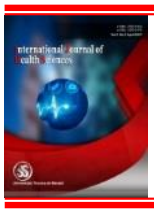

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Transcription Phenotype of Circulating Tumor Cells in Non-Metastatic Breast Cancer: Clinical and Prognostic Significance

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surgery;

Abstract

The objective of the study was to evaluate the clinical significance of circulating tumor cells (CTCs) and their transcriptional phenotype about overall and progression-free survival in patients with non-metastatic breast cancer. The presence of CTCs was studied before the start of special antitumor treatment and after its completion in 102 patients with primary non-metastatic breast cancer (BC) stage I–IIIC. The statistically significant increase in PFS in the group of patients without CTCs before the start of the treatment was established in 89.2 (87.9–92.4 confidence interval (CI) 95%) versus 79.9 (77.6–82.2 CI 95%) in the group with CTCs before treatment at p Log-Rank=0.01. The presence of CTCs expressing ABC transporter superfamily genes in the peripheral blood statistically significantly reduces the values of overall survival (OS) and progression-free survival (PFS). Three-year OS was 79.2 (77.1–82.3 CI 95%), and 90.8 (87.4–91.9 CI 95%) without the expression with p Log-Rank=0.04. The presence of circulating tumor cells expressing BIRC5 and HER2-neu genes, and ABC transporter genes, before the initiation of special treatment and the preservation of CTCs after the completion of adjuvant anticancer therapy, are independent risk predictors of disease recurrence.

Contents

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1 Introduction

Breast cancer (BC) is the most commonly diagnosed cancer in the female population worldwide, except for skin cancer. BC is the first cause of cancer death in women (Senkus et al., 2015). The proportion of breast cancer women survivors is the highest and is almost half of all cancer survivors (DeSantis et al., 2014; Cheng et al., 2012). In 1968, Bernard Fisher put forward a hypothesis and later formulated a theory according to which breast cancer should be considered not as a local disease, but as a systemic one. Tumor cells spread early even in the early stages of the disease. In this context, the problem of possible recurrence becomes urgent and, the importance of minimal residual disease (MRD) is emphasized. The term "minimal residual disease" (MRD) is defined as the presence of tumor cells in the body that cannot be detected with current routine diagnostic methods used to establish the stage of the tumor process in cancer patients after surgical removal of the primary tumor (Komilova et al., 2020). This concept came to the oncology of solid tumors from oncohematology (Afita et al., 2021). Nowadays, the term "minimal residual cancer" is also widely used, which implies the presence of circulating or disseminated tumor cells in radically treated patients (Yessentayeva et al., 2021).

The early spread of tumor cells from the primary tumor cannot usually be detected even with the help of modern imaging methods (MRI, PET), which prevents the initiation of timely and potentially effective treatment. Currently, metastatic breast cancer is incurable, and metastases lead to the death of most cancer patients (Shiau et al., 1997). It is well known that the metastatic process is very complicated. Today, many hypothetical models have been proposed that explain the metastatic cascade events, based on various characteristics of malignant cells of the primary tumor and metastatic foci (Fidler et al., 1978; Coghlin & Murray, 2010). Despite the extensive knowledge on the metastatic cascade, the "fine" mechanisms and details have not yet been elucidated (Rankin et al., 2016).

BC metastasizes mainly in certain "favourite" places of the human body, such as bones and internal organs (liver, lungs, brain) (Müller et al., 2001). The disease can also recur at the site of the primary tumor even after complete tumor resection with morphologically confirmed clear margins of surgical resection (Dharmayuda et al., 2021). Relapses or metastases can appear after many years, for example, women with luminal A breast cancer with positive hormone-receptor status, although they have a relatively favourable overall prognosis, are still at risk of the progression for many years (Müller et al., 2001; Jani et al., 2021). Basal-like or "triple-negative" breast cancer lacking hormone receptors and human epidermal growth factor receptor (HER2-neu), as a rule, has fewer treatment options and a higher potential for metastasis, and therefore a poorer prognosis and treatment results (Senkus et al., 2013).

Most relapses occur within the first 5 years after diagnosis and special treatment. Thus women who live longer will be deemed "cured". This should be considered in the context of the fact that autopsy demonstrated the presence of cancer in humans that was not diagnosed during life (Narod et al., 2015), and also that latent metastases not diagnosed by conventional imaging methods were found in patients with breast cancer (Kayser & Burkhardt, 1980). This, along with an understanding of the metastatic process, is the fundamental basis for the MRD study in breast cancer.

Currently, the research area, aimed at analyzing the characteristics of cells which can leave the primary tumor and survive in the peripheral blood (circulating tumor cells (CTCs)) or in the bone marrow (disseminated tumor cells (DTCs)), and the study of mechanisms, with which these processes are implemented are of great interest (Shin et al., 2007). In a tumor model, it has been demonstrated that about a million cells per gram of primary tumor tissue of breast carcinoma "leave" it every day, but almost all of them are very efficiently removed from the circulation within a few minutes (Allison et al., 2004). However, it has been shown in animal models that about 2.5% of cells that "leave" the tumor can survive as micrometastases and about 0.01% can progress to form macrometastases (Chang et al., 2000; Luzzi et al., 1998; Chambers et al., 2002). Therefore, it is important to know what factors give this very small minority of cells the ability to survive and ultimately lead to the death of the patient. An important point is to consider the characteristics of these cells that allow them to spread, survive and proliferate from a very small subpopulation of cancer cells

that can ultimately metastasize (Carchi et al., 2021; Suwananta et al., 2021). The purpose of the study was to assess the clinical significance of CTCs and their transcriptional phenotype to overall and progression-free survival in patients with non-metastatic breast cancer.

2 Materials and Methods

The average age of the patients was (M±SD) 58.0±12.7 with individual variations from 31 to 91 years. The incidence of involvement of both left and right mammary glands was almost the same, 50.5% and 49.5%, respectively. Most often, the tumor node was localized in the upper-outer quadrant (61.9%). T1 and T2 tumors were found predominantly; up to 5 cm in maximum size (53.9% and 43.1%, respectively). In 48.0% of cases, regional lymph nodes were not involved in the tumor process. In 39.2% of cases, there were 1 to 3 metastatic lymph nodes at the level I. Greater lesions of the regional lymphatic collector of the N2–N3 category were less common (3.9% and 8.8%, respectively). The overwhelming majority of patients in the experimental subgroup were women with breast cancer stages I and IIA (66.6%), the proportion of locally advanced forms IIB–IIIC was 33.4%.

According to the data of pathomorphological examination, invasive unspecified (ductal) carcinoma was diagnosed most often in 74.5%, lobular carcinoma was diagnosed more than three times less often (21.6%). Other types of cancer (tubular, medullar, mucinous carcinomas) were significantly less common and were 3.9%. The tumor was usually high (G3) or medium (G2) grade (52.9% and 44.1%, respectively) and had lymphovenous stromal invasion (LVSI+ – 84.3%). Luminal A cancer was identified in 48.0%, non-expressing luminal B HER2 – in 25.5%, expressing luminal B HER2 – in 5.9%, HER2 overexpressing – in 6.9%, and triple-negative subtype was diagnosed in 13.7% of cases. Clinical and anatomical, pathological and morphological, and molecular and biological characteristics of the primary tumor are presented in Table 1.

