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ROLE OF ABC-CASSETTE TRANSPORTERS (MDR1, MRP1, BCRP) IN THE DEVELOPMENT OF PRIMARY AND ACQUIRED MULTIPLE DRUG RESISTANCE IN PATIENTS WITH EARLY AND METASTATIC BREAST CANCER

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The *aim* of the research was to study the influence of multiple drug resistance (MDR1), multidrug resistance protein 1 (MRP1) and breast cancer resistance protein (BCRP) proteins expression on the effectiveness of chemotherapy in breast cancer (BC) patients. *Patients and Methods*: The retrospective analysis of the results of treatment of 77 women with invasive BC with different molecular subtypes at the age of 54.1±12.5, who received treatment in Zaporizhzhya Regional Clinical Oncological Dispensary during 2011–2013, has been carried out. 23 (29.8%) patients were in II stage of disease, 32 (41.5%) — in III stage, 22 patients (28.5%) — IV stage. Neoadjuvant therapy has been given to 34 (44.2%) patients, palliative therapy in connection with presence of hematogenous visceral and bone metastases — to 43 (55.8%) patients. Transport proteins BCRP, MRP and PGP have been studied in tissues of primary BC tumor, in tissues of visceral metastases, in metastatically altered regional lymph nodes, as well as in circulating in blood tumor cells (CTC). In every case, 1000 of tumor cells has been calculated. The intensity of staining (0, 1+, 2+, 3+), percentage of stained cells (threshold has constituted 10%) and homogeneity of tumor cells staining (homogeneous was considered 80% staining of cytoplasm or membrane of studied cells) has been taken in consideration. *Results*: Retrospective analysis has determined the dependence of results of polychemotherapy (PCT) on level and pattern of expression of BCRP, MRP and PGP proteins. In 1st group, in the absence thereof expression of transport proteins in BC cells, the objective response (CR + PR) from the tumor has been observed in 17 out of 18 patients (94.4%). In 2nd group (only cytoplasmic staining) the objective response has been observed in 36 patients (85.7%). Ineffectiveness of therapy and tumor progression in this group has been observed in 6 (14.2%) patients. In 3rd group (high membrane BCRP, MRP and PGP expression), in 14 out of 17 patients (82.3%), during the PCT occurred progression of the disease. Malignant phenotype of this tumor corresponded with the state of primary multiple drug resistance. In 80.0% of patients of this group, the neoadjuvant therapy turned out to be ineffective. *Conclusion*: Clinical trials for determination of role of ABC-transporters in the development of drug resistance of BC patients have not yet ended. The leading role in development of resistance of BC cells plays not only PGP, but also MRP1 and BCRP. Marker of resistance is not cytological, but membrane staining of cell.

***Key Words*: breast cancer, ABC-cassette transporters, multiple drug resistance.**

Today, personified treatment of breast cancer (BC) is based on the recognition of fact of molecular-genetic heterogeneity of tumors (Perou and Sorli classification, 2000) and does not take into consideration such characteristics, as primary and acquired resistance. Despite the personification of therapy, 30% of patients with clinically early an more than 50% metastatic BC remain refractor to the treatment. Overall response to the treatment in metastatic cancer is almost always transitory. This phenomenon in clinics is known as multiple drug resistance (MDR).

One of the main mechanisms of drug resistance in cancer is activation of system of ATP-dependent transporters, which increase transmembrane efflux of drugs from cells that causes the decrease of their concentration in cytoplasm and, in consequence of that, — insensitivity of cell to damaging impact of cytostatics.

Adenosine-triphosphate binding cassette (ABC) are represented by numerous family of large transmembrane proteins, which are divided into seven different families (from ABC-A to ABC-G).

Function of proteins of ABC-family on the cell level is ATP-dependent transport of wide range of xenobiotics (including cytostatics), as well as lipids and metabolic products of intracellular membranes. In addition to the transport of xenobiotics, members of ABC-family are responsible for intracellular transport of peptides and play role in presentations of antigens of I class of the main complex of histocompatibility.

Mostly has been well studied ABCB1 transporter (also known as MDR1 and P-glycoprotein — PGP), on which were focused major clinical studies, since this protein often expressed in chemoresistant tumors of large intestine, kidneys, adrenal cortex and in hepatocellular carcinomas.

