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TUMOR MICROENVIRONMENT-DERIVED miRNAs AS PROGNOSTIC MARKERS OF BREAST CANCER

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Aim: **To study expression of miRNA derived from tumor microenvironment in patients with breast cancer (BC) as the aspect of tumor-host interaction.** ***Materials and Methods:*** **The expression levels of estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2 (HER2/neu) were analyzed in tissue of BC using immunohistochemical method. Relative expression levels of the miR-155, -320a, and -205 were examined in tissue and sera from BC patients using quantitative reverse transcription polymerase chain reaction.** ***Results:*** **Serum and tissue miR-155, -320a, and -205 levels in patients with BC are of low diagnostic value as such for differentiation of malignant and non-malignant breast neoplasms. Nevertheless, we established the relation of circulating and tissue miR-155, -320a, and -205 to lymph node metastases and basal breast cancer subtype.** ***Conclusions:*** **Changes of miR-155, -320a, and -205 expression in tumor tissue and sera of BC patients provide information about major clinical-pathological characteristics of BC.**
Key Words**: miRNA, breast cancer.**

Cancer is complex systemic disease and tumor-host interaction is one of the most complicated and hard for understanding aspects. For example, microenvironment in metastatic niche affects metastases behavior, and the dormancy or development of secondary tumor depends on migrated cancer cell surrounding. It is well known that microenvironment is essential for cancer progression. At last decades, there were many successful attempts to create the molecular profile of tumor microenvironment [1–7].

It needs to be clearly understood that the tumor is a complex biological system closely associated with the organism where tumor originates and develops. It is fundamentally important to consider that tumor cells are surrounded by factors of different nature, which form the tumor microenvironment. Various cells and structures and complex macromolecules belong to this microenvironment. Four types of tumor microenvironment are currently delineated:

1. Pathophysiological microenvironment of the tumor: angiogenesis, perfusion and microcirculation, vascular permeability; lymphatic system (vessels); interstitial space, pressure of interstitial fluid (osmotic and oncotic).

2. Metabolic microenvironment of a tumor: oxygenation (hypoxia); glycolysis (lactate); nutrients restriction; extracellular acidosis *vs* intracellular alkalinity; bioenergetic status; redox status.

3. Stromal microenvironment of the tumor: non-tumor cells — endothelial cells, pericytes, smooth muscle, fibroblasts, myofibroblasts; extracellular molecules (non-classical elements of the stroma): adhesion molecules, growth factors, hormones, proteases and other enzymes, metabolites; extracellular matrix (connective tissue elements, for example, fibers).

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Abbreviations used: BBN – benign breast neoplasm; BC – breast cancer; ER – estrogen receptor; MMP – matrix metallopeptidase; PR – progesterone receptor; PTEN – phosphatase and tensin homolog.

4. Immunological microenvironment (active elements) of the tumor: cells — macrophages, dendritic cells, mast cells, NK-cells, naive lymphocytes and memory lymphocytes, B cells, effector T-cells (helper lymphocytes, regulatory T-cells, cytotoxic T-cells), eosinophils, mature myeloid cells; molecules — cytokines, chemokines; inflammation and its components.

Naturally, this classification does not claim to be final. The concept of tumor microenvironment always includes stromal elements and immunocompetent cells. Undoubtedly, the factors of all types of microenvironment are in close interaction, which is the basis of the formation of the tumor-host relationship that now is in focus of cancer biology [8]. The role of circulating miRNAs as mediators of tumor-host interactions in neoplastic process has been recently suggested.

In fact, among signaling molecules, the exosomal miRNAs are considered to be challenging predictive and prognostic markers of cancer. There is a lot of evidence about role of miRNAs, derived from tumor microenvironment, in carcinogenesis. Remarkable are the articles of Kuninty *et al*. [9] and Bell *et al*. [10], where authors summarized the main patterns of miRNAs expression in cells of tumor microenvironment and the role of extracellular miRNAs as drivers of cancer progression.

The heterogeneity of miRNAs that regulate neoplastic process is also associated with cells where they are expressed. For example, elevated miR-34a and miR-126 levels in the tumor are often associated with elevated miR-21 and miR-155 levels, while miR-126 and miR-155 are expressed by endothelial and immunocompetent cells that are present in the tumor area. MiR-155 targets are *SOCS1* and *APC* oncosuppressive genes, WEE1 kinase, which blocks the activity of Cdc2 and prevents entry into mitosis. TGF-β induced epithelial-mesenchymal transition preference is also regulated by miR-155 because its target is RhoA [11].