Table 1
Clinical and anatomical, pathological and morphological and molecular and biological characteristics of the primary tumor

Category, value		n=102	
		abs.	%
T	1	55	53.9%
	2	44	43.1%
	3	2	2.0%
	4	1	1.0%
N	0	49	48.0%
	1	40	39.2%
	2	4	3.9%
	3	9	8.8%
Stage	I	34	33.3%
	IIA	34	33.3%
	IIB	18	17.6%
	IIIA	5	4.9%
	IIIB	1	1.0%
	IIIC	10	9.8%
Histological structure of carcinoma	tubular	1	1.0%
	medullar	1	1.0%
	mucinous	2	2.0%
	unspecified	76	74.5%
	lobular	22	21.6%
Grade	G1	3	2.9%
	G2	45	44.1%
	G3	54	52.9%

LVSI	LVSI+	86	84.3%
	LVSI–	16	15.7%
Molecular biological tumor subtype	luminal A	49	48.0%
	luminal B HER2–	26	25.5%
	luminal B HER2+	6	5.9%
	overexpressing HER2	7	6.9%
	triple negative	14	13.7%

During treatment, a 5 ml sample of peripheral blood was collected from the cubital vein in the morning on an empty stomach from all patients into a sterile vacuum tube with K2EDTA for subsequent enrichment and isolation of CTCs and was stored at 4 °C until the study. Samples were processed immediately or not later than four hours after blood sampling. Enrichment and isolation of CTCs were carried out using the method of rapid isolation of tumor cells from whole blood based on covalently bound antibodies for CD326 on a non-magnetic polymer matrix of large microspheres with subsequent isolation of CTCs by size (S-pluriBead Maxi Reagent Kit and anti-human CD326 S- pluriBead, Germany).

Isolation of mRNA from lysed, enriched cells was carried out following the instructions of the manufacturer of kits for RNA isolation (SIVital LLC, Belarus); using reverse transcription technology, cDNA was synthesized, which was subsequently used to analyze gene expression in real-time by RT-PCR. To analyze the expression of the HER2-neu and BIRC5 genes, we used the originally developed test systems to determine the expression of Survivin (BIRC5) and HER2-neu cDNA transcription by REAL-TIME PCR.

A specially selected set of primers and probes Platinum PCR SuperMix (Invitrogen, USA) was used to analyze the expression of multidrug resistance genes. For the analysis of normalized expression, the following target drug resistance genes were selected: ABCB1 (encoded by it PGP/MDR1 protein), ABCC1 (encoded MRP1 protein), ABCC5 (encoded MRP5 protein), ABCC10 (encoded MRP7 protein), and ABCG2 (encoded BCRP/MXR protein). AdnaTest EMT-1/StemCell kit (Adna Gen AG, Langenhagen, Germany) was used to analyze EMT and the presence of a marker of breast cancer stem cells. This test system includes one breast cancer stem cell marker ALDH1 and 3 EMT markers (AKT2, PI3K, and TWIST). In all cases, the c-ABL "housekeeping" gene was used as a reference gene. A qualitative and quantitative analysis of expression (real-time PCR) was carried out for HER2-neu, BIRC5, ABC transporters genes, and qualitative analysis of PCR (presence or absence of a gene) for EMT and ALDH1 genes.

Due to the unique technology for enrichment and isolation of CTCs, the determination of the expression of the reference c-ABL gene confirmed the presence of cells expressing EpCAM on their surface (CD 326) in the sample. BIRC5 and HER2-neu have been identified by marker genes for the identification of CTCs. CTCs can be identified and their phenotype and functional activity can be evaluated using qualitative assessment and quantitative characterization of the normalized expression of targeted genes. All stages of PCR were performed on Bio-Rad equipment (USA).

Statistical processing of the data obtained was carried out following modern requirements for medical and biological studies. Qualitative attributes are presented in absolute and relative values. The distribution of quantitative signs for normality was checked using the Lilliefors and Shapiro-Wilk tests. Quantitative sigs that meet the normal distribution are presented as mean (M), standard deviation (SD), standard error of the mean (SE), minimum and maximum values (min, max).

Quantitative signs that do not meet the normal distribution are shown as median (Me), interquartile range (LQ/UQ), minimum and maximum values (min, max). Two groups were compared in terms of quantitative characteristics having equal general variances and corresponding to the normal distribution using the Student's test. The condition of equality was checked according to Leuven and Fisher's tests. Two groups were compared for quantitative characteristics that do not correspond to the normal distribution using the nonparametric Mann–Whitney test. Two groups were compared for qualitative ordinal characteristics according to the Mann–Whitney test, for qualitative nominal using the Pearson chi-square ($\chi 2$) test, for qualitative binary using the $\chi 2$ test with Yates' correction, and Fisher's exact test as applicable.

Pearson's correlation coefficient was calculated as a measure of the relationship for quantitative signs that met the normal distribution. Spearman's rank correlation coefficient was used for quantitative signs that do not meet the normal distribution, and for qualitative ordinal signs, and in some cases, the Mann-Whitney test

was applied. To determine the degree of heterogeneity, cluster analysis was used with the construction of a hierarchical tree (tree diagram) with an estimate of the Euclidean distance. In addition, the cluster analysis used the two-input combining method (Allison et al., 2006; Widana et al., 2021). To assess the long-term results of treatment, the values of overall and disease-free survival were calculated using the Kaplan-Meier method. The survival in the two groups was compared using the log-rank criterion.

Groups were also compared by long-term results of treatment using values of the relative risk of all-cause mortality and the relative risk of recurrence and progression of the disease (See, 2007). Risk ratios (RRs), 95% confidence intervals for the risk ratios, and the level of significance of various risks were calculated. The relative risk and its 95% confidence interval were calculated using the Cox proportional-hazards regression model. To identify indicators that affect the risk of recurrence and progression of the disease, a mono variant analysis was carried out for all individual indicators. Risk-related indicators with a statistical significance level of $p<0.05$ are included in the multivariate model as predictors.

Cox's proportional-hazards model is based on the following assumptions: all predictors (independent variables) are linearly related to the logarithm of the event risk function; the risks of an event occurring for any two objects at any time interval are proportional. This means that for two patients with different values of independent variables (predictors), the ratio of their risk functions does not depend on time. In all cases, the differences were considered statistically significant at a significance level of $p<0.05$. All p values were two-sided. Statistical processing of the results was performed using SPSS Statistics 10.0.

3 Results and Discussions

When examining the peripheral blood of patients with verified primary non-metastatic breast cancer in the morning before surgery, the expression of the c-Abl gene and at least one marker gene in the CTC was detected in 69 women of 102 examined women, which is 67.6%. The frequency of expression of targeted genes is shown in Table 2 and Figure 1.