However, not all cells of resistant malignant tumors express PGP in the same degree. In order primary and acquired resistance to be formed, tumor uses several types of ABC transporters (2). Study of the role of biological markers of ABC-transporters family (ABCB1 or MDR1, ABCC1 or MRP1, as well as ABCG2 or BCRP) in the development of primary and acquired chemoresistance in the different stages of treatment in patients with luminal, HER/2-positive and triple negative BC is relevant.

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Abbreviations used: ABC – adenosine-triphosphate binding cassette; BC – breast cancer; BCRP – breast cancer resistance protein; MDR – multiple drug resistance; MRP1 – multidrug resistance protein 1; PGP – P-glycoprotein.

The objective of study was to investigate the influence of expression of MDR1, MRP1 and BCRP proteins on the effectiveness of chemotherapy in BC patients.

MATERIALS AND METHODS

The retrospective analysis of results of 77 women with invasive BC, who received treatment in Zaporizhzhya Regional Clinical Oncological Dispensary during the 2011–2013, has been carried out. The age of patients has constituted 54.1±12.5. Twenty nine (37.6%) women were in menopause. Staging has been determined according to the TNM-6 classification. In 23 (29.8%) patients was II stage of disease, in 32 (41.5%) — III stage, in 22 patients (28.5%) — IV stage.

Neoadjuvant therapy has been given to 34 (44.2%) patients, palliative therapy as a result of presence of hematogenous visceral and bone metastases — in 43 (55.8%).

In 67 (87.0%) patients have been used anthracycline-containing schemes of polychemotherapy (PCT) (CAF/CEF). In 10 patients (13.0%) have been used taxane-containing schemes. All patients had 1st line of therapy.

Other components of comprehensive treatment (operative intervention, radiotherapy, hormonal therapy, bisphosphonates therapy) were applied in accordance with national standards and taking into account status of tumor.

Evaluation of effectiveness of chemotherapy in process of neoadjuvant and palliative treatment has been carried out by the dynamics of marker focuses (RECIST 1.1 criteria: Complete Response — CR; Partial Response — PR; Stable Disease — SD; Progressive disease — PD).

Trepan-biopsy material of mammary gland of size 0.2 × 1.5 cm have been fixed in neutral buffered formalin (pH 7.4), material has been put through the line of alcohols of ascending concentration and chloroform, than put in paraplast. For morphological evaluation of biopsy material, sections 0.3–0.5 mcm thick have been stained with hematoxylin-eosin. Stage of differentiation of BC cells has been calculated according to the Nottingham scoring (score evaluation based on three morphological criteria). Receptor status of tumor has been determined according to the existing standards: estrogen-receptor alpha (DAKO, clone SP1), progesterone-receptor (DAKO, clone Pg 636), HER2/neu (clone A0485), Ki-67(clone MIB-1). Biopsy material has been studied on three markers of chemoresistance. As primary antibodies have been used antibodies Anti-P-Glycoprotein (p170) — clone F4 (DBS company); for Anti–MRP — clone MRPm6 (Millipore Corporation) for Anti-BCRP — clone BXP-21 (Millipore Corporation). The EnVision FLEX+ system of visualization with standard staining process has been used. Visual evaluation of obtained results has been carried out on the microscope Imager A1m (ZEISS company) at ×100, ×200, ×400 magnification. Since there are no standardized rating scales of expression of chemoresistance markers, we have developed own rating scales of qualitative and quantitative evaluation of level of every marker. Parenchyma tissue of human liver was taken as positive control for Anti-MRP and Anti-BCRP, for Anti-P-Glycoprotein (p170) — tissue of adrenal cortex. In every case, 1000 tumor cells has been calculated. Intensity of staining (0, 1+, 2+, 3+), percentage of cells (threshold has constituted 10%) and homogeneity of tumor cells staining (homogeneous was considered 80% staining of cytoplasm or membrane of studied cells) has been taken into account. Transport proteins BCRP, MRP and PGP have been studied in tissues of primary BC tumor, in tissues of visceral metastases (mainly in liver), in metastatically altered regional lymph nodes, as well as in single circulating in blood tumor cells (CTC). Original procedure of identification and phenotypization of CTC, which is applied in ZRCOD, has been described by us earlier. For determination of CTC has been taken out patent of Ukraine № 75643.

RESULTS

According to the pattern of expression of studied proteins, patients have been divided into three groups.

In the 1st group (n = 18), there was no staining of membrane and cytoplasm of cell on the proteins BCRP, MRP and PGP or staining was too low (0/1+). Patients of this group have been evaluated as ABC-negative.