A recent study revealed specific miRNAs responsible for phosphatase and tensin homolog (PTEN) oncosuppressive properties. Among them, the main one

is oncosuppressive miR-320 family, whose targets are v-Ets erythroblastosis virus E26 oncogene homolog 2, matrix metallopeptidase (MMP) 9, and Elastin microfibril interface located protein. Increased expression of miR-320 is correlated with lower levels of MMP9, MMP2, bone morphogenetic protein, and Lysyl oxidase like 2 responsible for angiogenesis and invasion [11]. The PTEN deletion in stromal fibroblasts promotes oncogenesis in the epithelium of the mammary glands. Protein analysis of PTEN-zero fibroblasts by Bronisz *et al*. identified the miR-320 as the main regulator of the paracrine response [12]. The expression of miR-320 is significantly reduced in the mammary gland fibroblasts in PTEN-null mice. When these fibroblasts are introduced into the mammary gland into inbred mice with attenuated immunity, there is a fourfold increase in tumor growth compared with the growth of PTEN-positive fibroblasts. Restoring the expression of the miR-320 in the PTEN-zero fibroblasts cancels this effect, as well as reduces the invasiveness of tumors, reduces vascularization. In addition, Bronisz *et al*. analyzed the expression of miR-320 in stromal and epithelial cells of 126 samples of invasive breast cancer compared with normal tissue samples. It has been found that the low expression of this miRNA correlates with more malignant phenotype [12].

MiR-205 is known to be one of the main drivers of activation of breast normal fibroblasts into cancer-associated fibroblasts and enhances interleukin (IL)-11 and IL-15 expressions, regulating angiogenesis in different cancers due signal transducer and activator of transcription 3 (STAT3) signaling [13, 14].

The profile of miRNAs in tumor microenvironment compiled from the relevant data of the papers cited above, is illustrated in Fig. 1.

Several microRNAs representing tumor microenvironment (see Fig. 1), e.g. miR-155, -320a, and -205 are known to be involved in the natural history of breast cancer (BC) [15–17]. The aim of the study was to analyze the possible utility of tissue and circulating miR-155, -320a, and -205 as prognostic and diagnostic markers for BC in Ukrainian population.

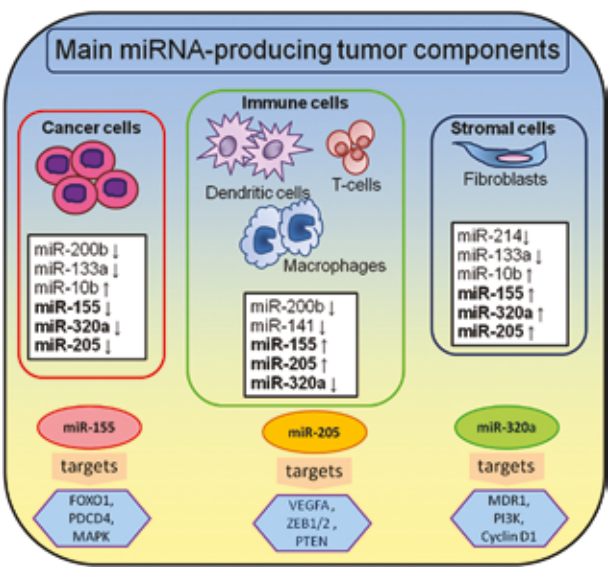

Fig. 1. MiRNA profile of tumor microenvironment components

MATERIALS AND METHODS

156 women were recruited for the study including 89 BC patients, 53 patients with benign breast neoplasms and 14 age-matched healthy individuals. 156 tumor samples and 14 samples of normal breast tissue obtained during surgery and 142 samples of blood serum of BC patients and 14 blood serum samples of healthy donors were studied. Tumor stage was determined by TNM staging system (2008). The histological type of tumor was verified by morphological study of paraffin-embedded tissue according to the WHO (2006). All patients before surgery received neither radiation nor chemotherapy. All patients were examined using conventional clinical and laboratory methods according to the standards of diagnosis and treatment of cancer patients, approved by the order of the Ministry of Health of Ukraine № 554 of 17.09.2007. All patients and donors were informed and agreed to the use of serum and surgical material for research purposes. All samples were encoded and depersonalized.