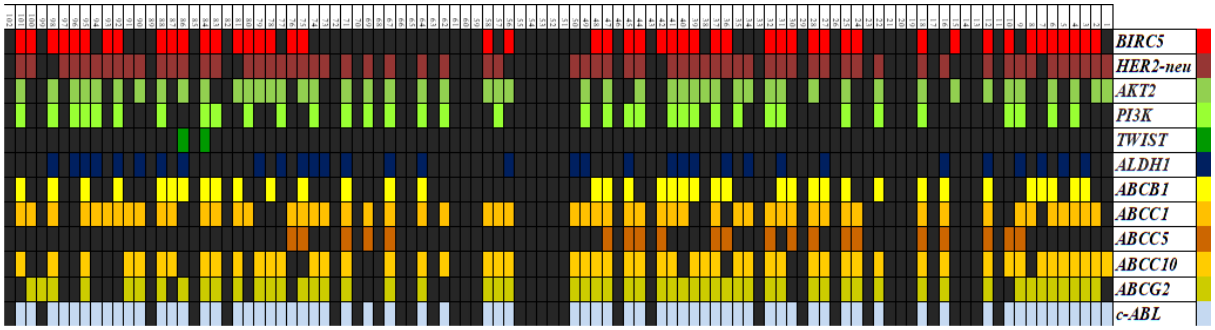

Figure 1. CTC transcriptional phenotype (colored marker – presence of expression, grey marker – absence of expression) in patients with primary breast cancer (n=102)

Table 2
Frequency of mRNA detection of targeted genes in CTCs in primary breast cancer

CSC genetic marker	Frequency (n=102) n1 – the presence of gene expression, abs	% of n	% of n1
c-Abl	69	67.6%	100.0%
HER2-neu	64	62.7%	92.8%
BIRC5	49	48.0%	71.0%
EMT	51	50.0%	73.9%
AKT2	48	47.1%	69.6%
PI3K	36	35.3%	52.2%

TWIST	2	2.0%	2.9%
ALDH1	32	31.4%	46.4%
ABC	59	57.8%	85.5%
ABCB1	36	35.3%	52.2%
ABCC1	55	53.9%	79.7%
ABCC5	21	20.6%	30.4%
ABCC10	55	53.9%	79.7%
ABCG2	57	55.9%	82.6%

Expression of genetic markers of cell differentiation and apoptosis of HER2-neu and BIRC5 in circulating tumor cells in primary breast cancer. Expression of the HER2-neu epidermal growth factor receptor gene in CTCs was detected in 64 of 69 CTC-positive patients (92.8%). Expression of the gene for the anti-apoptotic protein survivin BIRC5 was detected in 49 of 69 CTC-positive patients (71%). Indicators of normalized gene expression are presented in Table 3 and Figure 2.

Table 3
Indicators of normalized expression of *BIRC5* and *HER2-neu* genes in CTC

Indicator	Level of normalized expression of targeted genes	
	BIRC5	*HER2-neu*
Med	0.14	0.26
Min	0.01	0.11
Max	10.70	17.44
UQ	0.01	0.45
LQ	0.11	0.83

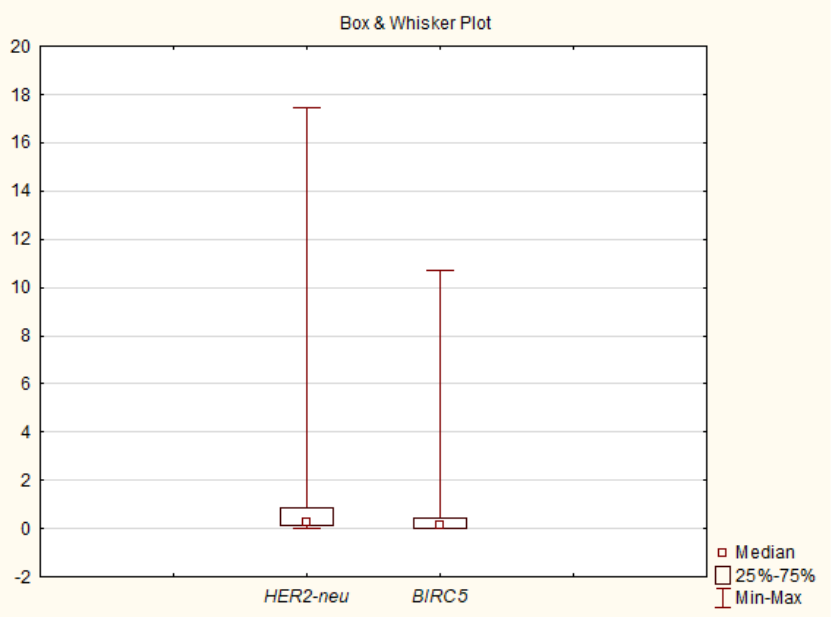

Figure 2. Indicators of normalized expression of BIRC5 and HER2-neu genes in CTC

BIRC5 positive and *HER2-neu* positive CTC mRNAs were more commonly detected at more advanced stages and, mainly, in the presence of metastatic regional lymph nodes, and with an increase in their number, the frequency of detecting CTCs expressing *BIRC5* and *HER2-neu* genes increased. However, the data were not statistically significant (p>0.05). When comparing the data on the frequency of identification of *BIRC5* positive and *HER2-neu* positive CTC mRNAs and the morphological and molecular biological characteristics of the

primary tumor, the change in the number of CTCs in positive patients did not reliably depend either on the histological structure of the primary carcinoma or on the degree of differentiation, presence or absence of lymphovenous stromal invasion. High frequency of CTC detection was observed in luminal B non-expressing *HER2-neu* cancer and triple-negative basal cancer. However, the data are not reliable. Besides, no significant differences were found in the frequency of CTC identification by target genes depending on other molecular biological tumor subtypes.

Expression of genetic markers of epithelial-mesenchymal transition and a stem cell marker in circulating tumor cells in primary breast cancer. A group of 51 women of 69 CTC-positive patients (74%) was positive for at least one of the EMT markers, and 32 of 69 patients (46.4%) were positive for *ALDH1*, respectively. When studying the comparability of groups of patients with positive peripheral blood samples for the presence of CTCs expressing EMT markers, the following patterns were established. The identification of EMT markers (*AKT2, PI3K, TWIST* genes) did not depend on the stage of the tumor process, determined by the tumor size and the presence of metastatic regional lymph nodes and their number. When comparing the data on the frequency of identification of EMT positive CTC mRNAs and the morphological and molecular biological characteristics of the primary tumor, the change in the frequency of CTC-positive cases did not reliably depend either on the histological structure of the primary carcinoma or on the degree of differentiation, the presence or absence of lymphovenous stromal invasion. Besides, no significant differences were found in the frequency of CTC identification by targeted EMT genes, depending on the molecular biological subtype of the tumor. When comparing the data on the frequency of identification of *ALDH1* positive CTC mRNAs and the stage of the tumor process, and the morphological and molecular biological characteristics of the primary tumor, the change in the number of CTC-positive patients reliably depended only on the histological structure of the primary carcinoma. No other statistically significant patterns were found.

Expression of multidrug resistance genes ABC transporters in circulating tumor cells in primary breast cancer. In 59 of 69 CTC-positive samples (86%), the expression of at least one targeted drug resistance gene was determined. It is fundamentally important not only to qualitatively determine the expression of drug resistance genes in CTCs but also to quantify the level of normalized expression, which can be assessed using real-time RT-PCR. The level of normalized expression of *ABC transporter genes* is considered to be elevated at > 1.0 and normal at ≤1.0. Based on the designated criterion, CTC-positive patients were conditionally divided into two groups. In 47 CTC positive samples, which amounted to 68%, and increased normalized expression of multidrug resistance genes was found. This group of patients can be conditionally characterized as "drug-resistance". In 22 (32%) women, the level of normalized expression was defined as normal, and this group can be conditionally characterized as "drug-sensitive". Indicators of normalized expression of genes of the *ABC transporter* family in CTCs are shown in Figure 3.

