.

54. **Ganapathy V, Ge R, Grazioli A, *et al.*** Targeting the transforming growth factor-β pathway inhibits human basal-like breast cancer metastasis. Mol Cancer 2010; **9**: 122–38.

55. **Piras F, Salani F, Bossu P, *et al.*** High serum level of transforming growth factor β1 are associated with increased cortical thickness in cingulate and right frontal areas in healthy subjects. J Neuroinfl 2012; **9**: 42–7.

56. **Todorovic-Racovic N, Ivanovic V, Demaj M, *et al.*** Elevated plasma levels of TGFbeta1 in patients with locally advanced breast cancer related to other clinical stages. Arch Oncol 2003, **11**: 131–3.

57. **Zhylchuk V, Lisnyak I, Vorontsova A, *et al.*** Inhibitory effect of interferon-alpha on the level of VEGF in the blood serum breast cancer patients with complex treatment. Oncologiya 2008; **10**: 321–6 (in Russian).

58. **Zhylchuk V, Vorontsova A, Klyuchevska O, *et al.*** Prognostic value of the level of TGF-beta in the blood serum breast cancer patients with interferon treatment. Oncologiya 2007; **9**: 337–41 (in Ukrainian).

59. **Moore LD, Isayeva T, Siegal GP, *et al.*** Silencing of transforming growth factor-B1 *in situ* by RNA interference for breast cancer: implications for proliferation and migration *in vitro* and metastasis *in vivo*. Clin Cancer Res 2008; **14**: 4961–70.

60. **Kallergi G, Markomanolaki H, Giannoukaraki V.** Hypoxia-inducible factor-1α and vascular endothelial growth factor expression in circulating tumor cells of breast cancer patients. Breast Cancer Research 2009; **11**: R84.

61. **Vorontsova A, Bezdenezhnykh N, Zhylchuk V, *et al.*** Inhibition expression VEGF in tumor cells and its level in serum of oncology patients by interferon-alpha. Urologiya 2009; **3**: 48–52 (in Ukrainian).

62. **Kudryavets Yu, Bezdenezhnykh N, Lukyanova N, *et al.*** Modyfying influence of prolonged action of interferon on phenotypic characteristics of human lung cancer cells *in vitro*. Exp Oncol 2008; **30**: 283–8.