Morphological examination of tumors. Tissue samples were fixed in 10% neutral formalin solution for 24 h followed by dehydration in spirits of increasing concentration and embedding into paraffin blocks. The histological sections were stained with hematoxylin and eosin. The histological type of tumors was evaluated according to the International Histological Classification of the WHO (2006). The degree of structural and cellular atypia was determined, on the basis of which the degree of differentiation of tumors was determined, dividing them into high (G1), moderate (G2) and low-differentiated (G3).

Immunohistochemical analysis. Immunohistochemical analysis was performed on series of 4–5 μm sections from paraffin-embedded tissue. Rabbit anti-human antibodies to estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2/neu), Ki-67 (DakoCytomation, Denmark) were used for staining according to manufacturer's instructions.

To estimate the results classic H-Score method was used:

$$H\text{-}Score\,/\,S = 1 \cdot N_{1+} + 2 \cdot N_{2+} + 3 \cdot N_{3+},$$

where S — "H-Score", N_{1+}, N_{2+}, and N_{3+} — number of cells with low, average and high expression. The end result is presented in the next grade: 50–100 points — low expression, 101–200 points — average expression, 201–300 points — high expression [18].

Samples collection and RNA isolation. 5 ml of blood was collected in a BD vacutainer (yellow top), and was centrifuged at 1,500 rpm. Serum was extracted and transferred to a conical bottom tube. Around 3–5 ml serum was obtained. Serum was stored at –80 °C until further use. Frozen tissue samples also were stored at –80 °C until further use. Total RNA was extracted from tissues/serum using "Ribozol" RNA Isolation Kit (Amplisens, Russia). RNA concentration was determined on a "NanoDrop 2000c" spectrophotometer (Thermo Scientific, USA). The purity of isolated RNA was controlled by using the ratio of optical absorption values at a wavelength of 260 and 280 nm. RNA was dissolved in TE buffer and stored at –20 °C. Single-stranded cDNA was synthesized from 100 ng of total RNA using TaqMan® MicroRNA Kit for reverse transcription.

Real-Time Quantitative Reverse Transcription polymerase chain reaction (qRT-PCR). Preparation of reverse transcription reaction mix was performed according to manufacturer's protocol. Reverse transcription was performed with "Tertsik" ("DNA tehnologiya", Russia). After the qRT-PCR product was added to the mixture of reagents to perform real-time PCR. qRT-PCR was performed on Applied Biosystems 7900HT Fast Real-Time PCR System using TaqMan® MicroRNA primers, Maxima SYBRGreen/ROX qPCR Master Mix (Thermo Scientific, USA). Small nucleolar RNA RNU48 was used as an endogenous control for normalization of the expression. Relative expression of the studied miRNAs was identified by comparative CT method. Experiment was performed in three parallels for each sample. The threshold cycle averaged in all technical and biological replicas within each sample. Fold change between the studied miRNAs expression relative to control was calculated by the formula $2^{-\Delta\Delta Ct}$ [19].

Statistical methods. Statistical analysis of the obtained data was performed using the program STATISTICA 6.0. All data were expressed as the mean ± SD of at least 3 independent experiments. The differences between the groups were analyzed using the Student's *t*-test and ANOVA; $p < 0.05$ was considered to indicate statistically significant results. To determine the variation of the selected miRNA expression in samples among different groups, the data of miRNA expression obtained by qRT-PCR were analyzed by Pearson's correlation coefficient (R).

RESULTS AND DISCUSSION

Clinical-pathological characteristics of BC patients. Expression of miRNAs was studied depending on the main clinical-pathological parameters of BC patients: patient's age, menstrual status, BC stage, the presence of metastases in the regional lymph nodes, the tumor differentiation degree, and molecular histological subtype.

The age of the patients ranged from 28 to 75 year. Most patients had menopause (72.5%), preserved menstrual function was in 27.5%. The number of patients with benign breast neoplasm was 53 and 89 were diagnosed with breast cancer. Lymph nodes metastases were found in 30 BC patients.

The morphological study of the surgical material showed that infiltrative duct carcinoma was diagnosed more often (73.0% of patients), infiltrative lobular cancer was observed in a smaller number of patients (37.0%). The degree of differentiation of the tumors was also different, with domination of moderate differentiation cases (45.9%).