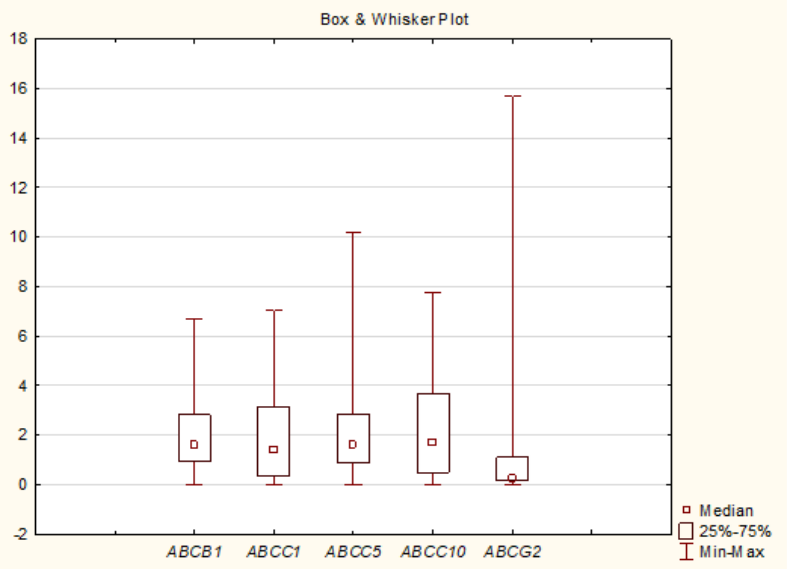

Figure 3. Indicators of normalized expression of the ABC transporter family genes in CTCs

Based on the analysis of the relationship between the normalized expression of this gene, encoding the PGP/MDR1 protein, which provides resistance to colchicine, doxorubicin, etoposide, vinblastine, and paclitaxel, a statistically significant relationship was found between the frequency of CTCs expressing this gene and the stage of the tumor process determined by metastatic lesions of regional lymph nodes and their number. In addition, a relationship with molecular biological subtypes of primary breast cancer was revealed. A similar situation was observed in the analysis of the normalized expression of the *ABCC* gene encoding the MRP1 protein, which provides resistance to doxorubicin, daunorubicin, vincristine, etoposide, colchicine, and methotrexate.

Based on the analysis of the relationship between the normalized expression of the *ABCC5* gene (MRP5 protein), which provides resistance to one agent fluorouracil, no significant relationships were found between the frequency of CTCs expressing this gene with various parameters of the primary tumor. Based on the analysis of the relationship between the normalized expression of the *ABCC10* gene encoding the MRP7 protein, which eliminates fluorouracil and paclitaxel from the tumor cell, a statistically significant relationship was established between the frequency of CTCs expressing this gene and the stage of the tumor process determined by metastatic lesions of regional lymph nodes and their number. In addition, the relationship with molecular biological subtypes of primary breast cancer has been determined.

Expression parameters of the *ABCG2* gene (BCRP/MXR protein), which provides multidrug resistance (doxorubicin, daunorubicin, irinotecan, topotecan, imatinib, methotrexate) are presented in Figure 5. Based on the analysis of the relationship of normalized gene expression, a statistically significant relationship was found in the frequency of CTC detection, expressing this gene, with the stage of the tumor process, determined by the metastatic damage of regional lymph nodes and the number of metastases. In addition, a relationship was revealed not only with molecular biological subtypes of primary breast cancer carcinoma but also with histological types of cancer.

Correlation analysis. Detailed correlation analysis of the expressed CTC genetic markers revealed certain patterns. For example, a strong statistically significant correlation was found between *AKT2* and *PI3K* ($r=0.78$; $p<0.01$). A moderate statistically significant correlation was found between the levels of normalized expression of the *BIRC5* and *HER2-neu* genes ($r=0.53$; $p<0.01$), and between *HER2-neu* and *PI3K* ($r=0.31$; $p<0.01$). A positive weak correlation was also revealed between the expression of the *BIRC5* gene and the EMT marker genes *TWIST* and the chemoresistance marker genes *ABCB1* and *ABCC1*. Expression of the epidermal growth factor receptor gene *HER2-neu* weakly correlated with the expression of the EMT genes and *ALDH1*. A weak correlation was found between the drug resistance genes of the *ABC transporter* family. The summary data on the correlation between the expressed genetic markers of cell proliferation and differentiation, EMT, and drug resistance are presented in Table 4.

Table 4
Correlation between the levels of expression of CTC gene markers

Spearman's correlation coefficient (r) and p-value	Genetic markers										
	BIRC5	*HER2-neu*	*AKT2*	*PI3K*	*TWIST*	*ALDH1*	*ABCB1*	*ABCC1*	*ABCC5*	*ABCC10*	*ABCG2*
BIRC5	1.000	0.526	0.012	-0.029	0.246	0.015	0.260	0.294	-0.124	0.083	0.010
p		0.000	0.875	0.699	0.001	0.845	0.002	0.001	0.142	0.341	0.882
HER2-neu	0.526	1.000	0.206	0.307	0.232	0.215	0.131	0.128	0.083	0.028	0.060
p	0.000		0.008	0.000	0.003	0.043	0.170	0.158	0.310	0.776	0.515
AKT2	0.012	0.206	1.000	0.782	0.011	-0.032	-0.042	0.070	0.019	-0.047	-0.036
p	0.875	0.008		0.000	0.868	0.638	0.596	0.388	0.797	0.559	0.655
PI3K	-0.029	0.307	0.782	1.000	0.047	-0.044	0.137	0.015	0.028	-0.055	0.047
p	0.699	0.000	0.000		0.486	0.516	0.083	0.853	0.717	0.494	0.560
TWIST	0.246	0.232	0.011	0.047	1.000	0.057	0.075	0.080	-0.135	0.007	-0.061
p	0.001	0.003	0.868	0.486		0.398	0.345	0.322	0.075	0.929	0.444
ALDH1	0.015	0.158	-0.032	-0.044	0.057	1.000	0.047	0.126	0.116	-0.129	0.066
p	0.845	0.043	0.638	0.516	0.398		0.554	0.120	0.127	0.110	0.411
ABCB1	0.260	0.131	-	0.137	0.075	0.047	1.000	0.261	0.112	0.121	0.084

p	0.002	0.170	0.042 0.596	0.083	0.345	0.554		0.004	0.178	0.203	0.406
ABCC1	0.294	0.128	0.070	0.015	0.080	0.126	0.261	1.000	0.029	0.294	- 0.071
p	0.001	0.158	0.388	0.853	0.322	0.120	0.004		0.714	0.002	0.477
ABCC5	-0.124	0.083	0.019	0.028	-0.135	0.116	0.112	0.029	1.000	0.104	0.288
p	0.142	0.310	0.797	0.717	0.075	0.127	0.178	0.714		0.222	0.002
ABCC10	0.083	0.028	- 0.047	-0.055	0.007	-0.129	0.121	0.294	0.104	1.000	- 0.086
p	0.341	0.776	0.559	0.494	0.929	0.110	0.203	0.002	0.222		0.450
ABCG2	0.010	0.060	- 0.036	0.047	-0.061	0.066	0.084	-0.071	0.288	- 0.086	1.000
p	0.882	0.515	0.655	0.560	0.444	0.411	0.406	0.477	0.002	0.450	

Cluster analysis. When constructing a hierarchical tree in the presence of expression of target genes in CTCs and assessing the Euclidean distance, significant heterogeneity of CTCs and a certain pattern manifested in the combination of CTCs into clusters were established (Figure 4). For example, the first CTC cluster includes CTCs expressing the *ABCC1* and *ABCC10* genes. The next cluster was formed by CTCs expressing the EMT genes *AKT2* and *PI3K*. The third cluster was formed by CTC expressing genes *BIRC5* and *HER2-neu*. The fourth cluster is represented by cells expressing the *TWIST* gene, which is responsible for EMT, and surprisingly, expressing the stem cell marker *ALDH1*. The Euclidean distance between clusters more than 2 indicates a severe heterogeneity of CTC clusters.