In the 2nd group (n = 42) was high (2+/3+) staining of cytoplasm of cell, but there was no staining of membrane. Patients of this group have been marked as ABC- Cit (+), Mem (-).

In 3rd group (n = 17) was apparent membrane expression (3+) of studied proteins. Patients of this group have been evaluated as ABC — Mem (+), Cit (-).

Examples of typical staining of BC cells on the presence of transport ABC proteins are represented in Fig. 1–3.

Retrospective analysis has determined the dependence of results of PCT on the level and character of expression of BCRP, MRP and PGP proteins (Table).

Table. Relation between PCT results and BCRP, MRP, and PGP expression

PCT results	Mem(+) Cit(+)		Mem(-) Cit(+)		ABC-negative	
	n	%	n	%	n	%
Total number of patients	17	100	42	100	18	100
Progression (PD)	14	82.3	6	14.3	1	5.5
Effect of PCT (CR, PR, SD)	3	17.6	36	85.7	17	94.5

In the 1st group (ABC-negative patients), i.e. in absence of expression of transport proteins in cells of BC, objective response (CR + PR) from tumor has been observed in 17 out of 18 patients (94.4%).

In 2nd group (only cytoplasmic staining) the objective response has been observed in 36 patients (85.7%). Ineffectiveness of therapy and tumor progression in this group has been observed in 6 (14.2%) patients.

In 3rd group (high membrane BCRP, MRP and PGP expression) in 14 out of 17 patients (82.3%) during the carrying out the PCT has come the progression of the disease. Malignant phenotype of this tumor corresponded with the state of MDR. It should be emphasized that 10 out of 17 patients of this group have been obtaining neoadjuvant therapy, which turned out

to be ineffective and, as the result, useless, in 8 patients (80.0%).

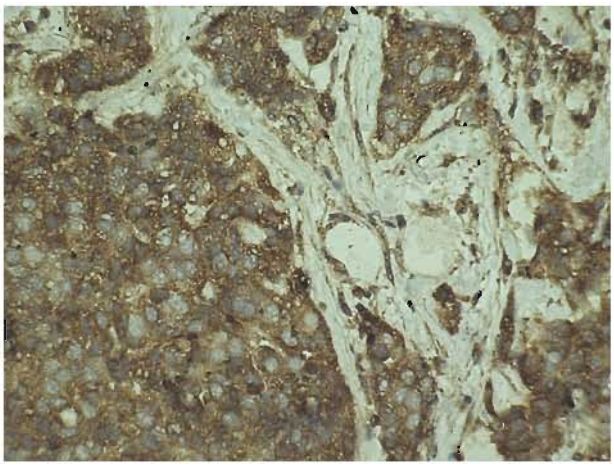

Fig. 1. Immunohistochemical analysis of BCRP expression in BC. ×200 magnification; expression level 3+, apparent staining of cytoplasm

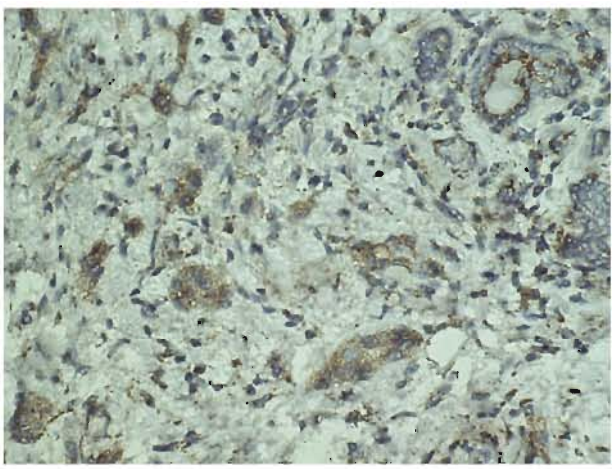

Fig. 2. Immunohistochemical analysis of BCRP expression in invasive tumor component, expression level 1+, right upper corner — ducts of non-neoplastic breast (no staining) — inner negative control