To determine the molecular subtype of tumors, immunohistochemical analysis of ER, PR and epidermal growth factor (HER2/neu) was performed. Based on this study, we identified four molecular subtypes of breast cancer: luminal A (ER+, PR+, HER2/neu–), luminal B (ER+, PR+, HER2/neu+), basal subtype (ER–, PR–, HER2/neu–) and HER2/neu-positive (ER–, PR–, HER2/neu+). The luminal A subtype was dominant (46.0%), the smallest number of cases were — HER2/neu+ (0.5%). The analysis of the dependence of tumor and serum miRNA expression on age and on the reproductive status of patients has

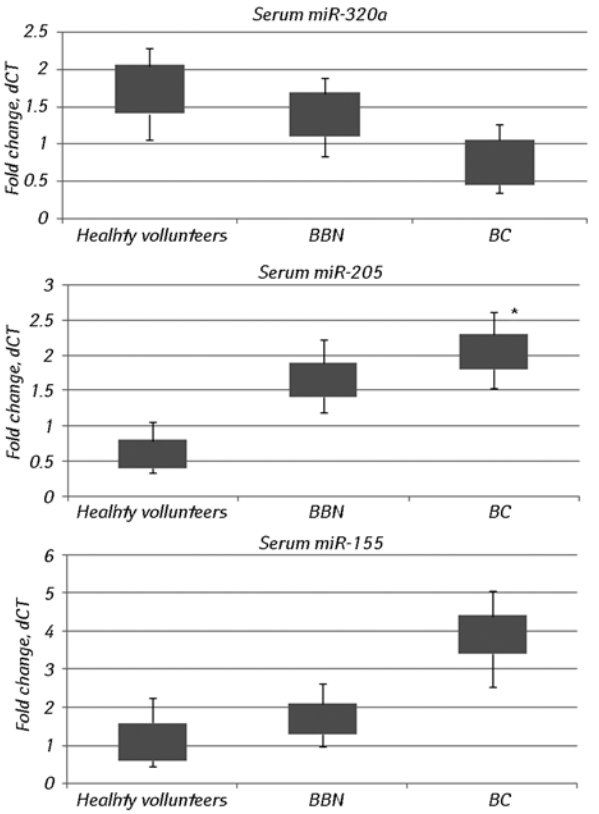

Fig. 2. Expression of miR-320a, -155, -205 in serum of healthy volunteers, BBN and BC patients. *$p \leqslant 0.05$ — compared to healthy volunteers

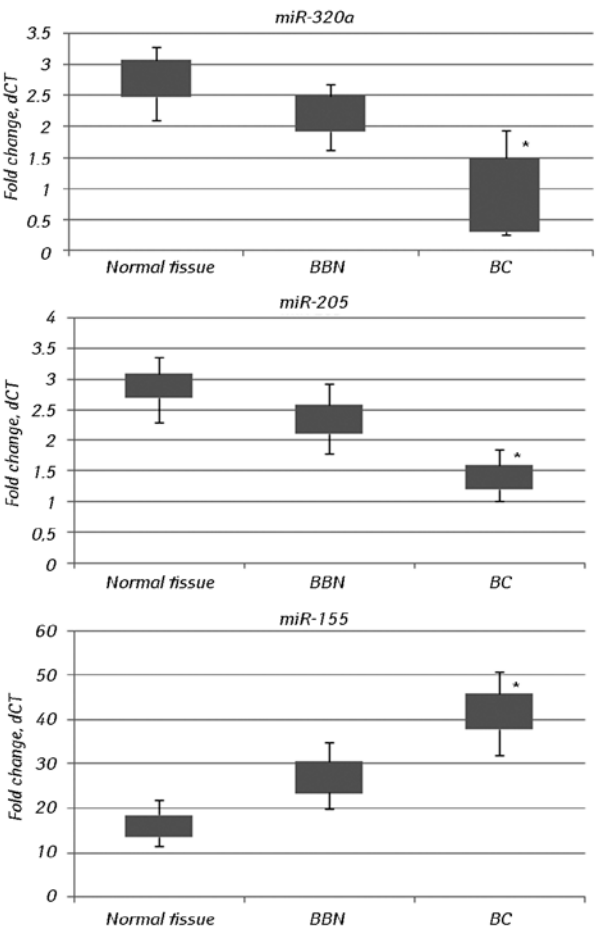

Fig. 3. Expression of miR-320a, -155, -205 in normal tissue, BBN and BC. *$p \leqslant 0.05$ — compared to conditionally normal tissue

shown that there are no significant differences in tissue and serum miR-155,-320a, and -205, while there is a tendency of increasing the serum miR-155 levels with the age.