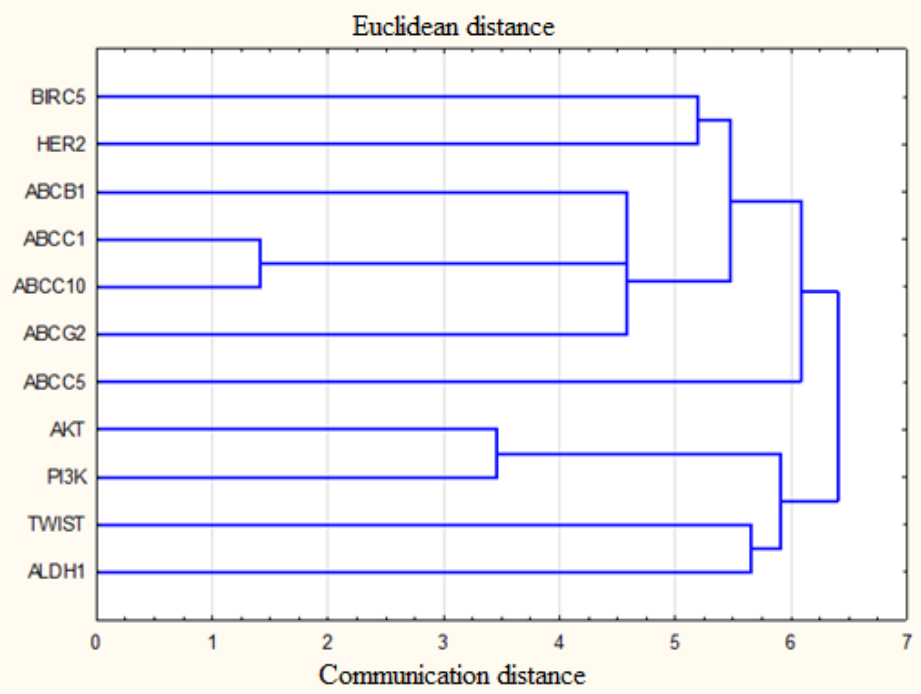

Figure 4. Cluster analysis of the hierarchical tree of the expression of target genes in CTCs

A two-way combination of the relationship between the presence of expression and the level of normalized gene expression in CTCs revealed a fairly homogeneous cluster (n=20), characterized by high levels of normalized expression of *BIRC5, HER2-neu* genes, and *ABC transporter* genes (Figure 5).

Figure 5. Cluster analysis of two-way combining of the presence of expression and levels of normalized expression (color scale) of target genes in CTC

Survival of patients with primary non-metastatic breast cancer. When analyzing the relative risks of breast cancer progression in the context of the presence of CTCs expressing the target survivin *BIRC5* gene and the epidermal growth factor receptor *HER2-neu*, before the start of special anticancer therapy, and in the context of MRD after completion of treatment, the following data were obtained. The average follow-up period for patients in the experimental subgroup was 38.8 months. After the combination treatment was completed, the disease progressed in 18 patients within one year with 4 patients dying during this year. Subsequently, the progress of the disease developed in 5 more women. In total, 23 of 102 (22.5%) patients had disease recurrence within three years. It should be noted that in this group of patients, all 5 had disseminated tumor cells before the start of treatment, confirmed by the identification of *BIRC5* and *HER2-neu* positive CTC mRNAs. In addition, after the completion of the entire program of anticancer therapy, tumor cells persisted in the peripheral blood in all these 25 women. The indicators of the relative risks of breast cancer progression after the treatment depending on the presence of *BIRC5* and *HER2-neu* positive CTC mRNAs before treatment and their persistence after treatment are presented in Table 5.

Table 5
Indicators of the relative risks of breast cancer progression after the treatment depending on the presence of *BIRC5* and *HER2-neu* positive CTC mRNAs before treatment and their persistence after treatment

Risk indicators	Indicator	
	presence of *BIRC5* and *HER2-neu* positive CTC mRNAs before treatment	presence of *BIRC5* and *HER2-neu* positive CTC mRNAs after treatment (MBP)
Absolute risk in the study group (EER)	0.31	0.55
Absolute risk in the control group (CER)	0.02	0.010
Relative risk (RR)	14.71	52.36
Standard error of relative risk (S)	0.99	1.00

Lower limit of 95% CI	2.08	7.36
Upper limit of 95% CI	104.23	372.55
Reduced relative risk (RRR)	13.71	51.36
Risk difference (RD)	0.29	0.52
Number of patients to be treated (NNT)	3.43	1.93
Sensitivity (Se)	0.97	0.97
Specificity (Sp)	0.37	0.74

The overall survival rate of all 102 patients of the first subgroup was 88.9 (77.5–94.1 CI 95%), and PFS was 77.1 (74.9–78.9 CI 95%) (Table 6).

Table 6
Overall survival and progression-free survival of patients (n=102)

Category	Survival rate, %, 95% CI		
	1-year	2-year	3-year
Overall survival	97.1 (95.9–98.9)	93.3 (91.3–95.5)	88.9 (77.5–94.1)
Progression-free survival	91.7 (89.3–93.2)	82.2 (80.1–85.4)	77.1 (74.9–78.9)

When comparing OS in groups of patients with CTCs expressing the *BIRC5* and *HER2-neu* genes, before the start of special treatment and with their absence, no significant differences were found (p=0.59) (Figure 6).

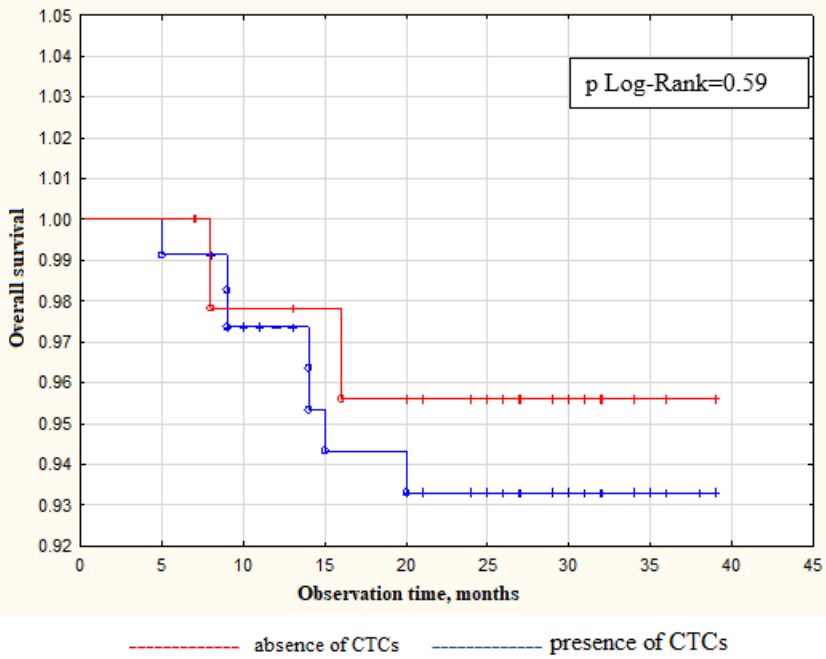

Figure 6. Comparison of OS in patients with and without CTCs expressing BIRC5 and HER2-neu genes before special treatment

When assessing PFS, statistically significant differences were obtained (p=0.01). There was an increase in indicators in all analysed time periods (Figure 7).