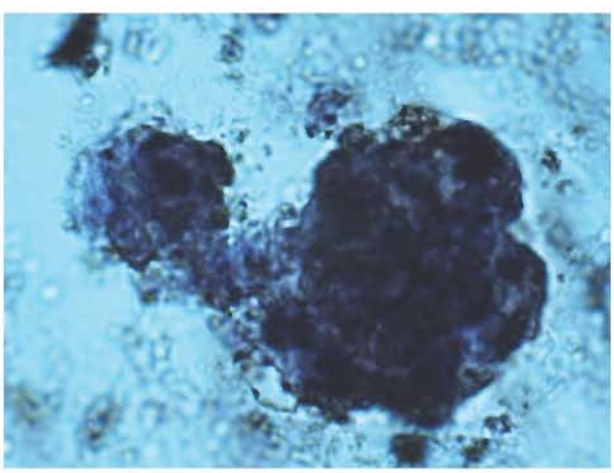

Fig. 3. Immunohistochemical analysis of expression of membrane MRP1in BC. Circulating tumor cell. ×1000 magnification

DISCUSSION

The cause of ineffectiveness of cytostatic therapy in cancer can be alterations of pharmacokinetics and/or pharmacodynamics of drug. To the pharmacokinetic are referred malabsorption, alteration of distribution in tissues, metabolism and elimination of drug. The important role at that plays stage of development of blood supply of tumor, level of interstitial pressure and the "geometry" of malignant cell itself.

To the pharmacodynamics are referred numerous alterations of intracellular damage mechanisms, which are conditioned by genetic and epigenetic alterations. To such are referred activation of system of detoxification of p450 proteins and system of DNA reparation, as well as errors in apoptosis signal pathways (p53 or ceramides).

In any population of malignant cells, which underwent the influence of chemotherapy, is always present more than one mechanism of drug resistance.

Increase of activity of family of proteins of ATP-binding cassette transporter (ABC), which causes the efflux of cytostatic from cells, are typical phenotype of MDR. Other mechanisms are considered to be atypical.

Despite about 30 years have passed from the moment of discovery of the main representative of ABC-transports (PGP), clinical trials for the determination of the role of these proteins in the development of drug resistance have not ended yet. It is known that family of transmembrane transporters is numerous and includes 48 members. The leading role in the development of resistance of BC cells plays not only PGP, but also MRP1 (multidrug resistance protein 1) and BCRP (breast cancer resistance protein).

Besides the cells of BC, the expression of MRP1 and BCRP is found in the leucosis and many solid tumors of gastrointestinal tract, womb, lungs and melanoma [1, 2].

High expression of transmembrane proteins of this superfamily is represented in stem cells of cancer [3].

The connection between BCRP expression, response of tumor to the chemotherapy and survival without progression has been determined [4].

The same data have been obtained in the respect of recurrence-free survival and MRP1 expression [5–7].

PGP, MRP1 and BCRP expression influences the efflux and pharmacodynamics of the main cytostatics, which are applied in clinical oncology: mitoxantrone, topotecan, methotrexate, doxorubicin, daunorubicin, actinomycin-D, vinblastine, vincristine, paclitaxel.

The prognosis of response of tumor to the therapy with cytostatics is especially important in carrying out the neoadjuvant component of treatment.

It has been showed that after three cycles of neoadjuvant chemotherapy according to the CAF scheme (cyclophosphamide 600 mg/m^2, adriamycin 50 mg/m^2, 5-fluorouracil 600 mg/m^2 every 3 weeks) in 72.5% of patients at initial absence of expression further has been observed increase of level of PGP that correlated with development of acquired resistance.

From the early eightieth of XX century numerous tries to inhibit the PGP with the aim to overcome the multidrug resistance have been set. For this purpose in clinical conditions have been used verapamil, phenothiazines, quinidine, acrichine, quinine,

amiodarone, neuroleptics, tamoxifen, progesterone, and cyclosporine. Given agents turned out to be poor inhibitors with high degree of toxicity.

Still there is no final answer, if inhibition of protein transporters can effectively overcome drug resistance *in vivo*.

Analysis is the evidence that fundamental role of ABC transporters in cancer is much more complicated, than realization of efflux of xenobiotics from cell. Expression of some proteins of this superfamily (PGP, MRP1 and BCRP) correlates with more aggressive phenotype of tumor and progression of disease [8, 9].

Methods of detection of PGP in BC cells need standardization of quantitative expression. Proteins of superfamily of ABC-transporters should be analyzed in clinical protocols within the context of personification of therapy in the stages of neoadjuvant and palliative therapy along with characteristics of molecular-genetic subtypes of tumor. Positive staining of circulating tumor cells on PGP can explain their refractivity to the cytostatic therapy as well as be the evidence of affiliation of this fraction of cells with stem cancer cells.

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