miRNAs expression in serum and tumor tissue of BC patients. Several studies demonstrated that expression of certain miRNAs may be used as biomarkers with respect to the disease prognosis in BC patients [20]. We have analyzed expression of miR-155, -320a, and -205 in blood serum and in tumor tissue of BC patients (Fig. 2, 3). We found an increase in the miR-205 expression in BC patients and patients with benign breast neoplasms (BBN) compared to healthy donors. Our data coincide with the results of several other studies [17, 21]. Nevertheless, there was no significant differences in circulating miR-320a and miR-155 levels between studied groups. It should be mentioned that increased expression of miR-155 was demonstrated in several studies in patients with nephrolithiasis [22] and hepatitis C [23]. Also, the levels of circulating miR-205 decrease in the serum of majority cancer patients [24–26].

Results of miR-155, -320a, and -205 analyses in tissue from BBN and BC patients are presented in Fig. 3. In total group of BC samples, we have found a significant increase in miR-155 (≈ 2 times) expression and decrease in miR-205 (≈ 2.6 times) levels compared to adjacent conditionally normal tissue (see Fig. 3). While levels of miR-320a in most samples were lower than in adjacent conditionally normal tissue (almost twice), there were no significant differences between all studied groups. The expression of miR-155 in tumor tissue was higher than in adjacent conditionally normal tissue.

Association of tumor and circulating miR-155, -320a, and -205 with major clinical-pathological characteristics of BC patients. We evaluated levels of miR-155, -320a, and -205 in terms of clinical-pathological characteristics of BC patients. As can be concluded from Fig. 3, expression of all studies miRNAs in cancer tissue differs from the one in adjacent normal tissues. Moreover, we found out the association of miR-320a with the stage on BC. Its levels in patients with stage III were twice lower than in patients with stage I. For miR-155 we observed tendency of increasing with the stage, but did not find statistical differences. We did not find any relation of miR-155, -320a, and -205 levels to the grade of BC.