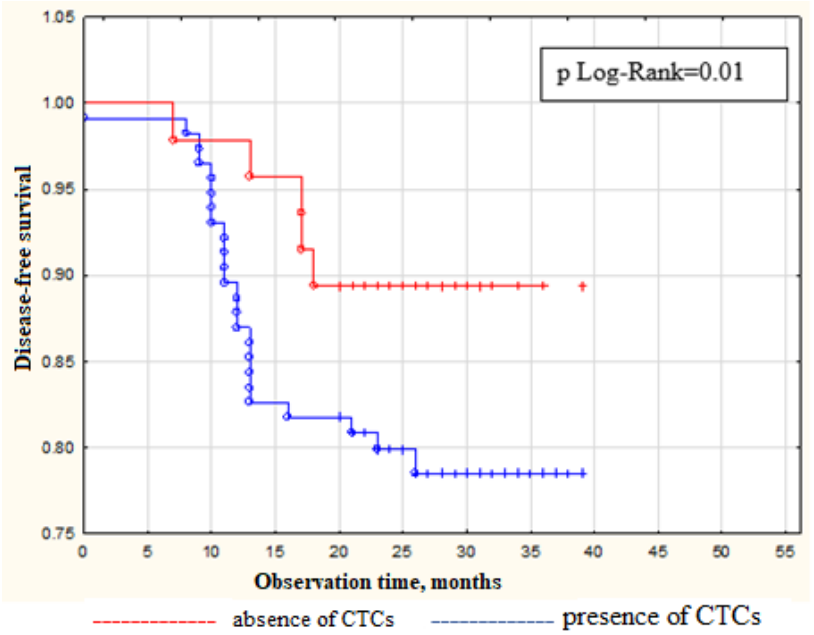

Figure 7. Comparison of PFS in patients with and without CTCs expressing BIRC5 and HER2-neu genes before special treatment

When comparing OS parameters of groups of patients with preservation of CTCs expressing genes *BIRC5* and *HER2-neu* after completion of special treatment and their absence, statistically significant differences (p=0.59) were not found (Figure 8).

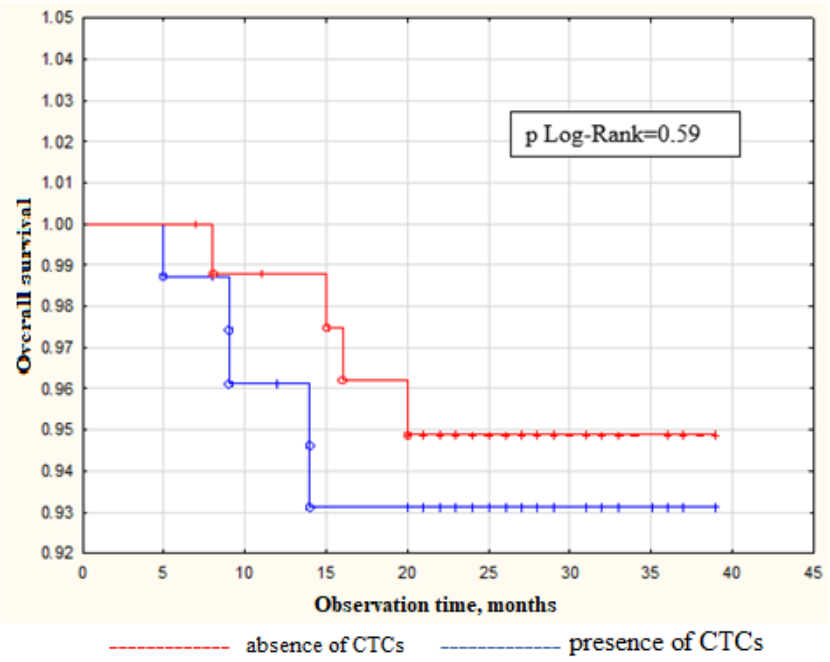

Figure 8. Comparison of OS in patients with present and absent CTCs expressing genes BIRC5 and HER2-neu after special treatment

When assessing PFS, statistically significant differences in 3-year survival were obtained (p=0.02). There was an increase in all analyzed time intervals (Figure 9).

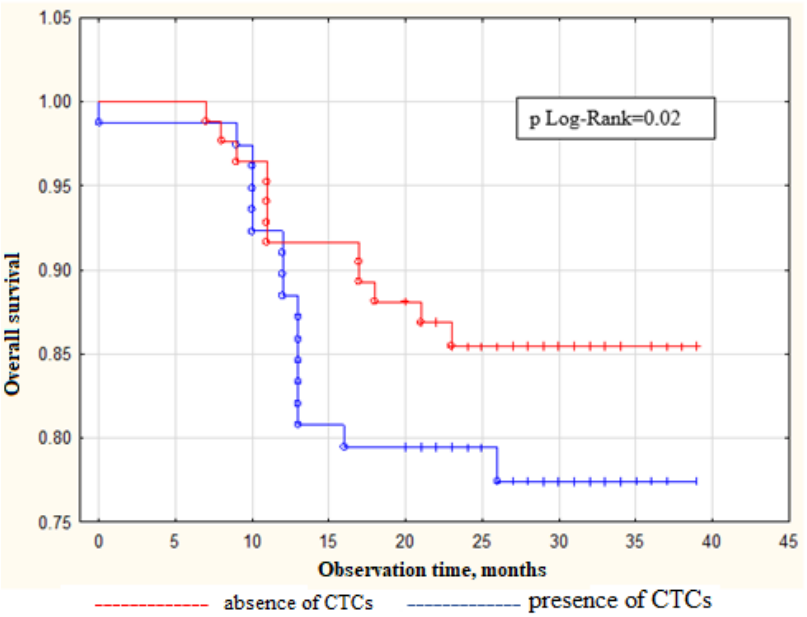

Figure 9. Comparison of PFS in patients with present and absent CTCs expressing BIRC5 and HER2-neu genes after special treatment

When comparing OS and PFS of groups according to the presence of an aggressive transcriptional CTC phenotype before treatment, in particular, the presence of expression of the EMT and ALDH1 genes, no statistically significant differences were found (p>0.05) (Figures 10–11).

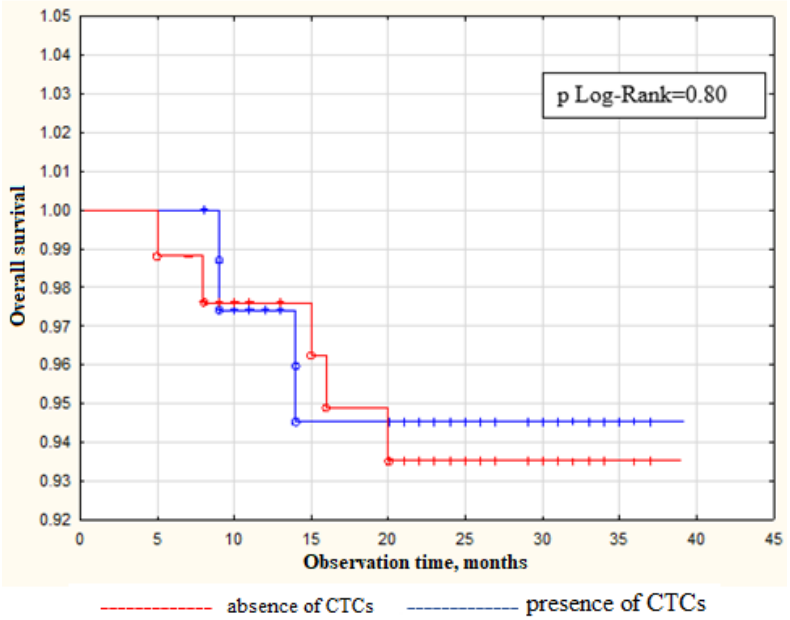

Figure 10. Comparison of OS of patients with and without expression of the EMT and ALDH1 genes in CTCs before the start of special treatment (n=162)

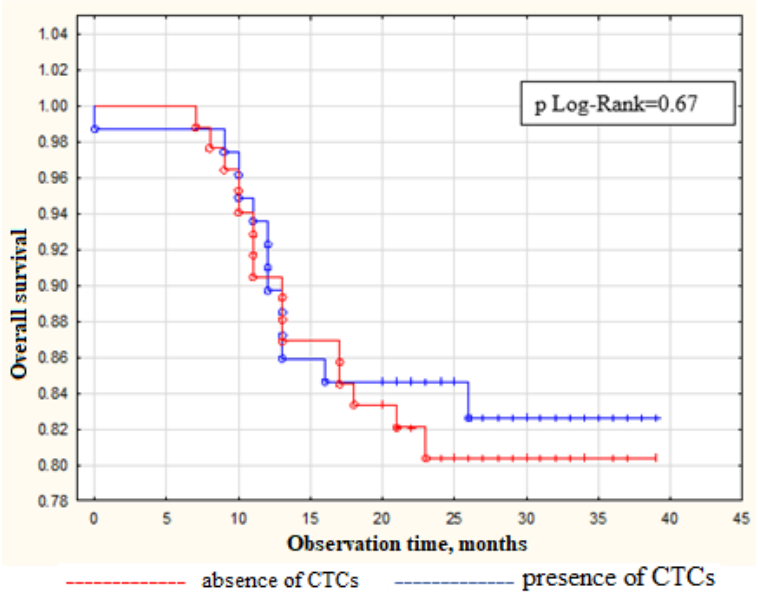

Figure 11. Comparison of PFS in patients with and without expression of EMT and ALDH1 genes in CTCs before the initiation of special treatment (n=102)

At the same time, the presence of CTCs expressing the *ABC-transporter* genes in the peripheral blood statistically significantly reduces OS and PFS. For example, the overall 3-year survival rate was 79.2 (77.1–82.3 CI 95%) for patients with expression of ABC transporters in CTCs, and 90.8 (87.4–91.9 CI 95%) for those without expression with p Log-Rank=0.04 (Figure 12).

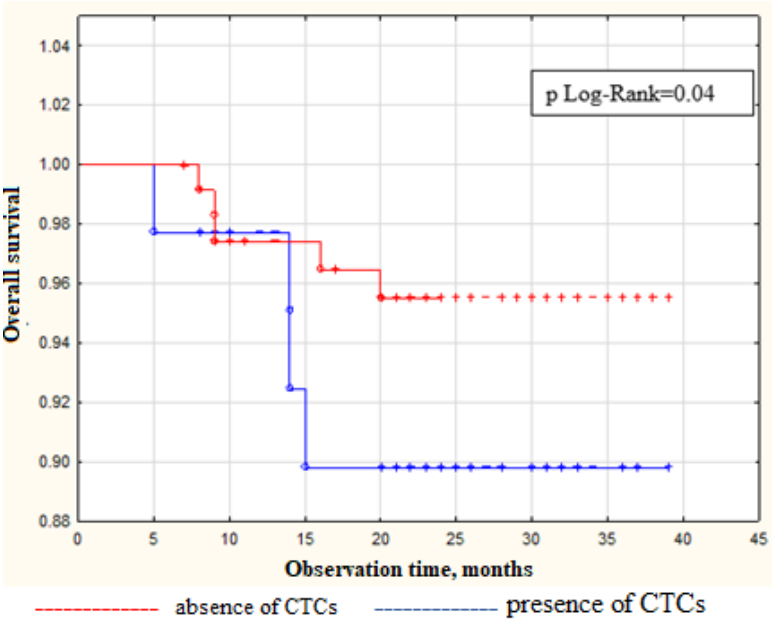

Figure 12. Comparison of OS in patients with and without expression of ABC transporter genes in CTCs (n=102)

3-year progression-free survival of patients was 75.2 (71.1–76.0 CI 95%) with the presence, and 84.5 (72.8–77.7 CI 95%) with the absence of gene expression of ABC transporters in CTCs with p Log-Rank=0.04 (Figure 13).

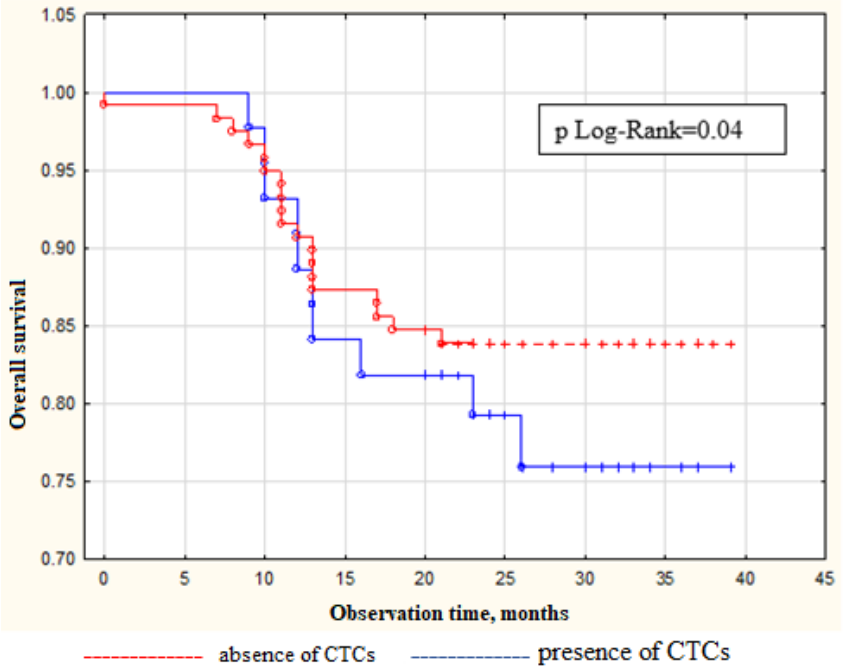

Figure 13. Comparison of PFS in patients with the presence and absence of expression of ABC transporter genes in CTCs (n=102)

Monofactorial analysis revealed that PFS is influenced by such factors as the presence of *BIRC5* and *ABC transporter* expressing CTCs in the peripheral blood before the start of special treatment for resectable breast cancer, and the preservation of CTCs *per se*, i.e. preservation of MRD after completion of special anticancer therapy. The results are presented in Table 7.