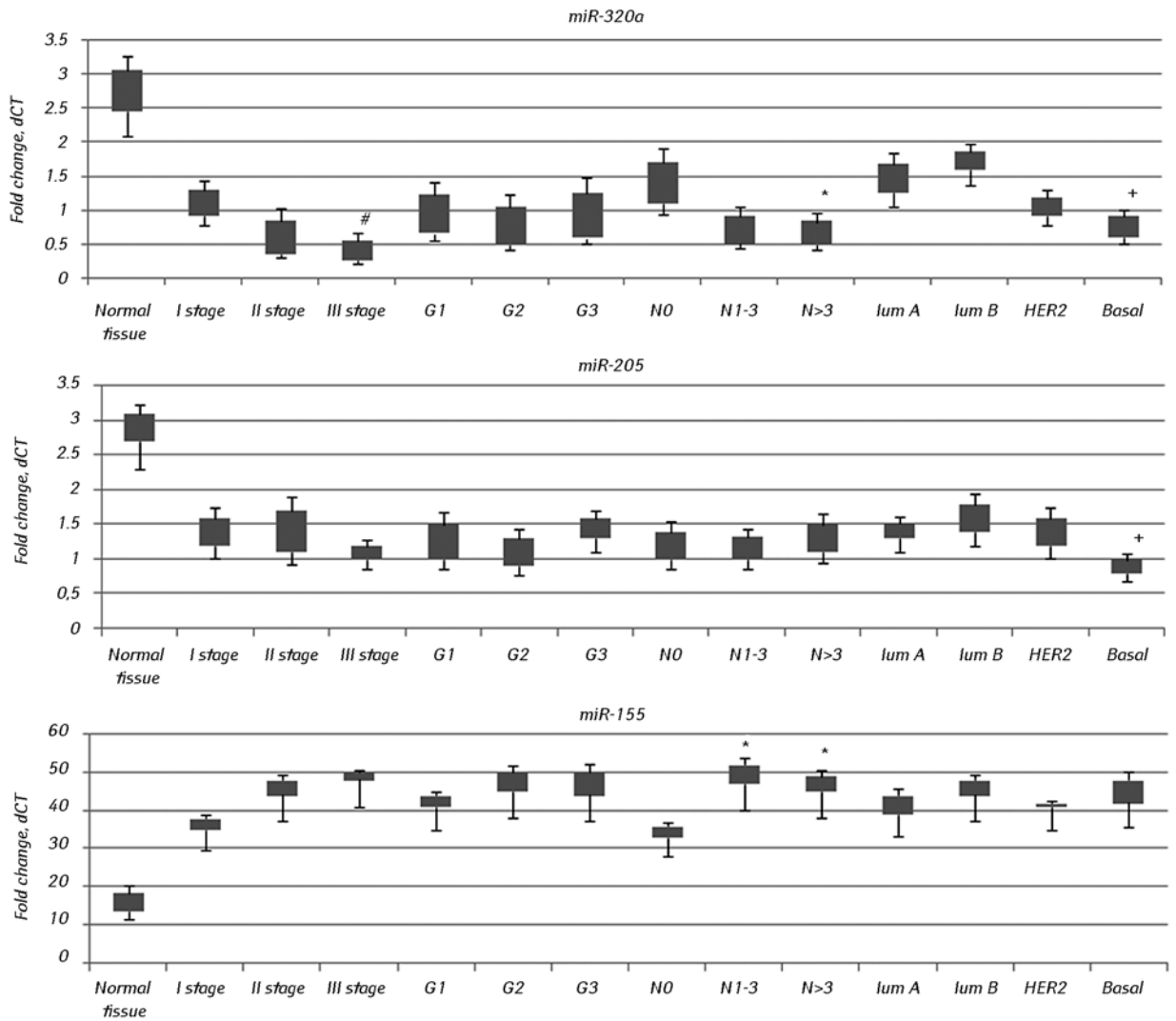

Fig. 4. Expression of miR-155, -320a and 205 in tumor tissue in association with major clinical-pathological characteristics of BC patients. $^{\#}p \leqslant 0.05$ compared to samples of lower stage; $^{*}p \leqslant 0.05$ compared to samples without metastases; $^{+}p \leqslant 0.05$ compared to hormone-positive samples