Table 7
Multivariate analysis results

Parameter	Statistical data							
	β	SE	e (β)	Wald-stat.	p	RR	CI	
							LL	UL
Expression of *BIRC5* in CTCs	–1.296	0.670	0.016	3.743	0.05	2.27	1.07	3.01
Expression of EMT markers in CTCs	0.529	0.561	1.630	0.889	0.35	1.69	0.56	5.10
Expression of *ALDH1*in CTCs	–0.076	0.614	1.128	0.015	0.90	0.93	0.27	3.09
Expression of *ABC transporters* in CTCs	–3.846	1.322	–1.254	8.461	0.03	0.97	1.01	1.28
Presence of CSCs before treatment	1.036	0.727	2.462	2.032	0.04	14.71	2.08	104.2
Preservation of CTCs after treatment	0.186	0.624	1.410	0.088	0.04	52.36	7.36	372.5

The presence of CTCs in primary non-metastatic breast cancer before the start of treatment confirms the fact that breast cancer is an initially disseminated systemic disease. In addition, the determination of the transcriptional CTC phenotype before treatment, and molecular genetic and immunohistochemical studies of the primary tumor can be the starting point for planning further systemic adjuvant treatment (Cristofanilli et al., 2004). The biological characteristics of CTCs are still poorly understood. It is assumed that only some CTCs can form true clinically significant metastases (Jolly et al., 2016). Moreover, the modern theory of breast

cancer progression is that EMT processes play a key role both in the primary tumor and at the stages of starting the metastatic cascade, and in the creation of the so-called BC stem cells (Khan et al., 2015).

Recently published data from small patient populations supports these findings. Raimondi et al. (2011), investigated the expression of EMT and stem cell markers in 92 breast cancer patients in all stages of the disease. Using the standard definition of CTCs as EpCAM+/CK+/CD45–, the authors could not find them in 31 of 92 (34%) patients. The absence of CTCs was characterized by scientists as a lack of expression in the enriched EpCAM fraction. Nevertheless, in 12/31 (38%) of these CK-negative cells, the authors found expression of EMT markers, in particular, vimentin and fibronectin. They concluded that their methods of detecting CTCs underestimate the most important subpopulations of CTCs involved in the spread of cancer (Raimondi et al., 2011).

In a retrospective study of 292 patients with metastatic breast cancer, the number of CTCs was counted before patients started a new line of treatment using the Cell-Search system. CTCs were not detected in 36% of patients. Multivariate analysis revealed that brain metastases and bone lesions remained independent variables associated with undetectable CTC status. In addition, in patients with a high degree of tumor malignancy with triple-negative cancer and inflammatory forms of breast cancer, the likelihood of undetectable CTC was increased. The authors suggested that these results may be due to the underestimation of CTC by CellSearch, which cannot capture CTCs undergoing EMT (Mego et al., 2011). At present, the question of whether the detection of CTCs in the peripheral blood can replace the detection of DTCs in bone marrow is very relevant. In a recently published study comparing DTCs and CTCs in patients with primary breast cancer, a weak correlation was found between the presence of these cells in the bone marrow and the peripheral blood, which may be due to the different biology of these cells and their genetic characteristics (Fehm et al., 2009). These findings are consistent with other comparative studies in which the correlation between DTCs and CTCs is low (Daskalaki et al., 2009). However, in two other recently published studies, scientists found that RT-PCR of CTC status conveyed clinically significant information that was not inferior to DTC status in breast cancer (Slade et al., 2009). There are various explanations for this phenomenon:

- The absence of CTCs may depend on the inadequacy of the procedures for selecting CTC standards. Most selection procedures are based solely on EpCAM, which may stop to express during EMT passage;
- EMT cells that destroy the surrounding matrix also allow non-EMT tumor cells to separate and spread. Thus, DTCs and CTCs can reflect different heterogeneous populations of primary tumor cells, including differentiated and more stem tumor-like cells;
- It should also be assumed that another mechanism of spread or epithelial differentiation is required for the formation of bone metastases. The PTEN/Akt/mTOR signaling has been described as one of the main pathways for the regulation of mammalian stem/progenitor cells. In addition, this pathway provides resistance to conventional therapy and plays a central role in the viability of cancer stem cells in the mammary gland, promoting proliferation and inhibiting apoptosis (Sabbah et al., 2008). At the same time, cells undergoing EMT develop resistance to antitumor agents (Kallergi et al., 2007).

Currently, only a few studies have attempted to investigate these issues with CTCs. An analysis of mononuclear cell cytospins using confocal laser scanning microscopy in peripheral blood samples from 28 patients with 17 CK-positive and 17 CK-negative CTCs, phosphorylated PI-3-kinase (PI3K) was detected in 15 of 17 CK-positive samples (Monteiro & Fodde, 2010). In a recent study, the authors analyzed peripheral blood mononuclear cells in 32 patients with *CK-19* positive CTC mRNA with early BC and 16 patients with metastatic BC and reported that *PI3K/AKT2* were identified in a significant proportion of CTCs (Kallergi et al., 2008). These data are consistent with the results obtained in this study.

Thus, it was found that the expression of stem cell markers and EMT markers in CTCs is associated with resistance to conventional anticancer drugs, thereby emphasizing the relevance of improving tools for the diagnosis and treatment of MRD (Bachelot et al., 2010). Therefore, stem cell detection can be recommended for diagnostic and therapeutic actions, and signaling pathways that support tumor stem cells are attractive targets for these studies. In addition, the search for new markers for both EMT and stem cells is important. The *BIRC5* gene and *BIRC5* encoded antiapoptotic protein survivin can be considered as a unique marker in this context. The suppression of apoptosis is the final stage of EMT; therefore, the use of an assessment of the

expression of this gene can serve as a unique marker not only for the search for CTCs and diagnostics of MRD but also for predicting the course of the tumor process and monitoring the efficacy of polychemotherapy.

All recent studies emphasize the need for molecular characterization of CTCs (beyond simple enumeration), which would provide critical information to distinguish between subpopulations of CTCs with different biological properties. Such information can influence the choice of the regimen and be useful for monitoring the effectiveness of systemic therapy and assessing the overall clinical outcome in patients with breast cancer (Wind & Holen, 2011). However, as noted earlier, all studies were conducted with the participation of patients with initially disseminated metastatic breast cancer (Gradilone et al., 2011; Gazzaniga et al., 2010).

There are some studies on the expression of multidrug resistance genes in a primary tumor (Wind & Holen, 2011; Tratsiak et al., 2015). The present study is innovative, since the potential sensitivity and resistance of CTCs, the immediate substrates of metastases in primary non-metastatic breast cancer, have been studied for the first time. MRD-expressing CTCs may represent a cell population with a greater propensity for intrinsic drug resistance. As you know, chemoresistance is an attribute of a tumor stem cell. Whether these MRD-positive CTCs are stem cells is currently unclear. However, the results of the Theodoropoulos et al. (2010), indicate a high expression of the tumor stem cell marker ALDH1 in primary BC tumors, combined with a high expression of various types of ATP-binding cassette ABC transporters.

4 Conclusion

CTCs isolated from the peripheral blood of patients with metastatic breast cancer have a pronounced heterogeneous transcriptional phenotype. CTCs express the anti-apoptotic protein survivin gene BIRC5 in 48.4%, and the epidermal growth factor receptor gene HER2-neu in 67.2%. CTCs export at least one of the EMT markers in 74%, and the tumor stem cell marker ALDH1 in 46.4%. In 86% of CTCs, the expression of at least one drug resistance gene of the ABC transporters was determined.

Independent prognostic factors for the recurrence of the disease include the presence of CTCs in the peripheral blood expressing the BIRC5 genes and the ABC transporter genes before the start of special treatment for resectable breast cancer, and the preservation of CTCs per se, i.e. preservation of MRD after completion of special anticancer therapy. The study of peripheral blood for the presence of CTCs during adjuvant antitumor therapy of breast cancer can serve as a reliable marker of the treatment efficacy.

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