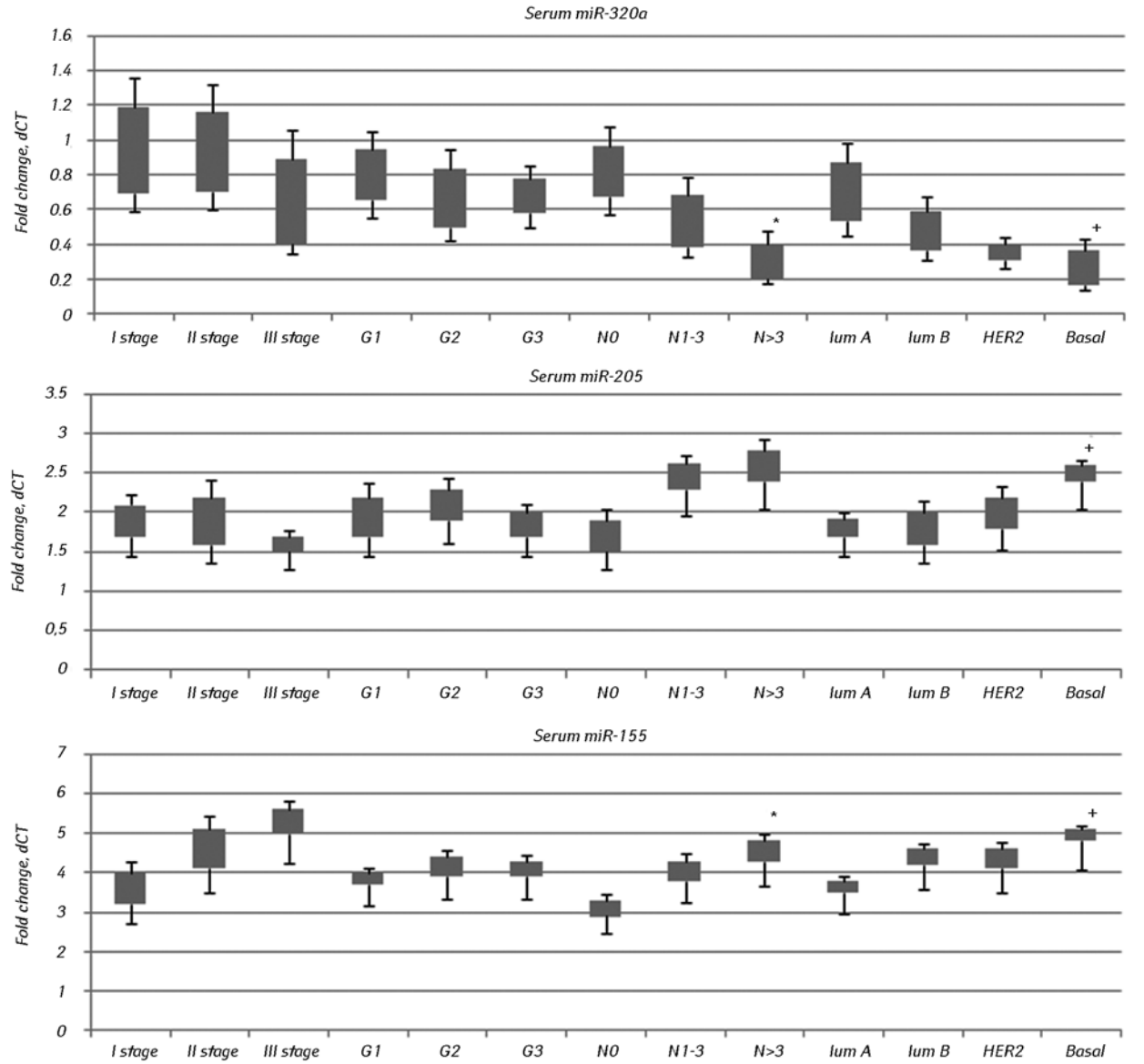

Fig. 5. Levels of serum miR-155, -320a, and -205 in association with major clinical-pathological characteristics of BC patients. $^{*}p \leqslant 0.05$ compared to samples without metastases; $^{+}p \leqslant 0.05$ compared to hormone-positive samples

For miR-320a and miR-155 expression, we established the correlation with lymph node metastasis. The levels of miR-320a in cancer tissue from patients with multiple metastases were significantly lower than in patients without any. The levels of miR-155 in cancer tissue from patients with metastases (N1, N2, N3, N>3) were much higher than in samples from patients without metastases (Fig. 4). These results suggest that miR-155 is involved in BC metastasis [27, 28]. Also, we found strong correlation of tissue miR-205 expression with the presence of hormonal receptors on cancer cells. These results are predictable, because there are a lot of *in vitro* and *ex vivo* studies, which prove the interdependence of miR-205, ER, and PR expression [29, 30].

The analysis of serum miR-155, -320a, and -205 showed similar results. We detected no correlation of the studied miRNAs levels in the serum of BC patients with the stage of the tumor process and tumor grade. The expression of miR-155 and miR-320a differ in patients with metastases in regional lymph nodes. As can be seen from Fig. 5, level of miR-320a was lower than 0.5 fold and expression of miR-155 was higher than 3.5 fold in patients with more than 3 lymph nodes with metastases. The expression of miR-205 increased in accordance with this parameter but we did not find significant differences with patients without metastases.

It is remarkable, that the levels of serum miR-155, -320a, and -205 in patients with triple-negative BC differed from the patients with other BC subtypes, especially luminal A (see Fig. 5). Patients with basal BC were characterized with the lowest miR-320a levels and highest miR-155 and -205 levels. These data demonstrate potential role of serum miR-155, -320a, and -205 as the additional non-invasive markers for BC course. However, it should be considered that these miRNAs separately have low specificity for basal BC diagnosis.

To sum up, while an analysis of serum and tissue miR-155, -320a, and -205 levels in patients with BC proved their low diagnostic value for differentiation of malignant and non-malignant breast disease, their expression in terms

of the major clinical and morphological characteristics suggests their usability as predictive BC markers for lymph node metastases and basal cancer subtype. The data obtained testify to the inclusion of miR-155, -320a, and -205 into a predictive panel for BC patients in order to individualize the strategy of treatment and to identify the individual characteristics of the neoplastic process.

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